Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 23 (2003) 223–230
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Novel halophilic 2-aminobutyrate dehydrogenase from *Halobacterium saccahrovorum* DSM 1137

Shinji Nagata [a], Yuki Kobayashi [a], Sanae Shinkawa [a], Reina Katoh [a], Toshihisa Ohshima [b], Haruo Misono [a,*]

[a] *Department of Bioresources Science, Kochi University, Nankoku, Kochi 783-8502, Japan*
[b] *Department of Biological Science and Technology, The University of Tokushima, Tokushima 770-8506, Japan*

Received 6 February 2003; received in revised form 10 April 2003; accepted 15 April 2003

Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

Abstract

A halophilic NAD^+-dependent 2-aminobutyrate dehydrogenase (EC1.4.1.1) was purified to homogeneity from a crude extract of an extreme halophile, *Halobacterium saccharovorum* DSM 1137, with a 30% yield. The enzyme had a molecular mass of about 160 kDa and consisted of four identical subunits. It retained more than 70% of the activity after heating at 60 °C for 1 h and kept it at 30 °C for 8 months in the presence of 2 M NaCl. The enzyme showed maximum activity in the presence of 2 M RbCl or KCl. The enzyme required NAD^+ as a coenzyme and used L-2-aminobutyrate, L-alanine, and L-norvaline as substrates. The best substrate was L-2-aminobutyrate. The optimum pH was 9.3 for the oxidative deamination of L-2-aminobutyrate and 8.6 for the reductive amination of 2-ketobutyrate. The Michaelis constants were 1.2 mM for L-2-aminobutyrate, 0.16 mM for NAD^+, 0.012 mM for NADH, 0.78 mM for 2-ketobutyrate, and 500 mM for ammonia in the presence of 2 M KCl. The K_m values for the substrates depended on the concentration of KCl, and the K_m values decreased under high salt conditions.

Keywords: 2-Aminobutyrate dehydrogenase; *Halobacterium saccharovorum*; Halophilic dehydrogenase; L-Alanine; L-2-Aminobutyrate

1. Introduction

Alanine dehydrogenase (L-alanine:NAD^+ oxidoreductase, deaminating, EC1.4.1.1) catalyzes the reversible deamination of L-alanine to pyruvate in the presence of NAD^+ (Fig. 1). The enzyme occurs ubiquitously in *Bacillus* and *Streptomyces* species and provides a route for the incorporation of ammonia into organic compounds [1]. It plays a pivotal role in the generation of energy during sporulation in *Bacillus* [2,3], while in cyanobacteria [4], *Rhodobactor capsulatus* [5], and *Streptomyces clavulogerus* [6], the enzyme functions in ammonia assimilation. The enzyme has been purified to homogeneity and characterized from various bacteria [5,7–20]. The primary structures of the enzymes [3,16,21–23] and the three-dimensional structure of the *Phormidium lapideum* enzyme [24] have been reported. Alanine dehydrogenase is applicable to the production of 3-fluoro-L-alanine [25], L-^{15}N-alanine, and stereoselectively deuterated NADH [1]. The multi-enzyme system containing alanine dehydrogenase has been

* Corresponding author. Tel.: +81-88-864-5187; fax: +81-88-864-5200.
E-mail address: hmisono@cc.kochi-u.ac.jp (H. Misono).

1381-1177/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S1381-1177(03)00084-5

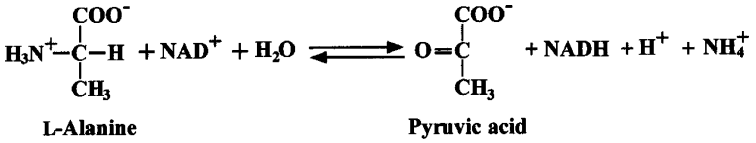

Fig. 1. Alanine dehydrogenase reaction.

developed for the production of D-amino acids, which are useful for the chemical synthesis of β-lactam antibiotics and bioreactive peptides [1,26]. The enzyme is also useful for the measurement of L-alanine and for the assay of several enzymes [1]. However, the enzyme is unfavorable for the measurement of L-alanine in the presence of high concentrations of salts.

