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Properties of an Alcohol Dehydrogenase from the Hyperthermophilic Archaeon *Aeropyrum pernix* K1

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A NAD^+-dependent medium-chain alcohol dehydrogenase from the hyperthermophilic archaeon *Aeropyrum pernix* K1 was expressed in *Escherichia coli* and purified. The recombinant enzyme was a homotetramer of molecular mass 1.6×10^2 kDa. The optimum pH for the oxidative reaction was around 10.5 and that for the reductive reaction was around 8.0. The enzyme had a broad substrate specificity including aliphatic and aromatic alcohols, aliphatic and aromatic ketones, and benzylaldehyde. This enzyme produced (*S*)-alcohols from the corresponding ketones. The enzyme was thermophilic and the catalytic activity increased up to 95°C. It maintained 24% of the original catalytic activity after incubation for 30 min at 98°C, indicating that this enzyme is highly thermostable.

[**Key words:** alcohol dehydrogenase, *Aeropyrum pernix*, Archaea, medium-chain, enantioselectivity, thermophilic, thermostable]

Alcohol dehydrogenases (ADHs) are widely distributed in nature and have been found in many animals, plants and microorganisms (1). They play important roles in a broad range of physiological process (1, 2). ADHs are generally subdivided into three groups (3), the medium-chain zinc-dependent ADHs (approximately 350 amino acids per subunit) such as horse liver ADH (4) and ADHs (isozymes I–III) from *Saccharomyces cerevisiae* (5), the short-chain zinc-independent ADHs (approximately 250 amino acids per subunit) such as ADH from *Lactobacillus brevis* (6), and the long-chain iron-activated ADHs (approximately 385 amino acids per subunit) such as ADH IV from *S. cerevisiae* (7).

ADHs catalyze the reversible oxidation of alcohols to the corresponding aldehydes or ketones. ADHs catalyzing the stereospecific reduction of carbonyl groups have been discovered in different organisms. For example, ADHs from *Rhodococcus erythropolis* (8) and *Thermoanaerobium brockii* (9) produce (*S*)-alcohols, and ADH from *Lactobacillus kefir* (10) produces (*R*)-alcohols. Optically active alcohols are important building blocks in the synthesis of a broad variety of natural compounds and drugs. However, many ADHs are generally unstable and the low stability often hampers their industrial application.

Recently, ADHs from thermophilic organisms have been isolated. These ADHs are thermostable and have broad substrate specificity (9, 11–16). In this report, we describe the purification and characterization of a zinc-containing medium-chain alcohol dehydrogenase from the hyperthermophilic archaeon *Aeropyrum pernix* K1 (17), of which structure was recently solved (18, 19). We report that this enzyme is highly thermostable, and has a broad substrate specificity and high enantioselectivity.

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MATERIALS AND METHODS

Construction of an expression vector for ADH from *A. pernix* K1 A shot-gun clone (A2GR7175) containing an alcohol dehydrogenase coding sequence (ORF: APE2239) was used as a template for PCR amplification. An N-terminal primer, 5′-CCGGGGTACCATATGAGAATAGAGCAAGACTTCTCGC-3′ and a C-terminal primer, 5′-CCCCCAAGCTTGGATCCGTTACGGTATCAGGACTGCCC-3′, containing *Nde*I and *Bam*HI sites (underlined in the sequences), were used. The fragment generated was gel-purified. This purified gene was digested with *Nde*I and *Bam*HI and ligated into the pET-11a vector (Novagen, San Diego, CA, USA) digested with the same restriction enzymes. The plasmid, pET-11a+apADH, was cloned and verified by DNA sequencing after transformation of *Escherichia coli* XL10-Gold with the ligated product.

Expression and purification of apADH A single colony of *E. coli* BL21(DE3) transformed with pET-11a+apADH was inoculated into 5 ml of LB media containing 50 μg/ml of ampicillin at 37°C. At OD_{660}=0.8, 50% (v/v) glycerol solution was added to the culture (20% glycerol, final concentration) and this glycerol mixture was stored at −80°C until use. Ten μl of the glycerol solution was added to 10 ml of LB media containing 50 μg/ml of ampicillin and incubated at 37°C. At OD_{660}=0.8, the culture was added to 1 *l* of TB media containing 100 μg/ml of ampicillin and the cells were grown overnight (15 h) at 37°C.

