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Purification, Characterization, and Overexpression of Psychrophilic and Thermolabile Malate Dehydrogenase of a Novel Antarctic Psychrotolerant, *Flavobacterium frigidimaris* KUC-1

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We purified the psychrophilic and thermolabile malate dehydrogenase to homogeneity from a novel psychrotolerant, *Flavobacterium frigidimaris* KUC-1, isolated from Antarctic seawater. The enzyme was a homotetramer with a molecular weight of about 123 k and that of the subunit was about 32 k. The enzyme required NAD(P)$^+$ as a coenzyme and catalyzed the oxidation of L-malate and the reduction of oxalacetate specifically. The reaction proceeded through an ordered bi–bi mechanism. The enzyme was highly susceptible to heat treatment, and the half-life time at 40 °C was estimated to be 3.0 min. The k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$) values for L-malate and NAD$^+$ at 30 °C were 289 and 2,790, respectively. The enzyme showed *pro-R* stereospecificity for hydrogen transfer at the C4 position of the nicotinamide moiety of the coenzyme. The enzyme contained 311 amino acid residues and much lower numbers of proline and arginine residues than other malate dehydrogenases.

Key words: malate dehydrogenase; *Flavobacterium*; psychrotolerant

Malate dehydrogenase (MDH, EC 1.1.1.37), requiring NAD (P)$^+$ as a coenzyme, catalyzes the reversible oxidation of malate to oxalacetate, and belongs to the NAD$^+$-dependent 2-ketoacid dehydrogenase family.[1] The biochemical and genetic properties of the MDHs from various organisms have been studied extensively, since MDH exists in most living organisms as an essential metabolic enzyme in the citric acid cycle.[2–6]

Most of Earth's environment is cold, since about three-quarters of its surface is covered by deep oceans, high mountains, and the Arctic and Antarctica, where the temperatures are permanently below 4 °C. Various psychrophilic microorganisms that have adapted not only to cold environments but also to other extreme environments, such as high osmotic pressure and high ion-strength environments, have been found in soils and waters.[7–9] These microorganisms generally produce various psychrophilic and thermolabile enzymes in order to grow effectively under cold conditions.[10–15] These psychrophilic enzymes have effective three-dimensional structures, show high catalytic activity under a cold environment, and usually lose their activities completely even at about 30 °C.[16] Psychrophilic enzymes show a character opposite to the thermostable enzymes, and much attention has been paid to their molecular structure, function, and detailed properties. To study NAD (P)$^+$-dependent psychrophilic enzymes, we isolated a psychrotolerant from Antarctic seawater. Taxonomic and 16S rDNA sequence analysis revealed that the organism belongs to a novel species of *Flavobacterium* and we named it *Flavobacterium frigidimaris* KUC-1.[17] This psychrotolerant produces an abundance of various NAD (P)$^+$-dependent dehydrogenases, such as alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH),[18] threonine dehydrogenase (ThrDH),[19] valine dehydrogenase (ValDH),[20] and glutamate dehydrogenase (GluDH). It is interesting that ThrDH, ValDH, and GluDH are psychrophilic and thermolabile, while ADH and ALDH are unexpectedly thermostable. Recently, we found NAD (P)$^+$-dependent MDH in the cell extract of *F. frigidimaris* KUC-1. The enzyme is structurally unique and the most psychrophilic and thermolabile enzyme of MDHs studied so far. In particular, the amino acid sequence is dissimilar to those of MDHs from both the hyperthermophile *Ther-*

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Abbreviations: MDH, malate dehydrogenase; ValDH, valine dehydrogenase; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; ThrDH, threonine dehydrogenase

Proteins and enzymes: alanine dehydrogenase (EC 1.4.1.1); alcohol dehydrogenase (EC 1.1.1.71); aldehyde dehydrogenase (EC 1.2.1.5); glutamate dehydrogenase (EC 1.4.1.2; 1.4.1.3; 1.4.1.4); malate dehydrogenase (EC 1.1.1.37); threonine 3-dehydrogenase (EC 1.1.1.103); valine dehydrogenase (EC 1.4.1.8)

mus aquaticus (identity, 21.1%) and the psychrophile *Aquaspirillum arcticum* (identity, 21.5%).

Here we describe the purification and characterization of *F. frigidimaris* KUC-1 MDH and its gene cloning, sequencing, and overexpression in *Escherichia coli*, with emphasis on comparison with other MDHs, especially with *A. arcticum* psychrophilic MDH.