The enzymes from halophilic bacteria require specific salts for their activity and stability and function under extremely high salt conditions. Thus, the enzyme from halophilic bacteria can be useful for the synthesis and determination of L-alanine in the presence of high concentrations of salts. Although alanine dehydrogenases from *Halobacterium salinarium* [27,28] and *Halobacterium cutirubrum* [29] have been reported, the detailed enzymological properties have not been studied. We found alanine dehydrogenase activity in an extreme halophile, *Halobacterium saccharovorum* DSM 1137, and purified the enzyme to homogeneity from a crude extract of the bacterium. The enzyme showed the highest activity with L-2-aminobutyrate as a substrate. Thus, we named the enzyme 2-aminobutyrate dehydrogenase. We here describe the purification and characterization of a novel 2-aminobutyrate dehydrogenase from *H. saccharovorum* DSM 1137.

2. Experimental

2.1. Materials

NAD^+ and NADH were purchased from Kohjin Biochemicals, Tokyo, Japan; amino acids and 2-keto acids (sodium salts) from Sigma, St. Louis, MO, USA; Sepharose CL-4B from Pharmacia, Uppsala, Sweden; Whatman HB-1 from Whatman Biosystems, Kent, UK; Gigapite from Toa Gohsei Kagaku, Tokyo, Japan; a TSK gel G3000SW column from Tosoh, Tokyo, Japan. The other chemicals were of analytical grade.

2.2. Medium and culture conditions

H. saccharovorum DSM 1137 was grown in a medium containing 0.75% casamino acids, 1.0% yeast extract, 0.3% trisodium citrate, 0.2% KCl, 2.0% $MgSO_4 \cdot 7H_2O$, 0.005% $FeSO_4 \cdot 7H_2O$, 2×10^{-5}% $MnSO_4 \cdot H_2O$, and 25% NaCl. The pH was adjusted to 7.4 with 3 M NaOH. The cells were cultured in 2-l flasks containing 800 ml of the medium at 37 °C for 5 days on a reciprocal shaker. The cells harvested by centrifugation were washed twice with 25% NaCl.

2.3. Enzyme and protein assays

2-Aminobutyrate dehydrogenase was assayed at 50 °C in a cuvette with a 1 cm light path. The standard reaction mixture (1.0 ml) for the oxidative deamination contained 10 mM L-2-aminobutyrate, 1 mM NAD^+, 0.2 M Tris–HCl buffer (pH 9.3), 2 M KCl, and the enzyme. The assay mixture (1.0 ml) for the reductive amination consisted of 10 mM sodium 2-ketobutyrate, 0.2 mM NADH, 1 M NH_4OH–NH_4Cl buffer (pH 8.6), 2 M KCl, and the enzyme. The substrate was replaced by water in a blank. The reaction was started by the addition of coenzyme to the reaction mixture previously incubated and continuously monitored by measuring the initial change in absorbance at 340 nm with a Shimadzu UV-140-02 double-beam spectrophotometer at 50 °C. One unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol of NADH per minute in the oxidative deamination with a molar absorption coefficient of $6220\,M^{-1}\,cm^{-1}$. Specific activity was expressed as units per mg of protein. Protein concentration was determined by the method of Lowry et al. [30] with egg albumin as the standard.

2.4. Electrophoresis

Gel electrophoresis was carried out according to the method of Davis [31]. Protein was stained with 0.04% Coomassie brilliant blue G-250 in 3.5% $HClO_4$. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the procedure of Laemmli [32]. Protein was stained for 0.05% Coomassie brilliant blue R-250 in methanol:acetic acid:water (5:1:5, v/v/v). Polyacrylamide gel electrophoresis in the presence of a cationic detergent, cetyltrimethylammonium bromide (CTAB-PAGE), was done according to the method of Eley et al. [33]. This electrophoresis has been used to obtain more accurate estimates of the molecular mass of certain highly charged protein subunits. Proteins were precipitated in a 12.5% trichloroacetic acid solution and washed by acetone to remove salts. The precipitate was dissolved in a buffer containing1% CTAB and then heated for 3 min in boiling water for the denaturation of the enzyme before electrophoresis. Protein was stained for 0.5% Coomassie brilliant blue R-250 in acetic acid:ethanol:water (10:45:45, v/v/v).

