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A cold-active and thermostable alcohol dehydrogenase of a psychrotorelant from Antarctic seawater, *Flavobacterium frigidimaris* KUC-1

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Abstract An NAD^+-dependent alcohol dehydrogenase of a psychrotorelant from Antarctic seawater, *Flavobacterium frigidimaris* KUC-1 was purified to homogeneity with an overall yield of about 20% and characterized enzymologically. The enzyme has an apparent molecular weight of 160k and consists of four identical subunits with a molecular weight of 40k. The p*I* value of the enzyme and its optimum pH for the oxidation reaction were determined to be 6.7 and 7.0, respectively. The enzyme contains 2 gram-atoms Zn per subunit. The enzyme exclusively requires NAD^+ as a coenzyme and shows the *pro-R* stereospecificity for hydrogen transfer at the C4 position of the nicotinamide moiety of NAD^+. *F. frigidimaris* KUC-1 alcohol dehydrogenase shows as high thermal stability as the enzymes from thermophilic microorganisms. The enzyme is active at 0 to over 85°C and the most active at 70°C. The half-life time and k_{cat} value at 60°C were calculated to be 50 min and 27,400 min^{-1}, respectively. The enzyme also shows high catalytic efficiency at low temperatures (0–20°C) (k_{cat}/K_m at 10°C; 12,600 mM^{-1} min^{-1}) similar to other cold-active enzymes from psychrophiles. The alcohol dehydrogenase gene is composed of 1,035 bp and codes 344 amino acid residues with an estimated molecular weight of 36,823. The sequence identities were found with the amino acid sequences of alcohol dehydrogenases from *Moraxella* sp. TAE123 (67%), *Pseudomonas aeruginosa* (65%) and *Geobacillus stearothermophilus* LLD-R (56%). This is the first example of a cold-active and thermostable alcohol dehydrogenase.

Keywords Alcohol dehydrogenase · Psychrotorelant · Thermostable · Cold-active · *Flavobacterium*

Abbreviations

AlcDH	Alcohol dehydrogenase
CHES	*N*-cyclohexyl-2-aminoethanesulfonic acid
conc.	Concentration
KPB	Potassium phosphate buffer
LMW	Low molecular weight
PVDF	Poly (vinylidene fluoride)
Tris	Tris (hydroxymethyl) aminomethane
UPGMA	Unweighted pair-group method with arithmetic mean

Introduction

Approximately 80% of the biosphere is under a cold environment in which the temperature is below 5°C (Russell 1998). Various cold-adapted microorganisms, which are classified as psychrophilic and psychrotorelant ones, grow widely both in natural and artificial environments (Wiedmann et al. 2000; Yumoto et al. 1999). Psychrophilic and psychrotorelant microorganisms produce various cold-active enzymes to carry out their metabolism efficiently under cold conditions (Russell 1998; Feller et al. 2003). Enzymologists and biotechnologists are very interested in the high catalytic efficiency of cold-active enzymes at low temperatures (Feller et al. 2003; Fujiwara 2002; Coker et al. 2003), and have studied them in detail from the viewpoint of structural biology and enzyme application (Russell 1998). The cold-active enzymes so far studied are thermolabile (Feller et al. 2003), whereas thermostable enzymes produced by thermophilic microorganisms show only slight or no

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activity under cold conditions (Fujiwara 2002; Innis et al. 1988; Vieille et al. 2001).

Alcohol dehydrogenase (AlcDH, EC 1.1.1.1; EC 1.1.1.2; EC 1.1.1.71) occurs in various organisms including extremophiles (Jornvall et al. 1987; Radianingtyas et al. 2003). The thermophilic and thermostable AlcDHs are produced by *Geobacillus stearothermophilus* LLD-R (Guagliardi et al. 1996; Liang et al. 2004a, b) and *Aeropyrum pernix* K1 (Hirakawa et al. 2004), and a cold-active and thermolabile AlcDH is by *Moraxella* sp. TAE 123 (Tsigos et al. 1998; Liang et al. 2004a, b). Recently, we observed the occurrence of a cold-active and thermostable NAD^+-dependent AlcDH in the cell extracts of a psychrotorelant, *Flavobacterium frigidimaris* KUC-1, isolated from Antarctic seawater (Nogi et al. 2005). The enzyme shows different thermal characteristics from AlcDHs previously reported: high catalytic efficiency at low temperatures and high stability even at high temperatures. We here describe the purification, properties, and primary structure of NAD^+-dependent AlcDH from *F. frigidimaris* KUC-1, which is exceptionally cold-active and thermostable, to compare its properties with those of other AlcDHs.

Materials and methods

Materials

Alcohols, aldehydes, ketones, and their substituted derivatives, NAD^+, NADH, $NADP^+$, and NADPH, were purchased from Wako Chemical (Osaka, Japan). DEAE-Toyopearl 650 M, Phenyl-Toyopearl 650 M, and Toyopearl HW-50 were products of Tosoh, Tokyo, and 5′-AMP Sepharose 4B was purchased from Amersham Biosciences, NJ, USA. The plasmid purification kit and gel extraction kit were purchased from Bio-Rad Laboratories, CA, USA, and LA-PCR reaction reagents were the products of Takara Bio, Kyoto, Japan. *Bacillus sphaericus* alanine dehydrogenase was purified according to the method previously described (Ohshima et al. 1979).

