J. Biochem. 120, 498-504 (1996)

A Thermophilic Alcohol Dehydrogenase from *Bacillus acidocaldarius* Not Reactive towards Ketones[1]

Sabato D'Auria,[2] Francesco La Cara, Filomena Nazzaro, Nunzia Vespa, and Mose' Rossi

Institute of Protein Biochemistry and Enzymology, C.N.R. Via Marconi, 10, 80125 Naples, Italy

Received for publication, March 4, 1996

An NAD-dependent alcohol-aldehyde oxidoreductase was purified to homogeneity and characterized from cell extracts of the thermophilic microorganism *Bacillus acidocaldarius*. The 500-fold purified homogeneous enzyme had a molecular mass of 154 kDa, as shown by gel filtration and glycerol gradient centrifugation. On sodium dodecyl sulfate polyacrylamide gel electrophoresis the protein showed one band of 38 kDa, indicating that the enzyme is a tetramer composed of subunits of identical molecular weight. Ethanol was the best substrate with the highest k_{cat}/K_m values, and the enzyme showed a substrate specificity that included linear, secondary and cyclic alcohols, as well as anisaldehyde, but it was not active on ketones. The protein contains eight zinc atoms per tetramer, four of which are removed by chelating agents with a concomitant loss of thermal stability. Circular dichroism spectra and determination of the NH_2-terminal sequence allowed structural and homology comparison with other alcohol dehydrogenases from animal and bacterial sources.

Key words: alcohol dehydrogenase, *Bacillus acidocaldarius*, circular dichroism, enzymatic activity, thermophilic enzymes.

Alcohol dehydrogenases (ADH; EC 1.1.1.1) of various origins play an important role in the biological metabolism of alcohols and hydroxysteroids. Some of these enzymes (*e.g.*, horse liver alcohol dehydrogenase) have been extensively studied, because they can provide new data on the relationship between structure and function, being oligomeric, coenzyme-dependent, and containing structural and functional metal atoms (*1-4*). The interest in ADHs isolated from thermophilic microorganisms is based on the peculiar properties of these enzymes in comparison to mesophiles. In fact, as reported for ADHs from *Bacillus stearothermophilus* (*5*), *Thermoanaerobacter ethanolicus* (*6*), *Thermoanaerobium brockii* (*7, 8*) and *Sulfolobus solfataricus* (*9*), these enzymes, in addition to being thermostable and thermophilic, show enhanced stability and activity in organic solvents and are more resistant to the common protein denaturants. Contrary to *T. brockii*, the *S. solfataricus* ADH shows a stereoselective preference to reduce (s)-alcohols from prokiral ketones and is more similar to horse liver and yeast enzymes. However, ADHs isolated from different thermophilic microorganisms show different thermostability and thermoactivity and different substrate specificity. For example, the enzyme from *T. brockii* (*8*) shows a stereoselectively temperature-dependence, the enzyme from *B. stearothermophilus* (*5*) is reactive towards methanol, while that described in this report is not active towards ketones. It is of great interest to compare the properties of these enzymes in the attempt to relate them to their structure.

This paper describes the purification to homogeneity, the characterization and the NH_2 terminal sequence of an NAD-dependent ADH isolated from the thermoacidophilic eubacterium *Bacillus acidocaldarius* (*Ba*ADH) that has the interesting property of not being active on ketones.

MATERIALS AND METHODS

Organism and Growth—B. acidocaldarius, a thermoacidophilic eubacterium with an optimal growth temperature of 60°C at pH 4.0, was originally isolated from hot springs at Agnano, near Naples (*10*). The microorganism was grown at 60°C with an aeration flux of 200 ml·min^{-1}·liter broth^{-1} in a 150-liter stainless steel prototype fermenter supplied by Bioindustrie Mantovane S.r.l. The fermentor was inoculated to 1 : 20 v/v with a 16-h broth culture. The complex culture medium contained (g/liter): KH_2PO_4, 5.0; $(NH_4)_2SO_4$, 1.3; $MgSO_4 \cdot 7H_2O$, 0.6; $CaCl_2 \cdot 2H_2O$, 0.15; yeast extract, 1.0; Casaminoacids, 1.0; Sucrose, 2.0. The pH was adjusted to 4.0 with 0.1 M H_2SO_4. The biomass obtained after a 19-h growth was 1.5 g/liter wet weight. Cells were harvested by centrifugation at 20,000 rpm, 14°C, in the logarithmic phase of growth, using a Padberg model Z41 continuous-flow centrifuge. Cell paste was stored at −80°C until use.

Chemicals and Reagents—All chemicals were of reagent grade. DEAE-Sepharose Fast Flow, Mono Q, and Phenyl Superose were purchased from Pharmacia; Matrex Gel Blue A from Amicon; Protein Pak Glass SW 300 from Waters; NAD, NADH, and molecular mass standard markers were from Boehringer Mannheim and Pharmacia.

All substrates were obtained from Aldrich. Byo-Lyte

[1] This work was supported by Progetto Finalizzato Biotecnologie, CNR, Italy.

[2] To whom correspondence should be addressed. Tel: +39-81-7257239, FAX: +39-81-7257240, E-mail: e021rn02@area.ba.cnr.it

Abbreviations: ADH, alcohol dehydrogenase; CD, circular dichroism.

ampholites were purchased from Bio-Rad.