The cells were harvested by centrifugation. The cell pellet was resuspended in 50 ml of 10 mM potassium phosphate buffer (pH 7.2) containing 0.1 mM AEBSF (Sigma, St. Louis, MO, USA), and disrupted by sonication at 4°C. The lysate was centrifuged and the supernatant was incubated first in the presence of Benzonase (Merck, Darmstadt, Germany; 40 units/ml of solution) and 6 mM $MgCl_2$ for 3 h at 37°C, and then in the presence of protamine sulfate from salmon (Sigma; 1 mg/ml of solution) at 4°C for 30 min. After the nucleic acid fragments were removed by centrifugation, the supernatant was heated at 60°C for 45 min. In addition, after centrifugation, the supernatant was heated at 75°C for 45 min, and the precipitated host proteins were removed by centrifugation. The supernatant was dialyzed against 10 mM potassium phosphate buffer (pH 7.2).

Saturated ammonium sulfate solution was added to the dialyzed enzyme to a final concentration of 50% saturation. The suspension was stirred for 30 min and then centrifuged. Solid ammonium sulfate was added to the resulting supernatant to a final concentration of 80% saturation. This mixture was stirred again and centrifuged as above. The resulting pellet was dissolved in a minimal volume of 10 mM potassium phosphate buffer (pH 7.2), and dialyzed against the same buffer.

The dialyzed enzyme was applied to a CIM QA disk column (12×3 mm; BIA Separations, Ljubljana, Slovenia) which had previously been equilibrated with the dialysis buffer. The column was eluted with 60 column volumes of a linear gradient of 0–0.3 M KCl in 10 mM potassium phosphate buffer (pH 7.2). The fractions that showed ADH activity were pooled and concentrated by ultrafiltration with a PLHK membrane (Millipore, Billerica, MA, USA).

The concentrated enzyme was applied to a Superdex 200 HR 10/30 column (1×30 cm; Amersham Biosciences, Piscataway, NJ, USA), and then eluted with 1.25 column volumes of 50 mM potassium phosphate buffer (pH 7.2) containing 150 mM potassium chloride. The fractions containing apADH were concentrated by ultrafiltration.

Enzyme assay The catalytic activity of apADH was determined at 60°C by monitoring the increase or decrease in absorbance at 340 nm (ε_{340}=6.22 mM^{-1} cm^{-1}), which is the characteristic absorption wavelength of NADH. The oxidation reaction mixture (2 ml) contained 0.18 μmol NAD^+, alcohol, and 0.2 nmol purified apADH in 100 mM potassium phosphate buffer (pH 8.0). The reduction reaction mixture (2 ml) contained 0.16 μmol NADH, aldehyde or ketones, and 0.2 nmol purified apADH in 100 mM potassium phosphate buffer (pH 8.0). Except when measuring the thermal activity, the reaction was initiated by the addition of an appropriate amount of coenzyme.

pH profiles of initial reaction rates The initial rates of the alcohol dehydrogenase reaction in both the oxidative and reductive directions were measured as a function of pH, from 3.8 to 11.5, using potassium citrate, potassium phosphate, glycylglycine–KOH, and glycine–HCl buffers. For the alcohol oxidation reaction assay, a 2.0 ml solution of an appropriate buffer (100 mM) with 100 nM apADH, 90 μM NAD^+ and 40 mM 2-pentanol was used. For the ketone reduction reaction assay, a 2.0 ml solution of an appropriate buffer (100 mM) with 100 nM apADH, 80 μM NADH and 40 mM 2-pentanone was used.

Thermal activity and stability The thermal activity of apADH was assayed at temperatures between 30°C and 95°C. The reaction mixture was composed of 90 μM NAD^+, 3.8 mM 2-pentanol and 100 nM apADH in 2.0 ml of 100 mM potassium phosphate (pH 8.0). The reaction was initiated by addition of 20 μl of the mixture of apADH and NAD^+.

The stability was studied by incubating apADH (4 μM) in 50 mM potassium phosphate buffer pH 7.2 containing 150 mM KCl at various temperatures. After incubation for 30 min, each sample was placed on ice and centrifuged at 4°C. The residual activity was assayed by the oxidation of 3.8 mM 2-pentanol under the reaction conditions described in the enzyme assay section.