Bacterial strain and culture conditions

We isolated an aerobic psychrotorelant from Antarctic seawater and classified it as *Cytophaga* sp. (Oikawa et al. 2001), but, recently, re-identified and termed it *Flavobacterium frigidimaris* KUC-1 (Nogi et al. 2005). This organism grows well between 2 and 26°C, and the maximum growth rate was observed at 15°C. The cells were cultivated in a medium containing 20 g polypepton (Wako Chemical. Cat No. 390-00117) and 10 g yeast extract per litter of tap water at 15°C. The pH was adjusted to 7.0 by addition of 10 N NaOH just before sterilization with an autoclave. The seed culture (200 ml) collected in the stationary phase (turbidity at 660 nm: about 10) was inoculated into the medium (7.0 l) and cultivated in a Jar fermenter MDL 1000 (Marubishi, Tokyo) until the same growth phase. The cells were harvested by centrifugation at 27,600*g* at 4°C, washed twice with an ice-chilled 10 mM potassium phosphate buffer (pH 7.0) containing 0.75% NaCl, and then suspended in a 10 mM potassium phosphate buffer, pH 7.0 (0.5 g-wet weight cells/ml). *Escherichia coli* (NovaBlue) was obtained from Merk KGaA (Darmstadt, Germany) and *E. coli* (NovaBlue)-pT7 Blue-T-Vector was grown aerobically at 37°C in a Luria-Bertani medium (Sambrook et al. 1989) supplemented with ampicillin (100 μg/ml).

Purification of alcohol dehydrogenases

All operations were carried out between 0 and 4°C unless otherwise stated. We disrupted the cells in an ice water bath by ultrasonication for 5 min, and repeated the treatment five times with 5-min cooling intervals. After centrifugation at 27,600*g* for 30 min, the supernatant solution (100 ml) was incubated at 50°C for 30 min, and centrifuged again under the same conditions to remove denatured proteins. The supernatant solution was dialyzed against a 10 mM potassium phosphate buffer (pH 7.0) containing 0.01% 2-mercaptoethanol (buffer A). The enzyme solution was applied to a DEAE-Toyopearl 650 M column ($\varphi 2.5 \times 20$ cm, Econo column; Bio-Rad Japan, Tokyo) equilibrated with buffer A, and the column was washed with 500 ml of the same buffer. The active fractions unabsorbed were combined, and ammonium sulfate was then added to the enzyme solution at a final concentration of 1.5 M. The mixture was cooled in an ice bath for 30 min, and centrifuged at 27,600*g* for 30 min. The supernatant solution was applied to a Phenyl-Toyopearl column ($\varphi 2.5 \times 18$ cm) equilibrated with buffer A containing 1.1 M ammonium sulfate. After washing with 450 ml of the buffer A containing 1.3 M ammonium sulfate, the adsorbed protein was eluted with 450 ml of buffer A containing 1.1 M ammonium sulfate. The active fractions were combined and concentrated by ultrafiltration. The enzyme solution was dialyzed against buffer A containing 0.15 M NaCl, and applied to a 5′-AMP Sepharose 4B column ($\varphi 1.0 \times 8$ cm) equilibrated with buffer A containing 0.15 M NaCl. After washing with 50 ml of buffer A containing 0.15 M NaCl, the enzyme was eluted with 50 ml of buffer A containing 0.5 mM NAD^+ and 0.15 M NaCl. The enzyme solution was concentrated by ultrafiltration, mixed with the same volume of 60% (w/v) sucrose in buffer A, and stored at −20°C in the Kelvinator U-190. NAD^+ and sucrose were removed from the enzyme solution by Toyopearl HW-50 gel filtration chromatography just prior to use.

Saccharomyces cerevisiae AlcDH I solution was purchased from Oriental Yeast, Co., Tokyo, and purified to homogeneity. The enzyme solution was dialyzed against buffer A containing 0.15 M NaCl, and applied to a 5′-AMP Sepharose 4B column ($\varphi 1.0 \times 8$ cm)

equilibrated with buffer A containing 0.15 M NaCl. After washing with 50 ml of buffer A containing 0.15 M NaCl, the enzyme was eluted with 50 ml of buffer A containing 0.5 mM NAD^+ and 0.15 M NaCl.

Enzyme assay

The enzyme was assayed spectrophotometrically at 340 nm by determination of the NADH produced or oxidized in a reaction mixture at 30°C. The standard reaction mixture for the oxidation reaction (3 ml) contained 10 mM ethanol, 1 mM NAD^+ in a 100 mM *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer, pH 9.0 and the enzyme solution. The standard reaction mixture for the reduction reaction (3 ml) contained 10 mM acetaldehyde, 0.2 mM NADH in 100 mM HEPES, pH 7.0 and the enzyme solution (20 µl). The enzyme reactions were started by the addition of an enzyme solution. The enzyme activity was estimated from an initial velocity, and each measurement was performed at least twice. One unit of enzyme was defined as the amount of enzyme that catalyzes the production or oxidation of 1 µmol of NADH per minute.

The effect of the temperature on the enzyme activity was examined in the temperature range from 0 to 85°C. The assay was carried out under standard assay conditions except for the reaction temperature. The effect of the temperature on the activity was examined as follows: the enzyme solution (100 µl; protein conc. 0.3 mg/ml) in the absence of NAD^+ in a microcentrifuge tube (cat. no. 616201; Greiner Bio-one, Tokyo) was incubated in 10 mM potassium phosphate buffer, pH 7.0, at 40, 50, 60, 65, 70 and 75°C. The enzyme solution (3 µl) was taken from the microcentrifuge tube at various incubation times, and the remaining activities were determined under the standard assay conditions.

The effect of the pH on the enzyme activity was examined in the pH range of 4–11 with the following 100 mM buffers under the standard assay conditions: citrate–phosphate buffer (pH 4–8), and glycine–NaOH buffer (pH 8–11). The effect of pH on the enzyme stability was examined by determination of the remaining activities under the standard assay conditions after incubation of the enzyme (500 µl; protein conc. 37 µg/ml) at 30°C in a microcentrifuge tube at various pH values. The effect of inhibitors was examined by determination of the remaining activities under standard assay conditions except for the addition of 1 mM solution of each inhibitor. The purified enzyme solution was pretreated with 1 mM inhibitor for 30 min in 10 mM potassium phosphate buffer (pH 7.0) before determination of the activity.

Steady-state kinetics

The kinetic constants were determined by measurement of the initial velocities at various concentrations of substrate under several fixed concentrations of coenzyme. The substrate and coenzyme concentrations used were as follows: ethanol 0.05–5 mM, and NAD^+ 0.05–1 mM.

