Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary X-ray diffraction studies of tetrameric malate dehydrogenase from the novel Antarctic psychrophile *Flavobacterium frigidimaris* KUC-1

Tomomi Fujii,[a] Tadao Oikawa,[b] Ikuo Muraoka,[b] Kenji Soda[a,b] and Yasuo Hata[a]*

[a]Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan, and [b]Department of Life Science and Biotechnology, Faculty of Chemistry, Materials and Bioengineering, Kansai University, Suita, Osaka 564-8680, Japan

Correspondence e-mail: hata@scl.kyoto-u.ac.jp

Received 14 September 2007
Accepted 18 October 2007

Flavobacterium frigidimaris KUC-1 is a novel psychrotolerant bacterium isolated from Antarctic seawater. Malate dehydrogenase (MDH) is an essential metabolic enzyme in the citric acid cycle and has been cloned, overexpressed and purified from *F. frigidimaris* KUC-1. In contrast to the already known dimeric form of MDH from the psychrophile *Aquaspirillium arcticum*, *F. frigidimaris* MDH exists as a tetramer. It was crystallized at 288 K by the hanging-drop vapour-diffusion method using ammonium sulfate as the precipitating agent. The crystal diffracted to a maximum resolution of 1.80 Å. It contains one tetrameric molecule in the asymmetric unit.

1. Introduction

The cold environment dominates the surface of the Earth, since approximately three quarters of its surface is covered by deep oceans, high mountains and the Arctic and Antarctic regions, where average temperatures are permanently below 277 K. Various psychrophilic microorganisms that grow at low temperatures unsuitable for most other organisms have been isolated from cold soils and waters (Tamegai *et al.*, 1997; Grant *et al.*, 1998; Kraegeloh & Kunte, 2002). In order to survive and grow effectively under such extreme environments, these microorganisms produce various psychrophilic and thermolabile enzymes (Breuil & Kushner, 1975; Kobori *et al.*, 1984; Vekovski *et al.*, 1990; Davail *et al.*, 1994; Feller *et al.*, 1994, 1996, 1997; Feller & Gerday, 1997; Gerike *et al.*, 1997; Tsigos *et al.*, 1998; Zakaria *et al.*, 1998; Bruni *et al.*, 1999). These enzymes show high catalytic activities in cold environments and usually completely lose their activities even at ∼303 K (Gianese *et al.*, 2002). This suggests that psychrophilic enzymes must possess unique three-dimensional structures suitable for cold adaptation. Psychrophilic enzymes have generated considerable interest since they can be used to improve the efficiency of industrial processes and in environmental applications (Herbert, 1992; Feller *et al.*, 1996). In order to understand the structural basis of the cold adaptation of psychrophilic enzymes, we have initiated studies on the tetrameric malate dehydrogenase (MDH) from a psychrophilic bacterium as a model of a structurally unknown psychrophilic enzyme. It may serve as a good template for understanding the unique structure-folding properties of psychrophilic proteins, as the crystal structures of other MDHs from various mesophiles and thermophiles have already been determined.

Malate dehydrogenase is an oligomeric enzyme that catalyzes the reversible oxidation of malate to oxaloacetate in the presence of NAD^+ and is present in most living organisms as an essential metabolic enzyme in the citric acid cycle (Goward & Nicholls, 1994). Various types of MDH from organisms representing archaea, bacteria and eukarya have been isolated and their genetic and biochemical aspects have been studied extensively (Madern, 2002). The MDHs are classified into two groups depending on their oligomeric state: homodimeric MDHs and homotetrameric MDHs. In most cases, including all eukaryotes, MDH exists as a dimer. In some prokaryotes (Sundaram *et al.*, 1980; Smith *et al.*, 1984) and in phototrophic bacteria (Tayeh & Madigan, 1987; Rolstad *et al.*, 1988), MDH is a tetramer in its native state. Crystal structures of dimeric and tetrameric MDHs have already been determined from various micro-

