Biochimie 82 (2000) 1143−1150

S0300908400011937/FLA

2-Hydroxyacid dehydrogenase from *Haloferax mediterranei*, a D-isomer-specific member of the 2-hydroxyacid dehydrogenase family

María-José Bonete*, Juan Ferrer, Carmen Pire, Magdalena Penades, José Luiz Ruiz

Departamento de Bioquímica, Facultad de Ciencias, Universidad de Alicante, Ap. 99, Alicante, Spain

(Received 15 May 2000; accepted 21 July 2000)

Abstract — An NAD-dependent D-2-hydroxyacid dehydrogenase (EC 1.1.1.) was isolated and characterized from the halophilic Archaeon *Haloferax mediterranei*. The enzyme is a dimer with a molecular mass of 101.4 ± 3.3 kDa. It is strictly NAD-dependent and exhibits its highest activity in 4 M NaCl. The enzyme is characterized by a broad substrate specificity 2-ketoisocaproate and 2-ketobutyrate being the substrates with the higher V_{max}/K_m. When pyruvate and 2-ketobutyrate were the substrates the optimal pH was acidic (pH 5) meanwhile for 2-ketoisocaproate maximum activity was achieved at basic pH between 7.5 and 8.5. The optimum temperature was 52 ºC and at 65 ºC there was a pronounced activity decrease. This new enzyme can be used for the production of D-2-hydroxycarboxylic acid. © 2000 Société française de biochimie et biologie moléculaire / Éditions scientifiques et médicales Elsevier SAS

Archaea / *Haloferax mediterranei* / D-hydroxyacid dehydrogenase

1. Introduction

The stereospecific reduction of carbonyl groups is of interest for the production of various chiral compounds such as hydroxyacids, amino acids or alcohol from prochiral precursors [1]. Such products have a high economic value and have applications in food and feed, or serve as building blocks in the synthesis of therapeutics, herbicides, insecticides, etc. [2–4]. NAD-dependent hydroxyacid dehydrogenases allow the synthesis of chiral hydroxyacids from their corresponding ketocarboxylic acids. They catalyze the stereospecific and reversible NADH-dependent reduction of 2-ketocarboxylic acids into the corresponding 2-hydroxy carboxylic acids, as depicted in equation:

$$\mathrm{RCOCOOH + NADH + H^+ \leftrightarrow RCH(OH)COOH + NAD^+}$$

A number of NAD-dependent 2-hydroxyacid dehydrogenases which seem to be specific for the D-isomer of their substrate have been shown [5–8] to be functionally and structurally related. One of these enzymes is D-2-hydroxyisocaproate dehydrogenase (EC 1.1.99.6), a bacterial enzyme that catalyses the reversible and stereospecific interconversion between 2-ketocarboxylic acids and D-2-hydroxy-carboxylic acids. Their actual substrate and function in vivo are not known [4]. NAD-dependent 2-hydroxyacid dehydrogenases of broad substrate specificity have been reported in many species [1, 9]. For example, L-2-hydroxyisocaproate dehydrogenase from *Lactobacillus confusus* is characterized by a broad substrate specificity and uses a wide range of 2-ketocarboxylic acids branched at the C4 atom [9], although the preferred substrates have an unbranched chain of 5–6 carbon atoms, 2-ketocaproate being the substrate tested with the highest V_{max}/K_M ratio. For the NAD-dependent 2-hydroxyisocaproate, the broad substrate specificity could be attributed to the presence of four extra residues in the mobile loop closing its active site, allowing accommodation of substrates larger than pyruvate [4, 10]. This flexible coenzyme loop closes over the active site like a lid after the ternary complex is formed, excluding water during catalysis [11]. However, the best-known 2-hydroxyacid dehydrogenases, lactate and malate dehydrogenases, suffer from a somewhat narrow substrate specificity. This is particularly a problem for the application of R-stereospecific enzymes, which are considerably more limited than the S-enzymes in their substrate specificity [3].

Archaea are a group of organisms phylogenetically distinct from Bacteria and Eukarya [12–14] that have evolved under extreme conditions. Extremely halophilic Archaea (haloarchaea) require 10–20% NaCl for optimal growth and predominantly use amino acids as their source of carbon and energy. However, some haloarchaea are able to use different sugars and *Haloferax mediterranei*, for example, grows in a minimal medium containing glucose as the only source of carbon [15, 16]. Enzymes from

* *Correspondence and reprints.*
E-mail address: mjbonete@ua.es (M.J. Bonete).

haloarchaea are often difficult to purify because they usually require high salt concentrations for stability and high-resolution purification procedures are therefore limited. The present paper deals with the characterization and purification of a D-2-hydroxyacid dehydrogenase from the halophilic Archaeon *Haloferax mediterranei.* This is the first example of an archaeal D-2-hydroxyacid dehydrogenase that has been purified. The new dehydrogenase can use for the production a variety of chiral D-2-hydroxycarboxylic acids.

