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Characterization of a Thermostable Short-Chain Alcohol Dehydrogenase from the Hyperthermophilic Archaeon *Thermococcus sibiricus*▽

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Short-chain alcohol dehydrogenase, encoded by the gene Tsib_0319 from the hyperthermophilic archaeon *Thermococcus sibiricus*, was expressed in *Escherichia coli*, purified and characterized as an NADPH-dependent enantioselective oxidoreductase with broad substrate specificity. The enzyme exhibits extremely high thermophilicity, thermostability, and tolerance to organic solvents and salts.

Alcohol dehydrogenases (ADHs; EC 1.1.1.1.) catalyze the interconversion of alcohols to their corresponding aldehydes or ketones by using different redox-mediating cofactors. NAD(P)-dependent ADHs, due to their broad substrate specificity and enantioselectivity, have attracted particular attention as catalysts in industrial processes (5). However, mesophilic ADHs are unstable at high temperatures, sensitive to organic solvents, and often lose activity during immobilization. In this relation, there is a considerable interest in ADHs from extremophilic microorganisms; among them, *Archaea* are of great interest. The representatives of all groups of NAD(P)-dependent ADHs have been detected in genomes of *Archaea* (11, 12); however, only a few enzymes have been characterized, and the great majority of them belong to medium-chain (3, 4, 14, 16, 19) or long-chain iron-activated ADHs (1, 8, 9). Up to now, a single short-chain archaeal ADH from *Pyrococcus furiosus* (10, 18) and only one archaeal aldo-keto reductase also from *P. furiosus* (11) have been characterized.

Thermococcus sibiricus is a hyperthermophilic anaerobic archaeon isolated from a high-temperature oil reservoir capable of growth on complex organic substrates (15). The complete genome sequence of *T. sibiricus* has been recently determined and annotated (13). Several ADHs are encoded by the *T. sibiricus* genome, including three short-chain ADHs (Tsib_0319, Tsib_0703, and Tsib_1998) (13). In this report, we describe the cloning and expression of the Tsib_0319 gene from *T. sibiricus* and the purification and the biochemical characterization of its product, the thermostable short-chain ADH (TsAdh319).

The Tsib_0319 gene encodes a protein with a size of 234 amino acids and the calculated molecular mass of 26.2 kDa. TsAdh319 has an 85% degree of sequence identity with short-chain ADH from *P. furiosus* (AdhA; PF_0074) (18). Besides AdhA, close homologs of TsAdh319 were found among different bacterial ADHs, but not archaeal ADHs. The gene flanked by the XhoI and BamHI sites was PCR amplified using two primers (sense primer, 5′-GTTCTCGAGATGAAGGTTGCTG TGATAACAGGG-3′, and antisense primer, 5′-GCTGGATCC TCAGTATTCTGGTCTCTGGTAGACGG-3′) and cloned into the pET-15b vector. TsAdh319 was overexpressed, with an N-terminal His_6 tag in *Escherichia coli* Rosetta-gami (DE3) and purified to homogeneity by metallochelating chromatography (Hi-Trap chelating HP column; GE Healthcare) followed by gel filtration on Superdex 200 10/300 GL column (GE Healthcare) equilibrated in 50 mM Tris-HCl (pH 7.5) with 200 mM NaCl. The homogeneity and the correspondence to the calculated molecular mass of 28.7 kDa were verified by SDS-PAGE (7). The molecular mass of native TsAdh319 was 56 to 60 kDa, which confirmed the dimeric structure in solution.