Enzyme Assay—Alcohol dehydrogenase activity was assayed spectrophotometrically at 65°C by measuring the change in absorbance at 340 nm in sealed quartz cuvette. The reaction mixture contained 50 mM glycine-NaOH (pH 10.0) and 1.0 μg protein/ml final volume. The concentrations of cofactors and substrates were 5.0 mM NAD and 3.0 mM ethanol for alcohol oxidation and 0.12 mM NADH and 3.0 mM *p*-anisaldehyde for aldehyde reduction, respectively.

One unit of enzyme activity was defined as the amount of the enzyme that catalyzes the reduction of 1 μmol/min of NAD at 65°C, using ethanol as substrate under the above conditions.

Protein Determination—Protein concentration was determined using the Bio-Rad Protein Assay kit by Bradford's method (*11*). Bovine serum albumin was used as standard.

Purification of Alcohol Dehydrogenase—All procedures were performed at room temperature.

(1) Preparation of cell extract: One hundred twenty grams of wet cell pellet was suspended in 360 ml of 10 mM Tris/HCl, 0.5 mM dithiothreitol (DTT), pH 9.0 (buffer A), and broken by five 1-min cycles of sonication at regular intervals, using an MSE Soniprep 150.

Cells debris was removed by centrifugation on Spinco Ti 50.2 rotor at 160,000×*g* for 90 min. The supernatant represented the crude extract.

(2) Column chromatography: The crude extract was applied to a DEAE-Sepharose Fast Flow (5.0×30.0 cm) previously equilibrated with buffer A. The column was washed with 1,000 ml of buffer A and eluted with a linear gradient from 0.0 to 0.3 M NaCl in buffer A, at a flow rate of 60 ml/h. Alcohol dehydrogenase activity was eluted at about 0.1 M NaCl.

The fractions containing enzymatic activity were pooled and loaded on a Phenyl-Superose FPLC column, at a flow rate of 30 ml/h, after addition of ammonium sulfate (1.0 M). After washing with 100 ml of buffer A, enzymatic activity was eluted with a gradient of ammonium sulfate from 1.0 to 0.0 M in buffer A. Fractions containing ADH activity were eluted at 0.3 M ammonium sulfate, pooled and dialyzed overnight against 10 liters of buffer A.

The active pool was loaded at a flow rate of 30 ml/h on a Mono Q FPLC column previously equilibrated with the same buffer. The column was washed with 30 ml of buffer A, and the enzymatic activity was eluted with a linear gradient from 0.0 to 0.5 M NaCl in buffer A, at a flow rate of 60 ml/h. Enzymatic activity, eluted at about 100 mM NaCl, was dialyzed overnight against 5 liters of 10 mM Tris/HCl, pH 7.4 (buffer B) and applied onto a Blue A affinity column (2.5×20 cm), previously equilibrated with the same buffer, at a flow rate of 20 ml/h. The column was washed with 150 ml of buffer B, and the homogeneous enzyme was eluted with a step of 2 mM NAD in the same buffer.

The homogeneous enzyme, stored at 4°C in buffer A, was stable for several weeks.

Glycerol Gradient and Gel Filtration—The molecular weight of the native enzyme was determined by glycerol gradient centrifugation and gel filtration.

The protein sample (50 μl, 50 μg enzyme) was layered onto a preformed 10-30% glycerol gradient in 10 mM Tris/HCl, 0.5 mM DTT, pH 9.0, 200 mM NaCl, and centrifuged for 17 h at 170,000×*g* (Beckman SW 65 K rotor, 4°C). Molecular mass standards were catalase (250 kDa), yeast alcohol dehydrogenase (150 kDa), and egg albumin (43 kDa). The molecular mass was determined according to Martin and Ames (*12*).

The estimation of molecular mass by gel filtration was carried out by the procedure of Andrews (*13*). The purified enzyme (2.0 mg) in a final volume of 0.1 ml was loaded onto a Protein Pak Glass 300 SW column by FPLC (Pharmacia) previously equilibrated with 10 mM Tris/HCl, 0.5 mM DTT, 0.3 M NaCl (pH 9.0), and calibrated with the following standards: thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa).

SDS-PAGE and Molecular Weight Determination—SDS-polyacrylamide gel electrophoresis (PAGE) was used to determine *Ba*ADH purity and the molecular weight of its subunits. A 12.0% (w/v) separation slab gel was used with Laemmli's discontinuous buffer system (*14*). Phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), egg albumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) were used as standards. Proteins were stained with Coomassie Brilliant Blue R-250.

Isoelectric Focusing—Isoelectric focusing was performed by the procedure of Robertson *et al.* (*15*), using a vertical minigel system (Bio-Rad Miniprotean II) in the pH range of 3.5–10.0. Polyacrylamide gel (7.5%) was prepared according to the manufacturer's instructions (Bio-Rad). The electrode solutions were 25 mM NaOH for the cathode and 20 mM acetic acid for the anode. Electrophoresis was performed at room temperature for 3.5 h at 200 V. Staining for enzymatic activity on polyacrylamide gel was performed as described by Rudge and Bickerstaff (*16*), using ethanol as substrate at the temperature of the standard assay.

pH Profiles—The optimum pH for ethanol oxidation was determined at 65°C in the pH range between 5.5 and 11.0, using the following buffers: sodium phosphate, sodium barbital, and glycine NaOH at a 25 mM final concentration.

The optimum pH for anisaldehyde reduction was tested under the same conditions.

About 1.0 μg of the enzyme was used in a total volume of 1.0 ml.