2. Materials and methods

2.1. Culture conditions

Haloferax mediterranei (R4, ATCC 33500) cells were grown aerobically at 37 ºC in a medium containing 25% (w/v) salts and 0.5% (w/v) yeast extract (Difco) (pH 7.0–7.3) as described by Rodriguez-Valera et al. [15], and supplemented with 1% (w/v) glucose as reported by Bonete et al [16].

2.2. Purification of 2-hydroxyacid dehydrogenase from Hf. mediterranei

Hf. mediterranei cells were harvested by centrifugation. Twenty-six grams of wet cells were resuspended in 50 mM sodium phosphate buffer, pH 6.6, containing 2.5 M $(NH_4)_2SO_4$ (buffer 1) (1g wet wt/2 mL) and lysed by sonication at 4 ºC. The disrupted suspension was clarified by centrifugation (60 min at 105 000 *g*). All subsequent steps were carried out at room temperature. The purification steps were carried out automatically with a Gradifrac System (Pharmacia).

- Step 1. The crude extract was applied to a Sepharose 4B column (2.6×53 cm) equilibrated with buffer 1. The 2-hydroxyacid dehydrogenase activity was eluted with a decreasing gradient of ammonium sulphate, from 2.5 M $(NH_4)_2SO_4$ (buffer 1) to 0.5 M $(NH_4)_2SO_4$.
- Step 2. The fractions with activity were pooled and applied to a DEAE-cellulose column (1.5×8.0 cm), previously equilibrated with buffer 1. After washing with the same buffer, 2-hydroxyacid dehydrogenase activity was eluted with 10 mM phosphate buffer, pH 7.3, containing 2 M NaCl and 2 mM EDTA.
- Step 3. The fractions with higher specific activity were pooled and desalted by gel filtration with a Sephadex G25 column (PD-10 from Pharmacia). This step was performed with 10 mM phosphate buffer, pH 7.3, 2 mM EDTA, 20% glycerol. The volume of the sample introduced every time in the column was 2.5 mL. The active fractions were pooled.
- Step 4. The obtained enzyme solution was loaded onto an anion-exchange column (2.5×5 cm) of Q-Sepharose (Pharmacia), equilibrated with phosphate buffer 10 mM, pH 7.3, 2 mM EDTA and 20% glycerol, and eluted with a linear gradient of 0–2 M KCl, in the same phosphate buffer.
- Step 5. The enzyme solution from the previous step was purified by gel filtration on a Sephacryl S-300 column (1.6×60 cm) from Pharmacia, the filtration was carried out in 50 mM phosphate buffer, pH 7.3, and 2 M KCl.
- Step 6. Finally, the high-specific-activity material previously pooled was again applied to a Sephacryl S-300 column, as in the previous step.

The purity of the enzyme solution was examined by SDS-PAGE according to Laemmli [17].

2.3. Enzyme assay and protein determinations

2-Hydroxyacid dehydrogenase was assayed spectrophotometrically at 340 nm and carried out at room temperature or, in any case, at 40 ºC. The reaction was assayed with three different substrates: 2-ketoisocaproate, 2-ketobutyrate and pyruvate for the reductive reaction. For the reverse reaction the substrates were L-2-hydroxycaproate, DL-2-hydroxybutyrate and the two stereoisomers of lactate: D-lactate and L-lactate. The concentration of ketocarboxylic acids in the assay mixture, for the reductive reaction, was 2.5 mM and 3.0 mM NADH. For the oxidative reaction NAD^+ concentration was 67.5 mM. Assay buffers for each ketocarboxylic acid was selected for optimal conditions. However, for routine assays the reaction mixture contained 0.1 M carbonate-bicarbonate buffer, pH 9.7, 4 M NaCl, 2.5 mM 2-ketoisocaproate and 3.0 mM NADH. The reaction was started by the addition of NADH or NAD^+, respectively. One unit of enzymatic activity was defined as the amount of enzyme which catalyses the consumption of 1 µmol of NADH/min under assay conditions. Protein concentrations were determined by the method of Bradford [18], using bovine serum albumin as a standard protein. Kinetic data were analyzed according to Cleland [19].

2.4. Determination of M_r values

After desalting by acetone precipitation, the M_r value for the subunit of purified 2-hydroxyacid dehydrogenase was determined by SDS-PAGE [17] and by CTAB-PAGE [20] using standard protein markers. The M_r value for native 2-hydroxyacid dehydrogenase enzyme was determined by gel filtration on a Pharmacia FPLC Sephacryl S-300 (1.6×60 cm) column, carried out in 50 mM sodium phosphate buffer, pH 7.3, and 2 M KCl. The column was calibrated with catalase (240 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa) and trypsin inhibitor (21.5 kDa).

3. Results

3.1. Purification of 2-hydroxyacid dehydrogenase

As reported for glucose dehydrogenase for the same microorganism [16], the level of 2-hydroxyacid dehydro-

Table I. Purification of D-2-hydroxyacid dehydrogenase from *Haloferax mediterranei*.