2.5. Enzyme purification

All operations were performed at room temperature in the standard buffer (50 mM potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol and 2 M NaCl).

The washed cells (about 50 g, wet weight) were suspended in 100 ml of the standard buffer and disrupted twice by ultrasonic oscillation in an ice bath for 10 min. After centrifugation, the extracts were dialyzed overnight against the buffer at room temperature. To the dialyzed solution, solid ammonium sulfate was added to 80% saturation with stirring. After being kept for 1 h, the precipitate was removed by centrifugation, and the supernatant was dialyzed overnight against the standard buffer containing 2 M ammonium sulfate. The solution was applied to a Sepharose CL-4B column (3.0 cm × 65 cm) equilibrated with the buffer containing 2 M ammonium sulfate. The column was developed with a linear gradient of ammonium sulfate (2–0 M). The active fractions were concentrated by ultrafiltration with an Amicon PM-10 membrane. The enzyme solution, which was dialyzed against the standard buffer containing 1.8 M ammonium sulfate, was applied to a Whatman HB-1 column (3.0 cm × 25 cm) equilibrated with the same buffer. The column was developed with a linear gradient of ammonium sulfate (1.8–1.0 M). The active fractions were concentrated by ultrafiltration with an Amicon PM-10 membrane and dialyzed against the standard buffer. The dialyzed solution was applied to a Gigapite column (1.3 cm × 13 cm) equilibrated with the standard buffer. After the column has been washed with the same buffer, the enzyme was eluted with 0.1 M potassium phosphate buffer (pH 7.4) containing 2-mercaptoethanol and 2 M NaCl. Active fractions were combined and concentrated by ultrafiltration with an Amicon PM-10 membrane.

2.6. Estimation of molecular mass

The molecular mass of the enzyme was estimated by high-performance liquid chromatography with a TSK gel G3000SW column (0.75 cm × 60 cm) [34]. Glutamate dehydrogenase (290 kDa), heart lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa), and cytochrome *c* (12.4 kDa) were used as marker proteins (Oriental Yeast Co., Tokyo, Japan). The subunit molecular mass was determined by SDS- or CTAB-PAGE. The following marker proteins (Pharmacia, Uppsala, Sweden) were used: catalase (60 kDa), ovalbumin (45 kDa), yeast alcohol dehydrogenase (37 kDa), α-chymotrypsinogen A (25 kDa), and myoglobin (17.2 kDa).

2.7. Steady-state kinetic analyses

The reactions were carried out under the standard condition as described above. Initial velocity experiments were done by varying the concentration of one substrate at different fixed concentrations of another substrate. The Michaelis constant was calculated from the secondary plot of intercepts versus the reciprocal concentration of the substrate.

3. Results and discussion

3.1. Enzyme purification

2-Aminobutyrate dehydrogenase was purified about 1380-fold from crude extracts of *H. saccharovorum*

Table 1
Purification of 2-aminobutyrate dehydrogenase from *H. saccharovorum* DSM 1137

Steps	Total protein (mg)	Total activity (U)	Specific activity ($U\,mg^{-1}$)	Yield (%)
Crude extract	2720	24.8	0.0091	100
Ammonium sulfate	952	30.6	0.0320	123
Sepharose CL-4B	58.7	29.8	0.510	120
Whatman HB-1	2.44	13.1	5.40	53
Gigapite	0.568	7.37	12.6	30

DSM 1137 with a 30% yield (Table 1). The final enzyme preparation was homogeneous by the criteria of SDS-PAGE and CTAB-PAGE.