Enzyme Thermophilicity and Thermostability—Enzyme thermophilicity was tested in assay buffer with ethanol as substrate at temperatures from 25 to 95°C, using 1.0 μg enzyme in a final volume of 1.0 ml.

Enzyme thermostability was tested by incubating the enzyme (1.0 mg/ml in 50 mM glycine NaOH buffer, pH 10.0) at 45, 55, 65, and 75°C. At intervals, aliquots of 1.0 μg of the pre-incubated enzyme were withdrawn from the mixture and assayed under standard conditions.

Kinetic Constants—K_m values for several substrates were determined with the standard assay. K_m values for the coenzymes NAD and NADH were determined using ethanol and *p*-anisaldehyde as substrates, respectively, and 1.0 μg of the enzyme.

The kinetic results were analyzed with the Enzfitter program (Elsevier-Biosoft, Cambridge, UK).

Circular Dichroism—Measurements were performed on homogeneous samples at a protein concentration of 0.2 mg/

ml, under the conditions given in the legend to the figure. A Jasco J-710 spectropolarimeter (Jasco, Tokyo) equipped with temperature-controlled liquid systems Neslab RTE-110 (Neslab Instruments, Portsmouth, USA) calibrated with a standard solution of (+)-10 camphorsulfonic acid was used. Spectra from 240 to 190 nm were recorded, using 0.1 cm light path quartz cuvettes (Hellma Jamaica, NY), under a nitrogen flow. A spectral acquisition spacing of 0.2 nm (1.0 nm band width) was used, and the photomultiplier absorbance did not exceed 600 V. Each spectrum was averaged 5 times and smoothed with Spectropolarimeter System Software ver. 1.00 (Japan Spectroscopy, Tokyo).

Zinc Analysis—Contamination of protein samples with zinc was minimized by employing buffers prepared with ultrapure water and by using plastic and glassware cleaned exhaustively with 20% HNO_3 and then rinsed with ultrapure water. Purified *Ba*ADH at a concentration of 1.0 mg/ml was dialyzed at 4°C for 12–24 h against five changes of 200 volumes of buffer A, containing 1.0 mM 1,10-phenanthroline (Sigma) or 3.0 mM EDTA. Residual chelating agent was removed by dialysis against 5 changes of 200 volumes of buffer A. Moreover, an enzyme sample (control) was dialyzed under the same conditions in the absence of chelating agents.

Both enzyme samples were stored at 4°C in metal-free plastic tubes and used for functional and structural studies.

The zinc content was estimated with a Perkin-Elmer 5100 PC atomic absorption spectrophotometer equipped with an AS 60 autosampler and a Zeeman 5100 furnace.

NH_2-Terminal Sequence—A lyophilized protein sample was subjected to automated Edman degradation by an Applied Biosystems 477 A Protein Sequencer, equipped on line with a model 120 A phenylthiohydantoin analyzer. Degradation proceeded for 25 cycles according to the manufacturer's instructions, and produced identifiable amino acid residues.

RESULTS

Enzyme Purification—The results of a typical purification procedure are summarized in Table I. The alcohol dehydrogenase was purified 500-fold with 61% recovery of the activity. The crucial step was the specific elution of enzyme activity by NAD from Blue A column. The presence of DTT in the buffers was necessary in order to avoid inactivation of the enzyme.

Analysis of the purified enzyme by SDS-PAGE revealed the presence of a single band of 38 kDa as shown in Fig. 1.

Molecular Mass and Subunit Composition—The enzyme molecular mass, determined by glycerol gradient centrifugation and gel filtration, was 150 and 154 kDa, respectively. SDS-PAGE gave a single band corresponding to a molecular mass of about 38 kDa, indicating a tetrameric structure for the enzyme.

pH Profiles and Isoelectric Point Determination—Activity dependence on pH is shown in Fig. 2. The optimum pH in 25 mM glycine NaOH buffer for ethanol oxidation and in 25 mM sodium phosphate for *p*-anisaldehyde reduction was 10.0 and 7.5, respectively. A single protein band with a pI of 5.6 was obtained for the purified alcohol dehydrogenase by isoelectric focusing.

Substrate Specificity—The kinetic constants of several substrates, determined at 65°C, are given in Table II. Ethanol, propan-1-ol, and pentan-1-ol showed similar K_m values, but ethanol showed the highest k_{cat}/K_m ratio. The study of the substrate specificity showed that the *p*-anisaldehyde was the only aldehyde reduced by the enzyme and moreover, interestingly, no enzyme activity was found on all ketones tested.

*Ba*ADH required NAD(H) as a coenzyme, and no activity was found when NADP(H) was used.

Zinc Analysis—The zinc analyses of the enzyme dialyzed

TABLE I. **Purification of alcohol dehydrogenase from *Bacillus acidocaldarius*.**

Purification step	Total protein (mg)	Specific activity (μmol·min^{-1}·mg^{-1})	Purification (fold)	Yield (%)
Crude extract	12,200	0.3	1	100
DEAE Fast Flow	1,100	3.3	11	99
Phenyl-Superose	500	6.1	20	83
Mono-Q	150	16	53	65
Blue A	15	150	500	61

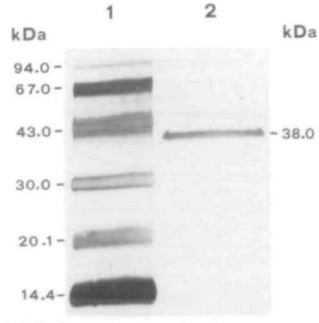

Fig. 1. **SDS/PAGE.** Lane 1: standard protein markers in the order of increasing molecular mass: α-lactalbumin, 14,400; soybean trypsin inhibitor, 20,100; carbonic anhydrase, 30,000; ovalbumin, 43,000; bovine serum albumin, 67,000; phosphorylase *b*, 94,000. Lane 2: *Bacillus acidocaldarius* alcohol dehydrogenase (10 μg).

