Sulfolobus tokodaii ST0053 Produces a Novel Thermostable, NAD-Dependent Medium-Chain Alcohol Dehydrogenase[▽]

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An alcohol dehydrogenase (ADH) gene, ST0053, from *Sulfolobus tokodaii* was expressed in *Escherichia coli*. The purified recombinant enzyme was an NAD-dependent medium-chain ADH with high thermostability and tolerance of a wide range of pHs. This is the first step in creating an experimental functionality library of 10 genes annotated as ADHs in the *S. tokodaii* genome.

NAD(P)-dependent alcohol dehydrogenases (ADHs; EC 1.1.1.1 and EC 1.1.1.2) catalyze the reversible oxidation of alcohols to their corresponding aldehydes or ketones. Many kinds of ADHs have been identified in a variety of microorganisms and are utilized for the production and detection of alcohols, aldehydes, and ketones (1, 11). Particularly useful are ADHs from thermophiles and hyperthermophiles, which are often more stable than their counterparts from mesophiles and psychrophiles (6, 7). These thermostable enzymes provide us with long-term functionality and novel reaction systems, such as the enzymatic vapor reaction under high temperature. Ten different genes have been annotated as NAD(P)-dependent ADHs in the genome of the hyperthermophilic and acidophilic archaeon *Sulfolobus tokodaii* strain 7 (13). We have been interested in compiling the structural and functional characteristics of their products into a library. Such a library would provide much useful information that could facilitate their application. We here describe the expression of ST0053, one of the 10 ADH genes in the *S. tokodaii* genome, and the subsequent purification and characteristics of its product.

Cloning and expression of ST0053. A hybrid DNA plasmid, pET11a/ST0053, whose ST0053 open reading frame was amplified by PCR and inserted into the NdeI-BamHI site of pET11a (Novagen), was provided by Seiki Kuramitsu (Department of Biology, Graduate School of Science, Osaka University, Osaka, Japan). The plasmid was transformed into *E. coli* strain Rosetta(DE3), after which the transformants were cultured for 16 h in 10 ml of Luria-Bertani medium containing ampicillin ($50 \mu\text{g ml}^{-1}$) at 37°C with shaking at 120 rpm. This culture medium was then added to 1 liter of Luria-Bertani medium containing the same amount of antibiotic in a 2-liter Erlenmeyer flask and incubated at 37°C with shaking at 220 rpm. Once the optical density at 660 nm reached 0.5, expression was induced by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG; 1 mM final concentration) and cultivation was continued at 37°C for an additional 4 h. The culture was harvested by centrifugation at $10,000 \times g$ for 10 min at 4°C , the

cells were washed with 0.85% NaCl and pelleted, and then the cell pellet was stored at -80°C .

Purification. To purify ADH, the stored cells (wet weight, about 2.55 g) were suspended in 10.2 ml of 20 mM Tris-HCl buffer (pH 8.0) containing 0.01% 2-mercaptoethanol (2-ME) (buffer A) and then disrupted by ultrasonication on ice (Tomy Ultrasonic Disruptor UD-201 set at an output level of 2 and a 50% duty cycle; three sonications for 3 min each with 5-min intervals). The resultant homogenate was centrifuged at $10,000 \times g$ for 15 min at 4°C , after which the supernatant was incubated at 70°C for 40 min, and the precipitate was removed by centrifugation at $10,000 \times g$ for 15 min at 4°C . Solid ammonium sulfate was then added to the supernatant up to 20% saturation, and the solution was loaded onto a Butyl Sepharose column (2.5 by 5.0 cm) previously equilibrated with 150 ml of buffer A containing 20% saturated ammonium sulfate. After the column was washed with 300 ml of the same buffer at room temperature, the ST0053 product was eluted with a linear gradient of 20% to 0% saturated ammonium sulfate in buffer A (120 ml) at room temperature. The column elution was monitored for protein at 280 nm and for ADH activity. The active fractions were collected, and the solution was concentrated by the addition of solid ammonium sulfate to 80% saturation. After centrifugation at $10,000 \times g$ for 15 min at 4°C , the precipitate was collected and dissolved in 2 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 0.01% 2-ME. This enzyme solution was dialyzed against 1,000 ml of the same buffer (three time changes, 5 h-5 h-overnight) at 4°C , and the dialyzate was stored at 4°C until used as the purified enzyme.

