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# Purification and characterization of leucine dehydrogenase from an alkaliphilic halophile, *Natronobacterium magadii* MS-3

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

**Abstract**

Leucine dehydrogenase (L-leucine: $NAD^+$ oxidoreductase, deaminating, EC 1.4.1.9) was purified to homogeneity from the crude extract of an alkaliphilic halophile, *Natronobacterium magadii* MS-3, with a yield of 16%. The enzyme had a molecular mass of about 330 kDa and consisted of six subunits identical in molecular mass (55 kDa). The enzyme required a high concentration of salt for stability and activity. It retained the full activity after heating at 50 °C for 1 h and about 50% activity after being kept at 30 °C for 2 months in the presence of 2.5 M NaCl. The enzyme required $NAD^+$ as a coenzyme and showed maximum activity in the presence of more than 3 M salt, as CsCl, RbCl, NaCl, or KCl. In addition to L-leucine, L-valine and L-isoleucine were also good substrates in the oxidative deamination. In the reductive amination, 2-keto analogs of branched-chain amino acids were substrates. The Michaelis constants were 0.69 mM for L-leucine, 0.48 mM for $NAD^+$, 4.0 mM for 2-ketoisocaproate, 220 mM for ammonia, and 0.02 mM for NADH in the presence of 4 M NaCl. The $K_m$ for L-leucine depended on the concentration of salt and increased with decreasing salt concentration. The *N. magadii* enzyme was unique in its halophilicity among leucine dehydrogenases studied so far.
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*Keywords:* L-Leucine dehydrogenase; *Natronobactrium magadii*; Halophilic dehydrogenase; Alkaliphilic halophile; L-Leucine

## 1. Introduction

Leucine dehydrogenase (L-leucine:$NAD^+$ oxidoreductase, deaminating EC 1.4.1.9) catalyzes the reversible deamination of L-leucine and some other branched-chain L-amino acids to their keto analogs (Fig. 1). The enzyme occurs mainly in endo-spore-forming *Bacillus* and thermophilic *Clostridium* species [1]. The enzyme has been purified to homogeneity from mesophiles (*Bacillus sphaericus* [2], *Bacillus cereus* [3], *Bacillus caldolyticus* [4], and *Corynebacterium pseudodiphtheriticum* [5]) and thermophiles (*Bacillus stearothermophilus* [6,7], *Bacillus licheniformis* [8], thermophilic *Bacillus* sp. [9], *Clostridium thermoaceticum* [10], and *Thermoactinomyces intermedius* [11]). It is applicable to the production [12–14] and determination [15,16] of branched-chain L-amino acids as well as the assay

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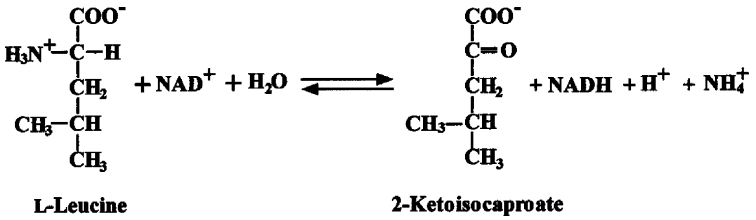


Fig. 1. Leucine dehydrogenase reaction.

of leucine aminopeptidase [17]. Leucine dehydrogenase has been used for the industrial production of L-*tert*-leucine, which is a component of an antibiotic [18]. For the synthesis of amino acids with dehydrogenases, high concentrations of ammonia are usually used, and a stable enzyme in high concentrations of ammonia is required.

In order to obtain a stable enzyme in high concentrations of salt, we screened for leucine dehydrogenase in halophilic bacterial strains including alkaliphiles from various sources and found the activity in a halophilic and alkaliphilic bacterium, *Natronobacterium magadii* MS-3. We describe here the purification and characterization of leucine dehydrogenase from *N. magadii* MS-3.

## 2. Experimental

### 2.1. Materials

$NAD^+$ and NADH were obtained from Kohjin Biochemicals, Tokyo, Japan; 2-keto acids (sodium salts), L-amino acids, bovine serum albumin, and ovalbumin from Sigma, St. Louis, MO, USA; marker proteins for molecular mass determination from Orient Yeast, Osaka, Japan; Sepharose CL-4B from Pharmacia, Uppsala, Sweden; Whatman HB-I from Whatman Biosystems, Kent, England; Gigapite from Toa Gohsei Kagaku, Tokyo, Japan; and a TSK gel G3000SW column from Tosoh, Tokyo, Japan. The other chemicals were of analytical grade.

### 2.2. Bacteria and culture conditions

*N. magadii* MS-3 was grown in a medium (pH 9.5) containing 1.0% casamino acids, 1.0% yeast extract, 0.3% trisodium citrate dihydrate, 0.2% KCl, 0.1% $MgSO_4{\cdot}7H_2O$, 0.005% $FeSO_4{\cdot}7H_2O$, 1.85% $Na_2CO_3$, and 20% NaCl. Cultivation was carried out in 2 l flasks containing 1 l of the medium at 37 °C for 5 days on a reciprocal shaker. The cells were harvested by centrifugation and washed twice with 2.5 M NaCl.