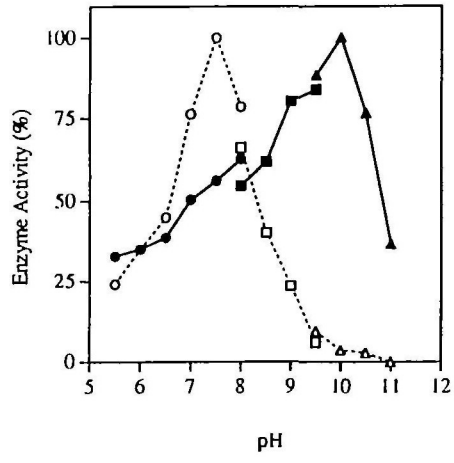

Fig. 2. ***Ba*ADH activity dependence on pH.** Ethanol oxidation in Na-phosphate (●), Na-barbital (■), and glycine-NaOH (▲) buffers; *p*-anisaldehyde reduction in Na-phosphate (○), Na-barbital (□), and glycine-NaOH (△) buffers. The reactions were carried out at 65°C in buffers (25 mM concentration) adjusted to each pH at room temperature.

against buffer A in the absence and in the presence of chelating agents revealed an average of 8 and 4 mol of zinc per mol of tetramer, respectively. *Ba*ADH dialyzed against chelating agents retained the same specific activity exhibited by the native enzyme.

Enzyme Thermophilicity and Thermostability—Enzyme thermophilicity is shown in Fig. 3. Enzymatic activity increased up to 80°C. The Arrhenius plot was not linear: the curve showed a break-point at about 50°C. The activation energy calculated was 145 kJ/mol at 35-50°C and 42 kJ/mol at 50-80°C.

Thermal stability studies, at four different temperatures, revealed that the activity was stable for at least 24 h at 45°C, while at 55, 65, and 75°C the half-life was 5.0, 3.0, and 1.5 h, respectively. Furthermore, thermal stability studies on the zinc-depleted *Ba*ADH revealed a significant decrease of the thermoresistence. In fact, the half-life values at the above temperatures were 240, 60, 20, and 5 min, respectively.

Circular Dichroism Studies—Figure 4 shows far UV circular dichroism spectra in the temperature range between 25 and 95°C after pre-incubation for 10 min at each temperature. The results indicate that the *Ba*ADH secondary structure content is temperature-dependent. At 95°C the enzyme lost almost all of its α helix content, and its melting point was about 80°C.

The prediction of the content of the *Ba*ADH secondary structure at 25°C (*17*) showed the following values: 22% α helix; 45% β structure; 8.1% turn structure; 24.9% random structure. The same secondary structure content was found for the zinc-depleted enzyme, suggesting that the removal of the four zinc atoms did not affect the protein secondary structure.

NH_2-Terminal Amino Acid Sequence—The NH_2-terminal amino acid sequence determined for *Ba*ADH was: Met-Lys-Ala-Ala-Val-Val-His-Gly-Phe-Arg-Glu-Pro-Leu-Arg-Ile-Glu-Asp-Val-Pro-Lys-Pro-Asp-Val-Gly-Glu. It revealed some homology with other known sequences of alcohol dehydrogenases from animal and bacterial sources. In fact,

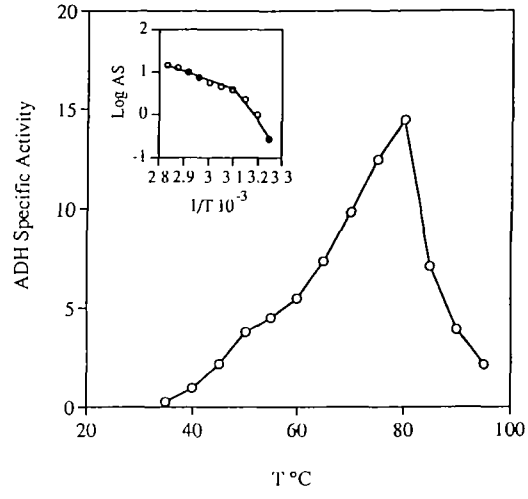

Fig. 3. **Temperature dependence of purified *Ba*ADH.** The reaction mixture contained (1.0 ml total volume): 50 mM glycine-NaOH buffer, pH 10.0, 5.0 mM NAD, 3.0 mM ethanol, and 1 μg of the purified enzyme; the temperature was as indicated. For full experimental details, see the text.

