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NOTE

Characterization of malate dehydrogenase from the hyperthermophilic archaeon *Pyrobaculum islandicum*

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Abstract Native and recombinant malate dehydrogenase (MDH) was characterized from the hyperthermophilic, facultatively autotrophic archaeon *Pyrobaculum islandicum*. The enzyme is a homotetramer with a subunit mass of 33 kDa. The activity kinetics of the native and recombinant proteins are the same. The apparent K_m values of the recombinant protein for oxaloacetate (OAA) and NADH (at 80°C and pH 8.0) were 15 and 86 μM, respectively, with specific activity as high as 470 U mg^{-1}. Activity decreased more than 90% when NADPH was used. The catalytic efficiency of OAA reduction by *P. islandicum* MDH using NADH was significantly higher than that reported for any other archaeal MDH. Unlike other archaeal MDHs, specific activity of the *P. islandicum* MDH back-reaction also decreased more than 90% when malate and NAD^+ were used as substrates and was not detected with $NADP^+$. A phylogenetic tree of 31 archaeal MDHs shows that they fall into 5 distinct groups separated largely along taxonomic lines suggesting minimal lateral *mdh* transfer between Archaea.

Keywords Malate dehydrogenase · *Pyrobaculum islandicum* · Archaea · Citric acid cycle · Autotrophy

Malate dehydrogenase (MDH) is commonly found in the archaea. Archaeal MDHs have been characterized from *Sulfolobus acidocaldarius* (Hartl et al. 1987), a moderately thermophilic member of the Crenarchaeota; from thermophilic and hyperthermophilic members of the Euryarchaeota (Graupner et al. 2000; Honka et al. 1990; Langelandsvik et al. 1997; Thompson et al. 1998); and from the extreme halophile *Haloarcula marismortui* (Bonnete et al. 1993; Hecht and Jaenicke 1989; Mevarech et al. 1977). Crystal structures have been resolved for the MDHs from *A. fulgidus*, *H. marismortui*, and *Methanocaldococcus jannaschii* (Irimia et al. 2003; Lee et al. 2001; Richard et al. 2000). These MDHs preferentially reduce oxaloacetate (OAA) using NAD(P)H as the electron donor and are nearly all homotetramers.

In this study, native and recombinant MDH was purified from heterotrophically grown *Pyrobaculum islandicum* and was the first hyperthermophilic MDH characterized from a member of the Crenarchaeota and from an archaeal autotroph that uses the reductive citric acid cycle. It shows several characteristics that differ from those found in other archaeal MDHs such as the lowest K_m measured for oxaloacetate, a strong preference for NADH as the reductant, and a significantly higher catalytic efficiency. These may be tied to its role in the reductive citric acid cycle. A phylogenetic comparison of the *P. islandicum* MDH with other archaeal MDHs suggest that this enzyme falls into five distinct groups among the archaea that follow taxonomic and environmental trends. It appears that there was little lateral transfer of the archaeal *mdh* gene within the archaea and among other organisms.

Pyrobaculum islandicum (DSM 4184) was grown heterotrophically at 95°C in a 20-l fermentor in a medium described previously (Hu and Holden 2006; Selig and Schönheit 1994) using 0.05% (wt vol^{-1}) casein hydrolysate (Difco, enzymatic) and 0.02% (wt vol^{-1}) yeast extract (Difco) as the carbon sources and 0.2% (wt vol^{-1}) $Na_2S_2O_3$ as the terminal electron acceptor. Cells from the fermentor

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Table 1 Purification of native *P. islandicum* malate dehydrogenase, from 2.5 to 3.0 g (wet weight) of cells

Step	Activity (U)	Protein (mg)	Sp Act (U mg^{-1})	Recovery (%)	Purification (fold)
Cytoplasmic extract	187	63	3.0	100	1
DEAE-Sepharose FF (pH 8)[a]	222	30	7.4	119	2.5
DEAE-Sepharose FF (pH 9)[b]	131	4	32.8	70	10.9
HiTrap Phenyl HP[c]	54	0.53	102	29	34
Superdex 200 10/300 GL[d]	26	0.083	310	14	103

Activities are expressed in units where 1 U is equivalent to 1 μmol of substrate transformed min^{-1}. Protein concentrations were determined spectrophotometrically using a protein determination kit (Bio-Rad) based on the Bradford assay (Bradford 1976)