### 2.3. Enzyme and protein assays

The standard reaction mixture for the oxidative deamination contained 10 μmol of L-leucine, 5 μmol of $NAD^+$, 200 μmol of glycine–NaCl–NaOH buffer (pH 9.5), 3 mmol of NaCl, and enzyme in a final volume of 1.0 ml. The assay system for the reductive amination consisted of 10 μmol of sodium 2-ketoisocaproate, 0.2 μmol of NADH, 750 μmol of $NH_4Cl$–$NH_4OH$ buffer (pH 9.5), 3 mmol of NaCl, and enzyme in a final volume of 1.0 ml. The substrate was replaced by water in a blank. Incubation was performed at 30 °C in a cuvette with a 1 cm light path. The reaction was started by addition of $NAD^+$ or NADH and monitored by measuring the initial changes in the absorbance at 340 nm with a Shimazu UV-140-02 double-beam spectrophotometer at 30 °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. [19] with crystalline bovine serum albumin as the standard.

### 2.4. Electrophoresis

Gel electrophoresis of the native enzyme was performed with a 7.5% polyacrylamide gel by the method of Davis [20]. 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 done with 10% polyacrylamide by the procedure of Laemmli [21]. Protein was stained with 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 carried out according to the method of Eley et al. [22]. This electrophoresis has been used to obtain more accurate estimates of the molecular mass of certain highly charged protein subunits. Protein was precipitated in a 12.5% trichloroacetic acid solution and washed by acetone to remove salts. The precipitate was dissolved in a buffer containing 1% CTAB and then heated for 3 min in boiling water for denaturation of the enzyme before electrophoresis. Protein was stained with 0.5% Coomassie brilliant blue R-250 in acetic acid:ethanol:water (10:45:45, v/v/v).

### 2.5. Enzyme purification

All procedures were carried out at room temperature in a standard buffer (50 mM potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol and 2.5 M NaCl).

The washed cells (about 50 g, wet weight) were suspended in the standard buffer and disrupted by ultrasonic oscillation in an ice bath for 10 min. The supernatant obtained by centrifugation was dialyzed overnight against the standard buffer. To the dialyzed crude extract, solid ammonium sulfate was added to 2.0 M with stirring. After being kept for 1 h, the precipitate was removed by centrifugation. The supernatant was dialyzed overnight against the standard buffer containing 1.8 M ammonium sulfate and put on a Whatman HB-1 column (3 cm × 32 cm) equilibrated with the standard buffer containing 1.8 M ammonium sulfate. The column was developed with a linear gradient of ammonium sulfate (1.8–1.0 M). The active fractions were concentrated with an Amicon ultrafiltration unit with a PM-10 membrane and dialyzed overnight against the standard buffer containing 1.8 M ammonium sulfate. The enzyme solution was put on a Sepharose CL-4B column (3 cm × 35 cm) equilibrated with the standard buffer containing 1.8 M ammonium sulfate. The column was developed with a linear gradient of ammonium sulfate (1.8–1.0 M). The active fractions were concentrated with an Amicon ultrafiltration unit with a PM-10 membrane and dialyzed overnight against the standard buffer. The dialyzed solution was applied to a Gigapite column (2 cm × 25 cm) equilibrated with the standard buffer. After the column had been washed with the buffer, the enzyme was eluted with 0.1 M potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol and 2.5 M NaCl. The active fractions were pooled and concentrated with an Amicon ultrafiltration unit with a PM-10 membrane and dialyzed overnight against the standard buffer.

### 2.6. Determination of molecular mass

The molecular mass of the enzyme was determined by high-performance liquid chromatography on a TSK gel G3000SW column (0.75 cm × 60 cm) at a flow rate of 0.7 ml/min with 0.1 M potassium phosphate buffer (pH 7.4) containing 2.5 or 0.1 M NaCl [23]. The molecular mass of the subunit was estimated by CTAB-PAGE with the following standard proteins (Pharmacia, Uppsala, Sweden): bovine serum albumin (68 kDa), ovalbumin (43 kDa), yeast alcohol dehydrogenase (37 kDa), α-chymotrypsinogen A (25.7 kDa), and myoglobin (17.2 kDa).

### 2.7. Steady-state kinetic analyses

The reactions were done under the standard condition as described above. Initial velocity experiments were carried out 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. Screening and purification of leucine dehydrogenase

We screened for leucine dehydrogenase in 25 strains of halophiles including *Halobacterium cutirubrum* NRC 34001, *Halobacterium halobium* CCM 2090 pink, purple, and whitish, *Halobacterium saccharovorum* ATCC 29252, *Halobacterium sodomense* ATCC

Table 1
Purification of leucine dehydrogenase from *N. magadii* NM-3

| Steps | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Yield (%) |
|---|---|---|---|---|
| Crude extract | 2360 | 88.8 | 0.038 | 100 |
| Whatman HB-1 | 479 | 71.0 | 0.150 | 78 |
| Sepharose CL-4B | 82.5 | 46.3 | 0.560 | 52 |
| Gigapite | 7.27 | 13.9 | 1.92 | 16 |

