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Production and Characterization of a Thermostable Alcohol Dehydrogenase That Belongs to the Aldo-Keto Reductase Superfamily

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The gene encoding a novel alcohol dehydrogenase that belongs to the aldo-keto reductase superfamily has been identified in the hyperthermophilic archaeon *Pyrococcus furiosus*. The gene, referred to as *adhD*, was functionally expressed in *Escherichia coli* and subsequently purified to homogeneity. The enzyme has a monomeric conformation with a molecular mass of 32 kDa. The catalytic activity of the enzyme increases up to 100°C, and a half-life value of 130 min at this temperature indicates its high thermostability. AdhD exhibits a broad substrate specificity with, in general, a preference for the reduction of ketones (pH optimum, 6.1) and the oxidation of secondary alcohols (pH optimum, 8.8). Maximal specific activities were detected with 2,3-butanediol (108.3 U/mg) and diacetyl-acetoin (22.5 U/mg) in the oxidative and reductive reactions, respectively. Gas chromatography analysis indicated that AdhD produced mainly (S)-2-pentanol (enantiomeric excess, 89%) when 2-pentanone was used as substrate. The physiological role of AdhD is discussed.

Alcohol dehydrogenases (ADHs) are present in all organisms. They display a wide variety of substrate specificities and play an important role in a broad range of physiological processes. However, it is often difficult to prove their physiological function (21). Alcohol dehydrogenases can be divided into different classes based on their cofactor specificity: (i) NAD(P), (ii) pyrrolo-quinoline quinone, heme, or cofactor F_{420}, and (iii) flavin adenine dinucleotide (FAD). The NAD- or NADP-dependent alcohol dehydrogenases are often subdivided into three major groups: the zinc-dependent ADHs, short-chain ADHs, and iron-activated ADHs (21). In addition, some of the NAD(P)-dependent dehydrogenases are related to yet another group, the aldo-keto reductase (AKR) superfamily (7, 10, 28). This enzyme superfamily consists of NAD(P)-dependent oxidoreductases, which share a common fold and are mostly monomeric proteins that bind nicotinamide cofactor without a Rossmann-fold motif (1, 14, 22, 33).

There is considerable interest in the use of stable alcohol dehydrogenases in the food, pharmaceutical, and fine chemicals industries for the production of aldehydes, ketones, and chiral alcohols. The production of chiral synthons is particularly desired because this is an increasingly important step in the synthesis of chirally pure pharmaceutical agents (3, 6, 20, 35). For certain applications, robust biocatalysts are desired. Enzymes from hyperthermophiles, i.e., microorganisms that grow optimally above 80°C, generally display an extreme stability at high temperature and high pressure, as well as high concentrations of chemical denaturants (32). These features make hyperthermophilic enzymes very interesting from both scientific and industrial perspectives.

The hyperthermophilic archaeon *Pyrococcus furiosus* grows optimally at 100°C by the fermentation of peptides and carbohydrates to produce acetate, CO_2, alanine, and H_2, together with minor amounts of ethanol. The organism will also generate H_2S if elemental sulfur is present (4, 12, 13). Three different alcohol dehydrogenases have previously been identified in *Pyrococcus furiosus*: a short-chain AdhA and an iron-containing AdhB encoded by the *lamA* operon (31) and an oxygen-sensitive, iron- and zinc-containing alcohol dehydrogenase that has been purified from cell extracts of *P. furiosus* (17). By careful analysis of the *P. furiosus* genome, 16 additional genes that potentially encode alcohol dehydrogenases have been identified (R. Machielsen, unpublished results).

The work reported here describes the functional production of one of the newly identified alcohol dehydrogenases, AdhD, in *Escherichia coli*. The enzyme was purified to homogeneity and characterized with respect to substrate specificity, kinetics, and stability. Since AdhD was found to exhibit high thermostability, high enantioselectivity, and a broad substrate specificity, it is an attractive candidate for industrial utilization.

MATERIALS AND METHODS

Chemicals and plasmids. All chemicals (analytical grade) were purchased from Sigma-Aldrich or Acros Organics. The restriction enzymes were obtained from Invitrogen and New England Biolabs. *Pfu* Turbo and T4 DNA ligase were purchased from Invitrogen and Stratagene, respectively. For heterologous expression, the vector pET-24d (Novagen) and the tRNA helper plasmid pSJS1244 (15, 29) were used.

