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Expression, purification and crystallization of a thermostable short-chain alcohol dehydrogenase from the archaeon *Thermococcus sibiricus*

A. V. Lyashenko,[a]* E. Y. Bezsudnova,[b] V. M. Gumerov,[c] A. A. Lashkov,[a] A. V. Mardanov,[c] A. M. Mikhailov,[a] K. M. Polyakov,[d] V. O. Popov,[b] N. V. Ravin,[c] K. G. Skryabin,[c] V. K. Zabolotniy,[a] T. N. Stekhanova[b] and M. V. Kovalchuk[a]

[a]Shubnikov Institute of Crystallography, RAS, Leninsky Prospekt 59, Moscow 119333, Russia, [b]Bach Institute of Biochemistry, RAS, Leninsky Prospekt 33, Moscow 119071, Russia, [c]Centre 'Bioengineering', RAS, Prospekt 60-let Oktyabrya 7-1, Moscow 117312, Russia, and [d]RSC 'Kurchatov Institute', Kurchatov Square 1, Moscow 123182, Russia

Correspondence e-mail: lyashenko@ns.crys.ras.ru

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Alcohol dehydrogenases belong to the oxidoreductase family and play an important role in a broad range of physiological processes. They catalyze the cofactor-dependent reversible oxidation of alcohols to the corresponding aldehydes or ketones. The NADP-dependent short-chain alcohol dehydrogenase TsAdh319 from the thermophilic archaeon *Thermococcus sibiricus* was overexpressed, purified and crystallized. Crystals were obtained using the hanging-drop vapour-diffusion method using 25%(*w*/*v*) polyethylene glycol 3350 pH 7.5 as precipitant. The crystals diffracted to 1.68 Å resolution and belonged to space group *I*222, with unit-cell parameters a = 55.63, b = 83.25, c = 120.75 Å.

1. Introduction

Alcohol dehydrogenases (ADHs; EC 1.1.1.1) belong to the oxidoreductase family and catalyze the cofactor-dependent reversible oxidation of primary and secondary alcohols to the corresponding aldehydes and ketones (Reid & Fewson, 1994). These enzymes are widely distributed throughout all domains of life: they are important for the cell detoxification and metabolism of ethanol and other alcohols and probably decrease the cell reductive potential *via* the formation of alcohols. Based on their cofactor specificity, several classes of ADHs have been distinguished: NAD-dependent, NADP-dependent, PQQ-dependent and FAD-dependent. According to their size, the NAD(P)-dependent enzymes can further be subdivided into three groups: long-chain (350–550 amino acids per subunit) iron-containing, medium-chain (~350 amino acids per subunit) zinc-dependent and short-chain (~250 amino acids per subunit) ADHs (Reid & Fewson, 1994).

ADHs show broad substrate specificity and are regioselective and these properties determine their applicability for the synthesis of chiral blocks and synthones. For practical purposes, biocatalysts that have increased stability in non-aqueous media and under elevated temperatures are required. However, the majority of ADHs are not sufficiently stable under real operational conditions. Enzymes from extremophilic microorganisms represent a promising source of catalysts that can be employed in industrial bioconversions, mainly owing to their high thermal and chemical stability. Representatives of all of the groups of NAD(P)-dependent ADHs have been detected in the genomes of extremophilic microorganisms, including archaea. Several long-chain and medium-chain ADHs from thermophiles have been identified and characterized, including the ADHs from *Sulfolobus solfataricus* (Ammendola *et al.*, 1992), *Hyperthermus butylicus* (Cowan, 1992), *Aeropyrum pernix* (Hirakawa *et al.*, 2004) and *Picrophilus torridus* (Hess & Antranikian, 2008). Later, the first short-chain ADH with broad substrate specificity was characterized from *Pyrococcus furiosus* (van der Oost *et al.*, 2001). This enzyme had a temperature optimum at 363 K with a half-life of 22.5 h.

Since the three-dimensional structure of horse liver alcohol dehydrogenase was reported in 1973 (Brändén *et al.*, 1973), ADHs from various organisms have been studied intensively and more than 40 crystal structures of NAD(P)-dependent enzymes have been solved.

The structures of two thermostable archaeal ADHs have been described: those from *A. pernix* (holo form with the inhibitor octanoic acid) at 1.62 Å resolution (PDB code 1h2b; Guy *et al.*, 2003) and from *S. solfataricus* (apo form) at 1.85 Å resolution (PDB code 1jvb; Esposito *et al.*, 2002). Both enzymes belong to the medium-chain zinc-dependent NAD-dependent ADHs and are composed of four identical subunits, each comprising catalytic and cofactor-binding domains. A specific fingerprint motif, the so-called Rossmann fold (Rossmann & Argos, 1981), is implicated in NAD(P) binding.