Stereochemical analysis of hydrogen transfer at the C4 position of the nicotinamide ring of the coenzyme

The stereospecificity for the C4 hydrogen transfer of NADH was analyzed by ^{1}H-NMR (Esaki et al. 1989; Nakajima et al. 1989). The reaction mixture (1 ml) contained CD_3CD_2OD (10 µmol), NAD^+ (1 µmol), sodium pyruvate (10 µmol), ammonium sulfate (20 µmol), *Bacillus sphaericus* alanine dehydrogenase (*pro-R*-specific, 1 U) (Ohshima et al. 1990), and *F. frigidimaris* KUC-1 AlcDH (1 U) in a 100 mM glycine–NaOH buffer (pH 9.0). After incubation at 30°C for 3 h, the enzymes were removed with an ultrafilter unit, and the reaction mixture was then dried by centrifugal vacuum evaporator. The residue was dissolved in D_2O, and the ^{1}H-NMR spectra at the C4 position of the nicotinamide ring of NAD^+ were recorded on a datum JNM-EX 270 FT NMR spectrometer (270 MHz, Nihon Denshi, Tokyo). 2,2-Dimethyl-2-silapentane-5-sulfonate was used as an internal standard.

N-terminal and internal amino acid sequence determination

Approximately 16 µg of protein was used to determine the N-terminal amino acid sequence. An internal peptide was sequenced as follows. About 160 µg of protein was digested at 37°C for 12 h with lysylendopeptidase in the presence of 2 M urea and 20 mM Tris–HCl buffer (pH 9.0), and the peptides produced were separated by reverse-phase HPLC (LC-10A system, Shimadzu, Kyoto, Japan) at a flow rate of 0.7 ml/min. The column was equilibrated with 0.1% trifluoroacetic acid in ultrapure water obtained by Milli-Q Labo, and eluted with 0.1% trifluoroacetic acid in acetonitrile. The following gradient program was used: acetonitrile conc. 0% (0–40 min); 0–60% (40–85 min); 60–100% (85–100 min) and 100% (100–120 min). The samples were transferred to a PVDF membrane (Immobilon-P, Millipore Japan, Tokyo), and the N-terminal and internal amino acid sequences were analyzed by Edman degradation with an automated sequencer (model 477A, Applied Biosystems Japan, Tokyo).

Cloning and sequence analysis of the gene encoding AlcDH

On the basis of the partial N-terminal and internal sequences, MLPKTMKA and DLVVNAK, two oligonucleotides, 5′-ATGYTICCIAARACIATGAARGC-3′ and 5′-YTTIGCRTTIACIACIARRT-3′, were synthe-

sized and used as forward and reverse PCR primers, respectively: I, R and Y mean inosine, adenine or guanine and cytosine or thymine, respectively. A PCR was performed by a thermal cycler (system 9700; Applied Biosystems Japan, Tokyo) with 100 pmol of each primer against 70 ng of chromosomal DNA isolated from the *F. frigidimaris* KUC-1 cells. The thermal program consisted of 30 cycles of denaturation at 94°C for 20 s., annealing at 40°C for 30 s., and extension at 68°C for 1 min. The 650 bp DNA fragment obtained was inserted into a pT7 Blue T-Vector (Merk KGaA, Darmstadt, Germany). The DNA sequence of the fragment was determined with a Big dye™ terminator cycle sequencing ready-reaction kit in a model 377A DNA sequencing system gel apparatus (Applied Biosystems Japan, Tokyo) according to the manufacturer's instructions.

Genome-walking PCR was used to determine the DNA sequences in the upstream and downstream region from the 650 bp insert to clarify the in-frame start and termination codons. Four primers, 5′-ACATAT CCTACGGCTTCA-3′, 5′-CCAATCTCCTTCTACTG CG-3′, 5′-GGTACAATATGCCAAAGC-3′, and 5′-GG AATCGGAGGTCTTGG-3′, were designed, and genome-walking PCR was performed with the Takara LA PCR™ in vitro Cloning kit (Takara, Kyoto, Japan) according to the manufacturer's instructions. The extracted chromosomal DNA was digested with *Hin*dIII or *Sal*I and ligated to the *Hin*dIII or *Sal*I cassette, respectively. The obtained DNA fragments were used as a template for PCR. The thermal program consisted of 30 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 2 min, and extension at 72°C for 1 min. The obtained PCR fragments were sequenced in the same way as described above.

Other methods

The protein concentrations were measured by the method of Bradford (Bradford 1976) with bovine serum albumin as a standard. The protein concentrations were determined by measurement of the absorption of the fractions at 280 nm on column chromatography. The molecular weight of the enzyme was estimated by gel filtration with a Superose 12 column (Amersham Biosciences, NJ, USA) at 4°C. The following proteins were used as molecular weight standards: ribonuclease (13.7k), ovalbumin (43k), aldolase (158k), and ferritin (440k). PAGE and SDS/PAGE were performed by the methods of Tulchin et al. (1976) and Laemmli (1970), respectively. The LMW marker kit (Amersham Biosciences, NJ, USA) was used as a molecular weight marker for SDS-PAGE. The phylogenenic tree was drawn by the UPGMA method with GENETYX-WIN version 4.0 (Software Development Co., Tokyo). Zn, Ni, Mn, Fe, and Cu were measured with Shimadzu AA-5400 atomic absorption spectrophotometer (Shimadzu, Kyoto, Japan) at room temperature (about 20°C). Absorption was monitored with deuterium-arc background correction. The enzyme solution was used after dialysis against three changes of 150 volumes of ultra-pure water TAMAPURE-AA (Tama Chemicals, Kanagawa, Japan) at 4°C for 8 h. The outer solution of dialysis tube was used as a blank. We took an average of at least two sets of independent determination.

Nucleotide sequence accession number

The nucleotide sequence for *F. frigidimaris* KUC-1 AlcDH was submitted to the DDBJ/EMBL/GenBank Data Bank as accession number AB084581.