[a] Sample suspended in 50 mM tris–HCl (pH 8.0); MDH collected in the pass-through fraction

[b] MDH eluted using a linear 0–0.5 M NaCl gradient over 10 column volumes (100 ml) in 50 mM glycine (pH 9.0)

[c] MDH eluted using a linear 1–0 M $(NH_4)_2SO_4$ gradient over 20 column volumes (20 ml) in 50 mM Trizma base (pH 8.0)

[d] MDH filtered in 50 mM potassium phosphate—0.15 M NaCl (pH 7.0)

were harvested when they reached late logarithmic growth phase (~10^8 cells ml^{-1}), concentrated, and frozen at –20°C as described previously (Feinberg and Holden 2006). Thawed cell suspensions were disrupted on ice by sonication, spun at 100,000×*g* for 45 min, and the supernatant was decanted and concentrated to less than 2 ml using a centrifugal filter device with a 5,000 Da molecular weight cutoff (Millipore, Amicon Ultra) and centrifugation at 5,000×*g*.

Native MDH was purified using an Äkta Fast Protein Liquid Chromatography (FPLC) system (Amersham Biosciences) and MDH activity was measured in fractions collected from each of the columns. MDH activity was determined at 80°C as described previously (Steen et al. 2001) using 50 mM EPPS (pH 8.4 at room temperature) unless otherwise stated. The columns and buffer solutions used for purification and an example of a typical protein purification run are shown in Table 1. The purified protein gave rise to one band with an apparent molecular mass of 35 kDa using SDS-PAGE analysis (Fig. 1). Gel filtration chromatography of the purified protein with molecular weight standards showed that the protein eluted with an apparent molecular mass of 150 kDa suggesting that the native holoenzyme is a homotetramer. Tryptic digestion (Promega) and peptide mass fingerprinting (Shevchenko et al. 1996) of the 35 kDa band resulted in eight well-defined peaks using MALDI-TOF mass spectrometry. The masses of seven of these fragments were within 1 Da of those predicted for the putative *P. islandicum* MDH sequence, and the eighth was within 3 Da when correlated with the *P. islandicum mdh* gene sequence using the web-based program PeptideMass (ca. http://www.expasy.org/tools/peptide-mass.html). The low yield of native MDH from a fermentor run prompted us to obtain recombinant MDH for our studies.

Cloning and expression of *P. islandicum mdh* (Pisl0612, accession number NC008701) were based on methods

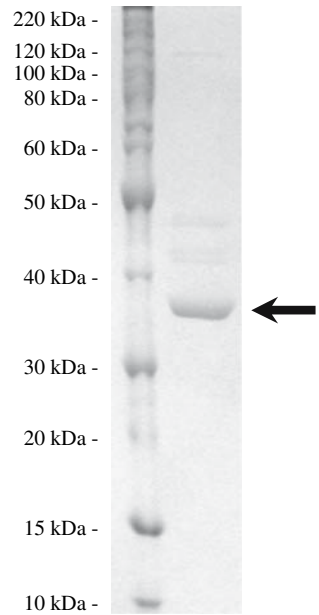

Fig. 1 SDS-PAGE of native *P. islandicum* malate dehydrogenase. The *right lane* contained purified malate dehydrogenase; the *left lane*, marker polypeptides with molecular masses indicated at the *left*. The *arrow* indicated the location of the purified protein

described previously (Weinberg et al. 2004). The forward PCR primer (5′-ACTTGGATCCATTACAATAATTGGAAGCGG-3′) omitted the start codon and encoded a *BamHI* restriction site. The reverse primer (5′-GACCACGTGCGGCCGCTTACTGGAGATAATTTGACG-3′) encoded a *NotI* site. The PCR product was cloned into a pET-24d expression vector with a His-6 tag at the N terminus (MAHHHHHHGS) and transformed into *E. coli* strain BL21-codon plus (DE3) RIL (Stratagene). The recombinant cells were grown at 37°C in 1 l of LB broth containing 50 μg each of chloramphenicol and kanamycin per ml, and 0.5 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG) was added when the optical density at 600 nm was

0.6. After 3 h, cells were harvested by centrifugation at 5,000×*g*. The cell pellet was resuspended in 15 ml of sample buffer (0.5 M NaCl and 50 mM NaH_2PO_4, pH 8.0) plus a few grains of lysozyme, sonicated, and spun at 5,000×*g*. The supernatant was applied to a column (5 ml column volume) of Ni-nitrilotriacetic acid His-binding affinity resin (Invitrogen) that had been equilibrated with sample buffer. After loading, the resin was rinsed with sample buffer plus 20 mM imidazole and recombinant MDH was eluted with the sample buffer plus 250 mM imidazole. Those fractions containing MDH activity were concentrated and passed through a Superdex 200 column as described in Table 1.