Gel electrophoresis. Disc gel electrophoresis of the native enzyme was carried out with a 7.5% polyacrylamide gel by the method of Davis (5), after which ADH activity staining was carried out for 10 min at 50°C in the presence of a mixture of 100 mM Tris-HCl buffer (pH 8.0), 10 mM benzyl alcohol, 1.25 mM NAD, 1 mM ZnSO_4 , 0.1 mM 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride, and 0.04 mM 1-methoxy-5-methylphenazinium methylsulfate (24). In addition, the protein band was stained with Coomassie brilliant blue (CBB) R-250. About $10 \mu\text{g}$ of purified protein was used for each gel. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a 10% polyacrylamide gel according to the procedure of Laemmli (14).

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Commercially available molecular marker proteins (Bio-Rad Laboratories) served as standards. The protein band was stained with CBB.

Determination of molecular mass. The molecular mass of ST0053 product was estimated by the gel chromatography method with ÄKTA explorer (GE Healthcare). The column was run with Tris-HCl (20 mM, pH 7.5) containing 0.2 M NaCl at a flow rate of 3 ml min⁻¹. The standard proteins used for the calibration were as follows: bovine α -chymotrypsinogen A type II (Sigma-Aldrich), 25 kDa; hen egg ovalbumin, 44 kDa; chicken egg white conalbumin, 75 kDa; rabbit muscle aldolase, 158 kDa; horse spleen ferritin, 440 kDa (gel filtration calibration kit HMW; GE Healthcare England).

N-terminal amino acid sequencing. The ST0053 product was electroblotted (constant potential of 30 V for 15 min) from the SDS-PAGE gel onto a polyvinylidene difluoride membrane in 10 mM CAPS [(3-cyclohexylamine)propanesulfonic acid, pH 11] containing 10% methanol. The membrane was then washed with 10 mM borate buffer (pH 8.0) containing 25 mM NaCl, stained with Ponceau S, and washed with distilled water. Finally, the protein band was excised and subjected to automated Edman degradation with a PPSQ-10 Protein Sequencer (Shimadzu, Kyoto, Japan).

Enzyme assay and determination of protein concentration. ADH activity was assayed by measuring the change in absorbance of NADH ($\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) at 340 nm with a Beckman DU530 spectrophotometer with a temperature-controlled cuvette holder. The standard mixture for the oxidative reaction included 2 mM 1-hexanol, 100 mM Gly-KOH buffer (pH 10.5), 1 mM ZnSO₄, 1.25 mM NAD, and enzyme in a final volume of 1.0 ml. After the reaction mixture was incubated for 3 min at 50°C in a cuvette with a 1.0-cm light path, the reaction was started by adding NAD. One unit of ADH activity was defined as the amount catalyzing the formation of 1 μmol of NADH per min. Alternatively, the standard reaction mixture for the reductive reaction included 10 mM benzaldehyde, 100 mM morpholinepropanesulfonic acid (MOPS)-NaOH buffer (pH 7.0), 1 mM ZnSO₄, 300 μM NADH, and enzyme in a final volume of 1.0 ml. After the mixture was incubated for 3 min at 50°C in a cuvette with a 0.4-cm light path, the reaction was started by adding NADH. Protein concentrations were determined by the method of Bradford (3) with Bio-Rad reagents; bovine serum albumin served as the standard.