Results

Enzyme purification

Flavobacterium frigidimaris KUC-1 AlcDH and *Saccharomyces cerevisiae* AlcDH I were purified to homogeneity: a single band of each enzyme was shown on both PAGE and SDS-PAGE (data not shown). As compared with *S. cerevisiae* AlcDH I , a cold-active AlcDH from *Moraxella* sp. TAE123 is more suitable for the reference enzyme, but the strain was not available from the collector. *F. frigidimaris* KUC-1 AlcDH was purified 120-fold with a yield of 20% (Table 1). The specific activity for the oxidation reaction was 43.0 U/mg when ethanol was used as a substrate. The enzyme occupied about 0.83% of the soluble cellular protein. The enzymes could be stored at −20°C in a 10 mM potassium phosphate buffer, pH 7.0 containing 0.5 mM NAD^+ without loss of activity for several months.

Molecular weight and subunit structure

The apparent molecular weights of the enzyme and the subunit were determined to be about 160 and 40 k, respectively by Superose 12 gel chromatography and SDS-PAGE. Accordingly, the enzyme is homotetrameric.

Metal ion content

The atomic absorption analysis of the enzyme revealed the presence of 2 gram-atoms Zn per subunit [calculated values (gram-atoms Zn per subunit): First, 1.5; Second, 1.8]. Other metals were not detected in a significant amount (< 0.1 gram-atoms/subunit).

Substrate and coenzyme specificities

The enzyme catalyzed the oxidation of various aliphatic and aromatic alcohols, and was most active on 1-decanol, but did not act on methanol and amino alcohols

Table 1 Purification of cold-active and thermostable alcohol dehydrogenase from *Flavobacterium frigidimaris* KUC-1

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	6,460	18,000	0.358	100	1
Heat treatment	5,400	2,690	2.02	84	5.64
DEAE-Toyopearl	3,920	420	9.34	61	26.1
Phenyl-Toyopearl	2,200	165	13.3	34	37.2
5′-AMP Sepharose	1,360	31.6	43.0	20	120

Table 2 Substrate specificity of alcohol dehydrogenase from *Flavobacterium frigidimaris* KUC-1

Substrate	Specific activity (U/mg)
(a) Oxidative reaction of alcohols	
Methanol	0
Ethanol	30.9
1-Propanol	30.9
1-Butanol	28.7
1-Pentanol	22.8
1-Hexanol	30.8
1-Heptanol	35.6
1-Octanol	38.4
1-Nonanol	40.3
1-Decanol	70.2
2-Propanol	3.01
2-Butanol	2.58
2-Pentanol	1.64
2-Hexanol	1.22
2-Heptanol	3.25
2-Octanol	7.14
2-Nonanol	7.53
(S)-(+)-2-Nonanol	15.2
(R)-(−)-2-Nonanol	0.01
2-Decanol	9.90
3-Decanol	2.30
Benzylalcohol	2.01
Allylalcohol	39.5
(b) Reductive reaction of aldehydes and ketones	
Formaldehyde	9.53
Acetaldehyde	327
Propionaldehyde	171
Butanal	168
Pentanal	147
Hexanal	104
Heptanal	95.3
Octanal	104
Nonanal	120
Decanal	161
Acetone	0.500
2-Butanone	0.472
2-Pentanone	0.589
2-Hexanone	0.258
2-Heptanone	0.430
2-Octanone	0.705
2-Nonanone	0.763
2-Decanone	1.04
Benzaldehyde	51.2

The activity was determined under standard assay conditions with various 1 mM substrates
Triton X-100 was added at a concentration of 0.025% to dissolve the substrate with low solubility. To eliminate the effect of Triton X-100 between the activities measured, it was added to all reaction mixtures

(Table 2a). In the reductive reaction, various aldehydes and ketones were reduced enzymatically, and acetaldehyde was the best substrate among compounds tested (Table 2b). The specific activities for primary alcohols were about 5–10 times higher than those for the counterparts of secondary alcohols, and the specific activities for aldehydes were about 150–300 times higher than those for the corresponding ketones. The enzyme showed 1,500 times higher specific activity for (S)-(+)-2-nonanol than for (R)-(−)-2-nonanol. NAD^+ was exclusively used as a coenzyme, and $NADP^+$ was inert.

Effect of the temperature

F. frigidimaris KUC-1 AlcDH and *S. cerevisiae* AlcDH I showed the maximum activities at 70 and 30°C, respectively (Fig. 1a). The *Flavobacterium* enzyme was active even at 85°C, and the activity at 85°C corresponds to 65% of the activity at 70°C. *F. frigidimaris* KUC-1 AlcDH was also active at 0°C as found for the *Saccharomyces* enzyme, although it is thermostable enzyme (Fig. 1a). *F. frigidimaris* KUC-1 AlcDH showed continuity in Arrhenius plots without any transition temperature (data not shown). In case of thermophilic AlcDH from *G. stearothermophilus* LLD-R, a transition temperature was observed at 30°C (91 kJ/mol, 4–30°C; 64 kJ/mol, 30–65°C) (Guagliardi et al. 1996) and *G. stearothermophilus* LLD-R AlcDH preferably acts over 30°C. From the linear part of the Arrhenius plots, the activation energy for *F. frigidimaris* KUC-1 AlcDH was calculated to be 40.0 kJ/mol. The value agreed well with that of the psychrophilic AlcDH from *Moraxella* sp. TAE123 (41 kJ/mol) (Tsigos et al. 1998). The thermal stability of *F. frigidimaris* KUC-1 AlcDH (protein conc. 0.3 mg/ml) was examined in the temperature range of 40–75°C in the absence of NAD^+ (Fig. 2a). The enzyme activity was not affected by incubation at 40°C for 210 min. The half-life times (protein conc. 0.3 mg/ml) in the absence of NAD^+ were estimated to be 304, 50, 20, and 6 min at 50, 60, 65, and 70°C, respectively (Fig. 1b). The half-life time of *S. cerevisiae* AlcDH I (protein conc. 0.3 mg/ml) was shown to be 2 min at 60°C in the absence of NAD^+. Accordingly, the half-life time of *F. frigidimaris* KUC-1 AlcDH was about 25 times longer than that of the *S. cerevisiae* AlcDH I at 60°C in the absence of NAD^+. The enzyme stabilities (protein conc.