Each recombinant MDH purification yielded 5 mg of pure protein that was used to determine its catalytic properties. The recombinant MDH also had an apparent monomer molecular mass near 35 kDa using SDS-PAGE. Specific activity was highest at 80°C and pH 8.0 (470 U mg^{-1}) when OAA and NADH were used. The K_m for OAA and NADH using the purified recombinant protein were 15 and 86 μM, respectively. The K_m of the native protein for OAA was 10 μM; therefore, the specific activity and K_m for OAA of the recombinant protein were not significantly different from that of the native protein. Specific activities were 7 and 28 U mg^{-1} when OAA and NADPH or malate and NAD^+, respectively, were used as substrates. The K_m for malate and NAD^+ were 500 μM and 28 μM, respectively. There was no activity detected when malate and $NADP^+$ were used as substrates. Specific activity was measured at temperatures between 40 and 92°C and was highest (826 U mg^{-1}) at this latter temperature (the highest temperature that could be tested with our system). The optimum temperature and maximum activity are likely to be higher than these since the optimum growth temperature of the organism is 100°C. Activity at 80°C was measured between pH 5.5 and 11.0 with highest specific activity (495 U mg^{-1}) at pH 10.0. Lactate dehydrogenase activity was not detected even though this and other archaeal MDHs show high sequence similarity to this enzyme.

All of the archaeal MDHs that have been purified and characterized share many common traits, such as similar monomer and holoenzyme sizes and highly conserved active site residues. However, there are several characteristics found within these MDHs that set them apart from each other (Table 2). For *P. islandicum*, the specific activity and catalytic efficiency for the use of OAA is significantly higher than those reported for other archaeal MDHs. The *P. islandicum* MDH K_m for oxaloacetate is significantly lower than any other reported for an archaeal MDH. It also has a strong preference for NADH as the reductant while those of *S. acidocaldarius* and *M. thermoautotrophicus* have nearly equal affinities for NADH and NADPH. The MDHs from *P. islandicum*, *S. acidocaldarius*, *M. jannaschii*, and *H. marismortui* are homotetramers while that of *A. fulgidus* is a homodimer, which may be due to a shortened secondary loop structure necessary for two dimers to form a tetramer (Madern et al. 2001). Other differences reflect the native environments of the organisms (e.g., temperature, salt requirements).

Table 2 Properties of recombinant *P. islandicum* malate dehydrogenase relative to other purified archaeal malate dehydrogenases

Property	Properties of MDHs for			
	Pyrobaculum islandicum	*Sulfolobus acidocaldarius*	*Methanothermo bacterthermoautotrophicus*	*Haloarcula morismortui*
Molecular mass (kDa)	150	128.5	ND	115
Number of subunits	4	4	ND	4
Subunit size (Da)	33,319	33,563	35,915	32,808
Predicted pI	6.8	6.7	7.4	4.0
Maximum activity (U mg^{-1})	470	277	60	104
K_m for oxaloacetate (μM)	15	52	30	500
K_m for NADH (μM)	86	4.1	90	20
Catalytic efficiency for OAA (M^{-1} s^{-1})	17.4×10^6	3.0×10^6	1.2×10^6	0.1×10^6
Activity with NADPH (U mg^{-1})	7	360	52	ND
Activity with malate and NAD^+ (U mg^{-1})	28	194	1	ND
Activity with malate and $NADP^+$ (U mg^{-1})	0	205	1	ND
Optimal temperature (°C)	≥ 92	ND	ND	60
Optimal pH	10.0	ND	ND	6.0
Sequence identity/similarity (%)	100/100	30/51	32/57	28/49

Data for the enzymes from *S. acidocaldarius* (Hartl et al. 1987), *M. thermoautotrophicus* (Thompson et al. 1998), and *H. morismortui* (Bonnete et al. 1993; Hecht and Jaenicke 1989; Mevarech et al. 1977) were taken from the indicated references