Effect of pH on ADH activity and stability. The effect of pH on the oxidation of alcohol was determined in a mixture of 10 mM 1-hexanol or 0.5 mM benzyl alcohol, 1 mM ZnSO₄, 1.25 mM NAD, and 100 mM buffer (Tris-HCl at pH 7.5 to 9.0 and Gly-KOH at pH 9.0 to 11.5) in a cuvette with a 1.0-cm light path. The effects of pH on the reduction of aldehydes were determined in a mixture of 10 mM benzaldehyde, 1 mM ZnSO₄, 300 μM NADH, and 100 mM buffer 2-(*N*-morpholino)ethanesulfonic acid (MES)-NaOH at pH 5.5 to 6.5 and MOPS-NaOH at pH 6.5 to 7.5 in a cuvette with a 0.4-cm light path. After the reaction mixture was incubated for 3 min at 50°C, the reaction was started by adding NAD or NADH. NADH was dissolved in 1 mM Tris-HCl (pH 8.0) to avoid its decomposition under acidic conditions. The effect of pH on enzyme stability was assessed by incubating the enzyme (about 1 mg ml⁻¹) in various 100 mM buffers for 30 min at 50°C. The buffers used were as follows: Gly-HCl (pH 2.5 to 3.5), sodium

TABLE 1. Purification of ST0053-ADH from recombinant *E. coli* cells^a

Step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Crude extract	167.0	24.7	0.15	100	1
Heat treatment	17.6	23.2	1.33	95	8.9
Butyl Sepharose ^b	6.8	9.6	1.41	39	9.4

^a Wet weight of cells, about 2.55 g.

^b The values were determined after concentration with ammonium sulfate.

acetate (pH 3.5 to 5.5), MES-NaOH (pH 5.5 to 6.5), MOPS-NaOH (pH 6.5 to 7.5), Tris-HCl (pH 7.5 to 9.0), Gly-KOH (pH 9.0 to 11.0), and phosphate-NaOH (pH 11.0 to 12.0). The mixture was then cooled on ice for 5 min, after which the residual activity in an aliquot of each mixture was assayed under the same experimental conditions used for the standard oxidative reaction. The pHs of all of the buffers used were adjusted at room temperature.

Effect of temperature on ADH activity and stability. The effect of temperature on enzyme activity was assayed in the standard oxidative reaction mixture at temperatures ranging from 30°C to 90°C. In order to avoid NAD degradation and enzyme inactivation under the conditions of higher temperature and higher pH, the reaction was started by the addition of the two solutions. The thermostability of the enzyme was examined by measuring the residual activity after incubating the enzyme (1 to 2 mg ml⁻¹) for 10 min in 10 mM Tris-HCl (pH 7.5) containing 0.01% 2-ME at temperatures ranging from 50°C to 95°C. The residual activity in an aliquot of the enzyme solution was then assayed by using the standard oxidative reaction.

Additive effect of various compounds on ADH activity. The effect of several salts, metals, and inhibitors (final concentration, 1 mM) on the enzyme activity was checked under the standard assay conditions for the oxidative reaction.

Steady-state kinetic constants. The apparent Michaelis constant (K_m) and maximum velocity (V_{max}) were determined for each substrate by Lineweaver-Burk plots. The assays for the forward and reverse reactions were carried out under the standard assay conditions, and the apparent K_m and V_{max} values for NAD and NADH were determined by using 0.5 mM benzyl alcohol and 3 mM benzaldehyde as the substrates, respectively.

According to DOGAN, a genome database of microorganisms sequenced at NITE (<http://www.bio.nite.go.jp/dogan/Top>) (13), 10 different genes in the *S. tokodaii* genome have been annotated as NAD(P)-ADH. We expressed one of those, ST0053, as a recombinant protein in *E. coli* and detected a high level of ADH activity in the cell extract prepared by sonication and heat treatment. The enzyme was then easily purified to homogeneity in a single column chromatography step (Table 1). Elution fractions containing ADH activity were collected from a Butyl Sepharose (GE Healthcare) column, after which the enzyme was concentrated as the precipitate by the addition of ammonium sulfate to 80% saturation. After dialysis, the purified ADH gave a single CBB band on SDS-PAGE (Fig. 1), and one band appeared upon ADH activity staining after non-denaturing polyacrylamide gel electrophoresis, which corresponded to the CBB-stained band.