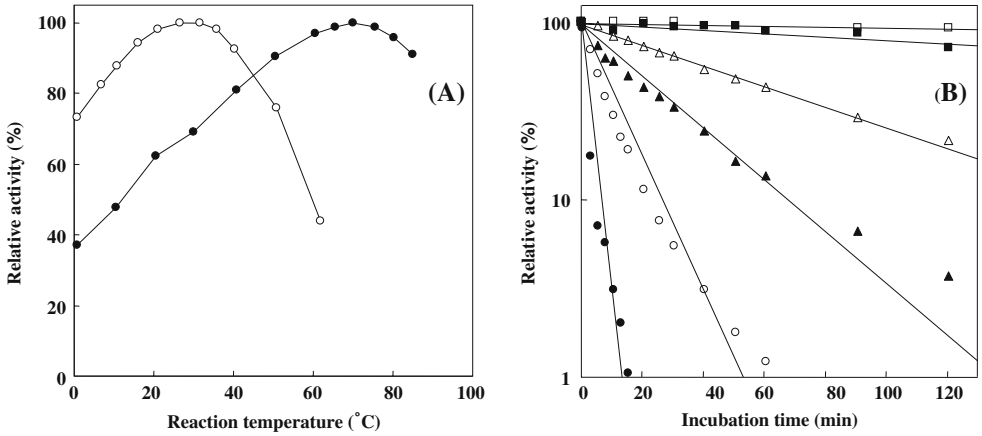

Fig. 1 Effect of temperature on the enzyme activity and stability of *Flavobacterium frigidimaris* KUC-1 alcohol dehydrogenase. **a** Effect of temperature on the activities for *Flavobacterium frigidimaris* KUC-1 alcohol dehydrogenase (*filled circle*) and *Saccharomyces cerevisiae* alcohol dehydrogenase I (*open circle*). **b** Effect of temperature on the stability for *Flavobacterium frigidimaris* KUC-1 alcohol dehydrogenase. *open square*, *filled square*, *open triangle*, *filled triangle*, *open circle*, and *filled circle* represent the remaining activities (protein conc. 0.3 mg/ ml) in the absence of NAD^+ at 40, 50, 60, 65, 70, and 75°C, respectively

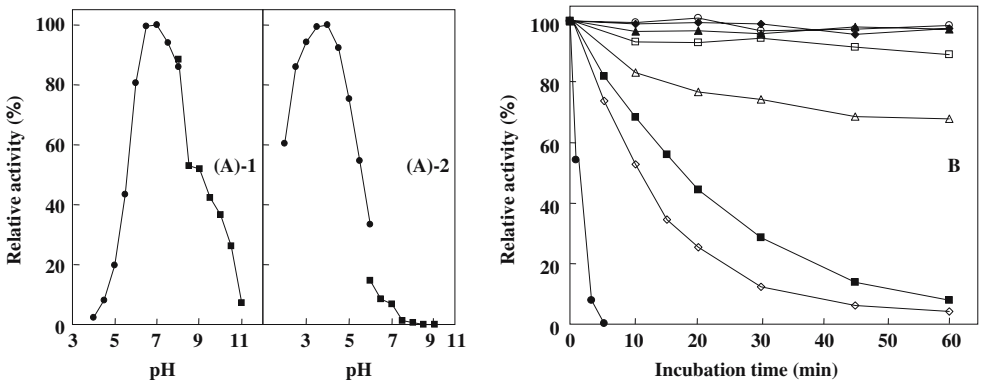

Fig. 2 Effect of pH on the enzyme activity and stability of *Flavobacterium frigidimaris* KUC-1 alcohol dehydrogenase. **a** Effect of pH on the enzyme activity. The effect of the pH on the enzyme activity was examined in the pH range of 4–11 with the following 100 mM buffers under the standard assay conditions: *filled circle*, citrate-phosphate buffer (pH 4–8); *filled square* glycine–NaOH buffer (pH 8–11). *A1* Oxidation reaction of ethanol to acetaldehyde. *A2* Reduction reaction of acetaldehyde to ethanol. **b** Effect of pH on the enzyme stability. The effect of the pH on the enzyme stability was examined by determination of the remaining activities under the standard assay conditions after incubation of the enzyme (protein conc. 37 μg/ml, 500 μl) at 30°C in a microcentrifuge tube (cat. no. 616201; Greiner bio-one, Tokyo) at various pH values: pH 4–5, citrate-sodium buffer; pH 6–7, Bis–Tris–HCl buffer; pH 8, TAPS–NaOH buffer; pH 9, CHES–NaOH buffer; pH 10–11, CAPS–NaOH buffer. Symbols *filled circle* pH 4, *filled square* pH 5, *filled triangle* pH 6, *filled diamond* pH 7, *open circle* pH 8, *open square* pH 9, *open triangle* pH 10, *open diamond* pH 11

0.3 and 1.0 mg/ml) were also examined at 60°C in the presence and absence of 10 μM NAD^+. The half-life times of *F. frigidimaris* KUC-1 AlcDH (0.3 mg/ml) at 60°C in the presence of 10 μM NAD^+ (143 min) was about three times longer than that of the enzyme (0.3 mg/ml) in the absence of NAD^+ (50 min). When 1.0 mg/ml of the enzyme was used in the absence of NAD^+, the half-life time increased to 80 min.

Effect of pH

The activities were determined at various pH values. *F. frigidimaris* KUC-1 AlcDH showed activity in the pH range between 4 and 11 for the reduction reaction (Fig. 2a1) and from 4 to 9 for the oxidation reaction (Fig. 2a2). The optimum pH values for the reduction of acetaldehyde and the oxidation of ethanol were determined to be pH 6 and 7, respectively. The enzyme was stable between pH 6 and 9 under the conditions used and characteristically inactivated at pH 5 and 10 (Fig. 2b).