Materials and Methods

Materials. DEAE-Toyopearl 650M, Phenyl Toyopearl 650M, and Butyl-Toyopearl were purchased from Tosoh (Tokyo), and Blue Sepharose CL-6B was purchased from Amersham Bioscience (Tokyo). A plasmid purification kit and gel extraction kit were purchased from Nippon Bio-Rad Laboratories (Tokyo), and LA PCR reaction reagents from Takara (Kyoto, Japan). Deuterated alcohol (Ethanol-D_6) were purchesed from Nacalai tesque (Kyoto, Japan). Malate and other chemicals were the best grade commercially available.

Organisms and growth conditions. We used a psychrotolerant, *Flavobacterium frigidimaris* KUC-1, which was isolated from Antarctic seawater and identified taxonomically.[17] This strain was grown aerobically at 15 °C in a medium containing 2% polypepton and 1% yeast extract (pH 7.0). A seed culture (200 ml) of the cells grown at 15 °C for 48 h (turbidity at 660 nm: about 10) was inoculated into 7.0 liters of a medium in a jar fermenter (10 liters, Marubishi, Tokyo) and cultured at 15 °C, 160 rpm for 48 h. The cells were harvested by centrifugation at 4 °C, washed twice with a chilled 10 mM potassium phosphate buffer (pH 7.0) containing 0.75% NaCl, and suspended in a 10 mM potassium phosphate buffer (pH 7.0) (0.5 g wet-weight cells/ml). *Escherichia coli* (NovaBlue) was obtained from Novagen (San Diego, CA) and grown aerobically at 37 °C in a Luria-Bertani medium supplemented with ampicillin (100 μg/ml).

Enzyme assays. MDH activity was determined spectrophotometrically with a Hitachi U-3210 spectrophotometer. The standard assay mixture (total volume, 3.0 ml) contained 10 mM L-malate, 1 mM NAD^+, and a 100 mM glycine–NaOH buffer (pH 10.0), and was preincubated at 30 °C. The reaction was started by the addition of an enzyme solution. The rate of NAD^+ reduction was measured spectrophotometrically at 340 nm. One unit of enzyme was defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH per min.

Purification of F. frigidimaris KUC-1 MDH. All procedures were done at 4 °C under aerobic conditions. After the cells were cultivated at 15 °C for 48 h, they were harvested by centrifugation (9,200 × *g*, 20 min) and washed twice with a 10 mM potassium phosphate buffer, pH 7.0, containing 0.01% 2-mercaptoethanol. The washed cells were suspended in a 10 mM potassium phosphate buffer, pH 7.0, containing 0.01% 2-mercaptoethanol (Buffer A), and disrupted at about 4 °C (5 min × 7 times, output 6) by ultrasonication (model UD-201, Tomy, Tokyo). The cell debris was removed by ultracentrifugation (27,600 × *g*, 30 min), and the supernatant was dialyzed against Buffer A and used as a crude enzyme. The crude enzyme was dialyzed against Buffer A and put on a column of DEAE-Toyopearl 650M (2.5 by 25 cm) equilibrated with Buffer A. After the column was washed with Buffer A (700 ml), the absorbed proteins were eluted with a 500-ml linear gradient of 10 to 150 mM potassium phosphate, pH 7.0, in Buffer A. Fractions containing MDH activity were combined and dialyzed against Buffer A containing 1.0 M $(NH_4)_2SO_4$ (Buffer B). This was loaded onto a column (2.5 by 15 cm) of phenyl-Toyopearl equilibrated with Buffer B. The column was eluted with Buffer B (500 ml), and the absorbed proteins were eluted with a 250-ml linear gradient of 1 to 0.5 M $(NH_4)_2SO_4$. Fractions containing MDH were combined and concentrated by ultrafiltration (Advantec Ultrafilter; PO200 membrane). The concentrated fractions were dialyzed against Buffer B and applied to a column (2.5 by 10 cm) of butyl-Toyopearl equilibrated with Buffer B. After the column was washed with Buffer B, the absorbed proteins were eluted with a 100-ml linear gradient of 1 to 0.4 M $(NH_4)_2SO_4$. Fractions containing the enzyme were combined and dialyzed against Buffer A. The enzyme solution was applied to a column of Blue Sepharose CL-6B (2.5 by 6 cm) equilibrated with Buffer A. After the column was washed with Buffer A (100 ml), the enzyme was eluted with Buffer A (100 ml) containing 1 mM NAD^+, 1 mM L-malate, and 40% ethylene glycol. The active fractions were pooled and dialyzed against Buffer A. The enzyme solution was concentrated by ultrafiltration and stored at −20 °C until use.

Steady-state kinetics. The initial-velocity experiments were carried out by varying the concentration of one substrate at different fixed concentrations of the other substrate.[21] The kinetic parameters were determined from the secondary plots of intercepts versus the reciprocal concentrations of the substrate.