Step	*Total protein (mg)*	*Specific activity (U/mg)*	*Enrichment (n-fold)*	*Yield (%)*
Cell extract	794	1.08	–	100
Sepharose-4B	170	2.62	2.4	52
DEAE-cellulose	41	4.44	4.1	21
Q-Sepharose	4.2	19.44	17.9	9
Sephacryl S-3000	0.07	269.4	249	2.2

genase was influenced by the composition of the culture medium. When this medium is supplemented with 1% glucose the specific activity is higher. The specific activity obtained in a culture with 0.5% yeast extract was 0.36 U/mg, whereas in a medium supplemented with 1% glucose it was 1.08 U/mg. The purification of 2-hydroxyacid dehydrogenase from *Haloferax mediterranei* is summarised in *table I*. All the steps were performed in buffers of high salt concentration and/or in the presence of glycerol (20%) to stabilize the protein structure [16]. The last step was repeated twice to increase the enzyme purity. Other alternative ways were tested but in all cases the purification achieved was rather worse than the proposed scheme.

3.2. Effect of pH on the enzyme activity

The influence of the pH on the reaction rate for the reduction of 2-ketocarboxylic acids was measured. When pyruvate and 2-ketobutyrate were the substrates the optimal pH was acidic (pH 5) meanwhile for 2-ketoisocaproate maximum activity was achieved at basic pH (8.5). However, the activity with the three substrates can be measured in a wide range of pH (5.0–9.0) with a very high activity *(figure 1)*.

3.3. Substrate specificity

Substrate specificity of the purified enzyme was established by following the NADH dependent reduction of a variety of 2-ketocarboxylic acids: pyruvate, 2-ketobutyrate, 2-ketoisocaproate, β-phenylpyruvate, 2-ketometil-n-valerate and 2-ketoisovalerate *(table II)*, but only pyruvate, 2-ketobutyrate, 2-ketoisocaproate showed significant activity. The activity shown by the others was less than 2% of the activity achieved with pyruvate. The concentration of the ketocarboxylic acids added to the assay mixture was 2.5 mM and NADH concentration was 6 mM. The reverse reaction was studied, using the related 2-hydroxyacid compound, i.e., L-2-hydroxyisocaproate (using concentrations ranging from 3.9 mM to 0.12 mM), 2-hydroxybutyrate and the two stereoisomers of lactate, D-lactate and L-lactate (with concentrations from 1.75 to 0.0625 mM). No reaction was detected for L-lactate, 2-hydroxybutyrate and L-hydroxyisocaproate, and low activity with D-lactate. Kinetic parameters of the reductive reaction for each of the possible substrates were determined in 0.1 M glycine-NAOH buffer, pH 9.0, containing 4 M NaCl, 6 mM NADH and variable amounts of the 2-ketocarboxylic acids. The reaction rate was measured as a function of the substrate concentration and K_M and V_{max} values calculated by non-linear regression analysis of the Michaelis-Menten equation [19]. Similarly the K_M value for NADH was determined using 25 mM of 2-ketoisocaproate, or 10 mM 2-ketobutyrate or 10 mM pyruvate. The results are summarised in *table III*

3.4. Determination of the product of the enzymatic reaction by mass spectrometry

Due to the low activity obtained for substrates in the reverse reaction, the product of reaction catalyses by 2-hydroxyacid dehydrogenase, using pyruvate as substrate, was determined by electrospray mass spectrometry on a Platform (Fisons) instrument. The m/z range from 50 to 500 Da/e was scanned continuously during 1 s. The

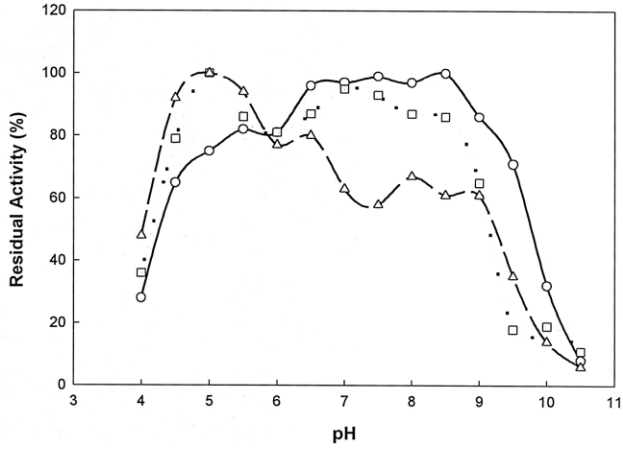

Figure 1. pH profiles for different substrates. Pyruvate (Δ), 2-ketobutyrate (□) and 2-ketoisocaproate (o). The pH range was 4–5.5 (acetate buffer), 5.5–8.0 (phosphate buffer), 8.0–9.0 (Tris-HCl buffer) and 9.0–10.5 (bicarbonate buffer). All buffers were 0.1 M, containing 4 M NaCl.

Table II. Substrate specificity of D-2-hydroxyacid dehydrogenase from *Hf. mediterranei*. The concentration of the ketocarboxylic acids added to the assay mixture for the reductive reaction was 2.5 mM and NADH concentration was 6 mM. Assays were performed in glycine-NaOH buffer 0.1 M, pH 9 containing 4 M NaCl.