FIG. 1. SDS-PAGE of purified ST0053-ADH (A) and a calibration curve for the determination of native molecular mass (B). The standard proteins used for the calibration curve were horse spleen ferritin (a; 440 kDa), rabbit muscle aldolase (b; 158 kDa), chicken egg white conalbumin (c; 75 kDa), hen egg ovalbumin (d; 44 kDa), and bovine α -chymotrypsinogen A type II (e; 25 kDa).

The N-terminal sequence of the purified enzyme was determined to be MKAML, which corresponds to that predicted from the nucleotide sequence of ST0053. Thus, ST0053 appears to encode an ADH (ST0053-ADH).

The molecular mass of ST0053-ADH calculated from the gene sequence was 37,477 Da (344 amino acid residues), which is consistent with the 38 kDa obtained in the SDS-PAGE analysis (Fig. 1A). Gel filtration chromatography (Superdex 200) revealed the molecular mass of the native form to be about 133 kDa (Fig. 1B), suggesting that the enzyme exists as a homotetramer. The subunit structure of ST0053-ADH is thus quite similar to those of the *S. solfataricus* DSM1617 ADH (38 and 140 kDa) (4) and the *Aeropyrum pernix* K1 ADH (40 and 160 kDa) (10).

NAD(P)-dependent ADHs have been generally divided into

the following three groups: (i) medium-chain zinc-dependent ADHs (about 350 amino acids per subunit), (ii) short-chain zinc-independent ADHs (about 250 amino acids per subunit), and (iii) long-chain iron-activated ADHs (about 385 amino acids per subunit) (12). From its chain length, ST0053-ADH appears to belong to the medium-chain zinc-dependent ADH group. To confirm that idea, we used ClustalW version 2.0 EBI (15) to examine amino acid sequence homology between ST0053-ADH and the other nine ADHs encoded in the *S. tokodaii* genome, as well as enzymes from various other organisms (Table 2). ST0053-ADH showed a high degree of sequence homology to medium-chain ADHs from other organisms, with the highest being 55% homology to known ADHs from *S. solfataricus* and *Sulfolobus* sp. strain RC3. In addition, the crystal structure of the *S. solfataricus* medium-chain ADH (8) shows the presence of two zinc atoms per subunit: a catalytic zinc atom bound by amino acid residues Cys38, His68, Cys154, and Glu69 and a structurally important zinc atom bound by Cys100, Cys103, Cys112, and Glu98. The sequence alignment showed that corresponding residues are conserved in the sequence of ST0053-ADH, suggesting that ST0053-ADH likely contains two zinc atoms. Judging from the number of amino acids, the sequence homology, and the conservation of amino acids in the active center, we suggest that ST0053-ADH should be classified as a medium-chain ADH similar to the ADH from *S. solfataricus*. However, the two ADHs from *S. solfataricus* and *Sulfolobus* sp. strain RC3 show higher sequence homology to the ST2577 product (89%) than to ST0053-ADH (55%) (Table 2). Thus, although ST0053-ADH belongs to the same medium-chain zinc-dependent ADH group, it is a novel thermostable ADH that is unlike those from *S. solfataricus* and *Sulfolobus* sp. strain RC3.