3.2. Molecular mass and subunit structure

The molecular mass of 2-aminobutyrate dehydrogenase was estimated to be about 160 kDa by gel filtration on a TSK gel G3000SW column with a buffer containing 2 M NaCl. A major peak (about 160 kDa) and a minor peak (about 95 kDa) were obtained by gel filtration performed in the presence of a low concentration of NaCl (0.5 M). The molecular mass of the subunit was calculated to be 54 kDa by SDS-PAGE and 46 kDa by CTAB-PAGE. These results suggest that the enzyme consists of four subunits with identical molecular masses and that the enzyme dissociates into dimers in a low concentration of NaCl, though the alanine dehydrogenases from *H. salinarium* and *H. cutirubrum* are monomers [27–29].

3.3. Stability

The enzyme required a high concentration of NaCl (more than 1.5 M NaCl) for heat stability (Fig. 2A). About 70% of the activity was retained on heating at 60 °C for 1 h in the presence of 2 M NaCl, but the activity was lost on heating at 65 °C for 1 h (Fig. 2B). The enzyme was most stable in a pH range from 6.7 to 8.0 when it was incubated at 60 °C for 10 min in the presence of 2.0 M NaCl (Fig. 3A). The enzyme could be stored at 30 °C for 8 months with a little loss of activity in 50 mM potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol and 2 M NaCl (Fig. 3B). KCl, RbCl, and CsCl were also useful for stability (data not shown).

3.4. Effects of pH and temperature on the enzyme activity

The optimum temperature of the enzyme reaction was 60 °C, and the optimum pH for the oxidative deamination of L-2-aminobutyrate or L-alanine was 9.3 in the presence of 0.2 M Tris–HCl buffer containing 2 M KCl (Fig. 4A). The optimum pH for the

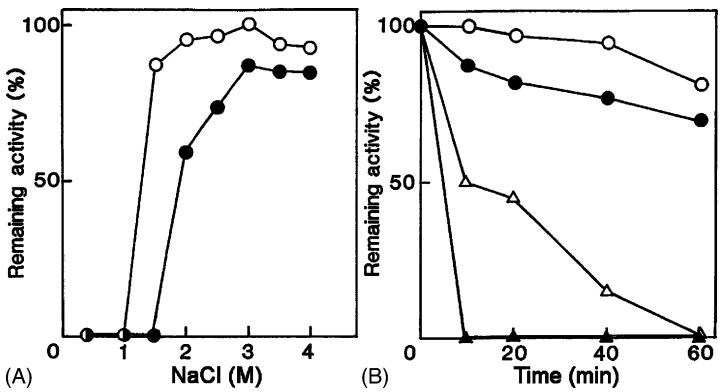

Fig. 2. Effects of NaCl and temperature on the heat stability of the enzyme. (A) The enzyme in 50 mM potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol and various concentration of NaCl was incubated at 60 °C (○) or 65 °C (●) for 10 min. After cooling of the enzyme solution in running water, the remaining activities were measured for the oxidative deamination of L-2-aminobutyrate under the standard condition. (B) The enzyme in 50 mM potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol and 2.0 M NaCl was incubated at 55 °C (○), 60 °C (●), 65 °C (△), or 70 °C (▲) for various times.