Steady-state kinetics

The double reciprocal plots of the initial velocity against various concentrations of substrate or NAD^+ measured under the various fixed concentrations of NAD^+ or substrate gave linear graphs (data not shown). These results show that *F. frigidimaris* KUC-1 AlcDH catalyzes

the enzyme reaction via the formation of a ternary complex with a substrate and a coenzyme, and that the enzyme reaction probably proceeds through the Theorell–Chance mechanism (Theorell et al. 1951). The kinetic parameters for ethanol at various temperatures were calculated from the secondary plots of the intercepts against the reciprocal concentrations of NAD^+. The K_m values for *F. frigidimaris* KUC-1 AlcDH and *S. cerevisiae* AlcDH I were highly influenced by the reaction temperature and the lowest K_m value was observed at 20°C for both enzymes (Table 3). The K_m values of *F. frigidimaris* KUC-1 were 40–50 times lower than those of *S. cerevisiae* AlcDH I under all the conditions tested. The k_{cat}/K_m values of *F. frigidimaris* KUC-1 AlcDH were much higher than those of *S. cerevisiae* AlcDH I in a wide temperature range including 10°C (Table 3).

Effect of inhibitors

We examined the effect of various reagents on the enzyme activity (Table 4). The enzyme was strongly inhibited by *p*-(chloromercuri) benzoic acid (inhibition, 100%), iodoacetic acid (94.0%), $HgCl_2$ (100%), and *N*-ethylmaleimide (100%). Accordingly, the thiol group or groups are directly or indirectly involved in the catalytic activity, as reported for other dehydrogenases (Cheng et al. 1992).

Table 4 Effects of inhibitors alcohol dehydrogenase on the activity for *Flavobacterium frigidimaris* KUC-1

Reagent	Concentration	Relative activity (%)
None	–	100
NaCl	1 mM	85
KCl	1 mM	84
$BaCl_2$	1 mM	73
$CoCl_2$	1 mM	67
$CuCl_2$	1 mM	32
$ZnCl_2$	1 mM	64
$Pb(CH3COO)_2$	1 mM	103
$FeCl_2$	1 mM	43
$MnCl_2$	1 mM	85
$AlCl_3$	1 mM	94
$CaCl_2$	1 mM	109
$MgCl_2$	1 mM	75
$NiCl_2$	1 mM	21
SDS	0.05%	9
Hexadecyltrimethyl-ammonium bromide	0.05%	21
Polyoxyethlene(10) octylphenyl ether	0.05%	57
EDTA	1 mM	74
EGTA	1 mM	88
1, 10-Phenanthroline	1 mM	62
2, 2′-Bipyridyl	1 mM	83
Semicarbazide hydrochloride	1 mM	76
Hydroxylamine hydrochloride	1 mM	66
Iodeacetic acid	1 mM	4
$HgCl_2$	1 mM	0
N-ethylmaleimide	1 mM	0
p-(Chloromercuri) benzoic acid	1 mM	0

Stereochemical analysis of the hydrogen transfer of a coenzyme

In general, NAD^+-dependent dehydrogenases show either *pro-R* or *pro-S* stereospecificity for hydrogen transfer at the C4 position of the nicotinamide moiety of NAD^+ and NADH. The stereospecificity of *F. frigidimaris* KUC-1 AlcDH for hydrogen transfer was examined by the in situ method (Esaki et al. 1989; Nakajima et al. 1989). If the stereospecificity of hydrogen transfer by the enzyme is the same as that of alanine dehydrogenase from *B. sphaericus* (*pro-R*-specific), C4 hydrogen of NAD^+ is fully retained, and the doublet for it appears at around δ 8.8 ppm. The resonance doublet of around δ 8.8 ppm for C4 hydrogen was retained in the ^{1}H-NMR spectrum. The result suggests that ^{2}H of deuterated ethanol is enzymatically transferred to the C4 position of the nicotinamide ring of NAD^+, and that the C4 deuterium in the [4R-^{2}H] NADH produced is transferred to pyruvate. Thus, *F. frigidimaris* KUC-1 AlcDH shows the *pro-R*-specificity for the hydrogen transfer.

Sequencing of the amino terminal region and the internal region

The N-terminal amino acid sequence was analyzed for 19 cycles and determined to be ^{1}MLPKTMKAAVI–FGSLLK^{19}. After separation by reverse-phase HPLC,

Table 3 Effects of temperature on kinetic parameters of alcohol dehydrogenases from *Flavobacterium frigidimaris* KUC-1 and *Saccharomyces cerevisiae*

Temperature (°C)	*F. frigidimaris* KUC-1 AlcDH			*S. cerevisiae* AlcDH I			k_2/k_1
	k_{cat} for ethanol (k_1) (min^{-1})	k_m for ethanol (mM)	k_{cat}/K_m (min^{-1} mM^{-1})	k_{cat} for ethanol (k_2) (min^{-1})	k_m for ethanol (mM)	k_{cat}/K_m ($min^{-1}mM^{-1}$)	
10	2,394	0.190	12,600	15,080	9.03	1,670	6.3
20	3,749	0.147	25,500	25,935	5.70	4,550	6.9
30	6,569	0.168	39,100	47,166	8.56	5,510	7.2
40	9,647	0.248	38,900	53,724	12.1	4,440	5.6
50	15,738	0.430	36,600	–	–	–	–
60	28,496	1.04	27,400	–	–	–	–

three lysylendopeptidase digested peptides were obtained. The amino acid sequences of these peptides were determined to be LGADLVVNAK, MPLIPGHEA VGY, and VIASGVCHTDLHAVEGDWPV.