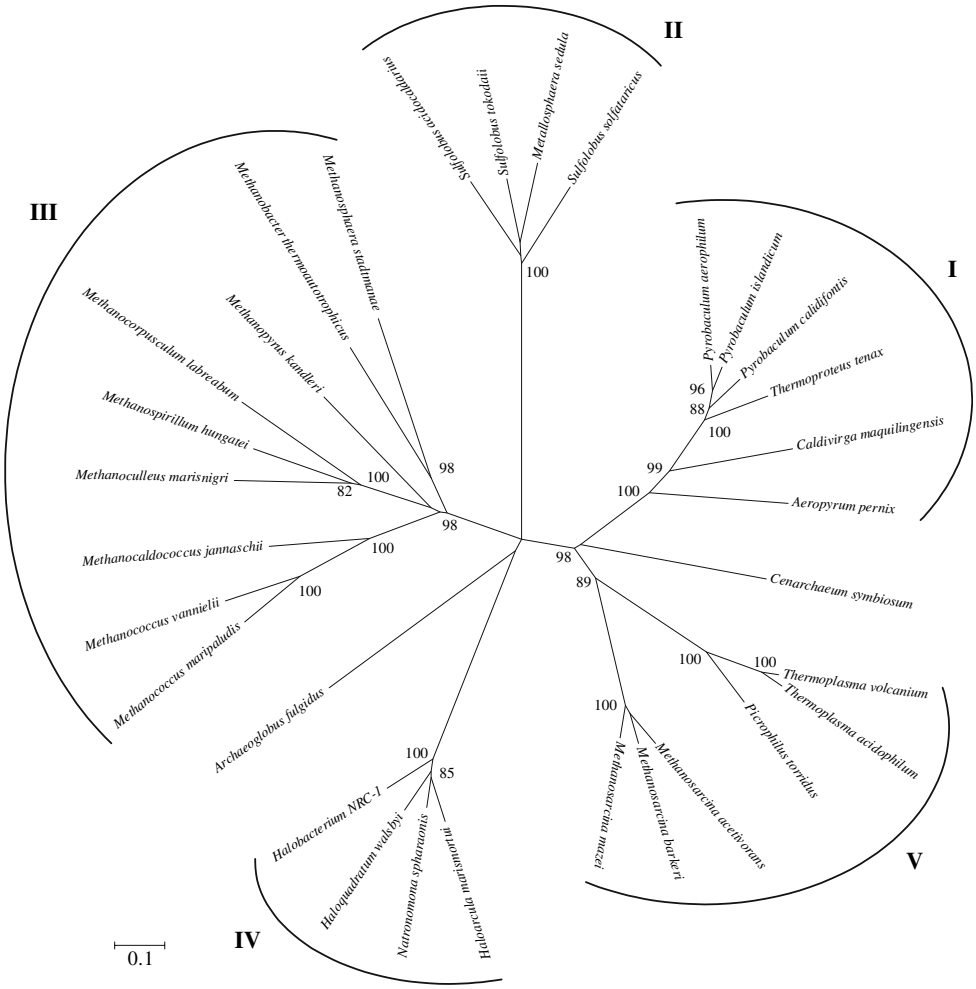

Fig. 2 Unrooted neighbor-joining tree of archaeal malate dehydrogenases. Bootstrap values (≥70%) are indicated at the *branch nodes*. *Lines* and *Roman numerals* denote the five groups. The *scale bar* represents 0.1 changes per amino acid. All sequences were obtained from the TIGR website (http://www.tigr.org) except for the *T. tenax* and *C. symbiosum* sequences, which came from accession numbers AJ621301 and DQ397579, respectively. Phylogenetic trees were constructed with the neighbor-joining method (Saitou and Nei 1987) using MEGA software version 3.0 (Kumar et al. 2004). Bootstrap values were calculated based on 1,000 replicates of the data (Felsenstein 1996)