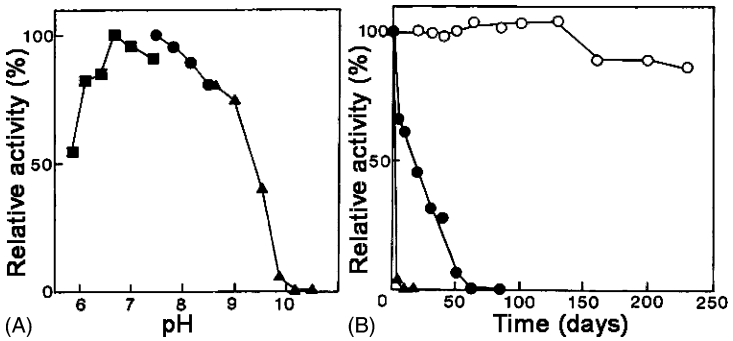

Fig. 3. Effects of pH and NaCl on the stability of the enzyme. (A) The enzyme in buffers of various pHs containing 0.01% 2-mercaptoethanol and 2 M NaCl was incubated at 60 °C for 10 min, and the remaining activities were then assayed for the oxidative deamination of L-2-aminobutyrate at 50 °C. The buffers used were 50 mM potassium phosphate buffer (■), Tris–HCl buffer (●), or glycine–KOH buffer (▲). The pH of the buffers was adjusted at 25 °C. (B) The enzymes were stored at 30 °C in 50 mM potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol and 0.5 M (▲), 1.0 M (●), or 2.0 M (○) NaCl, and the remaining activities were assayed for the oxidative deamination of L-2-aminobutyrate under the standard condition.

reductive amination of 2-ketobutyrate or pyruvate was 8.6 in the presence of 1 M NH_4Cl–NH_4OH buffer containing 2 M KCl (Fig. 4B). The optimum pH of 2-aminobutyrate dehydrogenase for the deamination of L-alanine is different from those of alanine dehydrogenases, though the optimum pH for the amination of pyruvate is similar in both the enzymes [1,13,14,18,19].

3.5. Substrate specificity

The enzyme catalyzed the oxidative deamination of L-2-aminobutyrate (relative activity, 100%), L-alanine (56.5%), and L-norvaline (14%), and the best substrate was L-2-aminobutyrate. L-Valine, L-leucine, L-serine, L-glutamate, L-phenylalanine, L-methionine, L-lysine, D-2-aminobutyrate, D-alanine, and D-norvaline were not substrates. 2-Ketobutyrate (100%), pyruvate (28%), and 2-ketovalerate (6.1%) served as substrates for reductive amination. The enzyme required NAD^+ or NADH as a coenzyme, which could not be replaced by $NADP^+$ or NADPH. Although alanine dehydrogenase catalyzes the deamination of L-2-aminobutyrate and L-serine in the presence of NAD^+, the reactivities of L-2-aminobutyrate and L-serine were lower than that of L-alanine [7–12,16].

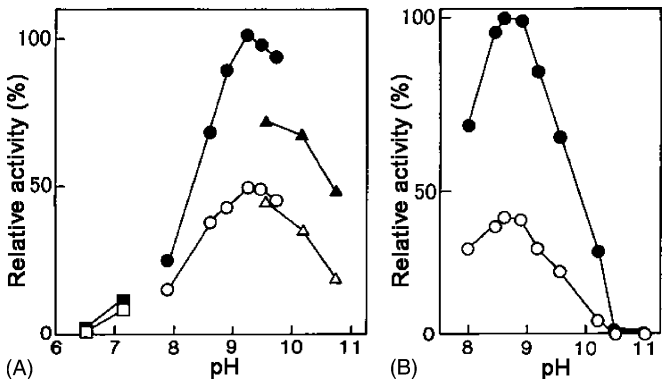

Fig. 4. Effect of pH on the enzyme activity. (A) The reaction mixture for the oxidative deamination contained 10 mM L-2-aminobutyrate (■, ●, ▲) or L-alanine (□, ○, △), 5 mM NAD^+, 2 M KCl, and 0.2 M potassium phosphate buffer (■, □), Tris–HCl buffer (●, ○), or glycine-KOH buffer (▲, △). (B) The reaction mixture for the reductive amination contained 0.2 mM NADH, 2 M KCl, 1 M NH_4Cl–NH_4OH buffer, and 10 mM 2-ketobutyrate (●) or 10 mM pyruvate (○).