Stereochemical analysis of hydrogen transfer at C4 of the nicotinamide ring of NADH. The stereospecificity of the enzyme for the hydrogen transfer of NADH was analyzed by ^1H NMR.[22] The reaction mixture contained *F. frigidimaris* KUC-1 ADH (*pro-R* stereospecificity, 0.5 U), deuterated alcohol (Ethanol-D_6, 5 μmol), NAD^+ (1 μmol), and a glycine–NaOH buffer, pH 9.0 (100 μmol), in H_2O (0.9 ml). It was incubated at 30 °C for 1.5 h, and then deuterated alcohol (5 μmol) was added to prevent substrate inhibition of ADH. After incubation at 30 °C for 1.5 h, ADH was removed with an ultrafilter unit (USY-1, Advantec, Tokyo), and oxalace-

tate (10 µmol) and *F. frigidimaris* KUC-1 MDH (1.0 U) were added to the filtrate and incubated at 30 °C for 3 h. After the solution was dried by centrifugal evaporation, the pellet was dissolved in ^2H_2O. The ^1H NMR spectra of the C4 position of the nicotinamide ring of NAD^+ produced were recorded on a Nihon Denshi datum JNM-EX 270 FT NMR spectrometer operating at 270 MHz with 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard.

Enzymatic cleavage and sequencing of N-terminal and internal peptides. The purified enzyme was digested with lysil-endopeptidase (Lys-C). The reaction mixture (total volume, 158 µl), which contained 16 µl of 1 M Tris–HCl (pH 9.0), 80 µl of 8 M urea, 42 µg of purified enzyme, and 2 µl of Lys-C, was incubated at 37 °C for 18 h. The clear solution obtained was acidified with 1% trichloroacetic acid to stop digestion, and dried by centrifugal evaporation. The Lys-C-digested peptides (2.0 nmol) were separated on a Wakosil $5C_{18}$-AR column (4.6 by 250 mm) in a Shimadzu LC 10A system (Shimadzu, Kyoto, Japan). A 55-min linear gradient from 0 to 60% (V/V) acetonitrile in 0.1% (V/V) trifluoroacetic acid was used to elute peptides at a flow rate of 0.7 ml/min. Peptides were monitored at 215 nm. Amino acid sequences of *N*-terminal and internal peptides were determined by automated Edman degradation with a protein sequencer model 477A (PE Applied Biosystems, Tokyo). Approximately 200 pmol of protein was used to determine an *N*-terminal peptide sequence.

Cloning and sequence analysis of the enzyme. On the basis of the *N*-terminal and internal peptide sequences, oligonucleotides mdh1 (5′-ATGAARGTIACIATHG-TIGGIGC-3′) and mdh2 (5′-ACCATIKCRTCICCRT-GICCICC-3′) were designed, and the MDH gene was amplified from *F. frigidimaris* KUC-1 genomic DNA (70 ng) with the oligonucleotides (100 pmol) by PCR. The thermal profiles for second PCR involved 30 cycles of denaturation at 94 °C for 30 sec, annealing at 45 °C for 1 min 30 sec, and extension at 74 °C for 1 min. PCR amplification was carried out with LA Taq polymerase (Takara, Kyoto, Japan) in a Gene Amp PCR system 9700 (PE Applied Biosystems). The resulting 270-bp fragment was sequenced with a DNA sequencing system, SQ5500 (Hitachi, Tokyo). The genome-walking PCR method was used to obtain upstream and downstream sequences from the 270-bp insert. Two primers, 5′-GTCGCACATTG CATAATATCC-3′ and 5′-ATAG-TTGTAGTTTCAAATCCAATGG-3′, were designed, and a genome-walking PCR was performed with a Takara LA PCR™ *in vitro* cloning kit (Takara, Tokyo). The *Flavobacterium* chromosomal DNA extracted was digested with *Hind* III and ligated to the *Hind* III cassette. The DNA fragments obtained were used as a template for PCR. The thermal profiles involved 30 cycles of denaturation at 62 °C for 30 sec, annealing at 63 °C for 30 sec, and extension at 74 °C for 1 min. The resulting fragments, 500-bp for upstream and 500-bp for downstream, were sequenced as described above. The start codon of the *mdh* gene was involved in the 500-bp fragment, but the termination codon did not exist in 500-bp. A primer, 5′-GAAGGA GAATACGGGCAAA-3′, was synthesized to obtain the sequences of the farther downstream region. The *Flavobacterium* chromosomal DNA extracted was digested with *EcoR* I and ligated to the *EcoR* I cassette. The DNA fragments obtained were used as a template for PCR. The thermal profiles involved 25 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, and extension at 74 °C for 1 min. The resulting approximately 1,000-bp fragment was sequenced to determine the full length of the *mdh* gene sequence.