Only a few crystal structures of short-chain ADHs from bacteria and eukaryotes (Ghosh *et al.*, 1991; Benach *et al.*, 1998; Zaccai *et al.*, 2007) are available. To our knowledge, no crystal structure of an archaeal short-chain ADH has been reported. Previously, we have characterized the genome of the hyperthermophilic euryarchaeon *Thermococcus sibiricus* (Mardanov *et al.*, 2009). At least four alcohol dehydrogenases, including three short-chain ADHs and one long-chain ADH, were found in this organism. A gene coding for a short-chain ADH, Tsib_0319, was found in the saccharolytic gene cluster (Mardanov *et al.*, 2009). The enzyme has 85% identity to a related ADH from *P. furiosus*; this was supposed to be the result of a lateral gene transfer in members of the order Thermococcales. The enzyme consists of 234 amino acids and has a molecular weight of 26 kDa. Analysis of the amino-acid sequence revealed an NADP-binding site at the N-terminus and several conserved residues that are responsible for catalysis (Ghosh *et al.*, 1991). Here, we report the overexpression, purification and preliminary crystallographic studies of the short-chain alcohol dehydrogenase TsAdh319 from *T. sibiricus*.

2. Expression and purification

The Tsib_0319 gene (GenBank No. CP001463) was cloned from *T. sibiricus* genomic DNA and inserted into the expression vector pET15b (Novagen). The encoded recombinant protein contained an N-terminal MGSSHHHHHHSSGLVPRGSHMLE tag followed by the amino-acid sequence of native Tsib_0319. The recombinant vector pET_Tsib0319 was introduced into *Escherichia coli* Rosetta-gami (DE3) strain. The transformants were grown at 310 K in Luria–Bertani medium containing 100 µg ml^{-1} ampicillin and 20 µg ml^{-1} chloramphenicol to an optical density of 0.5 at 600 nm. Isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 1 m*M* for induction of gene expression and cultivation was continued for an additional 15 h.

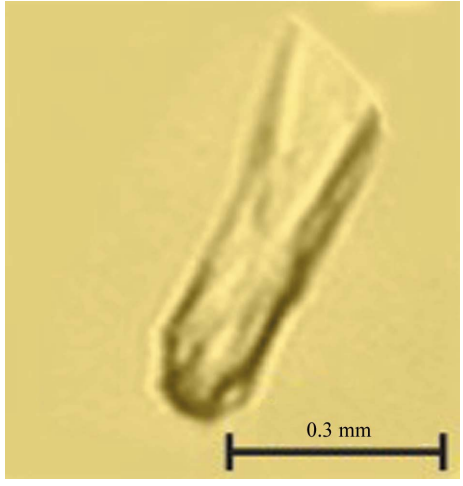

Figure 1
Crystal of recombinant TsAdh319.

The cells were harvested, resuspended in 50 m*M* Tris–HCl buffer pH 7.5 containing 200 m*M* NaCl, 20 m*M* imidazole, 10%(*v*/*v*) glycerol, 10 m*M* β-mercaptoethanol, 0.1%(*v*/*v*) Triton X-100 and 1 m*M* PMSF and sonicated. The crude cell extract was centrifuged for 25 min at 10 000*g*. The supernatant was applied onto a Hi-Trap chelating HP column (1 ml) equilibrated in 50 m*M* Tris–HCl pH 7.5 containing 500 m*M* NaCl, 20 m*M* imidazole, 0.1%(*v*/*v*) Triton X-100. The recombinant TsAdh319 protein was eluted with a linear gradient from 20 to 500 m*M* imidazole in the same buffer. The concentrated active pool was applied onto a Superdex 200 10/300 GL column (Amersham) equilibrated with 50 m*M* Tris–HCl pH 7.5 containing 200 m*M* NaCl. The protein purity was confirmed by 12%(*w*/*v*) SDS–PAGE. The purified TsAdh319 was able to catalyze NADP-dependent 2-propanol oxidation (1.1 U mg^{-1} at 333 K) and was used for crystallization.

3. Crystallization

Initial screening was performed at 298 K with Crystal Screen, Index Screen and Crystal Screen Cryo crystallization solutions (Hampton Research, USA). Crystallization was performed on siliconized glass slides (Hampton Research) in Linbro plates at 293 K using the hanging-drop vapour-diffusion method. Crystals of TsAdh319 (Fig. 1) were obtained by mixing 2 µl protein solution with 2 µl reservoir solution. The reservoir solution consisted of 0.1 *M* HEPES pH 7.5, 0.2 *M* sodium chloride, 25%(*w*/*v*) PEG 3350. The crystallization drop contained 2 µl TsAdh319 solution (10 mg ml^{-1}) in 25 m*M* Tris–HCl and 300 m*M* NaCl pH 7.5 and 2 µl reservoir solution. Crystals of dimensions 0.4 × 0.1 × 0.06 mm were obtained after 3–4 weeks and were used for X-ray diffraction analysis.