Substrate	*Specific activity*	*Relative rate (%)*
Pyruvate	1.462	100
2-ketobutyrate	0.799	55
2-ketoisocaproate	0.427	29
β-phenylpiruvate	0.025	1.7
α-ketomethyl-n-valerate	0.010	0.7
2-ketoisovalerate	0.002	0.13

mass spectra *(figure 2)* show that the peaks at m/z 98.6 Da/e only appear in the sample from enzymatic reaction and in the standard D-lactate and not in the standard L-lactate; it provides support for D-lactate as a product of the enzymatic reaction.

3.5. Kinetics

Initial velocities were analyzed with pyruvate as a variable substrate in the presence of several fixed concentrations of NADH. Plots of reciprocals of initial velocities against reciprocals of pyruvate concentrations gave a family of straight lines, which intersected in the left quadrant *(figure 3)*. When NADH was used as variable substrate, similar straight lines intersecting in the left quadrant were obtained. These results suggest that the reaction proceeds through a sequential mechanism. The K_M for NADH and pyruvate were calculated to be 0.022 and 0.41 mM, respectively. These values are different from those tabulated in *table III* because kinetic parameters were used in Tris-HCl buffer, pH 8.0.

3.6. Effect of salt on activity and stability of 2-hydroxyacid dehydrogenase

The activity of the halophilic 2-hydroxyacid dehydrogenase was markedly dependent on the concentration of NaCl or KCl. The effect of both salts are similar (KCl has a slightly higher stimulatory effect than NaCl at the same concentration) for the halophilic enzymes studied in available literature [16, 21–23]. A linear relation between enzyme activity and salt concentration was achieved, the optimal concentration being at 4 M, the concentration nearly saturating. Salts also affect the stability of halophilic proteins. The storage time was related to the residual activity exhibited by the enzyme in different conditions. When the extract was stored in absence of salts, the enzyme lost 40% of its activity in the first hour, in a day's time it exhibited only 20% of its initial activity and after a week its residual activity was only 9%. Meanwhile, the extracts stored with 1.5 M and 3 M NaCl preserved 100% of its initial activity. When salts are replaced by 20% (v/v) glycerol, enzyme extract also maintains 100% of its initial activity during all the time tested.

3.7. Effect of temperature on activity of 2-hydroxyacid dehydrogenase

The temperature dependence of 2-ketocarboxylic acid reduction was analyzed. The purified enzyme was incubated, for 3 min, at different temperatures and specific activity was measured for each substrate studied. The optimal temperature was found to be around 52 ºC. Above 60 ºC rapid thermal inactivation of the enzyme occurred and at 75 ºC activity was nearly over.

3.8. Molecular mass and subunit structure

The apparent molecular mass for the purified native 2-hydroxyacid dehydrogenase estimated by gel filtration was 101.4 ± 3.3 kDa. SDS-PAGE of the purified halophilic enzyme from *Hf. mediterranei* with anionic and cationic detergents revealed that the enzyme subunit migrated as a single protein band *(figure 4)*. The mobility of the enzyme in SDS-PAGE corresponds to a subunit M_r of 62.9 kDa. However, the M_r value determined by CTAB-PAGE was 59.5 kDa. According to these data enzyme protein must be a dimeric enzyme.

Table III. Kinetic parameter for the halophilic D-2-hydroxyacid dehydrogenase reaction. Assays were performed in glycine-NaOH buffer 0.1 M, pH 9, 4 M NaCl. 2-Ketoisocaproate was varied from 0.25 mM to 40 mM, 2-ketobutyrate from 0.125 mM to 40 mM and pyruvate from 0.05 mM to 40 mM. To calculate kinetic parameter for NADH, 2-ketoisocaproate concentration was 25 mM, and 2-ketobutyrate and pyruvate 10 mM. When the substrate was D-lactate (1.75 to 0.0625 mM) the buffer was 0.1 M acetate buffer, pH 5.0, containing 4 M NaCl and NAD^+ concentration was 67.5 mM.

Substrate	K_M *(mM)*	V_{max} *(U/mg)*	V_{max}/K_M *(min^{-1})*
Pyruvate	0.56 ± 0.07	2.92 ± 0.08	5.2 ± 0.5
2-ketobutyrate	0.36 ± 0.06	1.44 ± 0.05	5.2 ± 0.5
2-ketoicocaproate	2.3 ± 0.4	1.26 ± 0.06	0.54 ± 0.08
$NADH_{(pyruvate)}$	0.096 ± 0.016	3.6 ± 0.2	38 ± 4
$NADH_{(2\text{-ketobutyrate})}$	0.050 ± 0.004	1.70 ± 0.05	34 ± 2
$NADH_{(2\text{-ketoisocaproate})}$	0.086 ± 0.010	1.59 ± 0.08	18.5 ± 1.4
D-lactate	0.14 ± 0.02	0.163 ± 0.009	1.16 ± 0.16