We determined the effect of pH on the initial reaction rates for oxidation catalyzed by ST0053-ADH by using 1-hexanol and for reduction by using benzaldehyde. The optimum pH for

TABLE 2. Comparison of primary structures of the products from 10 putative ADH genes annotated in the genome of *S. tokodaii* and medium-chain ADHs so far found and characterized

Enzyme ^a	% Amino acid sequence homology with:													
	ST0053	ST0038	ST0075	ST0480	ST0569	ST2056	ST2252	ST2577	ST2605	STS015	SSADH	RC3ADH	APADH	YADH
STADHs														
ST0053	100	37	26	29	37	28	29	55	33	37	55	55	40	26
ST0038		100	24	19	28	30	26	36	29	24	35	34	48	24
ST0075			100	31	30	27	25	27	24	18	27	26	26	23
ST0480				100	22	29	26	29	26	29	29	28	26	26
ST0569					100	31	26	33	23	31	32	32	38	22
ST2056						100	25	32	28	22	31	30	29	27
ST2252							100	28	54	22	25	26	27	22
ST2577								100	30	33	89	89	40	28
ST2605									100	12	28	29	27	26
STS015										100	37	35	57	29
mADHs														
SSADH											100	94	39	29
RC3ADH												100	40	30
APADH													100	27
YADH														100
HLADH														

^a STADHs, ADH genes annotated in the genome of *S. tokodaii*; mADHs, medium-chain ADHs; SSADH, *S. solfataricus* DSM 1617 (1JVB_A); RC3ADH, *Sulfolobus* sp. strain RC3; APADH, *A. pernix* K1 (APE_2239.1); YADH, *Saccharomyces cerevisiae* (P00330); HLADH, horse liver (1P1R_A).

FIG. 2. Effect of pH on the activity (A) and stability (B) of ST0053-ADH. The buffers used were Gly-HCl (Δ), sodium acetate ($\blacksquare$), MES-NaOH ($\diamond$), MOPS-NaOH ($\square$), Tris-HCl ($\blacktriangle$), Gly-KOH ($\bullet$), and phosphate-NaOH ($\circ$).

oxidation was around 10.5, while that for reduction was around 7.0 (Fig. 2A). Similar optimum pHs were observed when benzyl alcohol was used instead of 1-hexanol (data not shown). Although a similar pH profile is observed with *A. permix* ADH (10), the optimum pH (pH 10.5) for oxidation catalyzed by ST0053-ADH is more alkaline than that for the *S. solfataricus* ADH (around pH 9) (22).

To test the effect of pH on ST0053-ADH stability, we incubated the enzyme for 30 min at 50°C in various pH buffers and then measured the remaining activity. No loss of activity was observed at pHs between 4.0 and 10.0 (Fig. 2B), while about half of the activity remained at pHs 3.25 and 12. Thus, ST0053-ADH is fairly stable at both acidic and alkaline pHs.

When we examined the effect of temperature on enzyme

activity, we found that at pH 10.5 the enzyme exhibited maximal activity at 85°C (Fig. 3A), which is somewhat lower than those for similar medium-chain ADHs from *S. solfataricus* (pH 10.5, 93°C) and *A. permix* (pH 8, 95°C) (9, 10) but is near the optimum growth temperature for *S. tokodaii*. The thermostability of ST0053-ADH was then examined by evaluating the activity remaining after incubation for 10 min at various temperatures. No loss of activity was observed at temperatures of up to 70°C (Fig. 3B), and half of the activity remained after 120 min at 70°C (Fig. 3C). By contrast, the half-life of *S. solfataricus* ADH at 70°C is 5 h (23), while *A. permix* ADH shows no loss of activity after incubation for 30 min at 75°C (10). In addition, both the long-chain ADH from *Thermococcus* strain ES-1 (half-life at 85°C, 35 h) (19) and the short-chain ADH from

FIG. 3. Effect of temperature on the activity and stability of ST0053-ADH. A, activity determined at the indicated temperatures; B, thermal stability assayed after the enzyme was incubated at the indicated temperatures for 10 min; C, time-dependent changes in enzyme activity at 70°C.