Organisms and growth conditions. *Escherichia coli* strain XL1-Blue (Stratagene) was used as a host for the construction of pET24d derivatives. *E. coli* strain BL21(DE3) (Novagen) harboring the tRNA helper plasmid pSJS1244 was used as an expression host. Both strains were grown under standard conditions (25) following the instructions of the manufacturers.

Cloning and sequencing of the alcohol dehydrogenase-encoding gene. The identification of the gene encoding an alcohol dehydrogenase was based on significant sequence similarity to several known alcohol dehydrogenases. The *P. furiosus adhD* gene (PF1960, GenBank accession number AE010289, region

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from nucleotides 7356 to 8192; National Center for Biotechnology Information) was identified in the *P. furiosus* database (http://www.genome.utah.edu). The *adhD* gene (837 bp) was PCR amplified from the chromosomal DNA of *P. furiosus* using the primers BG1287 (sense, 5′-GCGCGCCATGGCAAAAAG GGTAAATGCATTCAACGA) and BG1305 (antisense, 5′-GCGCGGGATCCTCACACACACCTCCTTGCCATCT), containing the NcoI and BamHI sites (underlined in the sequences). In order to introduce an NcoI restriction site, an extra alanine codon (GCA) was introduced in the *adhD* gene by the forward primer BG1287 (boldface in the sequence). The fragment generated was purified using the QIAquick PCR purification kit (QIAGEN). The purified gene was digested with NcoI-BamHI and cloned into *E. coli* XL1-Blue using an NcoI-BamHI-digested pET24d vector. Subsequently, the resulting plasmid pWUR85 was transformed into *E. coli* BL21(DE3) harboring the tRNA helper plasmid pSJS1244. The sequence of the expression clone was confirmed by sequence analysis of both DNA strands.

Production and purification of ADH. *E. coli* BL21(DE3) harboring pSJS1244 was transformed with pWUR85, and a single colony was used to inoculate 5 ml Luria-Bertani medium with kanamycin and spectinomycin (both 50 $\mu g \cdot ml^{-1}$) and incubated overnight in a rotary shaker at 37°C. Next, 1 ml of the preculture was used to inoculate 1 liter Luria-Bertani medium with kanamycin and spectinomycin (both 50 $mg \cdot liter^{-1}$) in a 2-liter conical flask and incubated in a rotary shaker at 37°C until an optical cell density at 600 nm of 0.6 was reached. The culture was then induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and incubation of the culture was continued at 37°C for 18 h. Cells were harvested, resuspended in 20 mM Tris-HCl buffer (pH 7.5), and passed twice through a French press at 110 MPa. The crude cell extract was centrifuged for 20 min at 10,000 × *g*. The resulting supernatant (cell extract) was heated for 30 min at 80°C and subsequently centrifuged for 20 min at 10,000 × *g*. The supernatant (heat-stable cell extract) was filtered (0.45 μm) and applied to a Q-Sepharose high-performance (Amersham Biosciences) column (1.6 by 10 cm) equilibrated in 20 mM Tris-HCl buffer (pH 7.8). Proteins were eluted with a linear 560-ml gradient from 0.0 to 1.0 M NaCl in the same buffer.

Size exclusion chromatography. Molecular mass was determined by size exclusion chromatography on a Superdex 200 high-resolution 10/30 column (24 ml; Amersham Biosciences) equilibrated in 50 mM Tris-HCl (pH 7.8) containing 100 mM NaCl. Two hundred fifty microliters of enzyme solution in 20 mM Tris-HCl buffer (pH 7.8), containing 872 μg enzyme, was injected on the column. Proteins used for calibration were Blue dextran 2000 (>2,000 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and RNase A (13.7 kDa).

SDS-PAGE. Protein composition was analyzed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE) (25) using a Mini-Protean 3 system (Bio-Rad). Protein samples for SDS-PAGE were prepared by heating for 30 min at 100°C in the presence of sample buffer (0.1 M sodium phosphate buffer, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, pH 6.8). A broad-range protein marker (Bio-Rad) was used to estimate the molecular mass of the proteins.

