Biochimica et Biophysica Acta 1794 (2009) 1496–1504

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Refolding, characterization and crystal structure of (*S*)-malate dehydrogenase from the hyperthermophilic archaeon *Aeropyrum pernix*

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ARTICLE INFO

Article history:
Received 27 January 2009
Received in revised form 15 June 2009
Accepted 16 June 2009
Available online 23 June 2009

Keywords:
Aeropyrum pernix
[LDH-like] MDH
Malate dehydrogenase
Hyperthermophilic archaea
Refolding
Crystal structure

ABSTRACT

Tartrate oxidation activity was found in the crude extract of an aerobic hyperthermophilic archaeon *Aeropyrum pernix*, and the enzyme was identified as (*S*)-malate dehydrogenase (MDH), which, when produced in *Escherichia coli*, was mainly obtained as an inactive inclusion body. The inclusion body was dissolved in 6 M guanidine–HCl and gradually refolded to the active enzyme through dilution of the denaturant. The purified recombinant enzyme consisted of four identical subunits with a molecular mass of about 110 kDa. NADP was preferred as a coenzyme over NAD for (*S*)-malate oxidation and, unlike MDHs from other sources, this enzyme readily catalyzed the oxidation of (2*S*,3*S*)-tartrate and (2*S*,3*R*)-tartrate. The tartrate oxidation activity was also observed in MDHs from the hyperthermophilic archaea *Methanocaldococcus jannaschii* and *Archaeoglobus fulgidus*, suggesting these hyperthermophilic MDHs loosely bind their substrates. The refolded *A. pernix* MDH was also crystallized, and the structure was determined at a resolution of 2.9 Å. Its overall structure was similar to those of the *M. jannaschii*, *Chloroflexus aurantiacus*, *Chlorobium vibrioforme* and *Cryptosporidium parvum* [lactate dehydrogenase-like] MDHs with root-mean-square-deviation values between 1.4 and 2.1 Å. Consistent with earlier reports, Ala at position 53 was responsible for coenzyme specificity, and the next residue, Arg, was important for NADP binding. Structural comparison revealed that the hyperthermostability of the *A. pernix* MDH is likely attributable to its smaller cavity volume and larger numbers of ion pairs and ion-pair networks, but the molecular strategy for thermostability may be specific for each enzyme.

1. Introduction

(*S*)-Malate dehydrogenases (EC 1.1.1.37, MDHs) catalyze the reversible conversion between (*S*)-malate, also known as L-malate, and oxaloacetate with strict substrate specificity in the presence of NAD or NADP. Because these enzymes play crucial roles in the citric acid cycle and malate/aspartate shuttle, the biochemical and structural properties of MDHs from eukaryal, bacterial and archaeal sources have been studied extensively [1–4]. MDHs and lactate dehydrogenases (LDHs) are members in a large family of dehydrogenases, which can be divided into three sub-families [5]: dimeric MDH, tetrameric LDH and [LDH-like] MDH, whose primary and quaternary structures are similar to those of LDHs. Several archaeal MDHs from *Sulfolobus acidocaldarius* [6], *Thermoplasma acidophilum* [7], *Haloarcula marismortui* [8], *Archaeoglobus fulgidus* (Af) [9,10], *Methanocaldococcus jannaschii* (Mj) [10,11] and *Pyrobaculum islandicum* [12] have been characterized. These enzymes all belong to the [LDH-like] MDH sub-family and are homotetramers with a subunit molecular mass of 33–37 kDa, except for *A. fulgidus* MDH, which is a dimer due to the absence of the sequence that mediates the dimer–dimer interaction [10]. Among these enzymes, the crystal structures of those from *H. marismortui*, *A. fulgidus* and *M. jannaschii* have already been determined [13–16].