Cloning and sequence analysis

Based on the amino acid sequences of N-terminal and internal peptides, the oligonucleotide primers, 5′-ATGYTICCIAARACIATGAARGC-3′ and 5′-YTTIGCR TTIACIACIARRT-3′, were synthesized and used as forward and reverse PCR primers, respectively. The amplified DNA fragment (about 650 bp) was inserted into a pT7 blue T-vector, and the DNA sequence was determined. The DNA sequence obtained lacked initiation and termination codons and showed an incomplete open reading frame. The flanking regions were amplified by the genome-walking PCR method, and the regions about 1.4 kb upstream and 1.6 kb downstream were sequenced to obtain the entire gene of alcohol dehydrogenase (*alcdh*). The *alcdh* sequence was preceded by a gene that is homologous to chloroacetaldehyde dehydrogenase from *Xanthobacter autotrophicus* (identity, 69%) (Bergeron et al. 1998) and acetaldehyde dehydrogenase from *Ralstonia eutropha* (identity, 68%) (Priefert et al. 1992). We found that the preceding gene encodes the thermostable aldehyde dehydrogenase gene (*alddh*; accession no. AB085823) previously reported (Yamanaka et al. 2002). The initiation codon, ATG, is located at the position 1,232 that is 79 bp after the termination codon of the preceding gene, whereas the termination codon, TAG is at the position of 2,266. The *alcdh* sequence is not preceded by the sequence of the putative bacterial Shine-Dalgano ribosome-binding site usually located upstream from the starting codon. The DNA fragment that forms a stem-loop structure of mRNA, GTAACAAACTTGTACTCTTATAAGTTTGTTAC, and the subsequent thymine-rich region, TTTTTTTT, co-existed in 16 bp downstream from the stop codon. Thus, *alcdh* is composed of 1,035 bp and codes 344 amino acid residues, and the molecular weight and p*I* values were estimated to be 36,823 and 6.7, respectively. The deduced amino acid sequence was used to find similar proteins in the GenBank and protein database with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The sequence identities were found with the amino acid sequences of AlcDHs from *Moraxella* sp. TAE123 (67%), *P. aeruginosa* (65%), *G. stearothermophilus* (56%), *S. cerevisiae* (42%), and horse liver (28%) (Fig. 3). The AlcDHs classified into the medium-chain alcohol dehydrogenase family contain two zinc atoms per subunit, the catalytic and structural zinc atoms (Akeson 1964; Drum et al. 1967). The catalytic zinc atom occurs in the active center of the AlcDHs, and is essential for the enzyme activity (Drum et al. 1967; Eklund et al. 1982). The structural zinc atom exists in the subunit boundary of the AlcDHs, and is considered to be important for the stability of the protein structure (Akeson 1964; Drum et al. 1967). *F. frigidimaris* KUC-1 AlcDH contains 2 gram-atoms Zn per subunit, and the corresponding amino acid residues for Zn binding are completely conserved in *F. frigidimaris* KUC-1 AlcDH. Ser48 of horse liver AlcDH in the proton release system is replaced by Thr45 in *F. frigidimaris* KUC-1 AlcDH (LeBrun et al. 2004).

Discussion

We isolated a psychrotorelant from Antarctic seawater and identified it as *F. frigidimaris* KUC-1 (Nogi et al. 2005). The organism produced the cold-active and thermostable NAD^+-dependent AlcDH. The cold-active and thermostable enzyme from a psychrophilic bacterium has been reported only for 3-isopropylmalate dehydrogenase of *Vibrio* sp. 15 (Svingor et al. 2001), and *F. frigidimaris* KUC-1 AlcDH is the first example of a cold-active and thermostable AlcDH. Heat treatment was very effective to purify the enzyme: approximately 85% of cellular proteins by weight was denatured and removed. The enzyme is composed of four identical subunits with a molecular weight of 40k, and contains 2 gram-atoms Zn per subunit. The amino acid residues that are essential to coordinate the catalytic and structural zinc atoms are well conserved (Fig. 3). In contrast to the optimum pH values of other AlcDHs for oxidation reaction (pH 8–11), the optimum pH of *F. frigidimaris* KUC-1 AlcDH was found at 7.0: this is similar to those of AlcDHs from *Thermococcus* sp. AN1 (optimum pH 6.8–7.0) (Li et al. 1997) and *Moraxella* (optimum pH pH 7.5) (Tsigos et al. 1998) (Fig. 2). The p*I* value (p*I* = 6.7) of *F. frigidimaris* KUC-1 AlcDH resembles those of AlcDHs from *G. stearothermophilus* LLD-R (p*I* = 6.0) and *S. cerevisiae* (p*I* = 6.3), and is different from those of *Moraxella* sp. TAE123 (p*I* = 4.9) and horse liver (p*I* = 7.9) AlcDHs.

The amino acid residues that are essential for catalytic activity in horse liver AlcDH are highly conserved in various AlcDHs (Fig. 3). The three conserved amino acid residues in horse liver AlcDH, Thr178, Asp223, and Lys228, correspond to Thr157, Asp200, and Lys205 in *F. frigidimaris* KUC-1 AlcDH, respectively, and were found to play an important role in the recognition of NAD^+ (Fan et al. 1991; Sekhar et al. 1988). The molecular weight and metal content of *F. frigidimaris* KUC-1 AlcDH are similar to those of AlcDHs from *Moraxella* sp. TAE123 (Tsigos et al. 1998), *S. cerevisiae* (Leskovac et al. 2002) and *G. stearothermohilus* (Cannio et al. 1994) and consequently *F. frigidimaris* KUC-1 AlcDH belongs to the medium-chain AlcDH family. We found that *alddh* from *F. frigidimaris* KUC-1 precedes *alcdh* (Yamanaka et al. 2002) on the genomic DNA, and that both gene products, alcohol and aldehyde dehydrogenases, probably function cooperatively to catabolize alcohols and aldehydes.