Expression of the enzyme gene in E. coli. E. coli BL21 (DE3) cells harboring a recombinant plasmid carrying the *mdh* gene were selected and grown in 5 ml of an LB medium at 30 °C for 10 h. The culture (0.5 ml) was transferred into 1l of an LB medium containing ampicillin and incubated at 15 °C for 24 h. The cells were allowed to grow for 3 h with vigorous shaking, collected by centrifugation ($5{,}000 \times g$, 5 min), and suspended in 10 ml of a 10 mM potassium phosphate buffer, pH 7.0.

Other methods. Protein concentrations were measured by the method of Bradford based on the calibration curve with a bovine serum albumin (Wako Chemical., Osaka, Japan, product No. 011-07493).[23] The molecular weight was estimated by gel filtration with a column (1.6 by 60 cm) of Superdex 200 Hiload (16/60) (Amersham Biosciences, Tokyo) with ferritin (440 k), catalase (232 k), aldolase (158 k), and albumin (67 k) as standard proteins. Polyacrylamide gel electrophoresis and sodium dodesyl sulfate polyacrylamide gel electrophoresis were carried out by the methods of Davis[24] and Laemmli[25] respectively. Gels were stained with Coomassie Blue R-250 or incubated at 37 °C in an activity-staining solution composed of 10 mM L-malate, 1 mM NAD^+, 0.1 mM phenazine methosulfate, and 0.12 mM *p*-nitroblue tetrazolium in a 100 mM glycine–NaOH buffer (pH 10.0).

Nucleotide sequence accession number. The DNA sequence of the gene encoding *F. frigidimaris* KUC-1 MDH is available from GenBank under accession no. AB16143.

Results

Purification of MDH

The enzyme was purified about 425-fold with a yield of 23% (Table 1). The purified enzyme was found to be homogeneous on polyacrylamide gel electrophoresis (PAGE) and sodium dodesyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The specific activity for forward reaction was 176 U/mg (at 30 °C).

Molecular weight and subunit structure

The purified enzyme migrated as a single band in SDS–PAGE with an apparent molecular weight of 32 k. The molecular weight of the native enzyme on gel filtration with Superdex-200 was 123 k, suggesting that it is a homotetramer.

Table 1. Purification of Malate Dehydrogenase from *Flavobacterium frigidimaris* KUC-1

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (Fold)
Crude Extract	2,510	1,690	0.673	100	1.00
DEAE-Toyopearl	606	1,570	2.59	92.8	3.84
Phenyl-Toyopearl	58.7	760	12.9	45.0	19.2
Butyl-Toyopearl	6.64	556	83.8	32.9	124
Blue-Sepharose	1.84	324	176	19.2	262

N-Terminal and internal amino acid sequence

Sixteen amino acid residues of the *N*-terminal position of the enzyme were determined to be [1]MKVTIVGAG-NVGATTAF-. The Lys-C-digests of the enzyme were separated by reversed phase high-performance liquid chromatography, and two internal peptide sequences were determined: K1, KVSGTNNYSK and K2, KNRII-GVGGALDSSR. These sequences were used for identification of the amplified DNA fragment.

Effect of temperature

The enzyme was quite thermolabile and psychrophilic. Its thermal stability was examined at 10, 15, 20, 25, 30, 35, 40, and 45 °C (Fig. 1). The half-life times at 35, 40, and 45 °C were estimated to be 26, 2.9, and 0.6 min respectively. The enzyme was active at temperatures from 5 to 60 °C (Fig. 2A), with highest initial velocity at 40 °C, similarly to other enzymes from a psychrotolerant.[26] An Arrhenius plot showed a break point at 21 °C in the slope of $\log v$ against $1/T \times 10^{-3}$, and the activation energies for the oxidation of malate changed from 40.8 (lower temperature region) to 23.7 kJ/mol (higher temperature region) at that point (Fig. 2B).

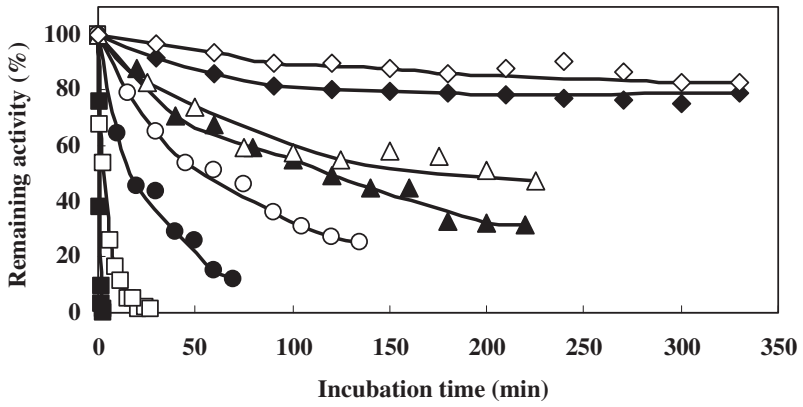

Fig. 1. Thermal Stability of the Enzyme.