TABLE II. **Substrate-specificity of alcohol dehydrogenase from *Bacillus acidocaldarius*.**[a]

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
Methanol	na	na	na
Ethanol	0.20	46.6	233.0
Propan-1-ol	0.17	19.4	111.5
Propan-2-ol	2.72	19.7	7.2
Butan-1-ol	0.29	19.3	66.5
Butan-2-ol	1.12	11.0	9.8
Pentan-1-ol	0.15	14.0	93.3
Pentan-2-ol	0.95	13.3	14.0
Cyclopentanol	0.66	16.0	24.2
Cyclohexanol	na	na	na
Benzyl alcohol	0.72	9.7	13.5
Formaldehyde	na	na	na
Acetaldehyde	na	na	na
Propanal	na	na	na
Butanal	na	na	na
Benzaldehyde	na	na	na
p-Anisaldehyde	2.01	10.2	5.1
Acetone	na	na	na
2-Butanone	na	na	na
2-Pentanone	na	na	na
Cyclopentanone	na	na	na
Cyclohexanone	na	na	na
Acetophenone	na	na	na
NAD	1.61	46.1	28.6
NADH	0.015	10.0	666.7
NADP	na	na	na
NADPH	na	na	na

[a]na, no activity. The K_m values for NAD^+ and NADH were determined at 65°C with 3 mM ethanol and *p*-anisaldehyde, as described in "MATERIALS AND METHODS."

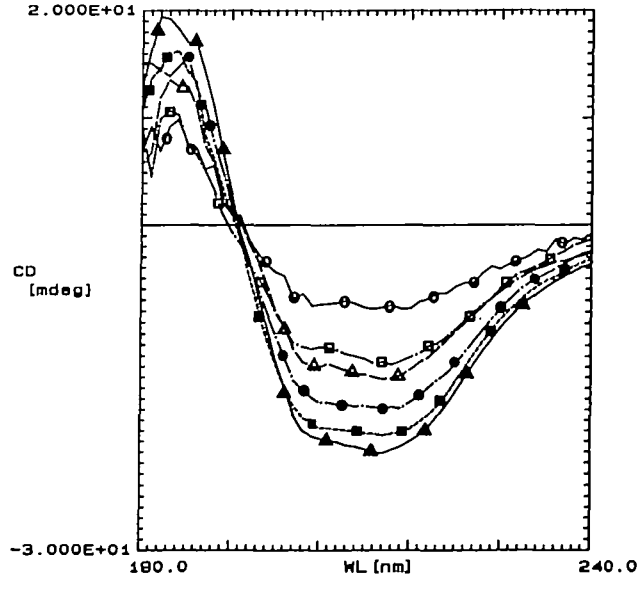

Fig. 4. **Temperature dependence of *Ba*ADH circular dichroism spectra.** Conditions: 0.2 mg/ml *Ba*ADH in 10 mM Tris-HCl, pH 8.1; spectra were registered at a scan speed of 5 nm/min with 5 accumulations. Temperature: 25°C (▲), 45°C (■), 65°C (●), 75°C (△), 85°C (□), 95°C (○). The samples were preincubated at the indicated temperatures for 10 min in sealed quartz cuvettes before circular dichroism analysis.

```
                1         10         20         30
                |         |          |          |
HLADH            STAGKVIKCKAAVLWEEKKPFSIEEVEVAP PKAHE
MZADH          MATAGKVIKCKAAVAWEAGKPLSIEEVEVAP PQAHE
YADH               SIPETQKGVIFYESHGKLEYKDIPVPK PKANE
TbADH                   MKGFAMLSIGKVGWIEKEKPAPGPFDAI
SsADH                   MRAVRLVEIGKPLSLQEIGVPK PKGPQ
BsADH                   MKAAVVEQFKKPLQVKEVEKPK ISYGE
BaADH                   MKAAVVHGFREPLRIED  VPK PDVGE
                        |         |          |
                        1         10         20
```

Fig. 5. **Comparison of NH_2-terminal amino acid sequences of horse liver (*HL*ADH), maize I (*MZ*ADH), yeast (*Y*ADH), *Thermoanaerobium brockii* (*Tb*ADH), *Sulfolobus solfataricus* (*Ss*ADH), *Bacillus acidocaldarius* (*Ba*ADH), and *Bacillus stearothermophilus* (*Bs*ADH) alcohol dehydrogenases.** Residue numbering of *HL*ADH is above the sequence, that of *Ba*ADH is below the sequence.

Pro 31 and Glu 35 (numbering of horse liver alcohol dehydrogenase), 2 of the 22 strictly conserved amino acid residues in 16 different alcohol/polyol dehydrogenases (*18*), were also present in the *Ba*ADH sequence (as shown in Fig. 5).

Effect of Metal Ions—No significant effect (*i.e.*, less than 10% variation in enzyme activity) was observed when the enzyme (1.0 μg/ml) was pre-incubated at 25°C for 5 min with 1 mM concentrations of each of the following salts and then assayed for activity in the presence of the salts at the same concentration as that used in the preincubation: NaCl, KCl, $CaCl_2$, $MgCl_2$, $MnCl_2$, and $ZnCl_2$.

The assay was performed as previously described by using the purified enzyme dialyzed against buffer A containing 1.0 mM 1,10-phenanthroline or 3.0 mM EDTA, overnight at 4°C.

The enzyme did not lose any activity on dialysis. None of the tested metal ions stimulated enzyme activity.

DISCUSSION

Alcohol dehydrogenases occur widely in nature and are produced by animals, plants, and microorganisms (bacteria, fungi, and yeasts). According to their source they differ in substrate and coenzyme specificity (*1*). It is commonly believed that these enzymes from eukaryotes are mainly NAD-dependent, while those from prokaryotic organisms are either NAD or NADP-dependent. In fact, two ADHs isolated from two different thermophilic bacteria utilize NADP as coenzyme (*19, 20*); we found that *Ba*ADH requires NAD, like the ADH from its closely related thermophilic eubacterium *B. stearothermophilus* (*Bs*ADH) (*21*) and ADH from archaeon *S. solfataricus* (*Ss*ADH) (*9*).