Activity assays. Rates of alcohol oxidation and aldehyde reduction were determined at 70°C, unless stated otherwise, by following either the reduction of NAD^+ or the oxidation of NADH at 340 nm using a Hitachi U2010 spectrophotometer, with a temperature-controlled cuvette holder. Each oxidation reaction mixture contained 50 mM glycine (pH 8.8), 100 mM alcohol, and 0.28 mM NAD^+. The reduction reaction mixture contained 0.1 M sodium phosphate buffer (pH 6.1), 100 mM aldehyde or ketone, and 0.28 mM NADH. In all assays, the reaction was initiated by the addition of an appropriate amount of enzyme. One unit of ADH was defined as the oxidation or reduction of 1 μmol of NADH or NAD^+ per min, respectively. Protein concentration was determined using Bradford reagents (Bio-Rad) with bovine serum albumin as a standard (2). The temperature-dependent spontaneous degradation of NADH was corrected for.

pH optimum. The pH optimum for alcohol oxidation was determined in a sodium phosphate buffer (100 mM; pH range, 5.4 to 7.9) and a glycine buffer (50 mM; pH range, 7.9 to 10.3), whereas the pH optimum for aldehyde reduction was determined in a sodium phosphate buffer (100 mM; pH range, 5.4 to 7.9). The pH of the buffers was set at 25°C, and temperature corrections were made using their temperature coefficients (−0.025 pH/°C for glycine buffer and −0.0028 pH/°C for sodium phosphate buffer).

Optimum temperature and thermostability. The thermostability of AdhD (enzyme concentration, 0.17 $mg \cdot ml^{-1}$ in 20 mM Tris buffer, pH 7.8) was determined by measuring the residual activity (2,3-butanediol oxidation according to the standard assay) after incubation of a time series at 100°C. The temperature optimum was determined in 50 mM glycine buffer, pH 8.8, by analysis of initial rates of 2,3-butanediol oxidation in the range of 30 to 100°C.

Kinetics. The AdhD kinetic parameters K_m and V_{max} were calculated from multiple measurements (at least eight measurements) using the Michaelis-Menten equation and the program Tablecurve (Tablecurve 2D, version 5.0). All the reactions followed Michaelis-Menten-type kinetics. The turnover number (k_{cat}, s^{-1}) was calculated as V_{max} × subunit molecular mass (32 kDa)/60.

Salts, metals, and inhibitors. The effects of several salts, metals (K^+, Mg^{2+}, Mn^{2+}, Na^+, Fe^{2+}, Fe^{3+}, Li^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}), and inhibitors (EDTA, dithiothreitol, 2-iodoacetamide) on the initial activity of AdhD were checked using 2,3-butandiol as substrate in the oxidation reaction and acetoin in the reduction reaction. Concentrations ranging from 1 to 25 mM were tested.

Enantioselectivity. The enantioselectivity of AdhD was determined by reduction of 2-pentanone with cofactor regeneration at 60°C for 24 h. The reaction mixture contained 1.0 mM NAD^+ or $NADP^+$, 250 mM 2-pentanone, 300 mM glucose, 350 mM $CaCO_3$, 10 U glucose dehydrogenase from *Bacillus megaterium* (for cofactor regeneration), and 4 nmol AdhD in 2 ml 50 mM Tris-HCl buffer (pH 7.0). Chiral gas chromatography was used to determine the enantiomeric excess (ee). All the samples were extracted with chloroform (1:1), and 1 μl was applied on a Chirasil Dex column (injector temperature, 250°C).

RESULTS

Analysis of nucleotide and amino acid sequences. The *P. furiosus* genome has been analyzed for genes encoding putative alcohol dehydrogenases, which resulted in the identification of 16 genes that potentially encode alcohol dehydrogenases. After successful production in *E. coli*, an initial screening for activity was performed in which two of the putative alcohol dehydrogenases, including AdhD, showed high activities (R. Machielsen, unpublished). The two enzymes were selected for a more detailed study; with respect to the other putative alcohol dehydrogenases, a more elaborate screening is currently being performed to gain insight into their substrate specificity and possibly into their physiological function. Here, we describe the production and characterization of the novel alcohol dehydrogenase AdhD (PF1960).