The enzyme solution was incubated in 10 mM phosphate buffer, pH 7.0, at various temperatures, and the remaining activity was determined under the standard assay conditions: ■ 10 °C, □ 15 °C, ● 20 °C, ○ 25 °C, ▲ 30 °C, △ 35 °C, ◆ 40 °C, ◇ 45 °C.

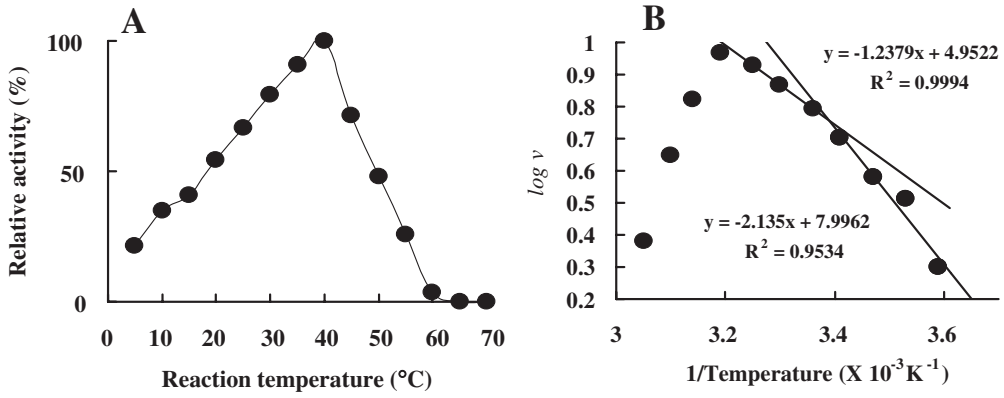

Fig. 2. Effect of Temperature on the Enzyme Activity.

A, Enzyme activity was measured every 5 °C at various temperatures ranging from 5 to 70 °C with the standard assay mixture. B, Arrhenius plot.

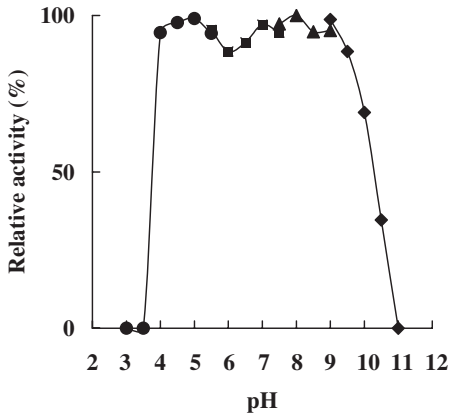

Fig. 3. pH Stability of the Enzyme.

The enzyme solution was incubated in various buffers (final conc., 50 mM) at 30 °C, and the remaining activity was determined under the standard assay conditions: ●, citrate–sodium buffer (pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5); ■, acetate buffer (4.0, 4.5, 5.0, 5.5); ▲, phosphate buffer (pH 5.5, 6.0, 6.5, 7.0, 7.5); ◆, tris–HCl buffer (pH 7.5, 8.0, 8.5, 9.0, 9.5), and glycine–NaOH buffer (pH 9.0, 9.5, 10.0, 10.5, 11.0).

Effect of pH

Activity was determined at various pHs. The buffers (final conc., 0.1 M) used were as follows: citrate–sodium buffer (pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5), acetate buffer (pH 4.0, 4.5, 5.0, 5.5), phosphate buffer (pH 5.5, 6.0, 6.5, 7.0, 7.5), tris–HCl buffer (pH 7.5, 8.0, 8.5, 9.0, 9.5), and glycine–NaOH buffer (pH 9.0, 9.5, 10.0, 10.5, 11.0, 11.5, 12.0). The enzyme showed activity in pH range of 6.5–12 for oxidation of malate, and of 4.0–10.0 for reduction of oxalacetate. The optimum pH for oxidation of malate (pH 10.5) was higher than that for the reduction of oxalacetate (pH 8.0). The enzyme showed high activity in alkaline conditions and was stable in broad pH range between 4 and 10.5 under the conditions tested (Fig. 3).

