Yu-Yung Chang,^{a,†} Chih-Hung
Hung,^{a,†} Tzann-Shun Hwang^{b,†}
and Chun-Hua Hsu^{a,c,d,*}

^aDepartment of Agricultural Chemistry, National
Taiwan University, Taipei 10617, Taiwan,

^bGraduate Institute of Biotechnology, Chinese
Culture University, Taipei 11114, Taiwan,

^cGenome and Systems Biology Degree Program,
National Taiwan University, Taipei 10617,
Taiwan, and ^dCenter for Systems Biology,

National Taiwan University, Taipei 10617,
Taiwan

† These authors contributed equally to this
work.

Correspondence e-mail: andyhsu@ntu.edu.tw

Received 7 July 2013

Accepted 4 September 2013

Cloning, overexpression, purification and crystallization of malate dehydrogenase from *Thermus thermophilus*

Malate dehydrogenase (MDH) has been used as a conjugate for enzyme immunoassay of a wide variety of compounds, such as drugs of abuse, drugs used in repetitive therapeutic application and hormones. In consideration of the various biotechnological applications of MDH, investigations of MDH from *Thermus thermophilus* were carried out to further understand the properties of this enzyme. The DNA fragment containing the open reading frame of *mdh* was amplified from the genomic DNA of *T. thermophilus* and cloned into the expression vector pET21b(+). The protein was expressed in a soluble form in *Escherichia coli* strain BL21(DE3). Homogeneous protein was obtained using a three-step procedure consisting of thermal treatment, Ni²⁺-chelating chromatography and size-exclusion chromatography. The purified MDH was crystallized and the crystals diffracted to a resolution of 1.80 Å on the BL13C1 beamline of the National Synchrotron Radiation Research Center (NSRRC), Taiwan. The crystals belonged to the orthorhombic space group *P*2₁2₁2₁, with unit-cell parameters *a* = 71.3, *b* = 86.1, *c* = 118.2 Å. The unit-cell volume of the crystal is compatible with the presence of two monomers in the asymmetric unit, with a corresponding Matthews coefficient *V*_M of 2.52 Å³ Da⁻¹ and a solvent content of 51.2%. The crystal structure of MDH has been solved by molecular replacement and is currently under refinement.

1. Introduction

Malate dehydrogenase (MDH; EC 1.1.1.37), an enzyme involved in the TCA cycle and the malate–aspartate shuttle, plays important roles in glucose metabolism and energy generation (Minárik *et al.*, 2002; Sundaram *et al.*, 1980). MDH catalyzes a dehydrogenation reaction from malate to generate oxaloacetate, accompanied by the reduction of NAD to generate NADH (Frieden & Fernandez-Sousa, 1975). The overall reaction catalyzed by MDH is reversible; therefore, the activity of MDH can easily be measured by detecting the decreasing amount of NADH. The convenient measurement method enables MDH to be applied in coupling assays for measuring the activities of enzymes whose product is oxaloacetic acid, such as activity assays of aspartate aminotransferase and aspartate oxidase. MDH is also used in medical biotechnology for enzyme immunoassay of a wide variety of compounds, such as drugs of abuse, drugs used in repetitive therapeutic application and hormones (Li *et al.*, 1996).

Although numerous thermophilic MDHs have been characterized, the structure of the enzyme without co-substrate binding and the molecular basis of the action mode at high temperature are still poorly understood. In consideration of the various applications of MDH, investigations of MDH from *Thermus thermophilus* were carried out to understand the structure–function relationship of this enzyme. Elucidation of the molecular architecture for future protein engineering on the enzyme's activity and thermostability would also be accomplished simultaneously. Here, we report the cloning, overexpression, purification and crystallization of malate dehydrogenase from *T. thermophilus*. In addition, the crystal structure of MDH has been solved by molecular replacement and is currently under refinement.

2. Experimental methods

2.1. Cloning

The *mdh* (malate dehydrogenase; UniProt accession No. P10584) gene was PCR-amplified using gene-specific primers (forward primer, 5'-GTG GCA **TAT** GAA GGC ACC CGT ACG CGT GG-3'; reverse primer, 5'-TCT GAG GCA **AAG CTT** TCG GAT GAG GCC CAG GG-3') and the genomic DNA of *T. thermophilus* HB8 genomic DNA as a template. The amplified DNA fragment flanked by *Nde*I and *Hind*III sites (bold) was cloned into the pGEM-T Easy vector (Promega, USA). Recombinant plasmids were introduced into *Escherichia coli* JM109 by transformation. Plasmid DNA was isolated from the transformants and digested with *Nde*I and *Hind*III and the fragment was then cloned into pET21b(+) (Novagen) linearized with the same restriction enzymes. The identity of the gene was confirmed by sequencing the clone using Sanger's method.