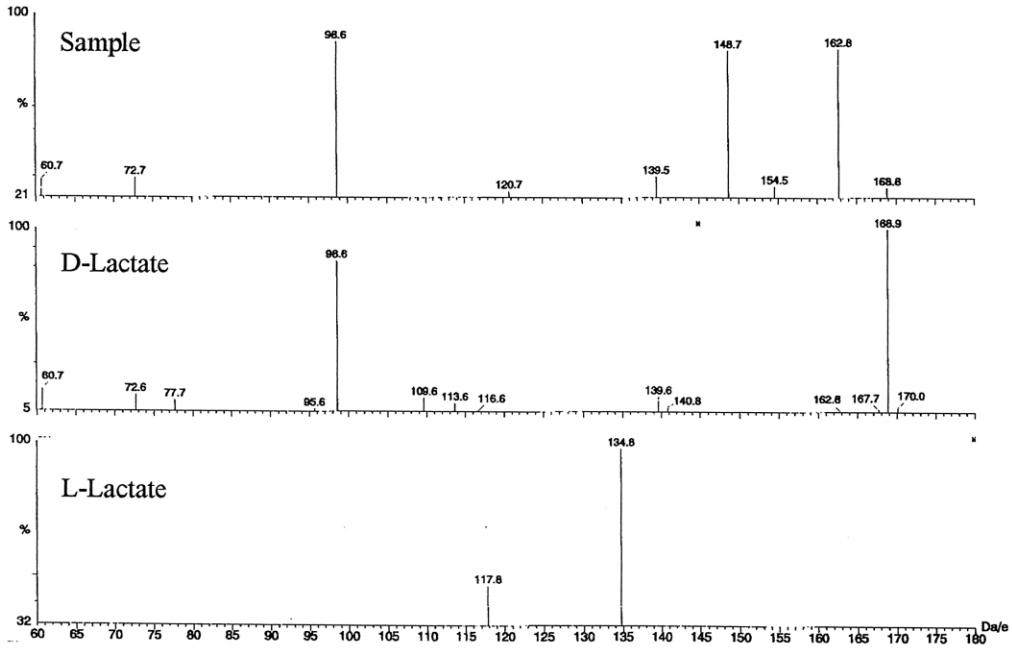

Figure 2. Electrospray mass spectra of the enzymatic reaction product (sample), D-lactate and L-lactate. Prior to analysis solutions were desalted using a ZipTipC18 (Millipore) and 25 µL were injected for the collection of the spectra. Mobile phase was acetronitrile, water and formic acid.

4. Discussion

In this study, we have purified 200-fold, higher than reported for other similar enzymes such as D-lactate dehydrogenase (34-fold) [24], nearly to homogeneity, and characterized a NAD-specific D-2-hydroxyacid dehydrogenase from *Haloferax mediterranei*. The halophilic enzyme is characterized by a broad substrate specificity. The same feature is reported for 2-hydroxyacid dehydrogenases from other sources, such as NAD-specific L-2-hydroxyisocaproate dehydrogenases from *Lactobacillus confusus* [9] (that uses a wide range of 2-ketocarboxylic acids branched at the C4 atom), *L. casei* [3] or *L. delbrueckii* [4]. D-lactate dehydrogenase from *L. casei* did not show this broad specificity [4]. In our case, the substrate with the higher V_{max}/K_m was pyruvate and 2-ketobutyrate followed by 2-ketoisocaproate. When pyruvate and 2-ketobutyrate were the substrates the optimal pH was acidic (pH 5) meanwhile for 2-ketoisocaproate maximum activity was achieved at basic pH between 7.5 and 8.5, although the enzyme is active in a wide range of pHs. For the oxidative reaction the related 2-hydroxyacids were tested and only D-lactate could act as substrate. With L-lactate and 2-hydroxybutyrate the enzyme did not show activity.

As can be observed in SDS-PAGE and CTAB-PAGE, the enzyme migrated as a single band (63.9 kDa and 59.5 kDa, respectively). The value obtained by CTAB-PAGE is seen as more reliable for highly acidic proteins [16]. According to our previous results, we concluded that *Hf. mediterranei* 2-hydroxyacid dehydrogenase is a dimeric enzyme. The molecular mass for the halophilic enzyme estimated by gel filtration was 101.4 ± 3.3 kDa is similar to L-isocaproate dehydrogenase from *L. confusus:* 125 kDa [25]. However, their structures are different, since this enzyme is tetrameric and the halophilic one is dimeric. The same enzyme, but obtained from *L. casei*, is also dimeric but with a lower molecular mass: 74 kDa [26], as well as the 2-hydroxyacid dehydrogenases from *L. curvatus* and *Streptococcus faecalis*, with 60 kDa and 72 kDa, respectively [1]. D-lactate dehydrogenase from *Leuconostoc mesenteroides* is also dimeric and each dimer has a molecular mass of 37 kDa [24]. The *Hf. mediterranei* 2-hydroxyacid dehydrogenase seems to have subunits bigger than for other reported enzymes.

