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# Expression, purification, crystallization and preliminary X-ray diffraction data of methylmalonate-semialdehyde dehydrogenase from *Bacillus subtilis*

**Hélène Dubourg,[a]‡ Claire Stines-Chaumeil,[b]‡ Claude Didierjean,[a]* François Talfournier,[b] Sophie Rahuel-Clermont,[b] Guy Branlant[b] and André Aubry[a]**

[a]Laboratoire de Cristallographie et Modélisation des Matériaux Minéraux et Biologiques, UMR 7036 CNRS-UHP, Groupe Biocristallographie, Faculté des Sciences et Techniques, BP 239, 54506 Vandoeuvre-lès-Nancy, France, and [b]Laboratoire de Maturation des ARN et Enzymologie Moléculaire, UMR 7567 CNRS-UHP, Faculté des Sciences et Techniques, BP 239, 54506 Vandoeuvre-lès-Nancy, France

‡ These authors contributed equally to this work.

Correspondence e-mail: claude.didierjean@lcm3b.uhp-nancy.fr

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Methylmalonate-semialdehyde dehydrogenase from *Bacillus subtilis* was cloned and overexpressed in *Escherichia coli*. Suitable crystals for X-ray diffraction experiments were obtained by the hanging-drop vapour-diffusion method using ammonium sulfate as precipitant. The crystals belong to space group $P2_12_12_1$, with unit-cell parameters $a$ = 195.2, $b$ = 192.5, $c$ = 83.5 Å, and contain one tetramer per asymmetric unit. X-ray diffraction data were collected to 2.5 Å resolution using a synchrotron-radiation source. The crystal structure was solved by the molecular-replacement method.

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## 1. Introduction

The members of the aldehyde dehydrogenase (ALDH) superfamily oxidize a wide variety of aldehydes to non-activated or CoA-activated acids *via* a two-step chemical mechanism. Mechanistic and structural aspects have been studied extensively for homotetrameric and homodimeric CoA-independent ALDHs (see, for example, Liu *et al.*, 1997; Steinmetz *et al.*, 1997; Marchal & Branlant, 1999; Marchal *et al.*, 2000; Cobessi *et al.*, 2000). In contrast, little information about the mechanistic and/or structural aspects of CoA-dependent ALDHs is available so far, except for the recent structure of a bifunctional aldolase-dehydrogenase (Manjasetty *et al.*, 2003). Methylmalonate-semialdehyde dehydrogenase (MSDH), which is one of the few ALDHs belonging to this family (Steele *et al.*, 1992; Leal *et al.*, 2003), has been found in a wide variety of organisms ranging from bacteria and archaea to mammals. *Pseudomonas aeruginosa* (Bannerjee *et al.*, 1970; Steele *et al.*, 1992) and *Streptomyces coelicolor* (Zhang *et al.*, 1996) MSDHs are involved in valine catabolism. Investigation of the *myo*-inositol catabolism of *Bacillus subtilis* (Yoshida *et al.*, 1997) and *Rhizobium leguminosarium* bv. *Viciae* (Fry *et al.*, 2001) revealed the putative implication of MSDH in this pathway. In mammals, MSDH is a mitochondrial enzyme that is involved in the distal portions of the valine and pyrimidine catabolic pathways (Goodwin *et al.*, 1989). Over the two last decades, a possible correlation of organic acidaemia with MSDH deficiency has been explored (Gray *et al.*, 1987; Chambliss *et al.*, 2000). Roe *et al.* (1998) proposed that a psychomotor delay associated with methylmalonyl aciduria, without any increase of propionylcarnitine in the blood or urine, could be a direct consequence of MSDH deficiency.

This NAD-dependent ALDH catalyzes the oxidation of methylmalonate semialdehyde (MMSA) and malonate semialdehyde (MSA) to propionyl-CoA and acetyl-CoA, respectively. Therefore, MSDHs show another difference with respect to the chemical mechanism of the CoA-independent ALDH family because the catalyzed reaction also includes a $\beta$-decarboxylation step. Here, we report the cloning, overexpression, purification, crystallization and preliminary X-ray diffraction analysis of the MSDH from *B. subtilis* (*Bs*_MSDH).