Substrate and coenzyme specificities

Substrate specificity was studied in the presence of 10 mM various substrates for oxidation, and 2-oxo acids for reduction. The enzyme was specific for the oxidation of D-malate, and only oxalacetate was reduced by the reverse reaction. The following substrates were inert: for the oxidative reaction, D-malate, malonate, L-glutamate, L-aspartate, D,L-2-hydroxybutyrate, D,L-3hydroxybutyrate, citrate, maleiate, succinate, L-tartrate, L-threonine, L-serine, L-hydroxymalonate, and D-glutamate; and for the reductive reaction, 2-oxocaproate, 2-oxoisocaproate, 2-oxovalerate, 2-oxoisovalerate, glyoxylate, 2-oxoglutarate, and 2-oxobutyrate. The enzyme required NAD^+ and $NADP^+$ as coenzymes for the oxidation reaction, and the relative activity for NAD^+ to that for $NADP^+$ was about 43.9%. $NADPH^+$ was inert for the reverse reaction.

Table 2. Effect of Inhibitors

Inhibitor (1 mM)	Relative activity (%)
None	100
NaCl	93.0
KCl	98.9
$BaCl_2$	75.1
$CoCl_2$	61.0
$CuCl_2$	10.2
$ZnCl_2$	27.4
$NiCl_2$	63.2
$CaCl_2$	70.9
$MgCl_2$	65.1
$MnCl_2$	71.4
$HgCl_2$	0
EDTA	47.8
EGTA	79.0
Phenanthroline	98.4
Semicarbazide	38.7
Hydroxylamine	42.9
Iodoacetate	16.4
N-Ethylmaleimide	0

Table 3. Kinetic Parameters

Substrate	Temperature (°C)	K_m (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
L-Malate	10	0.543	135	41.3	76
	20	0.269	160	48.4	180
	30	0.288	274	83.2	289
	40	0.740	456	138.0	187
NAD^+	10	0.0279	135	41.0	1,470
	20	0.0286	160	48.6	1,700
	30	0.0299	274	83.4	2,790
	40	0.0382	456	139.0	3,630

Effects of inhibitors

We examined the effects of various compounds on enzyme activity (Table 2). The enzyme was completely inhibited by *N*-ethylmaleimide and $HgCl_2$. Iodoacetic acid and $CuCl_2$ also strongly inhibited it. These results suggest that the thiol groups are directly or indirectly involved in the enzyme catalysis, as reported for other MDHs.[27]

Steady-state kinetics

The double-reciprocal plots of the initial velocity against the concentrations of malate and NAD^+ in the presence of various fixed concentrations of NAD^+ and malate respectively gave sets of straight intersecting lines. The results show that the reaction proceeds *via* the formation of a ternary complex of the enzyme with malate and NAD^+. The K_m and V_{max} values for L-valine and NAD^+ at various temperatures were calculated from the secondary plots of intercepts *versus* the reciprocal concentrations of the other substrate (Table 3). The product inhibition studies indicated that NAD^+ binds first to the enzyme and then oxalacetate is consequently released randomly *via* an ordered bi–bi mechanism.

Stereospecificity for hydrogen transfer of the coenzyme

NAD^+-dependent dehydrogenases show either pro-*S* or pro-*R* stereospecificity for hydrogen removal from the C4 position of the nicotinamide moiety of the reduced coenzyme. The stereospecificity for hydrogen transfer of NADH catalyzed by the enzyme was examined by the *in situ* method mentioned above, in which ADH from *F. frigidimaris* KUC-1 (*pro-R* stereospecificity) and deuterated NADH were used in H_2O. After the reaction, the resonance doublet around δ 8.8 ppm for hydrogen at the C4 position of NAD^+ appeared in the ^1H NMR spectrum (Fig. 4). This shows that the 4R-^1H of NADH is transferred to oxalacetate. Thus the enzyme is *pro-R* stereospecific.

Cloning and sequence analysis of the enzyme gene

The entire sequence of the *mdh* gene was determined for both strands. An open reading frame of 993 bp was identified, corresponding to 311 amino acid residues with a molecular weight of 32 k. The coding region of the *mdh* gene was not preceded by the sequence of a putative bacterial Shine-Dalgano ribosome-binding site, usually located upstream of the starting codon, ATG. The pyrimidine-rich region, ATTTT, was found immediately downstream of the stop codon TAA. The G + C content of *mdh* was 38.0%. The deduced amino acid sequence was used to search for identical sequences in the GenBank and protein databases with the BLAST program. Sequence identities were found with those of *Bacillus halodurans* MDH (identity, 47.3%), *Chlorobium tepidum* (thermophile) MDH (45.9%), *Thermoplasma volcanism* (thermophile) MDH (43.1%), *Staphylococcus epidermidis* LDH (42.1%), and *Aquaspirillum arcticum* MDH (21.5%). The alignment of the primary sequences of *F. frigidimaris*, *A. arcticum*, and *T. aquaticus* MDHs is summarized in Fig. 5. Important residues are fully conserved in the enzymes. These key residues include His^{195}, Asp^{168}, and Arg^{109}, and are essential for the catalytic activity of MDH. In addition, His^{195}, Asp^{168}, and Arg^{109} relate to the proton relay system, and Arg^{109} stabilizes the polarized carbonyl bond of the substrate during the transition state. The glycine-rich motif (GXGXXG) and Arg^{42} in the N-terminal region were involved in NAD^+-binding.