The high temperatures at which activity displays a maximum (52 ºC) and the enzyme loses its activity (75 ºC) are similar to the temperatures reported for other halophilic dehydrogenases such as NADP-glutamate dehydrogenase [22], NAD-glutamate dehydrogenase [21] from *H. salinarum*, isocitrate dehydrogenase from *Haloferax volcanii* [23] or NADP-glutamate dehydrogenase [27] and glucose dehydrogenase [16] from *Haloferax*

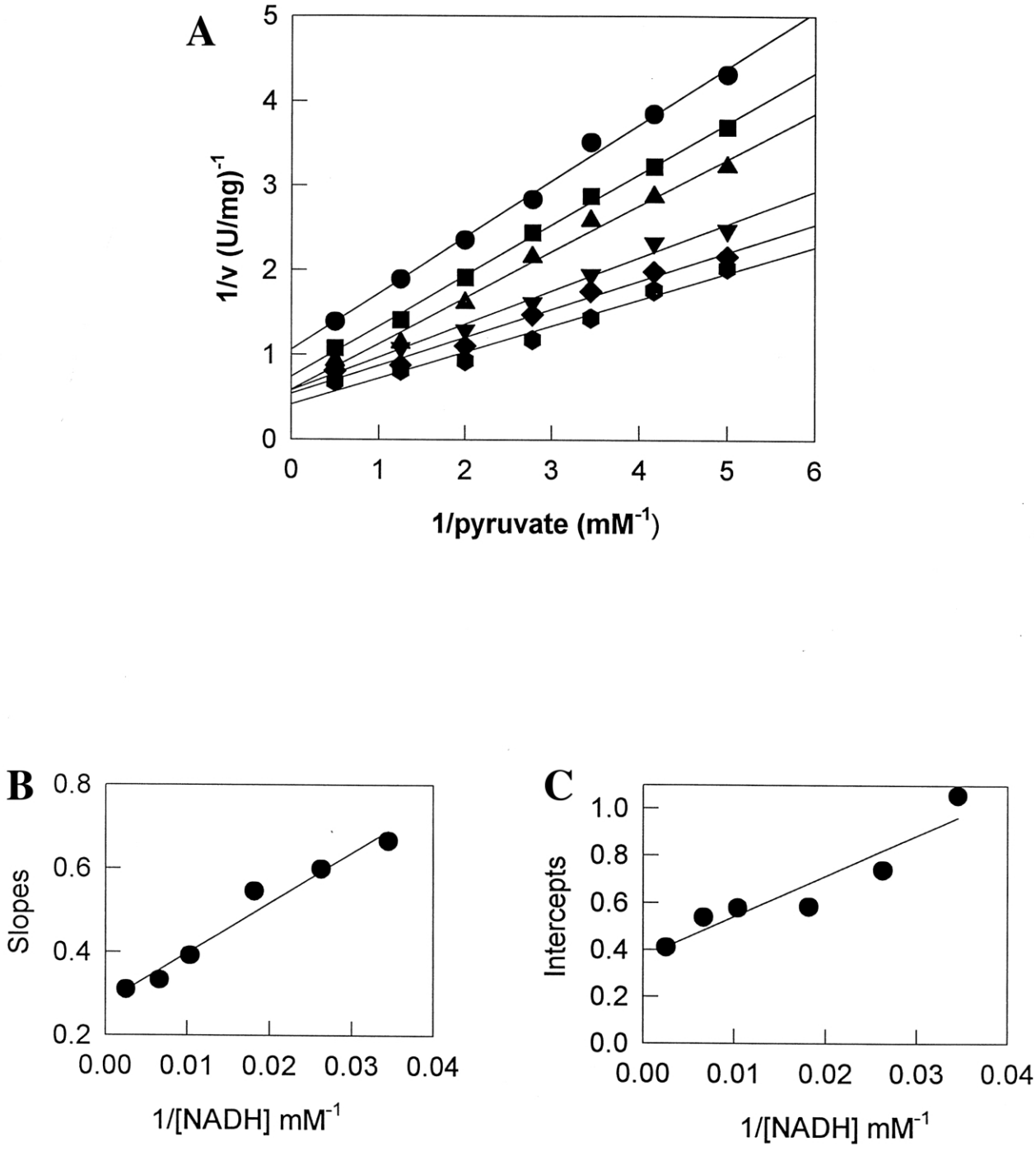

Figure 3. A. Initial velocity patterns with pyruvate as varied substrate (range 0.2–2 mM) and with NADH as fixed substrate 29–400 µM. **B.** Replot of slope versus 1/[NADH]. **C.** Replot of intercepts versus 1/[NADH].