## 2. Material and methods

### 2.1. Cloning procedure, expression and purification

The *iolA* ORF was amplified by PCR using the *B. subtilis* 168 genomic DNA as a template and the forward and reverse primers 5′-GAAGGAGGCAATA**CATATG**GCAGA-AATCAG-3′ and 5′-CTTACGCAACAAA-**GAGCTC**ATAGAGAAGC-3′, respectively. The forward primer contains an *Nde*I restriction site (in bold) and the reverse primer contains a *Sac*I restriction site (in bold). These restriction sites were used to clone *iolA* into a plasmid derived from pBluescriptSK+, yielding pSKmsdbsub for overexpression under control of the *lac* promoter. The cloned *iolA* ORF was sequenced in order to confirm that no mutations had been introduced in the amplification reaction.

*Escherichia coli* DH5$\alpha$ transformants containing the pSKmsdbsub plasmid were grown at 310 K for 24 h in Luria–Bertani medium supplemented with ampicillin (200 µg ml$^{-1}$). Cells were harvested by centrifugation (20 min at 3000$g$) at 277 K and the pellet was suspended in 50 m$M$ potassium

phosphate pH 8.2 (buffer *A*) containing 10 m*M* β-mercaptoethanol. Cells were disrupted by sonication, after which unbroken cells and debris were removed by centrifugation (45 min at 20 000*g*). MSDH was purified by ammonium sulfate fractionation (40–80%) and gel filtration on ACA 34 resin equilibrated in buffer *A*. Fractions containing MSDH activity were then applied to a Q-Sepharose column using a FPLC system (Amersham Biosciences) previously equilibrated with buffer *A*. MSDH was eluted at 330 m*M* KCl with a linear gradient of 0–1 *M* KCl in buffer *A* at 5 ml $min^{-1}$.

At this stage, wild-type MSDH was pure as checked by electrophoresis on 10% SDS–polyacrylamide gel (Laemmli, 1970) followed by Coomassie Brilliant Blue R-250 staining and electrospray mass-spectrometry analysis. The yield was estimated to be ~200 mg per litre of culture. The observed monomeric weight was 53 319 Da (the calculated weight without the N-terminal Met is 53 316 Da). The relative molecular weight of native wild-type MSDH was estimated by gel-filtration chromatography on a Superose 12 HR column as previously described (Roitel *et al.*, 1999). The obtained value of 223 ± 11 kDa is in good agreement with the theoretical value expected for a tetrameric enzyme, *i.e.* 213 kDa. Purified enzyme was stored at 253 K in the presence of 10 m*M* β-mercaptoethanol. MSDH was stable under these conditions for several weeks. The concentration was determined spectrophotometrically using an extinction coefficient of 280 nm of 51 000 $M^{-1}$ $cm^{-1}$ deduced by the method of Scopes (1974), Mach *et al.* (1992) and Gill & von Hippel (1989).

### 2.2. Crystallization

The purified enzyme was dialyzed against potassium phosphate buffer pH 7.8, 1 m*M* DTT, 1 m*M* EDTA and finally concentrated using a Centricon (Amicon/Millipore) with 30 kDa molecular-weight cutoff at 4500*g* and 277 K. Prior to crystallization experiments, the enzyme was incubated at 303 K for 30 min in phosphate buffer pH 7.8 containing 20 m*M* NAD. Crystallization experiments were performed using the hanging-drop vapour-diffusion method (Ducruix & Giegé, 1999) at room temperature. Drops were prepared by mixing 2 µl protein solution with an equal volume of reservoir solution and were suspended over 500 µl reservoir solution. The protein concentration used for the first screenings was 10 mg $ml^{-1}$. Initial crystallization trials were carried out at 277 and 293 K using commercially available sparse-matrix screens and a home-made ammonium sulfate kit (pH range 5.0–8.0 and ammonium sulfate concentration range 1.0–3.0 *M* in steps of 0.1). The commercially screens used were Crystal Screens I and II from Hampton Research and Clear Strategy Screens I and II from Molecular Dimensions Ltd. The crystallization experiment was repeated by increasing the protein concentration until crystals were obtained.