TABLE 3. Kinetic constants of major substrates

Substrate	$V_{\max}$ ($\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$)	K_m (mM)	$V_{\max}/K_m$ ($\text{mg}^{-1} \cdot \text{min}^{-1} \cdot \text{ml}$)
Oxidative reaction			
Ethanol	1.34	9.08	0.148
1-Propanol	1.15	1.21	0.950
1-Butanol	1.41	0.129	10.9
1-Pentanol	1.18	0.00754	156
1-Hexanol	1.32	0.0594	22.2
2-Butanol	3.76	632	0.00595
2-Pentanol	0.482	17.1	0.0282
2-Hexanol	0.860	13.3	0.0647
Cyclohexanol	0.511	5.72	0.0893
Benzyl alcohol	2.09	0.0265	78.9
1,3-Propanediol	1.16	16.8	0.0690
NAD (0.5 mM benzyl alcohol)	2.05	0.0415	49.4
Reductive reaction			
Acetaldehyde	16.1	29.7	0.542
1-Propanal	20.0	7.61	2.63
1-Butanal	40.5	2.29	17.7
1-Pentanal	24.2	0.613	39.5
1-Hexanal	13.5	1.82	7.42
Benzaldehyde	59.9	0.766	78.2
NADH (3 mM benzaldehyde)	39.1	2.10	18.6

Pyrococcus furiosus DSM 3638 (half-life at 80°C, 150 h) (25) show somewhat greater thermostability than ST0053-ADH.

The substrate specificity of ST0053-ADH was examined by using various alcohols, ketones, and aldehydes in the presence of NAD or NADH for oxidation or reduction, respectively. For oxidation of alcohols, the enzyme showed broad substrate specificity, acting on many primary and secondary alcohols and benzyl alcohol (Table 3). The highest $V_{\max}$ was obtained with 2-butanol, but the catalytic efficiency ($V_{\max}/K_m$) was low because of the very high K_m value. High catalytic efficiencies ($V_{\max}/K_m$) were obtained with 1-pentanol, 1-hexanol, and benzyl alcohol (Table 3). Overall, the catalytic efficiencies ($V_{\max}/K_m$) obtained with primary alcohols were much higher than those obtained with secondary alcohols. Methanol, 1,2-propanediol, glycerol, D-(+)-glucose, and some hydroxyl amino acids (e.g., L-serine and L-threonine) were inert as electron donors.

For the reverse reaction, the enzyme catalyzed the reduction of various aldehydes other than formaldehyde in the presence of NADH, but not ketones such as acetone, 2-butanone, 2-pentanone, 2-hexanone, and cyclohexanone (Table 3). The $V_{\max}$ values for the reduction of aldehydes were much higher than those for the corresponding alcohol oxidation. By contrast, the K_m values for good substrates such as 1-pentanol, benzyl alcohol, and 1-hexanol were rather lower than those for the corresponding aldehydes. The highest catalytic efficiency for the reductive reaction was obtained with benzaldehyde. The $V_{\max}$ and K_m values for the reduction of benzaldehyde were both about 30 times higher than those for the oxidation of benzyl alcohol, giving almost the same catalytic efficiency for the two reactions. ST0053-ADH utilized NAD, but not NADP, as an electron acceptor for benzyl alcohol oxidation. In addition, NADH was a much more effective cofactor than NADPH for

TABLE 4. Additive effects of various compounds on ST0053-ADH activity

Compound ^a	Relative activity (%)
None	100
NiSO ₄	88
K ₂ SO ₄	98
CuSO ₄	78
MgSO ₄	88
ZnSO ₄	139
BaCl ₂	90
ZnCl ₂	134
CoSO ₄	149
CaCl ₂	91
SrCl ₂	93
PbCl ₂	85
HgCl ₂	0
NaCl	96
EDTA	73
1,10-Phenanthroline	88
Iodoacetic acid	53

^a The concentration of each compound used was 1 mM.

benzaldehyde reduction; the reaction rate with NADPH was only about one-fourth of that with NADH.