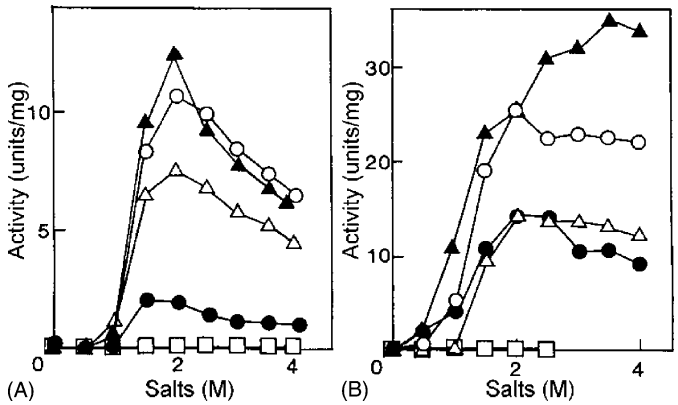

Fig. 5. Effects of various salts on the enzyme activity. The enzyme activity was assayed for the oxidative deamiantion of L-2-aminobutyrate (A) and the reductive amination of 2-ketobutyrate (B) under the standard conditions in the presence of various concentrations of RbCl (▲), KCl (○), CsCl (△), NaCl (●), or LiCl (□).

3.6. Effect of salts on the enzyme activity

The enzyme required more than 1.5 M of a neutral salt for activity (Fig. 5). The maximal activity in the oxidative deamination of L-2-aminobutyrate was obtained in the presence of 2 M RbCl or KCl and that in the reductive amination of 2-ketobutyrate was obtained by 3.5 M RbCl. The effective ions were as follows: Rb^+, K^+, Cs^+, and Na^+ as cations (Fig. 5) and Cl^-, CH_3COO^-, $HCOO^-$, Br^-, HPO_4^{2-}, and SO_4^{2-} as anions (data not shown). Li^+, Mg^{2+}, Ca^{2+}, Pb^{2+}, NO_3^-, SCN^-, ClO_4^-, CO_3^{2-}, and I^- had no effect on the enzyme activity.

3.7. Kinetics

Steady-state kinetic analyses were carried out to obtain K_m values for substrates in the presence of 2 M KCl. Initial velocity studies for the oxidative deamination were performed with NAD^+ as a variable substrate in the presence of several fixed concentrations of L-2-aminobutyrate. Double reciprocal plots of initial velocity against NAD^+ concentrations gave a family of straight lines, which intersected in the left quadrant. When L-2-aminobutyrate was used as a variable substrate, similar straight lines intersecting in the left quadrant were obtained. These results show that the reaction proceeds via the formation of a ternary complex of the enzyme with NAD^+ and L-2-aminobutyrate. The K_m values for L-2-aminobutyrate and NAD^+ were calculated to be 1.2 and 0.16 mM, respectively, from the secondary plots of intercept versus reciprocal concentrations of the other substrates. Kinetic analyses of the reductive amination were performed to investigate several possible reaction mechanisms. Double reciprocal plots of velocities against 2-ketobutyrate concentrations at several fixed concentrations of ammonia and a constant concentration of NADH gave straight lines intersecting in the left quadrant. At a high concentration of 2-ketobutyrate, the double reciprocal plots of velocities against NADH concentrations at several fixed concentrations of ammonia gave straight intersecting lines as well. However, with ammonia at a saturating concentration, the double reciprocal plots of velocities against NADH concentrations at several fixed concentrations of 2-ketobutyrate gave parallel lines. These observed kinetic patterns exclude the possibility of random addition of substrates and represent a sequential ordered mechanism in which ammonia binds to the enzyme between NADH and 2-ketobutyrate. The K_m values for NADH, 2-ketobutyrate, and ammonia were calculated to be 0.012, 0.78, and 500 mM, respectively.

The product-inhibition studies in oxidative deamination were performed to determine the order of substrate addition and product release according to the method of Cleland [35]. With NADH as an inhibitor, the double reciprocal plots of velocities against NAD^+ concentrations at a high and constant concentration of L-2-aminobutyrate showed competitive inhibition.