*Ba*ADH was purified to homogeneity by five simple steps. The crucial step in the purification procedure was the specific elution of enzymatic activity by NAD at pH 7.4 from a Blue A column. The presence of DTT in the buffers was necessary in order to avoid inactivation of the enzyme. The affinity chromatography step was useful for large-scale purification of the enzyme; a similar affinity chromatography procedure has been used for large-scale purification of horse liver alcohol dehydrogenase (*22*).

According to structure, alcohol dehydrogenases have been subdivided into different families: the short-chain family, which groups non-metalloenzymes with subunits of about 250 residues (*23*); the medium-chain family with subunits of 350-375 residues and, often, containing zinc (*18*); and the long-chain family with over 700 residues (*24*).

The zinc-dependent ADHs have a dimeric or tetrameric structure. Dimeric enzymes are the ADHs from mammals (*18*) and from higher plants (*25*). Tetrameric enzymes are the ADHs from yeasts and from bacteria (*26*). Recently, a detailed characterization of ADH from archaeon *S. solfataricus* has ascertained the presence of both dimeric and tetrameric forms, with the dimeric conformation showing the lower level of catalytic efficiency (*27, 28*).

*Ba*ADH appeared to have a tetrameric structure of 150 kDa, consisting of four similar or identical subunits of 38 kDa, like the ADHs of the yeasts *B. stearothermophilus* and *T. brockii* ADHs (*7, 21, 29*). Therefore, *Ba*ADH can be included in the family of tetrameric medium-chain alcohol dehydrogenases.

The temperature dependence of *Ba*ADH activity showed first-order kinetics, with increasing velocity, up to 80°C. At higher temperature the enzymatic activity decreased rapidly, probably due to thermal inactivation of the enzyme, as demonstrated by circular dichroism spectra, since at this temperature the melting of the protein structure is observed. The temperature dependence of the activity in the Arrhenius plot (insert panel of Fig. 3) showed a break point of the curve at about 50°C, indicating a probable conformational change of the protein, as previously reported for other thermophilic enzymes (*30, 31*). The activation energy calculated from the Arrhenius plot (at 50-80°C) was 42 kJ/mol, lower than that calculated for *S. solfataricus* ADH (47.8 kJ/mol) (*32*). Different activation energy could reflect a different conformational flexibility of the two enzymes in dependence on the temperature increase (*33*).

The enzyme thermal stability experiments revealed that *Ba*ADH possesses a thermoresistance comparable to that reported for *B. stearothermophilus* ADH (*21*), but it is less heat-stable than *S. solfataricus* ADH (*34*).

Investigations on the zinc content, combined with dialysis experiments, showed that the native *Ba*ADH contains 8 mol of zinc per mol of tetramer, and the chelating agents used removed only four of the eight zinc atoms. As was previously reported for *S. solfataricus* ADH (*9*), the zinc-depleted *Ba*ADH retained its catalytic activity but lost its thermal stability, suggesting that in the native enzyme four zinc atoms have a catalytic role and four have a structural role.

Horse liver ADH also contains two zinc atoms per subunit: one is bound in the center of the active site and is essential for the enzyme activity; the second is bound in a loop about 20 Å from the active site and plays a structural role (*35*).

Comparison between the predicted amounts of *Ba*ADH secondary structure obtained from far UV circular dichroism spectra and the well-known structure of horse liver enzyme (*36*) indicated significantly different contents of both α helix and β structure (22 and 45% against 28 and 41%, respectively).

Among the thermophilic enzymes which are utilized in synthetic processes, ADHs endowed with broad substrate specificity are valuable catalysts (*37*). Table II reports the kinetic constants towards different substrates, indicating the specificity of this enzyme.

*Ba*ADH was completely inactive towards methanol, whereas ethanol was the substrate with the highest value of k_{cat}/K_m. Moreover, at variance with other thermophilic ADHs whose substrate specificity has been investigated (*5, 6, 8, 9, 19, 21, 32, 38*), *Ba*ADH displays a higher activity towards primary alcohols than to the corresponding secondary alcohols. These results indicate the existence of a very selective site in the catalytic center of the enzyme, as was already hypothesized for the thermophilic ADHs from *T. brockii* (*8*) and *B. stearothermophilus* (*21*).

The high substrate selectivity is further supported by the kinetic behavior of the enzyme towards the aldehydes and ketones. In fact, of the aldehydes and ketones tested, only *p*-anisaldehyde was reduced with low efficiency and, even though the enzyme was active towards propan-2-ol, benzyl alcohol, and cyclopentanol, no activity was detected with acetone benzaldehyde and cyclopentanon. These unusual functional properties of *Ba*ADH differ from those described for other ADHs from mesophilic (*1, 39-41*) and thermophilic sources (*8, 9, 21*), which show a more broad substrate specificity. Recently it was shown (*42*) that the introduction of a negative charge in the active site of *S. solfataricus* ADH by selective carboxymethylation caused a significant increase in oxidation rate of aliphatic and aromatic alcohols, and a strong reduction rate of aromatic aldehydes. Moreover, it is worth noting that *Ba*ADH shows a very low K_m value toward NADH, and as a consequence its inability to reduce ketones could only be related to its active-site organization.

In its NH_2-terminal amino acid sequence, *Ba*ADH contains Pro and Glu at positions 31 and 35 (numbering of horse liver ADH). These two residues are strictly conserved in almost all alcohol dehydrogenases (*18*), and therefore we hypothesize that they also have an essential functional role *Ba*ADH. Furthermore, the analysis of sequence similarities between *Ba*ADH and *Bs*ADH showed positional identities of 60% (*43*). These results also indicate a common evolutionary origin for alcohol dehydrogenases from the two bacillus strains.