2.2. Overexpression

The expression vector containing the *mdh* gene fragment was introduced into the *E. coli* BL21(DE3) strain. 10 ml of an overnight culture of each recombinant clone was inoculated into Luria–Bertani medium containing 100 mg l⁻¹ ampicillin; the culture was grown at 310 K with shaking at 200 rev min⁻¹ to an OD₆₀₀ of 1.0. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce protein expression and the cultures were incubated for a further 2 h at 310 K. The cells were harvested by centrifugation at 9000g for 30 min.

2.3. Purification

The cell pellet was resuspended in resuspension buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 10 mM imidazole) and disrupted by ultrasonication. The cell debris was removed by centrifugation at 20 000g for 20 min at 277 K. The supernatant was further treated at 343 K for 30 min and centrifuged at 20 000g for 20 min at 277 K to remove the denatured proteins. The protein was purified from the supernatant by Ni²⁺-charged HiTrap chelating FF column chromatography (GE Healthcare, USA) in a buffer system consisting of 50 mM Tris–HCl pH 8.0, 100 mM NaCl, 200 mM imidazole. Fractions containing *T. thermophilus* MDH (TtMDH) were pooled and dialyzed against 50 mM Tris-buffered solution pH 8.0 containing 150 mM NaCl. The protein was further purified by size-exclusion chromatography using a Superdex S-200 HR10/300 column pre-equilibrated with size-exclusion buffer (50 mM Tris–HCl pH 8.0, 50 mM NaCl, 5 mM EDTA) on an ÄKTA FPLC system (GE Healthcare, USA). The purity of the TtMDH protein was monitored and appeared as a single band on SDS–PAGE (Fig. 1a). The protein concentration was determined by measuring the absorbance at 280 nm under denaturing conditions using a UV–Vis spectrometer (Hitachi, Japan). The predicted extinction coefficient based on the amino-acid sequence of the final protein construct (Gill & von Hippel, 1989) was 32 430 M⁻¹ cm⁻¹. Prior to crystallization, the purified protein was concentrated to 10.0 mg ml⁻¹ using a Centricon membrane filter (30 kDa cutoff, Millipore, USA). No His-tag removal was attempted.

2.4. Crystallization

Crystallization was performed by the vapour-diffusion method using a HoneyBee 963 robot (Genomic Solutions). Sitting drops were prepared by mixing 0.5 µl TtMDH solution at 10 mg ml⁻¹ with an equal volume of reservoir solution and were equilibrated against 100 µl reservoir solution at 283 K. Small crystals grew from a solution consisting of 0.1 M Tris–HCl pH 8.5, 30% (w/v) polyethylene glycol 4000, 0.2 M magnesium chloride. The crystals were then optimized manually by varying the pH or precipitant concentration in small steps. The best crystals grew in 5 d from a solution consisting of 0.1 M Tris–HCl pH 8.2, 22.5% (w/v) polyethylene glycol 4000, 0.2 M magnesium chloride, 5% (v/v) glycerol (Fig. 1b).

2.5. X-ray data collection and processing

Data collection was accomplished using a synchrotron-radiation X-ray source on protein crystallographic beamline BL13C1 equipped with the ADSC Quantum 315r CCD detector at the National Synchrotron Radiation Research Center (NSRRC) in Taiwan. The crystal was scooped up in a nylon CryoLoop (Hampton Research) and then flash-cooled in a nitrogen-gas stream at 100 K. Diffraction images were indexed, integrated and scaled using *DENZO* (Rossman & van Beek, 1999) and *SCALEPACK* (Borek *et al.*, 2003) from

Figure 1
(a) Purification of TtMDH. The calculated molecular weight of TtMDH is 37 kDa. Lane M, protein marker (labelled in kDa); lane 1, purified TtMDH. (b) Crystals of TtMDH in 0.1 M Tris–HCl pH 8.2, 22.5% (w/v) polyethylene glycol 4000, 0.2 M magnesium chloride, 5% (v/v) glycerol obtained using the sitting-drop vapour-diffusion method. The approximate dimensions of the crystals are 200 × 100 × 100 µm.