TABLE 1. Kinetic constants for oxidation of alcohols

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
Ethanol	0.23±0.03	13.7±3.3	0.017
1-Propanol	0.26±0.01	1.03±0.06	0.25
1-Butanol	0.41±0.02	0.596±0.097	0.69
1-Pentanol	0.45±0.02	0.396±0.057	1.1
1-Hexanol	0.37±0.03	0.147±0.037	2.5
2-Propanol	0.24±0.02	2.44±0.40	0.097
2-Butanol	0.48±0.01	1.05±0.09	0.46
2-Pentanol	0.60±0.02	0.752±0.093	0.79
Cyclohexanol	0.52±0.03	0.703±0.109	0.73
Benzylalcohol	1.02±0.01	5.43±0.16	0.189
4-Methoxybenzylalcohol	0.60±0.03	1.13±0.20	0.53
NAD^+	0.40±0.02	0.0010±0.0002	3.8×10^2

TABLE 2. Kinetic constants for reduction of benzylaldehyde and ketones

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
2-Pentanone	0.77±0.05	5.15±0.75	0.15
2-Hexanone	1.08±0.04	5.01±0.33	0.22
2-Heptanone	0.73±0.03	1.16±0.13	0.62
2-Octanone	0.74±0.01	0.286±0.018	2.6
2-Nonanone	0.71±0.02	0.215±0.016	3.3
2-Decanone	0.40±0.02	0.147±0.017	2.7
t-Butyl acetoacetate	0.072±0.002	0.694±0.073	0.10
Cyclohexanone	1.27±0.05	1.39±0.14	0.91
4-Methoxyphenyl acetone	0.071±0.004	0.131±0.023	0.54
Benzaldehyde	1.22±0.06	0.333±0.048	3.66
NADH	0.41±0.01	0.00040±0.00004	1.0×10^3

Kinetic constant measurements All the reactions followed Michaelis–Menten type kinetics under the appropriate experimental conditions. The Michaelis constant (K_m) and catalytic turnover (k_{cat}) were determined for each substrate summarized in Tables 1 and 2 with 90 μM NAD^+ or 80 μM NADH depending on the type of reaction studied. The k_{cat} and K_m values for NAD^+ and NADH were determined using 3.8 mM 2-pentanol and 10 mM 2-pentanone as the substrate, respectively. Other conditions were same as in the enzyme assay section.

Determination of enantiomeric excess The reduction of aliphatic ketones was conducted with cofactor regeneration at 60°C for 24 h. The reaction mixture contained 60 nmol NADH, 30 μmol ketone, 300 μmol cyclohexanol (for NADH regeneration) and 0.6 nmol purified apADH in 3 ml of 100 mM potassium phosphate buffer (pH 8.0). Chiral gas chromatography equipped with a flame ionization detector was used to determine the enantiomeric excess. All the samples were extracted with CH_2Cl_2 and were derivatized with trifluoroacetic anhydride. An aliquot (approximately 1 μl) was applied on a CHIRALDEX G-TA column (25 m×0.25 mm I.D.; Advanced Separation Technologies, Whippany, NJ, USA).

RESULTS

Enzyme expression and purification The recombinant apADH (ADH from *A. pernix* K1) was successfully expressed in *E. coli* without induction. The purified apADH gave a single band on SDS–PAGE. The molecular mass of apADH calculated from the gene sequence was 39.57 kDa and that obtained in the SDS–PAGE analysis was 40 kDa.

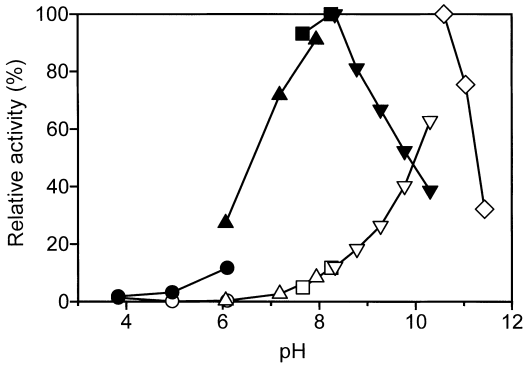

FIG. 1. pH dependence of the relative activities of apADH-catalyzed oxidation of 2-pentanol (open symbols) and reduction of 2-pentanone (closed symbols). The buffers used were citrate–KOH (circles), phosphate–KOH (triangles), glycylglycine–KOH (squares), glycine–KOH (inverted triangles), and phosphate–KOH (diamonds). Conditions are given in the text.