Studies are in progress to characterize the *B. acidocaldarius* enzyme structure in order to understand the molecular basis of the natural inability of the enzyme to reduce ketones, unlike all other ADHs.

We thank Dr. Antonio Capasso for the zinc analyses, Mr. Francesco Marulli, and Mr. Francesco Cavaliere for their technical assistance. Skillful assistance in protein N-terminal sequencing by Mr. Vitale Carratore is gratefully acknowledged.

REFERENCES

1. Branden, C.I., Jornvall, H., Eklund, H., and Furugren, B. (1975) Alcohol dehydrogenases in *The Enzymes* (Boyer, P.D., ed.) Vol. 11, pp. 103-190, Academic Press, New York
2. Brunger, A.T., Kuriyan, J., and Karplus, M. (1987) Crystallographic R factor refinement by molecular dynamics. *Science* **235**, 458-460
3. Brunger, A.T. (1993) Assessment of phase accuracy by cross validation: The free R value, methods and applications. *Acta Crystallogr. D* **49**, 24-36
4. Al-Karadaghi, S., Cedergren-Zeppezauer, E.S., Hovmoller, S., Petratos, K., Terry, H., and Wilson, K.S. (1994) Refined crystal structures of liver alcohol dehydrogenase-NADH complex at 1.8 Å resolution. *Acta Crystallogr. D* **50**, 793-807
5. Sheehan, M.C., Bailey, C.J., Dowds, B.C.A., and Mc Connell, D.J. (1988) A new alcohol dehydrogenase reactive towards methanol from *Bacillus stearothermophilus*. *Biochem. J.* **252**, 661-666
6. Bryant, R.O., Wiegel, J., and Ljungdahl, L.G. (1988) Purification and properties of primary and secondary alcohol dehydrogenases from *Thermoanaerobacter ethanolicus*. *Appl. Environ. Microbiol.* **54**, 460-465
7. Peretz, M. and Burstein, Y. (1989) Amino acid sequence of alcohol dehydrogenase from the thermophilic bacterium *Thermoanaerobium brockii*. *Biochemistry* **28**, 6549-6555
8. Keinan, E., Hafeli, E.K., Seth, K.K., and Lamed, R. (1986) Thermostable enzymes in organic synthesis. Asymmetric reduction of ketones with alcohol dehydrogenase from *Thermoanaerobium brockii*. *J. Am. Chem. Soc.* **108**, 162-169
9. Ammendola, S., Raia, C.A., Caruso, C., Camardella, L., D'Auria, S., De Rosa, M., and Rossi, M. (1992) Thermostable NAD^+ dependent alcohol dehydrogenase from *Sulfolobus solfataricus*: Gene and protein sequence determination and relationship to other alcohol dehydrogenases. *Biochemistry* **31**, 12514-12523
10. Millonig, G., De Rosa, M., Gambacorte, A., and Bu'Lock, J.D. (1975) Ultrastructure of an extremely thermophilic acidophilic microorganism. *J. Gen. Microbiol.* **86**, 165-173
11. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of Protein-dye binding. *Anal. Biochem.* **72**, 248-254
12. Martin, R.G. and Ames, B.N. (1961) A method for determining the sedimentation behaviour of enzymes: Application to protein mixtures. *J. Biol. Chem.* **236**, 1372-1379
13. Andrews, P. (1964) Estimation of the molecular weights of proteins by Sephadex gel-filtration. *Biochem. J.* **91**, 222-223
14. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**, 680-685
15. Robertson, E.F., Dannely, H.K., Mallay, P.J., and Reeves, H.C. (1987) Rapid isoelectric focusing in a vertical polyacrylamide minigel system. *Anal. Biochem.* **167**, 290-294
16. Rudge, J. and Bickerstaff, G.F. (1986) Purification and properties of an alcohol dehydrogenase from *Sporotrichum pulverulentum*. *Enzyme Microb. Technol.* **8**, 120-124
17. Young, J.T., Wu, C.-S.C., and Martinez, H.M. (1986) Calculation of protein conformation from circular dichroism. *Methods Enzymol.* **130**, 208-269
18. Jornvall, H., Persson, M., and Jeffery, J. (1987) Characteristics of alcohol/polyol dehydrogenases. *Eur. J. Biochem.* **167**, 195-201
19. Lamed, R.J. and Zeikus, J.G. (1981) Novel NADP-linked alcohol-aldehyde/ketone oxidoreductase in thermophilic ethanologenic bacteria. *Biochem. J.* **195**, 183-190
20. Lamed, R.J. and Zeikus, J.G. (1980) Ethanol production by thermophilic bacteria: Relationship between fermentation product yields of and catabolic enzyme activities in *Clostridium thermocellum* and *Thermoanaerobium brokii*. *J. Bacteriol.* **144**, 569-578
21. Guagliardi, A., Martino, M., Iaccarino, I., De Rosa, M., Rossi, M., and Bartolucci, S. (1996) Purification and characterization of the alcohol dehydrogenase from a novel strain of *Bacillus stearothermophilus* growing at 70°C. *Int. J. Biochem. Cell. Biol.* **28**, 239-246
22. Roy, S.K. and Nishikawa, A.H. (1979) Large-scale isolation of equine liver alcohol dehydrogenase on a Blue agarose gel. *Biotechnol. Bioeng.* **21**, 775-785
23. Persson, M., Krook, M., and Jornvall, H. (1991) Characteristics of short-chain alcohol dehydrogenases and related proteins. *Eur. J. Biochem.* **200**, 537-543
24. Goodlove, P.E., Cunningham, P.R., Parker, J., and Clark, D.P. (1989) Cloning and sequence analysis of the fermentative alcohol-dehydrogenase-encoding gene of *Escherichia coli*. *Gene* **85**, 209-214
25. Llewellyn, D.J., Finnegan, E.J., Ellis, J.G., Dennis, E.S., and Peacock, W.J. (1987) Structure and expression of an alcohol dehydrogenase 1 gene from *Pisum sativum* (cv. "Greenfast"). *J. Mol. Biol.* **195**, 115-123
26. Eklund, H., Branden, C., and Jornvall, H. (1976) Structural comparison of mammalian, yeast and bacillar alcohol dehydro-