Analysis of the *adhD* locus revealed that the start codon of a gene coding for a tungsten-containing aldehyde reductase, *wor4* (PF1961), is directly adjacent to the *adhD* stop codon (24), suggesting an operon-like organization. Interestingly, conserved context analysis with STRING (http://string.embl.de/) revealed that the clustering of these two genes is also observed in the related species *Pyrococcus abyssi* and *Pyrococcus horikoshii*; manual inspection identified a similar conserved gene pair in *Thermococcus kodakaraensis*.

The *adhD* gene encodes a protein of 278 amino acids and a calculated molecular mass of 31,794 kDa. The sequence belongs to the cluster of orthologous groups of proteins 0656 (aldo-keto reductases, related to diketogulonate reductase; http://www.ncbi.nlm.nih.gov/COG/). BLAST-P analysis (http://www.ncbi.nlm.nih.gov/BLAST/) revealed the highest similarity with hypothetical oxidoreductases and putative members of the aldo-keto reductase superfamily from hyperthermophilic archaea and bacteria. Some of these most significant hits of a BLAST search analysis were a hypothetical oxidoreductase/aldo-keto reductase of *Thermococcus kodakaraensis* KOD1 (85% identity; TK0845), a putative 2,5-diketo-D-gluconic acid reductase of *Pyrococcus abyssi* (84% identity; PAB2329), a putative dehydrogenase of *Sulfolobus solfataricus* P2 (53% identity; SSO2779), and an oxidoreductase/aldo-keto reductase of *Thermotoga maritima* (47% identity; TM1743). Together with an aldose reductase of *Sus scrofa* (pig) (22, 30) for which a structure has been determined (32% identity; ALR2; pdb identification, 1AH4), an alignment was made (Fig. 1). The AKR