organisms living in extreme environments: the hyperthermophile *Methanococcus jannaschii* (Lee *et al.*, 2001), the thermophiles *Thermus flavus* (Kelly *et al.*, 1993), *Chloroflexus aurantiacus* (Dalhus *et al.*, 2002) and *Chlorobium tepidum* (Dalhus *et al.*, 2002) and the psychrophile *Aquaspirillium arcticum* (Kim *et al.*, 1999). These dimeric and tetrameric MDHs have been extensively compared structurally with the mesophilic MDHs from *Escherichia coli* (Hall *et al.*, 1992; Hall & Banaszak, 1993) and *Chlorobium vibrioforme* (Dalhus *et al.*, 2002), respectively, in order to shed light on the adaptation of their structures to extreme environments. Good progress has been achieved in the elucidation of the adaptation mechanism of these enzymes to hot environments, but the molecular basis of cold adaptation is not very well understood (Goldman, 1995; Frolow *et al.*, 1996; Danson & Hough, 1997). In fact, only the crystal structure of the dimeric form of psychrophilic MDH from *A. arcticum* has been determined to date (Kim *et al.*, 1999). No structures of a tetrameric MDH from a psychrophile have yet been determined; the tetrameric enzymes probably differ from dimeric MDH in inter-subunit organization and interaction networks. Therefore, we have carried out preliminary structural studies on tetrameric malate dehydrogenase from a novel psychrotolerant bacterium, *Flavobacterium frigidimaris* KUC-1, which was isolated from Antarctic seawater (Nogi *et al.*, 2005).

MDH from *F. frigidimaris* KUC-1 is the most psychrophilic and thermolabile of the MDHs studied to date (Oikawa *et al.*, 2005). The enzyme is highly susceptible to heat treatment, with a half-life of 3.0 min at 313 K. It is estimated to be a tetramer with an overall molecular weight of about 130 kDa and a subunit weight of about 33 kDa. The subunit consists of 311 amino-acid residues and contains much lower numbers of proline and arginine residues, which are usable for thermostabilization, than other MDHs. The amino-acid sequence does not show a high similarity to that of MDH from *A. arcticum* (20.3% identity), which is only the psychrophilic MDH of determined structure. Here, we report the crystallization and preliminary X-ray diffraction studies of tetrameric malate dehydrogenase from the novel psychrophile *F. frigidimaris* KUC-1. The structure determination of the present MDH and intensive comparison of the MDH structures of microorganisms living in different environments are expected to provide more detailed structural information on the mechanism of cold adaptation.

2. Protein expression and purification

Expression of the protein was performed as described previously (Oikawa *et al.*, 2005). *E. coli* BL21 (DE3) cells harbouring a recombinant plasmid pET-3b carrying the *mdh* gene (GenBank accession No. AB161423) were grown in ampicillin-containing LB medium at 303 K. When the optical density at 600 nm of the culture medium reached 0.6, 1 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium. After 15 h incubation at 293 K, the cells were harvested by centrifugation (8000*g*, 20 min) and suspended in 10 m*M* potassium phosphate buffer pH 7.0 containing 0.01%(*v*/*v*) 2-mercaptoethanol (buffer *A*).

Purification of the protein was performed as described previously (Oikawa *et al.*, 2005). All purification procedures were performed at 277 K under aerobic conditions. The cultivated cells were disrupted by ultrasonication (model UD-201, Tomy, Tokyo). The cell debris was removed by centrifugation (27 600*g*, 30 min). The supernatant was dialyzed against buffer *A* and used as crude enzyme. The crude enzyme was applied onto a DEAE-Toyopearl 650M column equilibrated with buffer *A*. The adsorbed proteins were eluted with a linear gradient of 10–500 m*M* potassium phosphate buffer pH 7.0 containing 0.01%(*v*/*v*) 2-mercaptoethanol. The MDH activity of eluted fractions was assayed as described previously (Oikawa *et al.*, 2005). Fractions showing MDH activity were combined and dialyzed against buffer *A*. The enzyme solution was again applied onto a DEAE-Toyopearl 650M column equilibrated with buffer *A* and the column was washed with buffer *A* containing 30 m*M* NaCl. The adsorbed protein was eluted with a linear gradient of 30–100 m*M* NaCl. The active fractions were collected, dialyzed against buffer *A* and applied onto a SuperQ-Toyopearl column equilibrated with buffer *A* containing 40 m*M* NaCl. The adsorbed protein was eluted with a linear gradient of 40–80 m*M* NaCl. The active fractions were collected, dialyzed against buffer *A* and applied onto a Blue Sepharose CL6B column equilibrated with buffer *A*. The adsorbed protein was eluted with a linear gradient of 0.0–1.0 m*M* NAD^+ in buffer *A* containing 1 m*M* L-malate. The active fractions were dialyzed against buffer *A*. The enzyme solution was concentrated to 15–20 mg ml^{-1} by ultrafiltration and stored at 193 K until crystallization. Protein concentrations were measured by the method of Bradford based on a calibration curve with bovine serum albumin (Wako Chemical, Osaka, Japan; product No. 011-07493; Bradford, 1976).