**Table 1**
Statistics of X-ray data measurement for *B. subtilis* MSDH crystals.

Values in parentheses refer to the outermost resolution shell (2.50–2.59 Å).

| | |
|---|---|
| Space group | $P2_12_12_1$ |
| Unit-cell parameters (Å) | *a* = 195.2, *b* = 192.5, *c* = 83.5 |
| Resolution range (Å) | 20.0–2.5 |
| No. reflections measured | 786249 |
| No. unique reflections | 108345 |
| Completeness (%) | 99.5 (91.9) |
| $\langle I/\sigma(I)\rangle$ | 9.39 (3.58) |
| $R_{merge}$† (%) | 13.8 (37.2) |

† $R_{merge} = \sum_{hkl}\sum_j |I_{hklj} - \langle I_{hkl}\rangle| / \sum_{hkl}\sum_j I_{hkl}$, where $I_{hklj}$ is the intensity of observation *j* of Bragg reflection *hkl*.

### 2.3. Data collection and diffraction measurements

The cryoprotectant conditions for *Bs*_MSDH crystals were optimized using an in-house X-ray facility composed of an area detector (DIP2030b) with a φ goniometer using Cu *Kα* radiation from a rotating-anode generator (Bruker–Nonius FR591 Model) and an Oxford Cryosystems Cryostream. The X-ray diffraction data set was collected using synchrotron radiation from EMBL beamline X11 located at the DORIS storage ring, DESY, Hamburg. The wavelength used was 0.8126 Å and the data were collected at 100 K on a MAR CCD165 detector. 360 images were collected with a crystal-to-detector distance of 240 mm and 0.5° rotation per image. A 2.5 Å data set was processed and merged with the *XDS* suite (Kabsch, 1993).

### 2.4. Results and discussion

The first crystals were only obtained at a very high protein concentration (>150 mg $ml^{-1}$) using ammonium sulfate as precipitant. These initial crystallization conditions were further refined by changing the pH and the protein and precipitant concentrations. Finally, thin plates grew within 3–7 d to approximate dimensions of 0.3 × 0.3 × 0.05 mm under the following conditions: 2.0–2.3 *M* ammonium sulfate without buffer and a protein concentration of 200 mg $ml^{-1}$.

Prior to X-ray diffraction experiments, crystals were soaked briefly in reservoir solution supplemented with 18%(*v*/*v*) glycerol for flash-freezing. The diffraction pattern obtained using the home rotating-anode generator was too weak to determine the unit cell of the MSDH crystals. The diffraction pattern obtained using the synchrotron X-rays was consistent with the primitive orthorhombic Bravais lattice, with unit-cell parameters *a* = 195.2, *b* = 192.5, *c* = 83.5 Å. A total of 108 345 unique reflections (average multiplicity 8) were collected in the resolution range 20–2.5 Å. Detailed data-collection statistics are given in Table 1. The systematic absences unambiguously revealed screw twofold axes parallel to *a*, *b* and *c*, leading to the $P2_12_12_1$ space-group assignment. A reasonable Matthews coefficient of 3.6 $Å^3$ $Da^{-1}$ (Matthews, 1968) was obtained for four monomers in the asymmetric unit. The resulting calculated solvent content is 65%.

*Bs*_MSDH displays 18–34% sequence identity to non-phosphorylating ALDHs whose crystal structures are available in the Protein Data Bank. Consequently, the molecular-replacement method was first tested to solve the crystal structure of *Bs*_MSDH. Considering the oligomeric state of *Bs*_MSDH, trials were carried out using the crystal structures of tetrameric ALDHs only. The holo form of the cod liver betaine aldehyde dehydrogenase search model gave the best solution (Johansson *et al.*, 1998; PDB code 1bpw). In *CNS* (Brünger *et al.*, 1998), a cross-rotational search followed by a translational search were performed in the resolution range 10–4 Å. The top two solutions seem to be equivalent, exhibiting correlation coefficients of 34.2 and 33.9%, while that of the next solution was 25.1%. Crystallographic refinement and structure analysis will be published elsewhere (coordinates have been deposited in the PDB with code 1t90).

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