NADH showed noncompetitive inhibition with respect to L-2-aminobutyrate in the presence of a high and constant NAD^+. These results suggest that NAD^+ and NADH can bind to the free form of the enzyme. The other product-inhibition patterns for oxidative deamination observed with ammonia and 2-ketobutyrate as the inhibitors were identical with the predicted patterns for the sequential ordered binary–ternary kinetic mechanism whereby NAD^+ binds first to the enzyme followed by L-2-aminobutyrate and the products are released in the order of 2-ketobutyrate, ammonia, and NADH. The noncompetitive inhibition by 2-ketobutyrate with respect to L-2-aminobutyrate excluded the mechanism of the Theorell–Chance type. 2-Ketobutyrate showed competitive inhibition with respect to NAD^+ in the presence of a high and constant concentration of L-2-aminobutyrate. This result suggests that 2-ketobutyrate may be able to bind to the free enzyme. These results obtained from initial velocity and product-inhibition studies show that the sequence of addition of the substrates in the reductive amination is NADH, ammonia, and 2-ketobutyrate and that of the release of products is L-2-aminobutyrate and NAD^+. 2-Aminobutyrate dehydrogenase showed similar sequential ordered mechanism to that of alanine dehydrogenase [16,18,36].

Kinetic studies at several concentrations of KCl showed that the K_m for L-2-aminobutyrate in the oxidative deamination decreased under high-salt conditions (Fig. 6). The highest V_{max}, however, was obtained in the presence of 2 M KCl. The K_m values for L-alanine, L-norvaline, and pyruvate were 1.3, 13, and 0.77 mM, respectively, in the presence of 2 M KCl.

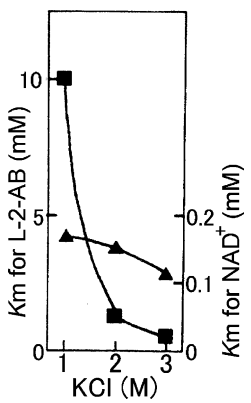

Fig. 6. Effect of KCl on the kinetic parameters of the enzyme in oxidative deamination. K_m values for L-2-aminobutyrate (L-2-AB (■)) and NAD^+ (▲) were measured in the presence of various concentrations of KCl.

The enzymes from extreme halophiles require salts for their stability and activity, since the halophilic enzymes contain many acidic amino acids. The 2-aminobutyrate dehydrogenase from *H. saccharovorum* DSM 1137 also required a high concentration of salt for its stability and activity. The enzyme dissociated into dimers in a low concentration of salts. The K_m value for L-2-aminobutyrate decreased under high salt conditions. These results suggest that a high concentration of salt is required for the formation of quaternary structure and proper conformation of the enzyme. 2-Aminobutyrate dehydrogenase from *H. saccharovorum* DSM 1137 can be applied for the synthesis of L-2-aminobutyrate and the determination of L-alanine and L-2-aminobutyrate in the presence of high concentrations of salts.

Acknowledgements

This work was supported in part by a Grant-in-Aid for the encouragement of Young Scientists (Grant No. 04856026 to S. Nagata) from the Ministry of Education, Science and Culture of Japan.

References

[1] T. Ohshima, K. Soda, Biochemistry and biotechnology of amino acid dehydrogenases, in: A. Fiechter (Ed.), Advances in Biochemical Engineering/Biotechnology, vol. 42, Springer, Berlin, 1990, p. 187.
[2] S.M. McCowen, P.V. Phibbs Jr., J. Bacteriol. 118 (1974) 590.
[3] K.J. Siranosian, K. Ireton, A.D. Grossmann, J. Bacteriol. 175 (1993) 6789.
[4] A.H. Neilson, M. Doudoroff, Arch. Microbiol. 89 (1983) 15.
[5] F.J. Caballero, J. Cardenas, F. Castillo, J. Bacteriol. 171 (1989) 3205.
[6] Y. Aharonowitz, C.G. Friedrich, Arch. Microbiol. 125 (1980) 137.
[7] A. Yoshida, E. Freese, Biochim. Biophys. Acta 92 (1970) 33.
[8] T. Ohshima, K. Soda, Eur. J. Biochem. 100 (1979) 29.
[9] H. Porumb, D. Vancea, L. Muresan, E. Presecan, I. Lascu, I. Peirescu, T. Porumb, R. Pop, O. Barzu, J. Biol. Chem. 262 (1987) 4610.
[10] Y. Sakamoto, S. Nagata, N. Esaki, H. Tanaka, K. Soda, J. Ferment. Bioeng. 69 (1985) 154.
[11] T. Ohshima, M. Sakane, T. Yamazaki, K. Soda, Eur. J. Biochem. 191 (1990) 715.