3. Crystallization

Crystallization experiments were performed using the hanging-drop vapour-diffusion method. The protein concentration was adjusted to 17 mg ml^{-1} in 10 m*M* potassium phosphate buffer pH 7.0. The temperature was initially set at 277 K considering the stability of the enzyme. Each of the drops consisting of 1 µl protein solution and 1 µl reservoir solution was equilibrated against 100 µl reservoir solution. Initial reservoir-solution conditions were screened using Crystal Screens I and II and Index Screen (Hampton Research) and crystals were obtained using condition No. 5 from Crystal Screen II [2.0 *M* ammonium sulfate, 5%(*v*/*v*) 2-propanol]. After improvement of the crystallization conditions, rod-shaped crystals of the enzyme with approximate dimensions of 0.70 × 0.10 × 0.02 mm were obtained at 277 K within 2–3 d by equilibrating the 2 µl protein drop against 500 µl reservoir solution consisting of 1.8 *M* ammonium sulfate, 2%(*v*/*v*) MPD, 50 m*M* sodium citrate buffer pH 5.5 (Fig. 1). However, this crystallization had poor reproducibility and did not produce any larger crystals. Moreover, the observation and manipulation of crystals at ordinary temperatures was not very convenient because

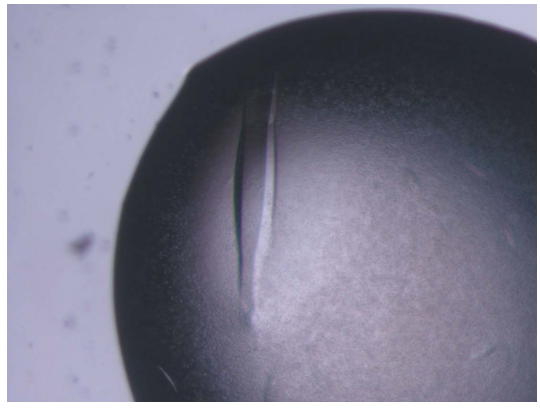

Figure 1
Crystal of psychrophilic MDH from *F. frigidimaris* KUC-1 obtained at 277 K. The dimensions of the crystal were 0.70 × 0.10 × 0.02 mm.

even a slight increase of the temperature in the crystallization cell caused the appearance of microcrystals and precipitation of the protein in the drop. Therefore, improvement of the crystallization conditions was performed using the temperature and the concentration of precipitant as the main parameters. Consequently, good rod-shaped crystals of the enzyme with maximum dimensions of 1.1 × 0.15 × 0.05 mm were reproducibly obtained at 288 K within 2–3 d by equilibrating the 2 µl protein drop against 500 µl reservoir solution consisting of 1.4 *M* ammonium sulfate, 5%(*v*/*v*) MPD, 2 m*M* NAD^+, 50 m*M* sodium citrate buffer pH 5.5 (Fig. 2).

4. X-ray analysis

Diffraction experiments were initially performed using the crystals obtained at 277 K. A crystal with approximate dimensions 0.40 × 0.10 × 0.02 mm was soaked in reservoir solution containing 26%(*v*/*v*) glycerol for several tens of seconds and then mounted in a nylon loop and flash-cooled in a nitrogen stream at 100 K. Diffraction data were collected on a Rigaku R-AXIS IV image-plate detector using Cu $K\alpha$ radiation (λ = 1.5418 Å) produced by a Rigaku RU-300 rotating-anode generator, which was equipped with confocal optics and operated at 40 kV and 100 mA. The crystal-to-detector distance was set to 150 mm. A data set (the RU data set) was collected from a single crystal with 1.0° oscillation steps over a range of 90°. The exposure time was 2.0 min per frame. All diffraction images were processed at 2.8 Å resolution with the program *HKL*-2000 (Otwinowski & Minor, 1997). Crystal data and intensity statistics of the RU data collection are summarized in Table 1.

The crystal belonged to space group $P3_221$, with unit-cell parameters $a = b = 147.5$, $c = 165.1$ Å. Based on the molecular weight and crystallographic data, it was assumed that the crystal contained one or two tetrameric molecules in the asymmetric unit. This assumption gives a V_M value (Matthews, 1968) of 3.97 or 1.99 $Å^3$ Da^{-1} and a solvent content of 69.1 or 34.6% for one or two tetrameric molecules per asymmetric unit, respectively. The structure determination of the enzyme was initiated using the RU data set. Phase calculation was attempted by molecular replacement (MR) using the program *MOLREP* (Vagin & Teplyakov, 1997) from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). The preliminary MR calculation gave an initial result indicating the presence of one tetramer of MDH per asymmetric unit.