The broad substrate specificity for aliphatic and aromatic alcohols and aldehydes shown by ST0053-ADH is similar to the substrate profile of *S. solfataricus* ADH, but the low catalytic efficiency of ST0053-ADH for cyclohexanol is different from the high value seen with the *S. solfataricus* enzyme (21). In addition, *A. pernix* ADH (10) is reported to efficiently catalyze the reduction of ketones such as 2-pentanone, cyclohexanone, and 2-nonanone whereas ST0053-ADH cannot. ST0053-ADH also differs from long-chain ADHs from *Thermococcus* strains (2, 16, 18, 19), which efficiently catalyze the oxidation of primary alcohols such as 1-hexanol, 1-pentanol, and 1-butanol but not secondary alcohols such as 2-propanol. And the enzymes from a few *Thermococcus* strains specifically utilize NADP as an electron acceptor and do not use NAD. NAD-dependent ST0053-ADH is totally different from *Thermococcus* enzymes in this respect.

Another anaerobic hyperthermophilic archaeon, *P. furiosus*, reportedly expresses three different types of ADH, including an oxygen-sensitive, iron and zinc-containing enzyme (17), a short-chain enzyme (25), and an aldo-keto reductase superfamily enzyme (20). The substrate specificity of the oxygen-sensitive iron- and zinc-containing ADH is similar to that of ST0053-ADH, but the coenzyme specificity differs. The short-chain ADH and the aldo-keto superfamily enzyme differ from ST0053-ADH in that they exhibit strong activity toward secondary alcohols (e.g., 2,3-butanediol and acetoin) but not toward primary alcohols (e.g., 1-pentanol). These enzymes are also NADP dependent. Moreover, the subunit structures of the *P. furiosus* enzymes are different from that of ST0053-ADH.

ST0053-ADH was activated by ZnSO₄, ZnCl₂, or CoSO₄ (Table 4). The effect was concentration dependent, with maximum activation achieved at concentrations above 1 mM (ZnSO₄, 139%; ZnCl₂, 134%) or 3 mM (CoSO₄, 161%). Conversely, the enzyme was markedly inhibited by 1 mM CuSO₄, EDTA, or iodoacetate and completely blocked by 1 mM HgCl₂ (Table 4). Inhibition by iodoacetate and HgCl₂ suggests that a

thiol group(s) is directly or indirectly involved in the catalytic activity. Interestingly, *S. solfataricus* ADH is known to be activated by iodoacetate via modification of Cys38 (22), and the sequence alignment showed that the corresponding cysteine residue is conserved in ST0053-ADH (Cys38). The different effects of iodoacetate on the two ADHs may be attributable to a difference in the structure surrounding the residue. Although the details remain unclear, this suggests that the structure of the ST0053-ADH active site differs from that of *S. solfataricus* ADH, reflecting the difference in their amino acid sequences.

Conclusion. We have examined the function of ST0053-ADH, which is the product of one of 10 ADH genes annotated in the genome of the hyperthermophilic archaeon *S. tokodaii*. Among the ADHs characterized so far, the sequence of ST0053-ADH exhibited the greatest similarity (55%) to an ADH from *S. solfataricus*, although the *S. solfataricus* enzyme showed much greater sequence homology to the ST2577 product (89%). Moreover, we found that the substrate specificity of ST0053-ADH differs somewhat from that of *S. solfataricus* ADH, as do the effects of pH and iodoacetic acid on its activity. Taken together, these findings suggest that ST0053-ADH is a novel NAD-dependent medium-chain ADH. It is also noteworthy that this enzyme was effectively purified to homogeneity in a single column chromatography step and was fairly stable under a variety of conditions, including high temperature and a wide range of pHs. We are now investigating the structure of this enzyme along with the characteristics of other genes annotated as ADHs in the *S. tokodaii* genome with the aim of constructing a functionality library. We anticipate that such a library will facilitate better understanding of the different physiological functions of ADHs in *S. tokodaii* cells and further the development of these enzymes for industrial application.

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