F. frigidimaris KUC-1 AlcDH requires only NAD^+ as a coenzyme, and showed a low substrate specificity in both oxidation and reduction reactions (Table 2). It preferably

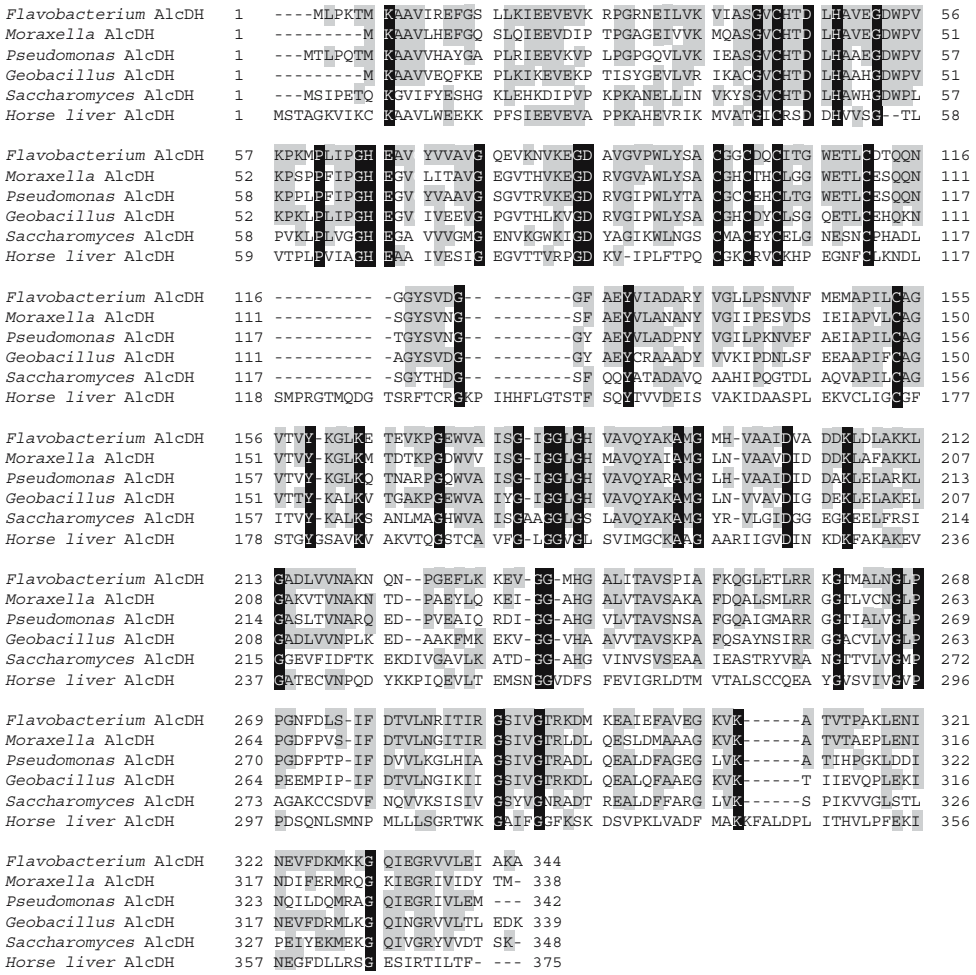

Fig. 3 Comparison of primary structure of various alcohol dehydrogenases. *Flavobacterium frigidimaris* KUC-1 (AB084581); *Pseudomonas aeruginosa* (1LLU_A); *Moraxella* sp. TAE123 (AJ441109); *G. stearothermophilus* LLD-R (P42328); *S. cerevisiae* (P00330); and Horse liver (1P1R_D). The conserved amino acid residues were shaded in black and the identical amino acid residues between *F. frigidimaris* KUC-1 AlcDH and other AlcDHs were shaded in gray. Gaps (–) were introduced to obtain maximum matching

acts on various primary alcohols except for methanol. The substrate specificity is similar to that of the *G. stearothermophilus* enzyme (Guagliardi et al. 1996). The enzyme shows the *pro-R*-stereospecificity for the hydrogen transfer at the C4 position of the nicotinamide moiety of NAD^+ as reported for AlcDHs from horse liver (Eklund et al. 1982), *S. cerevisiae* (Weinhold et al. 1991), *Moraxella* sp. TAE123 (Velonia et al. 1999), and *Thermoanaerobium brockii* (Peretz et al. 1993).

The k_{cat}/K_m value of *F. frigidimaris* AlcDH (12,600 mM^{-1} min^{-1} at 10°C) is approximately eight times larger than that of *S. cerevisiae* AlcDH I (1,670 mM^{-1} min^{-1} at 10°C) (Table 3). This probably reflects the high catalytic efficiency of the *Flavobacterium* enzyme at low temperatures. The kinetic parameters of *Flavobacterium* AlcDH depend on the reaction temperatures (Table 3). The lowest K_m value is found at about 20°C, which is close to the cultivation temperature (15°C) of *F. frigidimaris* KUC-1 and is 40–50 times lower than that of *S. cerevisiae* AlcDH I. Although the k_{cat} values of *F. frigidimaris* KUC-1 AlcDH (k_1) at 10–40°C are lower than those of *S. cerevisiae* AlcDH I (k_2), the k_2/k_1value increases from 10 to 30°C and decreases from 30 to 40°C with the maximum value at 30°C. This suggests that *F. frigidimaris* KUC-1 AlcDH shows relatively high turnover number below and above 30°C as compared with *S. cerevisiae* AlcDH I and is one of the features of cold-active and thermostable AlcDH from *F. frigidimaris* KUC-1. The high catalytic efficiency under low-temperature conditions also arises from the low activation energy, which is generally derived from the structural flexibility of the enzyme, in particular, in or in the vicinity of the active center (Fields et al. 1998; Lonhienne et al. 2000). The increased flexibility results also in the thermolability of enzyme (Feller et al. 1997), but that is not the case. The activation energy of *F. frigidimaris* KUC-1 AlcDH (40 kJ/mol) is close to that of the AlcDH from *Moraxella* sp. TAE123 (41 kJ/mol) (Tsigos et al. 1998), and much lower than that of the thermophilic AlcDH from *G. stearothermophilus* LLD-R (91 kJ/mol, 4–30°C; 64 kJ/mol, 30–65°C) (Guagliardi et al. 1996). However, the optimum temperature of *F. frigidimaris* KUC-1 AlcDH (about 70°C, Fig. 1) is substantially identical with that of *G. stearothermophilus* LLD-R AlcDH (about 70°C). We are currently studying the three-dimensional structure of enzyme in order to shed light on the apparently conflicting characteristics; namely, the enzyme is cold-active and thermostable.

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