All eight citric acid cycle enzyme activities were measured previously in *P. islandicum* cultures grown autotrophically (Hu and Holden 2006). Their presence and the absence of other CO_2 assimilation enzyme activities led to the suggestion that this organism assimilates CO_2 via the reductive citric acid cycle (Hügler et al. 2003), which is consistent with previous studies performed on *Thermoproteus neutrophilus*, a member of the same family (Schäfer et al. 1986, 1989; Strauss et al. 1992). The high catalytic efficiency ($k_{cat}\ K_m^{-1}$) of the *P. islandicum* MDH ($1.7 \times 10^7\ M^{-1}\ s^{-1}$) coupled with the very low K_m for OAA may be a reflection of this organism's need to rapidly convert low intracellular concentrations of oxaloacetate into malate during autotrophic growth. In *P. islandicum*, citrate lyase and ATP citrate lyase, which produce OAA during autotrophic growth, have specific activities of 25 and 12 nmol min^{-1} mg^{-1}, respectively, at 80°C in cell extracts (Hu and Holden 2006). High substrate affinity and catalytic efficiency for OAA by MDH would be necessary to maintain operation of the reductive citric acid cycle and prevent a bottleneck caused by low OAA production rates. Furthermore, the relatively high K_m for NADH suggests that a large pool of NADH, produced largely by the oxidation of H_2 on the membrane, must be present within the cell in order for the reactions of the reductive citric acid cycle and biosynthesis to occur.

The differences between the *P. islandicum* MDH and those from other archaea are its low K_m value, high catalytic efficiency value for OAA, and the strong preference for NADH and OAA over other substrates. These contrasts with those measured for MDHs from *S. acidocaldarius*, *M. thermoautotrophicus*, and *H. marismortui*, which use the citric acid cycle for substrate oxidation during heterotrophic growth and for the synthesis of biosynthetic precursors. Therefore, a low K_m for OAA may not be necessary in these organisms. Thermodynamically, OAA reduction is favored over malate oxidation ($\Delta G^{\circ\prime} = -7.1$ kcal mol^{-1}). However, the difference between the oxidative and reductive reaction rates in *P. islandicum* is larger than what is observed in other archaea and there is a strong preference for NADH over NADPH as the reductant in *P. islandicum*. The specific activity of MDH increased significantly in *P. islandicum* cultures grown heterotrophically relative to those grown autotrophically (Hu and Holden 2006). This

may reflect its need to produce higher cellular concentrations of MDH to compensate for the low oxidative activity of the enzyme when the cycle operates in the oxidative direction.

The genes for all eight of the citric acid cycle enzymes are present in genome sequences from representatives of the Crenarchaeota and the Euryarchaeota: namely five Thermoproteales, four Sulfolobales, three Thermoplasmales, and three Halobacteriales genomes and that of *Aeropyrum pernix*. The distribution of these genes across the archaea suggests that the last archaeal common ancestor contained the complete citric acid cycle, although it is uncertain whether this cycle operated in the reductive or oxidative direction. The cycle is incomplete in all methanogens, the Thermococcales (including *Nanoarchaeum equitans*), and *Staphylothermus marinus* and *Hyperthermus butylicus* (both in the Desulfurococcales). Since the divergence of the Crenarchaeota and the Euryarchaeota, portions of the citric acid cycle were lost which currently only serve to produce intermediates for some biosynthesis reactions in these organisms. The citric acid cycle enzyme most common to all archaea is malate dehydrogenase. Therefore, it may provide insight into the natural history of the citric acid cycle in the archaea.

A sequence alignment of all available archaeal MDHs shows that they fall into five distinct taxonomically similar groups (Fig. 2). Groups I and II consist only of Crenarchaeota while Groups III through V consist only of Euryarchaeota. Group I contains MDHs from the Thermoproteales and *A. pernix*; Group II, only the Sulfolobales; Group III, only methanogens (except the Methanosarcinales); Group IV, only the Halobacteriales; and Group V, which consists of two subgroups that contain the Methanosarcinales and the Thermoplasmales. Furthermore, archaeal MDHs align separately from nearly all bacterial and eukaryotic MDH sequences (Madern 2002). Therefore, there appears to have been very little lateral gene transfer of archaeal *mdh* within the archaea and among other organisms (with the possible exception of exchange between the Thermoplasmales and the Methanosarcinales). If, as proposed, the complete citric acid cycle was present in the last archaeal common ancestor, then this would suggest that MDH has evolved since the divergence of the archaea to fit the metabolic needs of the cell and remained relatively conserved. In conclusion, detailed characterization of citric acid cycle enzymes and related enzymes coupled with physiological studies of this pathway in organisms will continue to expand our knowledge of the history of this pathway in archaea.

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