

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

Tatiana N. Stekhanova,^{1*} Andrey V. Mardanov,² Ekaterina Y. Bezsudnova,¹ Vadim M. Gumerov,² Nikolai V. Ravin,² Konstantin G. Skryabin,² and Vladimir O. Popov¹

Bach Institute of Biochemistry Russian Academy of Sciences, Leninsky pr. 33, Moscow 119071, Russia,¹ and Centre Bioengineering, Russian Academy of Sciences, Prosp. 60-let Oktyabrya, Bld. 7-1, Moscow 117312, Russia²

Received 19 November 2009/Accepted 12 April 2010

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 thermostability, 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 differ-

ent bacterial ADHs, but not archaeal ADHs. The gene flanked by the XhoI and BamHI sites was PCR amplified using two primers (sense primer, 5'-GTTCTCGAGATGAAGGTTGCTGTGATAACAGGG-3', and antisense primer, 5'-GCTGGATCC TCAGTATTCTGGTCTCTGGTAGACGG-3') and cloned into the pET-15b vector. TsAdh319 was overexpressed, with an N-terminal His₆ tag in *Escherichia coli* Rosetta-gami (DE3) and purified to homogeneity by metalchelating 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 (R)-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 Me²⁺ for 30 min before measuring the activity in the presence of 1 mM Me²⁺ 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

* Corresponding author. Mailing address: Bach Institute of Biochemistry, RAS, Leninsky pr. 33, Moscow 119071, Russia. Phone: (7) 495 9545283. Fax: (7) 495 9542732. E-mail: tstekh@yandex.ru.

[∇] Published ahead of print on 23 April 2010.

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
(<i>RS</i>)-(\pm)-2-Butanol.....	86
(<i>S</i>)-(+)-2-Butanol.....	196
2-Pentanol.....	67
1-Phenylmethanol.....	180
1,3-Butanediol.....	91
Ethylene glycol.....	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
	10	105
	10	100
	10	79
	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-

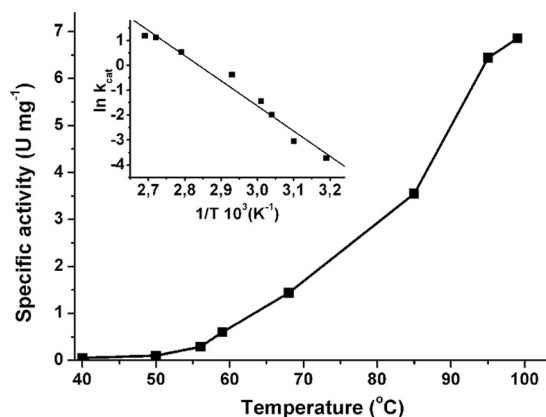


FIG. 1. Temperature dependence of the initial rate of the 2-propanol reduction by TsAdh319. The reaction was initiated by enzyme addition to a prewarmed 2-propanol–NADP mixture. The inset shows the Arrhenius plot of the same data.

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*
<i>n</i> -Hexane	105	60*	118*
<i>n</i> -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 to 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.

This work was supported by the Federal Agency for Science and Innovations of Russia (contract 02.512.12.2001).