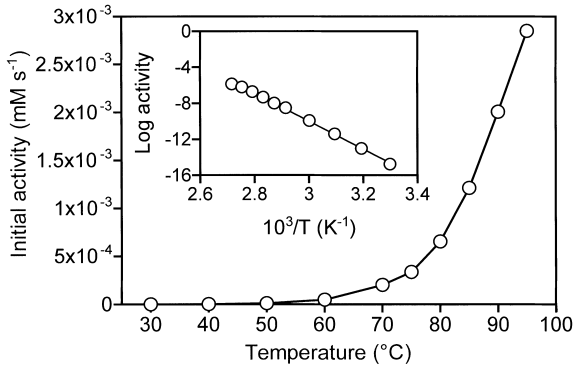

FIG. 2. Temperature-dependence of the initial rate of apADH. The initial rate was measured in 100 mM potassium phosphate buffer (pH 8.0) containing 0.09 mM NAD^+ and 3.8 mM 2-pentanol. The inset shows the Arrhenius plot of the same data.

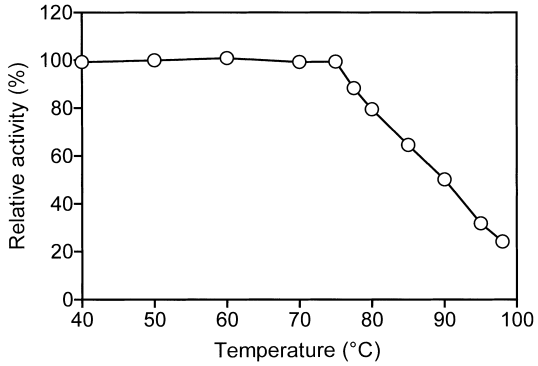

FIG. 3. Thermal denaturation of apADH monitored by the relative residual activity after incubation at each temperature for 30 min.

The molecular mass of the native apADH was estimated as 1.6×10^2 kDa by gel filtration chromatography (Superdex 200 HR 10/30), suggesting a tetrameric structure in aqueous solution.

Effect of pH on apADH activity The effect of pH on the initial reaction rates of apADH was investigated for the oxidation of 2-pentanol and the reduction of 2-pentanone (Fig. 1). The optimal pH for the oxidation was around pH 10.5, while that for the reduction was around pH 8.0. The initial rate of the oxidation was about 18-fold faster than that of the reduction measured in buffers at the respective pH optimums.

Thermal activity and stability of apADH The effect of temperature on the activity of apADH is shown in Fig. 2. The reaction rate increased up to 95°C. An Arrhenius plot showed no obvious transition point between 30°C and 95°C. The activation energy for oxidation of 2-pentanol was calculated to be 127 kJ mol^{-1}.

The thermal denaturation of apADH was monitored by the activity after incubation for 30 min at different temperatures (Fig. 3). The activity was completely maintained up to 75°C, after which its activity gradually decreased.

Substrate specificity of apADH The substrate specificity of apADH in the oxidative reaction was studied using a range of alcohols, including aliphatic, cyclic, and aromatic alcohols (Table 1). For aliphatic linear chain alcohols, a broad range of primary alcohols were oxidized by apADH. The K_m values decreased as the alkyl chain became longer. Similarly, in secondary alcohols, apADH preferred alcohols with long alkyl chains. The highest k_{cat} was found with 2-pentanol (0.60 s^{-1}). For aromatic alcohols, the K_m values became smaller as the alkyl chain length increased. Therefore, it appeared that alcohols with long chains were preferable substrates.

The substrate specificity of apADH in the reductive reaction was examined using a range of ketones including aliphatic, cyclic, and aromatic ketones, and benzylaldehyde (Table 2). For aliphatic ketones, the K_m values decreased as the alkyl chain became longer. The highest k_{cat} was found with 2-hexanone (1.08 s^{-1}). Therefore, it seemed that aromatic ketones were not good substrates for apADH. For example, it was hard to quantify the reduction rate of acetophenone due to the very small substrate conversion.

Enantioselectivity Table 3 shows the enantioselectivity of apADH for various aliphatic ketones. This enzyme preferably reduced aliphatic ketone to (*S*)-alcohol. The values of the enantiomeric excess increased with the increase of chain length except for the reduction of 2-hexanone. The highest enantioselectivity was showed with the reduction of 2-nonanone.