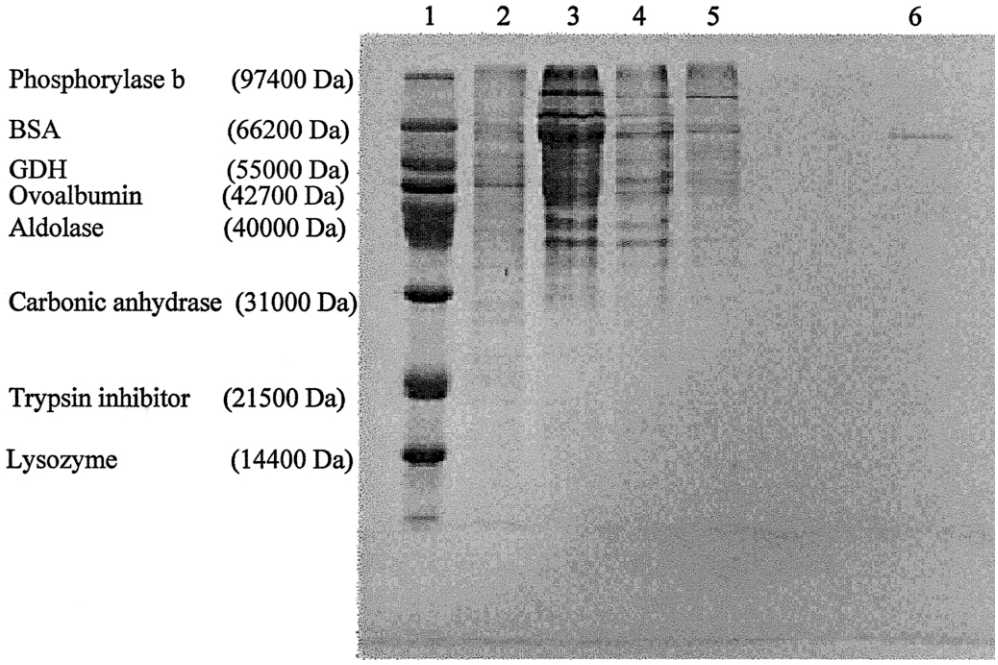

Figure 4. SDS-PAGE of *Haloferax mediterranei* 2-hydroxyacid dehydrogenase. Lane 1, 5 µg of protein markers (middle range of Promega); lanes 2, 3, 4, 5 and 6, 5 µg of protein obtained from purification steps 1, 2, 4, 5 and 6 respectively.

mediterranei. Although these temperatures are very high, they are lower than the optimal temperatures for extreme thermophiles, such as the thermophilic bacterium *Thermus thermophilus*, whose optimal temperature for glutamate dehydrogenase was around 85–90 ºC [28]. The thermophilic nature of halophilic enzymes has been noted in several systems and, from the crystal structure of *Haloarcula marismortui* malate dehydrogenase, Dym et al. [29] point out that several of the structural features conferring halophilicity (increased number of salt bridges and introduction of negatively charged amino acids near the amino termini of helices) are the same as those contributing to stability of thermophilic enzymes.

The other essential halophilic characteristics exhibited by our 2-hydroxyacid dehydrogenase is the nearly saturating concentration of salt, 4 M NaCl, at which maximum activity is reached, and its stability at high saline concentrations. Protein fragility is one of the major drawbacks of enzyme technology. Therefore, the capability of certain enzymes to work in stressful environments is considered an essential feature for several industrial applications. Haloarchaeal enzymes can work in conditions of very low water activity, for example in the presence of considerable amounts of organic solvents. All these features, added to the broad substrate specificity of D-2-hydroxyacid dehydrogenase from *Hf. mediterranei*, are of potential interest for enzyme technology.

Acknowledgments

This work was supported by CICYT grant PB95-0695.

References

[1] Hummel W., Kula M.R., Dehydrogenases for the synthesis of chiral compounds, Eur. J. Biochem. 184 (1989) 1–13.

[2] Schadewaldt P., Hummel W., Trautvetter U., Wendel U., A convenient enzymatic method for the determination of 4-methyl-2-oxopentanoate in plasma: comparison with high performance liquid chromatographic analysis, Clin. Chim. Acta 183 (1989) 171–182.

[3] Kallwass H.K.W., Potential of R-2-hydroxyisocaproate dehydrogenase from *Lactobacillus casei* for stereospecific reductions, Enzyme Microb. Technol. 14 (1992) 28–35.

[4] Bernard N., Johnsen K., Ferain T., Garmyn D., Hols P., Holbrook J.J., Delcour J., NAD-dependent D-2-hydroxycaproate dehydrogenase of *Lactobacillus delbrueckii* subsp. *bulgaricus*. Gene cloning and enzyme characterization, Eur. J. Biochem. 224 (1994) 439–446.

[5] Grant G.A., A new family of 2-hydroxyacid dehydrogenases, Biochem. Biophys. Res. Commun. 165 (1989) 1371–1374.

[6] Kochhar S., Hunziker P., Leong-Morgenthaler P.M., Hottinger H., Evolutionary relationship of NAD-dependent D-lactate dehydrogenase: comparison of primary structure of 2-hydroxy acid dehydrogenases, Biochem. Biophys. Res. Commun. 184 (1992) 60–66.

[7] Taguchi H., Ohta T., D-Lactate dehydrogenase is a member of the D-isomer-specific 2-hydroxyacid dehydrogenase family, J. Biol. Chem. 266 (1991) 12588–12594.