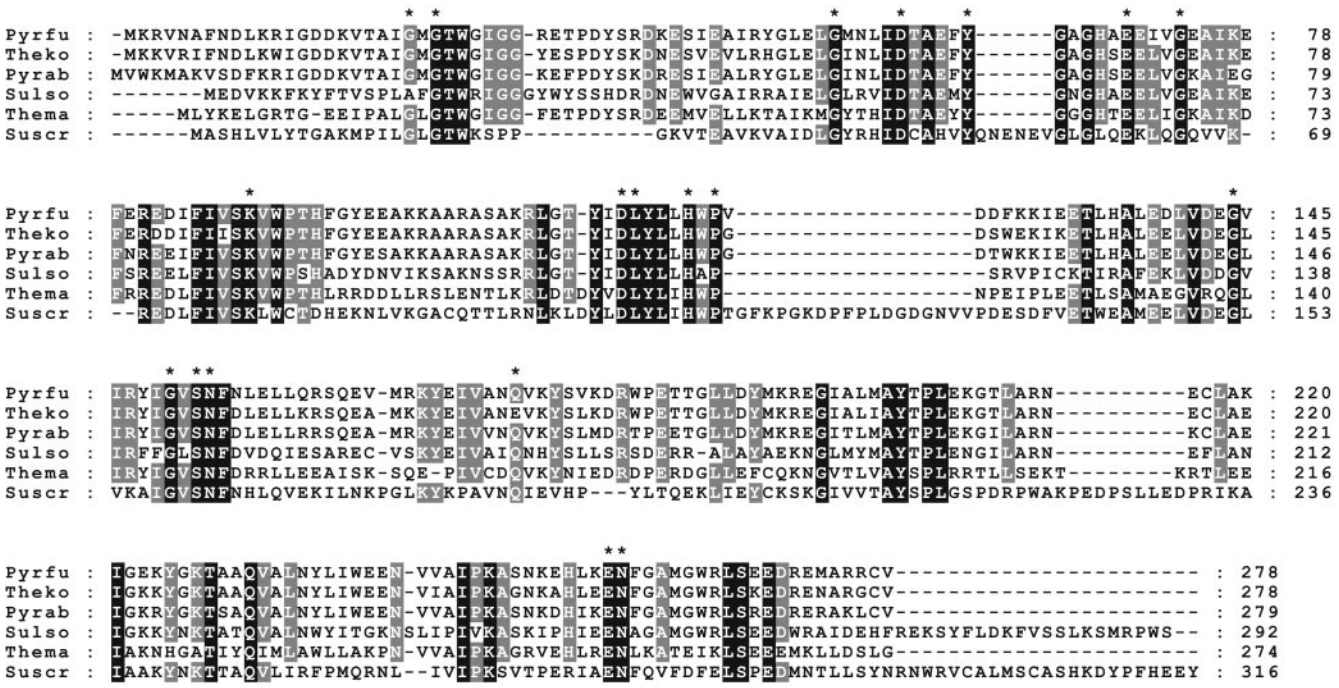

FIG. 1. Multiple sequence alignment of the *P. furiosus* AdhD with (hypothetical) members of the aldo-keto reductase superfamily. The following abbreviations are used: Pyrfu, *P. furiosus*; Theko, *T. kodakaraensis*; Pyrab, *P. abyssi*; Sulso, *S. solfataricus*; Thema, *T. maritima*; Suscr, *S. scrofa*. The sequences were aligned using the CLUSTAL program. Asterisks indicate highly conserved residues within the AKR superfamily.

superfamily shares a common $(\alpha/\beta)_8$-barrel fold and a catalytic tetrad (Asp, Tyr, Lys, and His), and their members bind the cofactor NAD(P)H in an extended conformation without a Rossmann-fold motif. Highly conserved residues within the AKR superfamily are indicated in Fig. 1 with an asterisk, and they are presumably involved in the three shared properties: Asp58, Tyr63, Lys89, and His121 in the catalytic tetrad; Asp58, Ser152, Asn153, Gln175, Glu257, and Asn258 in cofactor binding; and the remaining conserved residues probably in a structural role (*P. furiosus* numbering) (3, 8, 9, 26, 34).

Purification of recombinant AdhD. The pyrococcal AdhD was purified to homogeneity from heat-treated cell extracts of *E. coli* BL21(DE3)/pSJS1244/pWUR85 by anion-exchange chromatography. Active AdhD eluted between 0.35 and 0.52 M NaCl (peak at 0.41 M NaCl). Fractions containing the purified enzyme were pooled. The migration of AdhD on an SDS-PAGE gel confirmed a molecular subunit mass of 32 kDa (Fig. 2). The molecular mass of the native AdhD was estimated to be 30 kDa by size exclusion chromatography, which indicated a monomeric structure.

Substrate and cofactor specificity. The substrate specificity of AdhD in the oxidation reaction was analyzed using a range of alcohols, including primary alcohols (methanol to dodecanol, C_1 to C_{12}), secondary alcohols (2-propanol to 2-decanol, C_3 to C_{10}), aromatic alcohols, and alcohols containing more than one hydroxyl group (Table 1). AdhD showed low activity toward primary alcohols, and a broad range of secondary alcohols could be oxidized, which included substrates with 3 to 10 carbon atoms (Fig. 3). The highest specific activity of AdhD in the oxidative reaction was found with 2,3-butanediol (V_{max}, 108.3 U · mg^{-1}).

The substrate specificity of the reduction reaction was analyzed by using aldehydes, ketones, and aldoses as substrates (Table 2). The highest reduction rate of AdhD was observed with diacetylacetoin (3-hydroxy-2-butanone, V_{max} of 22.5 U · mg^{-1}).

AdhD could use both NAD(H) and NADP(H) as cofactor but has a clear preference for NAD(H). Measurements with 2,3-butanediol as substrate showed that the activity with $NADP^+$ as cofactor was only 14% of that found with NAD^+ as cofactor. Measurements with other substrates exhibited different ratios but always a preference for NAD(H).

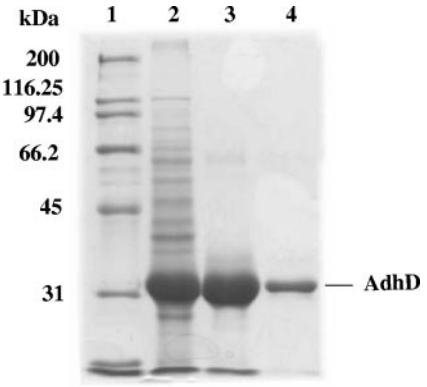

FIG. 2. SDS-PAGE analysis of heterologously produced AdhD in each purification step. Lane 1, broad-host-range protein marker; lane 2, cell extract; lane 3, heat-stable cell extract; lane 4, pooled Q-Sepharose fractions. Molecular mass markers are given to the left of the gel.