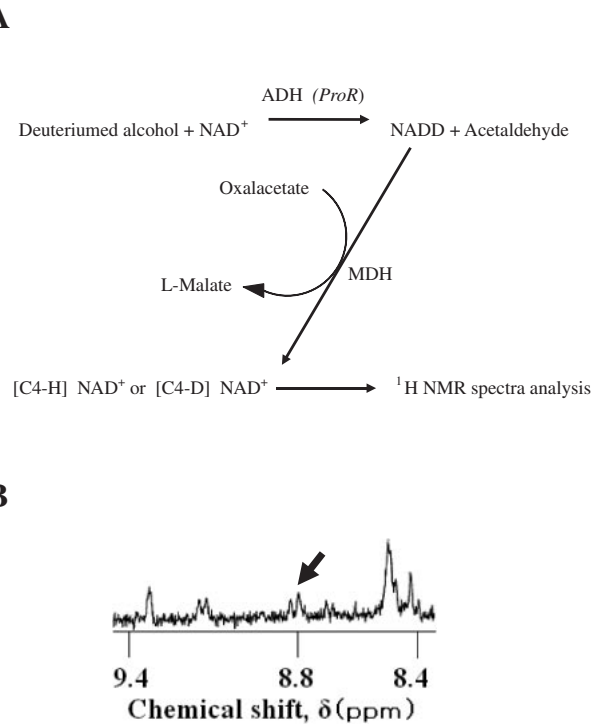

Fig. 4. Stereospecificity for Hydrogen Transfer of Coenzyme.

A, The reaction mechanism for determination of stereospecificity for hydrogen transfer of NAD^+. B, The aromatic region of the ^1H NMR spectra of NAD^+. If the stereospecificity of hydrogen transfer is *pro-R*, the C-4 hydrogen of NAD^+ fully retains, a doublet for it appears around δ 8.8 ppm, and is shown by an arrow.

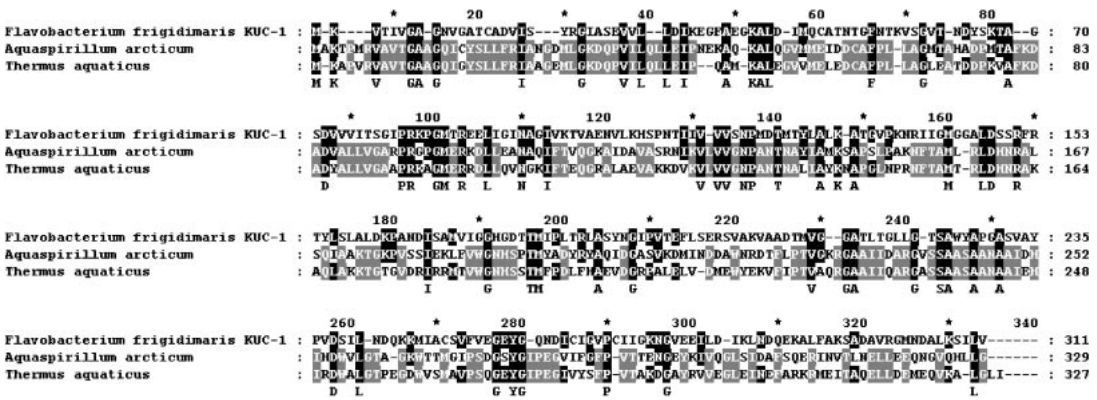

Fig. 5. Comparison of the Primary Structure of the Malate Dehydrogenases from *Flavobacterium frigidimaris* with Those from *Aquaspirillum arcticum* and *Thermus aquaticus*.

The residues conserved in all three sequences are shadowed in black, while the residues conserved in both *Aquaspirillum arcticum* MDH and *Thermus aquaticus* MDH are shadowed in gray.