[8] Goldberg J.D., Yoshida T., Brick P., Crystal structure of a NAD-dependent D-glycerate dehydrogenase at 2.4 Å resolution, J. Mol. Biol. 236 (1994) 1123–1140.
[9] Feil I.K., Hendle J., Schomburg D., Modified substrate specificity of L-hydroxyisocaproate dehydrogenase derived from structure-based protein engineering, Prot. Engin. 10 (1997) 255–262.
[10] Wilks H.M., Moreton K.M., Halsall D.J., Hart K.W., Session R.D., Clarke A.R., Holbrook J.J., Design of a specific phenyllactate dehydrogenase by peptide loop exchange on the *Bacillus stearothermophilus* lactate dehydrogenase framework, Biochemistry 31 (1992) 7802–7806.
[11] Niefind K., Hecht H.J., Schomburg D., Crystal structure of L-2-hydroxyisocaproate dehydrogenase from *Lactobacillus confusus* at 2.2 Å resolution. An example of strong asymmetry between subunits, J. Mol. Biol. 251 (1995) 256–281.
[12] Woese C.R., Kandler O., Wheelis M.L., Towards a natural system of organisms: proposal for the domains Archaea, Bacteria and Eukarya, Proc. Natl. Acad. Sci. USA 87 (1990) 4576–4579.
[13] Kates M., Kushner D.J., Mathenson A.T., The Biochemistry of the Archaea (New Comprehensive Biochemistry vol. 26), Elsevier Science Publishers, Amsterdam, The Netherlands, 1993.
[14] De Long E., Archaeal means and extremes, Science 280 (1998) 542–543.
[15] Rodriguez-Valera F., Juez G., Kushner D.J., *Halobacterium mediterranei* spec. nov., a new carbohydrate-utilizing extreme halophile, Syst. Appl, Microbiol. 4 (1983) 369–381.
[16] Bonete M.J., Pire C., Llorca F.I., Camacho M.L., Glucose dehydrogenase from the halophilic archaeon *Haloferax mediterranei*: enzyme purification, characterisation and N-terminal sequence, FEBS Lett. 383 (1996) 227–229.
[17] Laemmli U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[18] Bradford M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[19] Cleland W.W., Statistical analysis of enzyme kinetic data, Methods Enzymol. 63 (1979) 103–138.
[20] Eley M.H., Burns P.C., Kannapell C.C., Campbell P.S., Cetyltrimethylammonium bromide polyacrylamide gel Electrophoresis: estimation of protein subunits molecular weights using cationic detergents, Anal. Biochem. 92 (1979) 411–419.
[21] Bonete M.J., Camacho M.L., Cadenas E., Purification and some properties of NAD-dependent glutamate dehydrogenase from *Halobacterium halobium*, Int. J. Biochem. 18 (1986) 785–789.
[22] Bonete M.J., Camacho M.L., Cadenas E., A new glutamate dehydrogenase from *Halobacterium halobium* with different coenzyme specificity, Int. J. Biochem. 19 (1987) 1149–1155.
[23] Camacho M.J., Brown R.A., Bonete M.J., Danson M.J., Hough D.W., Isocitrate dehydrogenases from *Haloferax volcanii* and *Sulfolobus solfataricus*: enzyme purification, characterisation and N-terminal sequence, FEMS Microbiol. Lett. 134 (1995) 85–90.
[24] Shu H.C., Guoqiang D., Kaul R., Mattiasson B., Purification of the D-lactate dehydrogenase from *Leuconostoc mesenteroides* ssp. *cremoris* using a sequential precipitation procedure, J. Biotechnol. 34 (1994) 1–11.
[25] Schütte H., Hummel W., Kula M.R., L-2-hydroxyisocaproate dehydrogenase. A new enzyme from *Lactobacillus confusus* for the stereospecific reduction of 2-ketocarboxylic acids, Appl. Microbiol. Biotechnol. 19 (1984) 167–176.
[26] Hummel W., Schütte H., Kula M.R., D-2-hydroxyisocaproate dehydrogenase from *Lactobacillus casei*. A new enzyme suitable for the stereospecific reduction of 2-ketocarboxylic acids, Appl. Microbiol. Biotechnol. 21 (1985) 7–15.
[27] Ferrer J., Pérez-Pomares F., Bonete M.J., NADP-glutamate dehydrogenase from the halophilic archaeon *Haloferax mediterranei*: enzyme purification, N-terminal sequence and stability, FEMS Microbiol. Lett. 141 (1996) 59–63.
[28] Ruiz J.L., Ferrer J., Camacho M., Bonete M.J., NAD-specific glutamate dehydrogenase from *Thermus thermophilus* HB8: purification and enzymatic properties, FEMS Microbiol. Lett. 159 (1998) 15–20.
[29] Dym O., Mevarech M., Sussman J.L., Structural features that stabilize halophilic malate dehydrogenase from archaebacterium, Science 267 (1995) 1344–1346.