REFERENCES

- Antoine, E., J.-R. Rolland, and J. Dietrich. 1999. Cloning and over-expression in *Escherichia coli* of the gene encoding NADPH group III alcohol dehydrogenase from *Thermococcus hydrothermalis*. *Eur. J. Biochem.* **264**: 880–889.
- Daniel, R., M. Danson, D. Hough, C. Lee, M. Peterson, and D. Cowan. 2007. Enzyme stability and activity at high temperatures, p. 1–35. In K. S. Siddiqui and T. Thomas (ed.), *Protein adaptation in extremophiles*. Nova Biomedical Books, New York, NY.
- Giordano, A., F. Febbraio, C. Russo, M. Rossi, and C. A. Raia. 2005. Evidence for co-operativity in coenzyme binding to tetrameric *Sulfolobus solfataricus* alcohol dehydrogenase and its structural basis: fluorescence, kinetic and structural studies of the wild-type enzyme and non-cooperative N249Y mutant. *Biochem. J.* **388**:657–667.
- Hess, M., and G. Antranikian. 2008. Archaeal alcohol dehydrogenase active at increased temperatures and in the presence of organic solvents. *Appl. Microbiol. Biotechnol.* **77**:1003–1013.
- Honda, K., T. Ishige, M. Kataoka, and S. Shimizu. 2006. Microbial and enzymatic processes for the production of chiral compounds, p. 535–542. In R. N. Patel (ed.), *Biocatalysis in the pharmaceutical and biotechnology industries*. CRC Press, Boca Raton, FL.
- Klibanov, A. 2001. Improving enzymes by using them in organic solvents. *Nature* **409**:241–246.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
- Li, D., and K. J. Stevenson. 1997. Purification and sequence analysis of a novel NADP(H)-dependent type III alcohol dehydrogenase from *Thermococcus* strain AN1. *J. Bacteriol.* **179**:4433–4437.
- Ma, K., F. T. Robb, and M. W. W. Adams. 1994. Purification and characterization of NADP-specific alcohol dehydrogenase from the hyperthermophilic archaeon *Thermococcus litoralis*. *Appl. Environ. Microbiol.* **60**:562–568.
- Machielsen, R., N. G. H. Leferink, A. Hendriks, S. J. J. Brouns, H.-G. Hennemann, T. Daußmann, and J. van der Oost. 2008. Laboratory evolution of *Pyrococcus furiosus* alcohol dehydrogenase to improve the production of (2S,5S)-hexanediol at moderate temperatures. *Extremophiles* **12**:587–594.
- Machielsen, R., A. R. Uria, S. W. M. Kengen, and J. van der Oost. 2006. Production and characterization of a thermostable alcohol dehydrogenase that belongs to the aldo-keto reductase superfamily. *Appl. Environ. Microbiol.* **72**:233–238.
- Machielsen, R., C. H. Verhees, B. E. Dutilh, M. A. Huynen, W. M. De Vos, and J. van der Oost. 2002. Distribution of alcohol dehydrogenases in *Pyrococcus furiosus*, p. 229. In M. Rossi, S. Bartolucci, M. Ciaramella, and M. Morracchi (ed.), *Extremophiles. Proceedings of the 4th International Congress on Extremophiles*. Abstract Books, Naples, Italy.
- Mardanov, A. V., N. V. Ravin, V. A. Svetlitchnyi, A. V. Beletsky, M. L. Miroshnichenko, E. A. Bonch-Osmolovskaya, and K. G. Skryabin. 2009. Metabolic versatility and indigenous origin of the archaeon *Thermococcus sibiricus*, isolated from a Siberian oil reservoir, as revealed by genome analysis. *Appl. Environ. Microbiol.* **75**:4580–4588.
- Marino-Marmolejo, E., A. De Leon-Rodriguez, A. P. B. De la Rosa, and L. Santos. 2009. Heterologous expression and characterization of an alcohol dehydrogenase from the archaeon *Thermoplasma acidophilum*. *Mol. Biotechnol.* **42**:61–67.
- Miroshnichenko, M. L., H. Hippe, E. Stackebrandt, N. A. Kostrikina, N. A. Chernyh, C. Jeanthon, T. N. Nazina, S. S. Belyaev, and E. A. Bonch-Osmolovskaya. 2001. Isolation and characterization of *Thermococcus sibiricus* sp. nov. from a Western Siberia high-temperature oil reservoir. *Extremophiles* **5**:85–91.
- Raia, C. A., A. Giordano, and M. Rossi. 2001. Alcohol dehydrogenase from *Sulfolobus solfataricus*. *Methods Enzymol.* **331**:176–195.
- Serdakowski, A., and J. Dordick. 2008. Enzyme activation for organic solvents made easy. *Trends Biotechnol.* **26**:48–54.
- van der Oost, J., W. G. B. Voorhorst, S. W. M. Kengen, A. C. M. Geertling, V. Wittenhorst, Y. Gueguen, and W. M. de Vos. 2001. Genetic and biochemical characterization of a short-chain alcohol dehydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Eur. J. Biochem.* **268**:3062–3068.
- Yanai, H., K. Doi, and T. Ohshima. 2009. *Sulfolobus tokodaii* ST0053 produces a novel thermostable, NAD-dependent medium chain alcohol dehydrogenase. *Appl. Environ. Microbiol.* **75**:1758–1763.