TABLE 1. Substrate specificity of *P. furiosus* AdhD in the oxidation reaction

Substrate[a]	Relative activity (%)
1-Pentanol	1
2-Pentanol	9
1,2-Propanediol	3
Glycerol	1
3-Phenyl-1-propanol	1
2-Methyl-1-propanol	2
2-Propen-1-ol	1
1-Butanol	1
2-Butanol	7
1,2-Butanediol	2
1,3-Butanediol	3
2,3-Butanediol	100
Acetoin	18
3-Phenyl-1-butanol	0.4
3-Methyl-1-butanol	0.4
D-Arabinose	66
L-Arabinose	0
D-Xylose	3
D-Glucose	2
Cyclohexanol	12
Benzyl alcohol	3

[a] Substrates were present in 100 mM concentrations.

TABLE 2. Substrate specificity of *P. furiosus* AdhD in the reduction reaction

Substrate[a]	Relative activity (%)
Acetone	0.1
Dihydroxyacetone	36
Dihydroxyacetone phosphate	82
Hexanal	16
2-Hexanone	0.2
Decanal	1
Acetoin	100
Diacetyl	~150[b]
DL-Glyceraldehyde	31
Pyruvic aldehyde	81
D-Arabinose	25
D-Xylose	2
D-Glucose	0
D-Ribose	2
D-Mannose	2
Cyclohexanone	38

[a] Substrates were present in 100 mM concentrations, except for dihydroxyacetone phosphate, which had a concentration of 50 mM.
[b] Due to experimental difficulties, no kinetic data could be obtained and maximal activity was not reached.

Enantioselectivity. The enantioselectivity of AdhD was tested using 2-pentanone as substrate, and after 24 h of conversion, the products formed were measured by gas chromatography analysis. This enzyme preferably reduced 2-pentanone to (S)-2-pentanol. When NAD^+ was used as the cofactor, an ee value of 89.4% was obtained, and when $NADP^+$ was used, an ee value of 84.8% was obtained.

Effects of salts, metals, and inhibitors. The salts, metals, and inhibitors tested caused no significant inhibition or activation. In the oxidation reaction, only high concentrations of Mg^{2+} caused slightly lower activities, and in the reduction reaction, 2 mM Ni^{2+}, Co^{2+}, and Ca^{2+} had the same small inhibitory effect. There was no inhibition when dithiothreitol, EDTA, or 2-iodoacetamide was added to the reaction, confirming that the protein does not require metals for its activity and that no essential disulfide bridges or thiol groups are involved.

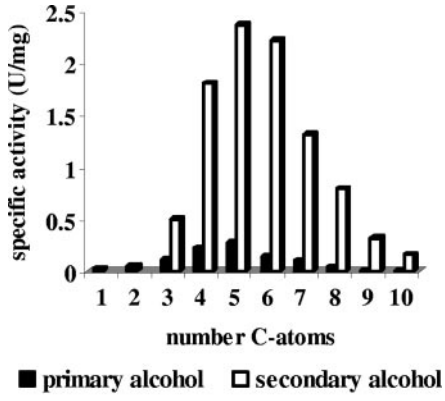

FIG. 3. Specific activity of *P. furiosus* AdhD toward primary and secondary alcohols.

Thermostability and pH optima. The oxidation reaction catalyzed by AdhD showed a pH optimum of 8.8, and the aldehyde reduction by AdhD showed a high level of activity over a wide pH range, with maximal activity at pH 6.1. The reaction rate of AdhD increased with increasing temperature from 30°C (6.3 U/mg) up to 100°C (38.1 U/mg), but due to instability of the cofactors at that temperature, all other activity measurements were performed at 70°C. At this temperature, the activity was only 15% lower than that at 100°C. AdhD has a high resistance to thermal inactivation, which was shown by a half-life value of 130 min at 100°C.