TABLE 3. Enantiomeric excess (ee) for reduction of aliphatic ketones

Substrate	Product	ee (%)
2-Pentanone	(*S*)-2-Pentanol	60
2-Hexanone	(*S*)-2-Hexanol	37
2-Heptanone	(*S*)-2-Heptanol	79
2-Octanone	(*S*)-2-Octanol	92
2-Nonanone	(*S*)-2-Nonanol	95
2-Decanone	(*S*)-2-Decanol	92

DISCUSSION

In thermophilic archaea, several kinds of ADHs have been discovered. The ADH from *Pyrococcus furiosus* (11) is a short-chain ADH, while those from *Thermococcus litoralis*

(12), *Thermococcus* strain ES-1 (13), *Thermococcus hydrothermalis* (14), *Thermococcus* strain AN1 (20), and *Pyrococcus furiosus* (21) are long-chain ADHs. Medium-chain ADHs have been discovered in *Sulfolobus solfataricus* (15, 22) and *Sulfolobus* strain RC3 (23). apADH (ADH from *A. pernix* K1) is a medium-chain alcohol dehydrogenase. This enzyme is a homotetramer with a molecular mass of 1.6×10^2 kDa, while the *S. solfataricus* ADH was found to be a homodimer with a molecular mass of approximately 70 kDa (15).

Similar to many ADHs, the optimum pH for the oxidation reaction was higher than that for the reduction reaction (8, 12, 14, 16, 24, 25). In the oxidation reaction, the pH profile showed a narrow peak in the alkaline region and less than 20% of its maximum activity below pH 9.0. Similar results were found for the NADPH-dependent long-chain ADH from *T. hydrothermalis* (14). In the reductive reaction, apADH showed catalytic activity in a broader pH range compared to the oxidative reaction. It showed more than 20% of its maximum activity between pH 6.0 and 10.2.

apADH was thermophilic and thermostable. Similar to *S. solfataricus* ADH, which has been known as the most thermostable medium-chain alcohol dehydrogenase (15) identified so far, the initial activity of apADH increased up to 95°C. However, apADH is more stable than *S. solfataricus* ADH. apADH maintained 24% of the initial activity after incubation for 30 min at 98°C, while *S. solfataricus* ADH lost 90% of the original activity after incubation for 30 min at 95°C (22). Guy *et al*. also examined the thermostability of apADH and reported that this enzyme had a half-life time for activity of over 2 h at 90°C (19), while that of 30 min at 90°C was observed in our study. This inconsistency may be caused by the difference in experimental conditions, however, which were not shown at all in the previous report. Consequently, apADH is now the most thermostable medium-chain alcohol dehydrogenase reported to date.

apADH shows broad substrate specificity and prefers aliphatic alcohols and ketones. Concerning the apADH preference for alcohols, there were no large differences in the reactivities between primary and secondary alcohols. The K_m values decreased with longer chains, and the higher values for the k_{cat}/K_m ratio were obtained for 1-hexanol and 2-nonanone. apADH prefers long-chain aliphatic alcohols and ketones. As for other ADHs from thermophilic bacteria, *P. furiosus* ADH and *T. brockii* ADH prefer secondary alcohols than primary alcohols (11, 26), while *T. litoralis* ADH and *S. solfataricus* ADH prefer primary alcohols (12, 15). The highest catalytic activities of *P. furiosus* ADH, *T. litoralis* ADH, and *T. brockii* ADH were found with C_4, C_6, and C_5 alcohols, respectively (11, 12, 26).

The k_{cat} values for aromatic ketones were small, while those for aromatic alcohols were larger than those for aliphatic alcohols. Acetophenone, in particular, was not estimated due to the low reaction rate, while benzaldehyde, which has the acetophenone structure without the methyl group, was a preferable substrate.

apADH reduced aliphatic C_8–C_{10} ketones to (*S*)-alcohols with high enantioselectivity. The enantioselectivity of *T. brockii* ADH is explained by 'two-site' model with a large and a small binding pockets (9). This model cannot explain that the enantioselectivity for 2-hexanone was lower than that for 2-pentanone, while the high enantiomeric excess of long-chain ketones seems to fit this model. Since cyclohexanol is one of good substrates, a large and a small binding pockets of apADH might not be definitely separated.

No ADHs from hyperthermophilic organisms that show enantioselectivity have been reported to date. Some ADHs from thermophilic bacteria, which are less stable than ADHs from hyperthermophilic organisms, showed high enantioselectivity (9, 27, 28). However, these ADHs are dependent on NADP(H) that is rather expensive than NAD(H). Valuable properties of more inexpensive cofactor NAD(H)-dependent apADH such as thermal stability, reversibility, broad substrate specificity and high enantioselectivity will make this enzyme one of potential biocatalysts for industrial chiral aliphatic alcohol syntheses.

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