Subsequently, high-resolution diffraction data collection for structure refinement was performed using a crystal obtained at 288 K on beamline BL-5A at Photon Factory, Tsukuba, Japan. A crystal with typical dimensions 0.35 × 0.15 × 0.05 mm was soaked in reservoir solution containing 30%(*v*/*v*) glycerol for several tens of seconds and then mounted in a nylon loop and flash-cooled in a nitrogen stream at 100 K. Diffraction data were collected at a wavelength of 1.000 Å using a Quantum 315 CCD detector (ADSC) set to a crystal-to-detector distance of 249.2 mm. A data set (the SR data set) was collected from a single crystal with 1.0° oscillation steps over a range of 180°. The exposure time was 1.5 s per frame. All diffraction images were processed with the program *MOSFLM* (Collaborative Computational Project, Number 4, 1994; Leslie, 1992). The crystal diffracted to 1.8 Å resolution. Crystal data and intensity statistics of the SR data collection are summarized in Table 1.

The structure determination of tetrameric malate dehydrogenase from the psychrophile *F. frigidimaris* KUC-1 is under way using the molecular-replacement method.

Table 1
Crystal data and X-ray data-collection statistics.

Values in parentheses are for the highest resolution shell.

	RU data set	SR data set
X-ray source	Rigaku RU-300	PF BL-5A
Wavelength (Å)	1.5418	1.000
Temperature (K)	100	100
Space group	$P3_221$	$P3_221$
Unit-cell parameters (Å)	$a = b = 147.5$, $c = 165.1$	$a = b = 147.8$, $c = 165.1$
Subunits per ASU	4	4
V_M ($Å^3$ Da^{-1})	3.97	3.99
Solvent content (%)	69.1	69.2
Resolution range (Å)	50.0–2.80 (2.90–2.80)	50.0–1.80 (1.90–1.80)
No. of observed reflections	282260	2099880
No. of unique reflections	51599 (5095)	192407 (27839)
Completeness (%)	99.9 (99.8)	100 (99.7)
R_{sym}† (%)	18.5 (66.8)	8.0 (37.0)
Multiplicity	5.5 (5.2)	10.9 (10.6)
Average $I/\sigma(I)$	7.4 (1.9)	24.7 (5.4)

† $R_{sym} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the intensity of reflection h, $\sum_h$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection h.

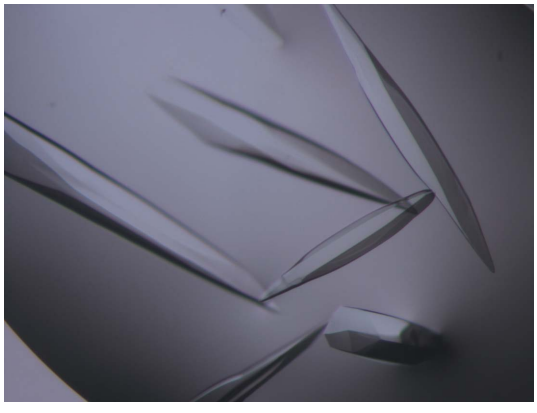

Figure 2
Crystals of psychrophilic MDH from *F. frigidimaris* KUC-1 obtained at 288 K. The dimensions of the largest crystal were 1.0 × 0.15 × 0.05 mm.

This work was supported in part by a grant from the National Project on Protein Structural and Functional Analyses, Ministry of Education, Culture, Sports, Science and Technology of Japan. The diffraction experiments at Photon Factory were approved by the Photon Factory Advisory Committee and the High Energy Accelerator Research Organization (KEK; proposal Nos. 2004G359 and 2006G387).

References

Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
Breuil, C. & Kushner, D. J. (1975). *Can. J. Microbiol.* **21**, 423–433.
Bruni, V., Gugliandolo, C., Maugeri, T. & Allegra, A. (1999). *New Microbiol.* **22**, 357–363.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Dalhus, B., Saarinen, M., Sauer, U. H., Eklund, P., Johansson, K., Karlsson, A., Ramaswamy, S., Bjørk, A., Synstad, B., Naterstad, K., Sirevåg, R. & Eklund, H. (2002). *J. Mol. Biol.* **318**, 707–721.
Danson, M. J. & Hough, D. W. (1997). *Struct. Comput. Biochem. Physiol. A*, **117**, 307–312.
Davail, S., Feller, G., Narinx, E. & Gerday, C. (1994). *J. Biol. Chem.* **269**, 17448–17453.
Feller, G. & Gerday, C. (1997). *Cell. Mol. Life Sci.* **53**, 830–841.
Feller, G., Narinx, E., Arpigny, J. L., Aittaleb, M., Baise, E., Genicot, S. & Gerday, C. (1996). *FEMS Microbiol. Rev.* **18**, 189–202.