The standard ADH activity measurement was made spectrophotometrically at the optimal pH by following either the reduction of NADP (in 50 mM Gly-NaOH buffer; pH 10.5) or the oxidation of NADPH (in 0.1 M sodium phosphate buffer; pH 7.5) at 340 nm at 60°C. The enzyme exhibited a strong preference for NADP(H) and broad substrate specificity (Table 1). The highest oxidation rates were found with pentoses D-arabinose (2.0 U mg^{-1}) and D-xylose (2.46 U mg^{-1}), and the highest reduction rates were found with dimethylglyoxal (5.9 U mg^{-1}) and pyruvaldehyde (2.2 U mg^{-1}). The enzyme did not reduce sugars which were good substrates for the oxidation reaction. The kinetic parameters of TsAdh319 determined for the preferred substrates are shown in Table 2. The enantioselectivity of the enzyme was estimated by measuring the conversion rates of 2-butanol enantiomers. TsAdh319 showed an evident preference, >2-fold, for (*S*)-2-butanol over (*RS*)-2-butanol. The enzyme stereoselectivity is confirmed by the preferred oxidation of D-arabinose over L-arabinose (Table 1). The fact that TsAdh319 is metal independent was supported by the absence of a significant effect of TsAdh319 preincubation with 10 mM Me^{2+} for 30 min before measuring the activity in the presence of 1 mM Me^{2+} or EDTA (Table 3). TsAdh319 also exhibited a halophilic property, so the enzyme activity increased in the presence of NaCl and KCl and the activation

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TABLE 1. Substrate specificity of TsAdh319

Substrate[a]	Relative activity (%)
Oxidation reaction[b]	
Methanol	0
2-Methoxyethanol	0
Ethanol	36
1-Butanol	80
2-Propanol	100
(*RS*)-(±)-2-Butanol	86
(*S*)-(+)-2-Butanol	196
2-Pentanol	67
1-Phenylmethanol	180
1.3-Butanediol	91
Ethyleneglycol	0
Glycerol	16
D-Arabinose*	200
L-Arabinose*	17
D-Xylose*	246
D-Ribose*	35
D-Glucose*	146
D-Mannose*	48
D-Galactose*	0
Cellobiose*	71
Reduction reaction[c]	
Pyruvaldehyde	100
Dimethylglyoxal	270
Glyoxylic acid	36
Acetone	0
Cyclopentanone	0
Cyclohexanone	4
3-Methyl-2-pentanone*	13
D-Arabinose*	0
D-Xylose*	0
D-Glucose*	0
Cellobiose*	0

[a] Substrates were present in 250 mM or 50 mM (*) concentrations.
[b] Relative rates, measured under standard conditions, were calculated by defining the activity for 2-propanol as 100%, which corresponds to 1.0 U mg^{-1}. Data are averages from triplicate experiments.
[c] Relative rates, measured under standard conditions, were calculated by defining the activity for pyruvaldehyde as 100%, which corresponds to 2.2 U mg^{-1}. Data are averages from triplicate experiments.

was maintained even at concentration of 4 M and 3 M, respectively (Table 3).

The most essential distinctions of TsAdh319 are the thermophilicity and high thermostability of the enzyme. The optimum temperature for the 2-propanol oxidation catalyzed by TsAdh319 was not achieved. The initial reaction rate of oxidation increased up to 100°C (Fig. 1). The Arrhenius plot is a straight line, typical of a single rate-limited thermally activated

TABLE 2. Apparent K_m and V_{max} values for TsAdh319

Coenzyme or substrate	Apparent K_m (mM)	V_{max} (U mg^{-1})	k_{cat} (s^{-1})
NADP[a]	0.022 ± 0.002	0.94 ± 0.02	0.45 ± 0.01
NADPH[b]	0.020 ± 0.003	3.16 ± 0.11	1.51 ± 0.05
2-Propanol	168 ± 29	1.10 ± 0.09	0.53 ± 0.04
D-Xylose	54.4 ± 7.4	1.47 ± 0.09	0.70 ± 0.04
Pyruvaldehyde	17.75 ± 3.38	4.26 ± 0.40	2.04 ± 0.19

[a] Activity was measured under standard conditions with 2-propanol. Data are averages from triplicate experiments.
[b] Activity was measured under standard conditions with pyruvaldehyde. Data are averages from triplicate experiments.