Aeropyrum pernix is a strict aerobic hyperthermophilic archaeon isolated from a coastal thermal vent in Japan [17]. Its genome was originally sequenced in 1998 [18] and reannotated in 2006, yielding 1700 open reading frames in the sequence [19]. All enzymes comprising the citric acid cycle, including MDH, are annotated in the genome database, though functional and structural information on these enzymes remains limited. At the outset of this study, we found an enzyme having strong activity toward (2*S*,3*S*)-tartrate and (2*S*,3*R*)-tartrate with NAD(P) and identified it as an MDH. Almost all MDHs show a high degree of specificity for (*S*)-malate, but some also show oxidative activity toward other substrates. For instance, MDHs from

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doi:10.1016/j.bbapap.2009.06.014

bovine heart mitochondria, *Pseudomonas testosteroni*, *Moraxella lwoffi* and *Rhodobacter capsulatus* can oxidize (2*S*,3*R*)-tartrate, (2*S*,3*S*)-tartrate and/or (*S*)-2-hydroxyglutarate [20–23], though at lower rates. Because there have been no previous reports of an MDH having strong catalytic activity toward tartrate, we endeavored to express the gene in *Escherichia coli* and characterize the enzyme produced. In this paper, we describe the refolding of the *A. pernix* MDH (ApeMDH) produced as an inclusion body in *E. coli* and the biochemical properties of the refolded enzyme. In addition, we determined the crystal structure of this enzyme at a resolution of 2.9 Å, analyzed important residues for coenzyme and substrate binding, and compared the structure with those of other [LDH-like] MDHs to evaluate the structural features responsible for its high thermostability.

2. Materials and methods

2.1. Screening for dehydrogenase activity in A. pernix

A. pernix was cultivated aerobically for 48 h at 90 °C, as described previously [24]. The cells were then collected by centrifugation (10,000 ×*g*, 10 min), washed with 3% NaCl, suspended in 10 mM phosphate buffer (pH 7.2) and disrupted by sonication. After another centrifugation (20,000 ×*g*, 10 min), the supernatant was dialyzed against 10 mM phosphate buffer (pH 7.2) and used as the crude extract.

Screening for dehydrogenase activity was performed at 50 °C using a microplate reader system (Benchmark, BioRad). The reaction mixture (0.2 ml), which contained 50 mM glycine/NaOH buffer (pH 9.0), 10 mM substrate and the crude extract, was pre-incubated for 3 min at 50 °C, after which the reaction was started by addition of 1 mM NAD(P), and the increase of absorbance at 340 nm was monitored for 30 min.

2.2. Gene cloning and expression of MDH from A. pernix

We initially carried out PCR to amplify an ApeMDH gene fragment using the following primer pair: 5′-GGGACAG**CATATG**ATAACAATAC-3′, containing a unique NdeI restriction site overlapping the 5′ initial codon, and 5′-TGGTT**GGATCC**TTACTCTCTGAGCTG-3′, containing a unique BamHI restriction site proximal to the 3′-end of the termination codon. Chromosomal DNA from *A. pernix* was isolated as previously described [25] and used as the template. The amplified 0.9-kb fragment was confirmed from its sequence, digested with NdeI and BamHI and ligated into pET11a expression vector (Novagen) linearized with NdeI and BamHI, yielding pET/ApeMDH.

2.3. Protein expression, refolding and purification of ApeMDH

E. coli BL-21 CodonPlus (DE3)-RIL cells (Stratagene) were transformed with pET/ApeMDH, after which the transformants were cultivated for 8 h at 37 °C in LB medium supplemented with 50 μg/ml ampicillin. Expression was then induced by adding 1 mM isopropyl-β-D-thiogalacto-pyranoside to the medium, and cultivation was continued for an additional 3 h at 37 °C. The cells were then harvested by centrifugation (10,000 ×*g*, 10 min), washed with 0.85% NaCl, suspended in 10 mM Tris/HCl (pH 7.5) buffer and lysed by sonication. After sonication, the suspension was centrifuged at 20,000 ×*g* for 10 min at 4 °C, and the pellet was resuspended in 10 mM Tris/HCl (pH 7.5) buffer containing 1 mM EDTA and 4% Triton X-100, incubated at room temperature for 30 min and centrifuged again. This procedure was repeated twice. The resultant pellet was washed twice with Milli Q water and then solubilized in the 50 mM Tris/HCl (pH 7.5) buffer containing 1 mM EDTA, 0.2 M NaCl and 6 M guanidine–HCl. The solubilized ApeMDH (200 mg of enzyme in 200 ml solution) was added to refolding buffer (1.5 L of 0.1 M Tris/HCl (pH 7.5) containing 2 mM EDTA and 0.4 M L-arginine) and then incubated for 36 h at 4 °C. The resultant enzyme solution (1.7 L) containing the refolded ApeMDH was concentrated to a volume of 50 ml, dialyzed against 50 mM Tris/HCl (pH 7.5) buffer containing 0.2 M NaCl, and then subjected to gel filtration on a Superdex 200 column (2.6×60 cm, GE Healthcare) equilibrated with 50 mM Tris/HCl (pH7.5) buffer containing 0.2 M NaCl. The active fractions were pooled and used for biochemical and structural experiments. All solutions used for the refolding procedures were filtered through 0.45-μm membrane filter (Advantec) to remove dust and any other impurities.