Feller, G., Payan, F., Theys, F., Qian, M., Haser, R. R. & Gerday, C. (1994). *Eur. J. Biochem.* **222**, 441–447.
Feller, G., Zekhnini, Z., Lamotte-Brasseur, J. & Gerday, C. (1997). *Eur. J. Biochem.* **244**, 186–191.
Frolow, F., Harel, M., Sussman, J. L., Mevarech, M. & Shoham, M. (1996). *Nature Struct. Biol.* **3**, 452–458.
Gerike, U., Danson, M. J., Russell, N. J. & Hough, D. W. (1997). *Eur. J. Biochem.* **248**, 49–57.
Gianese, G., Bossa, F. & Pascarella, S. (2002). *Proteins*, **47**, 236–249.
Goldman, A. (1995). *Structure*, **3**, 1277–1279.
Goward, C. R. & Nicholls, D. J. (1994). *Protein Sci.* **3**, 1883–1888.
Grant, W. D., Gemmell, R. T. & Mcgenity, T. J. (1998). *Extremophiles*, **2**, 279–287.
Hall, M. D. & Banaszak, L. J. (1993). *J. Mol. Biol.* **232**, 213–222.
Hall, M. D., Levitt, D. G. & Banaszak, L. J. (1992). *J. Mol. Biol.* **226**, 867–882.
Herbert, R. A. (1992). *Trends Biotechnol.* **10**, 395–402.
Kelly, C. A., Nishiyama, M., Ohnishi, Y., Beppu, T. & Birktoft, J. J. (1993). *Biochemistry*, **32**, 3913–3922.
Kim, S.-Y., Hwang, K. Y., Kim, S.-H., Sung, H.-C., Han, Y. S. & Cho, Y. (1999). *J. Biol. Chem.* **274**, 11761–11767.
Kobori, H., Sullivan, C. W. & Shizuya, H. (1984). *Proc. Natl Acad. Sci. USA*, **81**, 6691–6695.
Kraegeloh, A. & Kunte, H. J. (2002). *Extremophiles*, **6**, 453–462.
Lee, B. I., Changsoo, C., Cho, S.-J., Eom, S. H., Kim, K. K., Yu, Y. G. & Suh, S. W. (2001). *J. Mol. Biol.* **307**, 1351–1362.
Leslie, A. G. W. (1992). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **26**.
Madern, D. (2002). *J. Mol. Evol.* **54**, 825–840.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Nogi, Y., Soda, K. & Oikawa, T. (2005). *Syst. Appl. Microbiol.* **28**, 310–315.
Oikawa, T., Yamamoto, N., Shimoke, K., Uesato, S., Ikeuchi, T. & Fujioka, T. (2005). *Biosci. Biotechnol. Biochem.* **69**, 2146–2154.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Rolstad, A. K., Howland, E. & Sirevåg, R. (1988). *J. Bacteriol.* **170**, 2947–2953.
Smith, K., Sundaram, T. K. & Kernick, M. (1984). *J. Bacteriol.* **157**, 684–687.
Sundaram, T. K., Wright, I. P. & Wilkinson, A. E. (1980). *Biochemistry*, **19**, 2017–2022.
Tamegai, H., Li, L., Masui, N. & Kato, C. (1997). *Extremophiles*, **1**, 207–211.
Tayeh, M. A. & Madigan, M. T. (1987). *J. Bacteriol.* **169**, 4196–4202.
Tsigos, I., Velonia, K., Smonou, I. & Bouriotis, V. (1998). *Eur. J. Biochem.* **254**, 356–362.
Vagin, A. & Teplyakov, A. (1997). *J. Appl. Cryst.* **30**, 1022–1025.
Vekovski, V., Schlatter, D. & Zuber, H. (1990). *Biol. Chem. Hoppe-Seyler*, **371**, 103–110.
Zakaria, M. M., Ashiuchi, M., Yamamoto, S. & Yagi, T. (1998). *Biosci. Biotechnol. Biochem.* **62**, 655–660.