Table 1

Data-collection statistics for TtMDH crystals.

Values in parentheses are for the highest resolution shell.

X-ray source	BL13C1, NSRRC, Taiwan
Wavelength (Å)	0.97622
Detector	ADSC Q315r
Temperature of data collection (K)	100
Crystal-to-detector distance (mm)	350
Rotation range per image (°)	1
Total rotation range (°)	150
Exposure time per image (s)	60
Resolution range (Å)	27.86–1.80 (1.86–1.80)
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 71.3$, $b = 86.1$, $c = 118.2$
Mosaicity (°)	0.565
Total No. of measured intensities	515221 (43075)
Unique reflections	68001 (6665)
Multiplicity	4.7 (4.9)
Completeness (%)	99.9 (99.2)
$R_{\text{merge}}^{\dagger}$ (%)	6.1 (30.1)
Average $I/\sigma(I)$	27.7 (6.1)

$^{\dagger} R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ are the intensities of the individual replicates of a given reflection hkl and $\langle I(hkl) \rangle$ is the average intensity over all replicates of that reflection.

the *HKL*-2000 program suite (Otwinowski & Minor, 1997). The orthorhombic space group $P2_12_12_1$ was derived by auto-indexing, with unit-cell parameters $a = 71.3$, $b = 86.1$, $c = 118.2$ Å. A complete data set was obtained to 1.80 Å resolution, corresponding to an R_{merge} of 6.1%. Details of the data-collection statistics are summarized in Table 1.

3. Results and discussion

Assuming the presence of one dimer in the crystallographic asymmetric unit, the Matthews coefficient V_M (Matthews, 1968) and solvent content were calculated as $2.52 \text{ Å}^3 \text{ Da}^{-1}$ and 51.2%, respectively, based on a subunit of molecular weight 37 kDa. The *BALBES* program (Long *et al.*, 2008) was used for molecular-replacement calculation with *T. flavus* MDH (PDB entry 1bmd; Kelly *et al.*, 1993)

as a search model and *REFMAC5* (Murshudov *et al.*, 2011) was used for refinement. The R_{work} of the unrefined structure was found to be 33.8%. Examination of the molecular-replacement solution structure revealed good crystal packing and no clashes with the symmetry-related molecules. The initial σ_A -weighted electron-density map with $2F_o - F_c$ Fourier coefficients and molecular-replacement phases was of interpretable quality almost throughout the chain. This preliminary model is currently being refined. TtMDH cocrystallization or soaking with substrates is under way.

This work was supported by the National Science Council (NSC101-2113-M-002-017) and National Taiwan University (NTU-ERP-101R8600-1 and NTU-ICRP-102R7560-5) in Taiwan. We also thank the Technology Commons in College of Life Science and Center for Systems Biology, National Taiwan University for instrumental support of protein crystallization. Portions of this research were carried out at beamlines BL13B1 and BL13C1 of the National Synchrotron Radiation Research Center, Taiwan.

References

- Borek, D., Minor, W. & Otwinowski, Z. (2003). *Acta Cryst.* **D59**, 2031–2038.
- Frieden, C. & Fernandez-Sousa, J. (1975). *J. Biol. Chem.* **250**, 2106–2113.
- Gill, S. C. & von Hippel, P. H. (1989). *Anal. Biochem.* **182**, 319–326.
- Kelly, C. A., Nishiyama, M., Ohnishi, Y., Beppu, T. & Birktoft, J. J. (1993). *Biochemistry*, **32**, 3913–3922.
- Li, G., Liu, J., Yin, M., Guo, J. & Xie, S. (1996). *Ann. N. Y. Acad. Sci.* **799**, 476–481.
- Long, F., Vagin, A. A., Young, P. & Murshudov, G. N. (2008). *Acta Cryst.* **D64**, 125–132.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Minárik, P., Tomášková, N., Kollárová, M. & Antalík, M. (2002). *Gen. Physiol. Biophys.* **21**, 257–265.
- Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst.* **D67**, 355–367.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Rossmann, M. G. & van Beek, C. G. (1999). *Acta Cryst.* **D55**, 1631–1640.
- Sundaram, T. K., Wright, I. P. & Wilkinson, A. E. (1980). *Biochemistry*, **19**, 2017–2022.