[12] S. Nagata, H. Misono, S. Nagasaki, N. Esaki, H. Tanaka, K. Soda, Biochimie 71 (1988) 559.
[13] Z. Vali, F. Kilar, S. Lakatos, S.A. Venyaminov, P. Zavodszky, Biochim. Biophys. Acta 615 (1980) 34.
[14] N. Itoh, R. Morikawa, Agric. Biol. Chem. 47 (1983) 2511.
[15] E. Bellion, F. Tan, Biochm. J. 244 (1987) 565.
[16] E.K. Chowdhury, T. Saitoh, S. Nagata, M. Ashiuchi, H. Misono, Biosci. Biothechnol. Biochem. 62 (1998) 2357.
[17] Y. Sawa, M. Tani, K. Murata, H. Shibata, H. Ochiai, J. Biochem. 116 (1994) 995.
[18] A. Vančura, I. Vančurova, J. Volc, S.K.T. Jones, M. Flieger, G. Basařova, V. Běhal, Eur. J. Biochem. 179 (1989) 221.
[19] I. Vančurova, A. Vančura, J. Vloc, J. Neuzil, M. Flieger, G. Basařova, V. Běhal, Arch. Microbiol. 150 (1988) 438.
[20] M.T. Smith, S.W. Emerich, Arch. Biochem. Biophys. 304 (1993) 379.
[21] S. Kuroda, K. Tanizawa, Y. Sakamoto, H. Tanaka, K. Soda, Biochemistry 29 (1990) 1009.
[22] A. Galkin, L. Kulakova, H. Ashida, Y. Sawa, N. Esaki, Appl. Environ. Microbiol. 65 (1999) 4014.
[23] A.B. Andersen, P. Andersen, L. Ljungqvist, Infect. Immun. 60 (1992) 2317.
[24] P.J. Baker, Y. Sawa, H. Shibata, S.E. Sedelnikova, D.W. Rice, Nat. Struct. Biol. 5 (1998) 561.
[25] T. Ohshima, C. Wandrey, D. Conrad, Biotechnol. Bioeng. 34 (1989) 394.
[26] K. Soda, T. Oikawa, K. Yokoigawa, J. Mol. Catal. B: Enzym. 11 (2001) 149.
[27] K. Keradjopoulos, A.W. Holldorf, Biochim. Biophys. Acta 570 (1979) 1.
[28] K. Keradjopoulos, A.W. Holldorf, FEBS Lett. 112 (1980) 183.
[29] E.K. Kim, P.S. Fitt, Biochem. J. 161 (1977) 313.
[30] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[31] B.J. Davis, Ann. N. Y. Acad. Sci. 121 (1964) 404.
[32] U.K. Laemmli, Nature 227 (1970) 680.
[33] M.H. Eley, P.C. Burns, C.C. Kannapell, P.S. Campbell, Anal. Biochem. 92 (1979) 411.
[34] T. Miyaji, M. Ashiuchi, K. Packdibamrung, S. Nagata, H. Misono, J. Mol. Catal. B: Enzym. 12 (2001) 77.
[35] W.W. Cleland, Steady state kinetics, in: P.O. Boyer (Ed.), The Enzymes, vol. 2, third ed., Academic Press, New York, 1970, p. 1.
[36] C.E. Grimshaw, W.W. Cleland, Biochemistry 20 (1981) 5600.