4. X-ray analysis

Preliminary X-ray diffraction measurements were made at the Kurchatov Institute synchrotron-radiation source (Moscow, Russia). A complete data set was collected to 1.68 Å resolution (Fig. 2) on

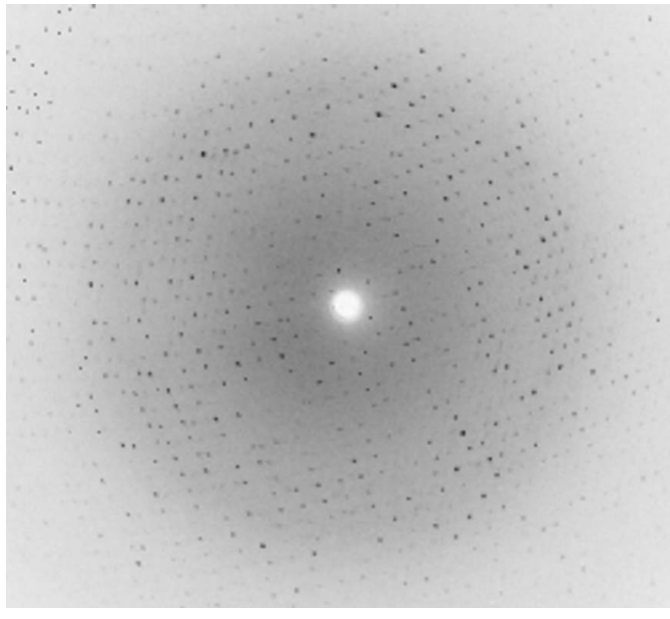

Figure 2
A typical 0.4° oscillation image obtained during data collection from TsAdh319 crystals. The edge of the oscillation image corresponds to 1.68 Å resolution.

Table 1
X-ray data statistics.

Values in parentheses are for the highest resolution shell.

Molecules in asymmetric unit	1
Space group	*I*222 [No. 23]
Temperature (K)	100
Unit-cell parameters (Å, °)	$a = 55.63$, $b = 83.25$, $c = 120.75$, $\alpha = \beta = \gamma = 90$
Molecular weight (kDa)	28.7
Oscillation (°)	0.4
Wavelength (Å)	0.8148
Resolution limits (Å)	7.14–1.68 (1.73–1.68)
No. of reflections	165950
No. of unique reflections	27700 (2185)
Completeness (%)	88.77 (85.1)
$I/\sigma(I)$	21.3 (4.7)
Redundancy	4.88 (4.95)
V_M (Å^3 Da^{-1})	2.43
Solvent content (%)	49.46
R_{merge}	0.05 (0.35)

beamline X11 of the Deutsches Electronen Synchrotron (EMBL/DESY, Hamburg, Germany) using a MAR CCD 555 detector (MAR Research, Germany).

The crystals were directly flash-cooled in a stream of cold nitrogen gas at 100 K using an Oxford Cryosystems cooling device (Oxford Cryosystems, England). The wavelength used was 0.8148 Å. Prior to freezing in liquid nitrogen, the crystals were transferred into a cryoprotectant solution consisting of 0.1 *M* HEPES pH 7.5, 0.2 *M* sodium chloride, 25%(*w*/*v*) PEG 3350 and 20%(*v*/*v*) anhydrous glycerol. All data were processed and scaled using *XDS* (Kabsch, 1988). 315 images were obtained during the collection of X-ray diffraction data. All data were indexed, merged and processed using the *XDS* program. For a semiautomatic determination of the space group, the minimal value of the *XDS* 'quality of fit' function was used.

The crystals of TsAdh319 belonged to space group *I*222. Detailed data statistics are presented in Table 1. The structure was solved by the molecular-replacement technique using the *MOLREP* (Vaguine *et al.*, 1999) program from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994) with the rigid-body refinement option. X-ray diffraction data from 20 to 3 Å resolution were used in this step. The 2.1 Å resolution X-ray structure of monomer *A* of ligand-free clavulanic acid dehydrogenase (PDB code 2jap; MacKenzie *et al.*, 2007) was used as a search model; the sequence homology between the model and the target protein was 51%. Water molecules and other ions were removed from the model. One molecule was found in the asymmetric unit. The Matthews coefficient (Matthews, 1968) was 2.44 Å^3 Da^{-1} and the solvent content was 49.53% (Table 1). Only one solution was evident. The *R* factor at this stage was 48.6%. Before refinement, 5% of the observations were chosen at random and set aside for cross-validation analysis and to monitor the various refinement strategies. After ten cycles of restrained refinement in *REFMAC* (Murshudov *et al.*, 1997), the *R* factor fell to 39.7% and R_{free} was 44.5%. σ_a-weighted electron-density maps with coefficients $(2|F_{obs}| - |F_{calc}|)$ and $(|F_{obs}| - |F_{calc}|)$ were then obtained using *PHENIX* (Adams *et al.*, 2002). Using the $(2|F_{obs}| - |F_{calc}|)$ electron-density map in the *Coot* program (Emsley & Cowtan, 2004), we changed all the amino-acid residues of the starting model to the amino-acid residues of TsAdh319 from *T. sibiricus*. After four cycles of restrained refinement in *REFMAC* and the synthesis of σ_a-weighted $(2|F_{obs}| - |F_{calc}|)$ and $(|F_{obs}| - |F_{calc}|)$ electron-density maps the *R* factor was 24.5% and R_{free} was 28.9%.

Refinement of the structure is currently in progress and will be published elsewhere.

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