TABLE 3. Effect of various ions and EDTA on TsAdh319[a]

Compound	Concn (mM)	Relative activity (%)
None	0	100
NaCl	400	206
	600	227
	4,000	230
KCl	600	147
	2,000	200
	3,000	194
$MgCl_2$	10	78
$CoCl_2$	10	105
$NiSO_4$	10	100
$ZnSO_4$	10	79
$FeSO_4$	10	74
EDTA	1	100
	5	80

[a] The activity was measured under standard conditions with 2-propanol; relative rates were calculated by defining the activity without salts as 100%, which corresponds to 0.9 U mg^{-1}. Data are averages from duplicate experiments.

process, but there is no obvious transition point due to the temperature-dependent conformational changes of the protein molecule. The activation energy for the oxidation of 2-propanol was estimated at 84.0 ± 5.8 kJ · mol^{-1}. The thermostability of TsAdh319 was calculated from residual TsAdh319 activity after preincubation of 0.4 mg/ml enzyme solution in 50 mM Tris-HCl buffer (pH 7.5) containing 200 mM NaCl at 70, 80, 90, or 100°C. The preincubation at 70°C or 80°C for 1.5 h did not cause a decrease in the TsAdh319 activity, but provoked slight activation. The residual TsAdh319 activities began to decrease after 2 h of preincubation at 70°C or 80°C and were 10% and 15% down from the control, respectively. The determined half-life values of TsAdh319 were 2 h at 90°C and 1 h at 100°C.

Protein thermostability often correlates with such important biotechnological properties as increased solvent tolerance (2). We tested the influence of organic solvents at a high concen-

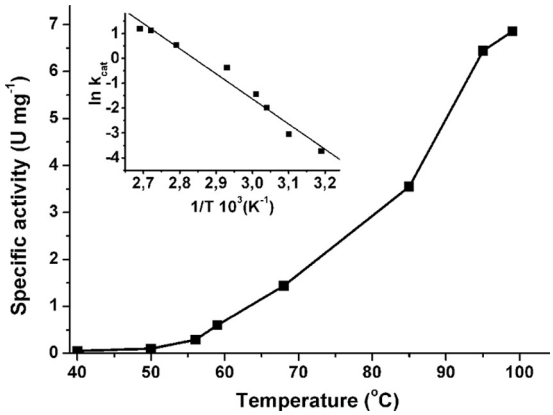

FIG. 1. Temperature dependence of the initial rate of the 2-propanol reduction by TsAdh319. The reaction was initiated by enzyme addition to a prewarmed 2-propanol–NADP mixture. The inset shows the Arrhenius plot of the same data.

TABLE 4. Influence of various solvents on TsAdh319 activity[a]

Solvent	Relative activity (%)[b]	Relative activity (%)[c]	
		Buffer without NaCl	Buffer with 600 mM NaCl
None	100	100	100
DMSO[d]	98	0	40
DMFA[e]	101	13	41
Methanol	98	25	9
Acetonitrile	95	0	0
Ethyl acetate	47	0*	33*
Chloroform	105	79*	81*
n-Hexane	105	60*	118*
n-Decane	36	91*	107*

[a] The activity measured at the standard condition with 2-propanol as a substrate. Data are averages from triplicate experiments.
[b] Preincubation for 4 h at 55°C in the presence of 50% (vol/vol) of solvent prior the activity assay.
[c] Without preincubation, solvent addition to the reaction mixture up to 50% (vol/vol) or using the buffer saturated by a solvent (*).
[d] DMSO, dimethyl sulfoxide.
[e] DMFA, dimethylformamide.

tration (50% [vol/vol]) on TsAdh319 by using either preincubation of the enzyme at a concentration of 0.2 mg/ml with solvents for 4 h at 55°C or solvent addition into the reaction mixture to distinguish the effect of solvent on the protein stability and on the enzyme activity. TsAdh319 showed significant solvent tolerance in both cases (Table 4), and the effects of solvents could be modulated by salts, acting apparently as molecular lyoprotectants (17). Furthermore, TsAdh319 maintained 57% of its activity in 25% (vol/vol) 2-propanol, which could be used as the cosubstrate in cofactor regeneration (6).

From all the aforesaid we may suppose TsAdh319 or its improved variant to be interesting both for the investigation of structural features of protein tolerance and for biotechnological applications.

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