Enzyme kinetics. The kinetic properties of AdhD were determined for the substrates that were converted with relatively high rates in the oxidation and reduction reaction, as well as for the cofactors used in these reactions. This showed that AdhD has a relatively high affinity for acetoin (K_m = 6.5 mM, V_{max} = 22.5 U/mg, k_{cat}/K_m = 1.8 $s^{-1} \cdot mM^{-1}$) and NADH (K_m = 97 μM, V_{max} = 22.5 U/mg) in the reduction reaction and clearly a lower affinity for 2,3-butanediol (K_m = 86.8 mM, V_{max} = 108.3 U/mg, k_{cat}/K_m = 0.7 $s^{-1} \cdot mM^{-1}$) and NAD^+ (K_m = 600 μM, V_{max} = 108.3 U/mg) in the oxidation reaction.

DISCUSSION

In (hyper)thermophilic archaea, several types of alcohol dehydrogenases have been discovered. Almost all are NAD(P) dependent and are classified into three different groups, the zinc-dependent ADHs, the short-chain ADHs, and the iron-activated ADHs (20). The enzyme described here, AdhD, represents a novel NAD(P)H-dependent alcohol dehydrogenase from *Pyrococcus furiosus* that does not show similarity with these groups but rather with the aldo-keto reductase superfamily.

AdhD was functionally produced in *E. coli*, and due to its stability at high temperatures, only two steps were needed for purification. It showed a preference for NAD(H) as cofactor and had a broad substrate specificity in the oxidation and

reduction reaction. A preference was observed for secondary alcohols compared with the primary alcohols, but the highest activities were detected when polyols were used as substrate.

Alcohol dehydrogenases can be involved in a wide range of metabolic processes, which makes it often difficult to determine their physiological role. The presence of the *wor4* gene, encoding a tungsten-containing aldehyde reductase (24), directly adjacent to the *adhD* gene suggests a role in which AdhD might use or produce aldehydes/ketones associated with the WOR4 activity. The fact that the clustering of these two genes is also observed in the related species *P. abyssi*, *P. horikoshii*, and *T. kodakaraensis* strengthens this suggestion. WOR4 was purified from *P. furiosus* cells that were grown in the presence of elemental sulfur (S^0) (24), and DNA microarray analyses showed that *wor4* and *adhD* were both moderately upregulated, three- and fourfold, respectively, in maltose-grown *P. furiosus* when S^0 was present (27). This is in contrast to an iron alcohol dehydrogenase from *Thermococcus* strain ES-1 (ES-1 ADH), which is down-regulated by the presence of S^0 (16). It was proposed that, under S^0 limitation, ES-1 ADH reduces (toxic) aldehydes that are generated by fermentation, thereby disposing of some of the excess reductant as alcohol. This function is also assigned to an iron/zinc-containing alcohol dehydrogenase from *P. furiosus* (17).

Kinetic data showed high catalytic efficiency for the reduction reaction and high affinity for the substrate and cofactor involved in this reaction, which suggest that the physiological function of AdhD is the reduction of aldehydes or ketones. Although it cannot be ruled out that these substrates are formed by WOR4, this appears unlikely because all described tungsten-containing aldehyde oxidoreductases (AOR, FOR, GAPOR) are known to catalyze the unidirectional oxidation of aldehydes (5, 11, 18, 19, 23). Assuming a physiological link between AdhD and WOR4, based on the conserved context, this gives rise to two other options. First of all, it is conceivable that AdhD oxidizes an alcohol to produce an aldehyde or ketone which is subsequently oxidized further by WOR4. However, because of the apparent preference of AdhD for the reduction reaction and WOR4 for the oxidation reaction, another putative scenario can be envisaged as well. It might be possible that an unidentified aldolase produces the substrates for AdhD and WOR4, for instance, a ketone and an aldehyde. Subsequently, the ketone could be reduced to an alcohol by AdhD and the aldehyde could be oxidized by WOR4. However, since no activity has yet been ascribed to WOR4, more experiments are required to establish the physiological roles of both enzymes.

Apart from the scientific interest in the physiological role of alcohol dehydrogenases, there is also interest from an industrial perspective. AdhD can be produced in relatively high amounts by heterologous expression in *E. coli*, and if necessary, it can be easily purified. It is extremely thermostable, which is shown by a half-life value of 130 min at 100°C, and it is highly S-enantioselective. These properties, together with a broad substrate specificity and a preference for the more inexpensive cofactor NAD(H), make this enzyme a potential catalyst for industry, especially for the production of chiral compounds. Further study to test AdhD for the production of interesting chiral compounds is in progress.

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