2.4. Enzyme assay and determination of protein concentration

MDH activity was determined spectrophotometrically using a Shimadzu UV-1200 spectrophotometer equipped with a thermostat. All assays were performed at 50 °C. The standard reaction mixture (1 ml) consisted of 100 mM glycine/NaOH (pH 10.0), 50 mM (*S*)-malate, 1 mM NAD(P) and the enzyme. The appearance of NAD(P)H was monitored from the absorbance at 340 nm (extinction coefficient $\varepsilon = 6.22\ \mathrm{mM}^{-1}\cdot\mathrm{cm}^{-1}$). For the kinetic analyses, various concentrations of (*S*)-malate (0.05 to 2 mM with NAD and 0.02 to 0.5 mM with NADP), (2*S*,3*S*)-tartrate (0.5 to 10 mM with NAD and 10 to 200 mM with NADP) or (2*S*,3*R*)-tartrate (0.2 to 5 mM with NAD and 2 to 50 mM with NADP) were used in the presence of 4 mM NAD or 1 mM NADP. In addition, various concentrations of NAD (0.2 to 5 mM) and NADP (0.005 to 0.1 mM) were used in the presence of 2 and 0.5 mM (*S*)-malate, respectively. The turnover number (kcat) and apparent Km values were estimated from Lineweaver–Burk plots.

Protein concentrations were determined using the Bradford method with bovine serum albumin serving as the standard [26]. The concentration of ApeMDH protein during purification was determined by the absorption at 280 nm (1% absorption coefficient $A_{280} = 6.23\ \mathrm{cm}^{-1}$) calculated from the amino acid sequence of the protein [27].

2.5. Electrophoresis and determination of molecular mass

Native-PAGE was carried out on a 7.5% polyacrylamide gel using the method of Davis [28], after which the proteins were stained with Coomassie Brilliant Blue R-250. Active staining was performed at 50 °C using a mixture containing 300 mM Tris/HCl (pH 8.0), 50 mM (*S*)-malate, 0.1 mM *p*-iodonitrotetrazorium violet, 0.04 mM phenazine methosulfate and 0.25 mM NAD(P). SDS-PAGE was carried out on a 15% polyacrylamide gel using the method of Leammli [29] with protein markers from Bio Rad serving as the standards. The molecular mass of the native ApeMDH was determined by Superose 6 gel filtration chromatography using 10 mM phosphate buffer (pH 7.0) and 0.2 M NaCl as the elution buffer. Thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), albumin (67 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa) were used as the molecular mass standards (GE Healthcare).

2.6. Crystallization, X-ray data collection, structure determination, refinement and structural analysis

The crystallization conditions were screened using the sitting drop vapor diffusion method with Wizard 1, 2 (Emerald Biosystems) and Crystal Screen 1, 2 (Hampton Research). Drops (2 μl) of protein solution (8.9 mg/ml) were mixed with an equal volume of the reservoir solution and equilibrated against 0.15 ml of reservoir solution at 20 °C. The crystals (ca. 0.1×0.2×0.4 mm) were grown for 2 weeks in the reservoir solution, which was comprised of 0.1 M CHES (pH9.5) and 40% PEG 600.

The X-ray diffraction data were collected at room temperature on an R-AXIS VII detector system using an in-house rotating copper anode generator (Micromax 007, Rigaku) operating at 40 kV and 20 mA. The crystal belonged to the orthorhombic space group $P2_12_12_1$