genases. *J. Mol. Biol.* **102**, 61-73
27. Raia, C.A., D'Auria, S., and Rossi, M. (1994) NAD-dependent alcohol dehydrogenase from *Sulfolobus solfataricus*: Structural and functional features. *Biocatalysis* **11**, 143-150
28. Cannio, R., Fiorentino, G., Carpinelli, P., Rossi, M., and Bartolucci, S. (1996) Cloning and overexpression in *Escherichia coli* of the genes encoding NAD-dependent alcohol dehydrogenase from two *Sulfolobus* species. *J. Bacteriol.* **178**, 301-305
29. Jornvall, H., Eklund, H., and Branden, C.I. (1978) Subunit conformation of yeast alcohol dehydrogenase. *J. Biol. Chem.* **253**, 8414-8419
30. Bartolucci, S., Rella, R., Guagliardi, A., Raia, C.A., Gambacorta, A., De Rosa, M., and Rossi, M. (1987) Malic enzyme from archaebacterium *Sulfolobus solfataricus*. *J. Biol. Chem.* **262**, 7725-7731
31. Pisani, F.M., Rella, R., Rozzo, C., Raia, C.A., Nucci, R., Gambacorta, A., De Rosa, M., and Rossi, M. (1990) Thermostable β-galactosidase from the archaebacterium *Sulfolobus solfataricus*. Purification and properties. *Eur. J. Biochem.* **187**, 321-328
32. Rella, R., Raia, C.A., Pensa, M., Pisani, F.M., Gambacorta, A., De Rosa, M., and Rossi, M. (1987) A novel archaebacterial NAD-dependent alcohol dehydrogenase. Purification and properties. *Eur. J. Biochem.* **167**, 475-479
33. Wrba, A., Schweiger, A., Schultes, V., and Jaenicke, R. (1990) Extremely thermostable D-glyceraldehyde-3-phosphate dehydrogenase from the eubacterium *Thermotoga marittima*. *Biochemistry* **29**, 7584-7592
34. Raia, C.A., D'Auria, S., Guagliardi, A., Bartolucci, S., De Rosa, M., and Rossi, M. (1995) Characterization of redox proteins from extreme thermophilic archaebacteria: Studies on alcohol dehydrogenase and thioredoxins. *Biosensor Bioelectron.* **10**, 135-140
35. Eklund, H., Jones, A., and Schneider, G. (1986) Active site in alcohol dehydrogenase in *Zinc Enzymes* (Bertini, I., ed.) Vol. 1, pp. 377-392, Birkhauser Boston, Boston
36. Ribas de Pouplana, L., Atrian, S., Gonzales-Duarte, R., Fothergill-Gilmore, L.A., Kelly, S.M., and Price, N.C. (1991) Structural properties of long- and short-chain alcohol dehydrogenase. *Biochem. J.* **276**, 433-438
37. Colbear, T., Daniel, R.M., and Morgan, H.W. (1992) The enzymes from extreme thermophiles: Bacterial sources, thermostabilities and industrial relevance. *Adv. Biochem. Eng. Biotechnol.* **45**, 57-98
38. Simpson, H.D. and Cowan, D.A. (1992) Secondary alcohol dehydrogenases from extremely thermophilic bacteria. *Ann. N.Y. Acad. Sci.* **672**, 145-151
39. Adolph, H.W., Maurer, P., Schneider-Bernlohr, H., Sartorius, C., and Zeppezauer, M. (1991) Substrate specificity and stereoselectivity of horse liver alcohol dehydrogenase. *Eur. J. Biochem.* **201**, 615-625
40. Dalziel, K. and Dickinson, F.M. (1966) Substrate activation and inhibition in coenzyme-substrate reactions cycloexanol oxidation catalyzed by liver alcohol dehydrogenase. *Biochem. J.* **100**, 491-500
41. Ramaswamy, S., El Ahmad, M., Danielsson, O., Jornvall, H., and Eklud, H. (1996) Crystal structure of cod liver class I alcohol dehydrogenase: Substrate pocket and structurally variable segments. *Protein Sci.* **5**, 663-671
42. Raia, C.A., Caruso, C., Marino, M., Vespa, N., and Rossi, M. (1996) Activation of *Sulfolobus solfataricus* alcohol dehydrogenase by modification of cysteine residue 38 with iodoacetic acid. *Biochemistry* **35**, 638-647
43. Bridgen, J., Kolb, E., and Harris, J.I. (1973) Amino acid sequence homology in alcohol dehydrogenase. *FEBS Lett.* **33**, 1-3