Comparison of the amino acid composition of F. frigidimaris KUC-1 MDH with that of the psychrophilic MDH of A. arcticum

The amino acid composition of *F. frigidimaris* KUC-1 MDH was compared with that of the MDH from *A. arcticum*, a psychrophile. *F. frigidimaris* KUC-1 MDH showed a characteristic amino acid composition. The *F. frigidimaris* enzyme contained fewer Pro (11 residues, 3.53%) and Arg (9 residues, 2.88%) residues than the enzymes from *A. arcticum* (Pro, 16 residues, 4.86%; Arg, 13 residues, 3.95%). The Arg/Arg + Lys ratio of the *F. frigidimaris* KUC-1 enzyme (0.321) was much lower than that of the psychrophilic enzyme from *A. arcticum* (0.433).

Expression of the mdh gene in E. coli

A 933-bp *Nde* I-*Bam* HI fragment containing the *F. frigidimaris* KUC-1 enzyme gene was ligated to pET 17b, and the pMDH obtained was used for production of the enzyme in *E. coli* BL 21 (DE3) cells under the control of the *T7* promoter. The *F. frigidimaris* KUC-1 enzyme was detected in the soluble fractions of the cell extract.

Discussion

We found that a psychrotolerant, *F. frigidimaris* KUC-1, isolated from Antarctic seawater, abundantly produces NAD (P)$^+$-dependent MDH, and we purified the enzyme to homogeneity, for the first time from a psychrotolerant. The enzyme occupies about 0.3% of the total soluble protein produced under the conditions tested. The enzyme is a constitutive enzyme and is not induced by the addition of L-malate. Therefore, the optimum temperature for enzyme production agreed well with that of the optimum growth temperature of the parent cell (15 °C). *Flavobacterium* MDH was highly susceptible to heat treatment. When the enzyme was incubated with a 10 mM potassium phosphate buffer (pH 7.0) at 40 °C for 2.9 min, more than 50% of the initial activity was lost. An Arrhenius plot showed a break point at 21 °C in the slope of $\log v$ against $1/T \times 10^{-3}$, which is characteristic of *Flavobacterium* MDH. The structure of the enzyme probably changes at about this temperature. The kinetic parameters changed depending on the reaction temperatures (Table 3). The lowest K_m for L-malate was observed at 20 °C, near the optimum growth temperature of *F. frigidimaris* KUC-1. Although the primary structure of *Flavobacterium* MDH is highly similar to that of a mild thermophile, *Chlorobium tepidum* MDH (identity, 45.9%), the thermal stability of these enzymes is quite different:[28] after incubation at 55 °C for 30 min, the *Chlorobium* enzyme showed more than 90% of initial activity, whereas the *Flavobacterium* enzyme lost activity completely.

In contrast, the primary structure of *Flavobacterium* MDH is totally different from that of a psychrophile, *A. arcticum* MDH (identity, 21.5%), previously reported (Fig. 5).[28] The low Arg/Lys ratio and the low content of Pro and hydrophobic amino acid residues observed for *Flavobacterium* MDH probably lead to fewer intramolecular salt bridges and hydrophobic interactions of the enzyme than those of the *A. arcticum* MDH, and result in conformational flexibility. *Flavobacterium* MDH has a homotetramer structure. This is quite different from the subunit structure of *A. arcticum* MDH, which has a homodimer. The difference in these enzymes in subunit structure also reflects the difference in the thermolability and psychrophilicity of *Flavobacterium* MDH. To our knowledge, *Flavobacterium* MDH is the most psychrophilic, thermolabile, and cold-active enzyme of MDHs reported so far. *Flavobacterium* MDH showed broad pH stability between 4 and 10.5. This is highly characteristic of the enzyme, and probably derived from its character.

The Gly residue-rich motif of MDH is directly involved in coenzyme binding and is important for the classification of the enzyme. The primary structure of MDH purified from cytoplasm and mitochondria contains a GXXGXG motif, while the GXGXXG motif is found in MDH, which is structurally similar to LDH (LDH-like MDH). The primary structure of *Flavobacterium* MDH resembles that of *Staphylococcus epidermidis* LDH (identity, 42.1%), and is classified into LDH-like MDH. His^{195}, Asp^{168}, and Arg^{109} are essential for the catalytic activity of both MDH and LDH. His^{195}, Asp^{168}, and Arg^{109} are related to the proton relay system, and Arg^{109} stabilizes the polarized carbonyl bond of the substrate during the transition state.[29–32]

Similar to other MDHs, *Flavobacterium* MDH shows high substrate specificity: *Pseudomonas testosterone* MDH shows no activity on D-malate or L-aspartate, and D-malate and D,L-2-hydroxybutyrate do not serve as a substrate for *Rhodobacter capsulate* MDH.[33,34] This suggests that *Flavobacterium* MDH is applicable in the detection and production of malate under cold conditions.

We are currently trying to determine why *Flavobacterium* enzyme is psychrophilic and thermolabile by means of x-ray crystallographic analysis.

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