33755, *Haloarcula vallismortis* ATCC 29715, *Haloarcula sinaiiensis* ATCC 33800, *Haloarcula trapanicum* NCMB 767, *Haloferax volcanii* NCMB 2012, *Haloferax mediterranei* ATCC 33500, *Haloferax gibbonsii* ATCC 33959, *Haloferax denitrificans* ATCC 35960, *Halococcus morrhuae* NRC 14032, 16006, 16008, 16012, and 16018, *Natronobacterium gregoryi* SP-2 NCMB 2189, *N. magadii* MS-3 NCMB 2190, *Natronobacterium pharaonis* SP-1 NCMB 2191, and halophilic four strains. We found a high activity of leucine dehydrogenase in the crude extract of an alkaliphilic halophile, *N. magadii* MS-3 (NCMB 2190), but the other tested halophilic strains did not show the activity.

We purified the enzyme to homogeneity from the crude extract of *N. magadii* MS-3, which required more than 2 M NaCl for its growth. A summary of the purification procedures is shown in Table 1. The enzyme was purified about 51-fold from the crude extract with a 16% yield. The purified enzyme showed a single band on SDS-PAGE and CTAB-PAGE.

### 3.2. Molecular mass and subunit structure

The molecular mass of the *N. magadii* enzyme was estimated to be about 330 kDa by gel filtration on a TSK gel G3000SW column with the buffer containing 2.5 M NaCl. In general, halophilic enzymes contain many acidic amino acid residues; therefore, PAGE was carried out in the presence of CTAB, a cationic surfactant. The molecular mass of the subunit was calculated to be 55 kDa by CTAB-PAGE. These results suggest that the enzyme consists of six identical subunits. Leucine dehydrogenases studied so far are hexamers [6,10] or octamers [3,4,11], except for the *C. pseudodiphthericum* enzyme (a monomer) [5]. The molecular mass and the subunit structure of the *N. magadii* enzyme are similar to those of the *C. thermoaceticum* enzyme [10]. When the molecular mass of the *N. magadii* enzyme was examined by gel filtration on a TSK gel G3000SW column with the buffer containing 0.1 M NaCl, two minor peaks (about 185 and 120 kDa) in addition to a major peak (about 330 kDa) were obtained. This result indicates that the *N. magadii* enzyme dissociates into trimers and dimers in a lower concentration of salt.

### 3.3. Stability

The enzyme required a high concentration (more than 2.5 M) of NaCl for stability. When heated for 1 h in the presence of 2.5 M NaCl or 2.5 M KCl, the enzyme was stable up to 50 °C (Fig. 2A). The enzyme retained about 50% activity after being kept at 30 °C for 2 months in the presence of 2.5 M NaCl (Fig. 2B).

### 3.4. Effect of salts on the enzyme activity

The enzyme required more than 2.0 M salt for activity (Fig. 3A). The maximal activity in the oxidative deamination of L-leucine was obtained in the presence of 3 M CsCl. High activity was obtained in the presence of 4 M RbCl, 3 M NaCl, or 3 M KCl; Li and bivalent cations were inert. As an anion, $CH_3COO^-$, $HCOO^-$, and $HPO_4^{2-}$ were more effective than $Cl^-$ (Fig. 3B). Maximal effects of the phosphate were observed at a concentration of 2 M and the enzyme activity was depressed at a concentration higher than 2 M. Nitrate, thiocyanate, and iodinate ions did not affect the enzyme activity.

### 3.5. Effects of temperature and pH on the enzyme activity

The optimum temperature of the enzyme reaction was 65 °C, and the pH optima for the oxidative deamination of L-leucine and for the reductive amination of 2-ketoisocaproate were about 9.5 in the presence of 3 M NaCl (Fig. 4). Although the optimum pH for the

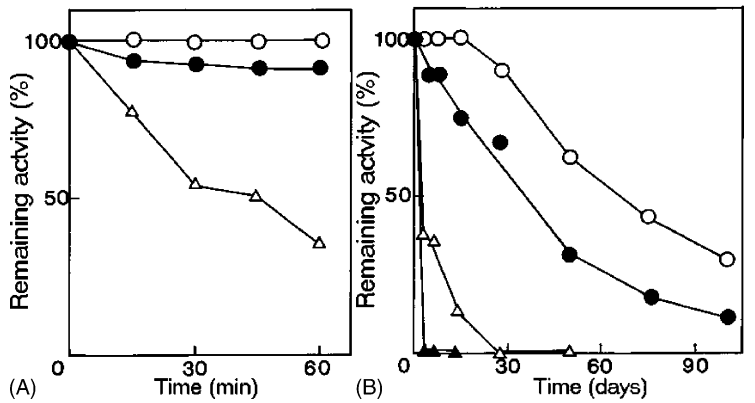


Fig. 2. Effect of NaCl and temperature on stability of the enzyme. (A) The enzyme in 50 mM potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol and 2.5 M NaCl was incubated at 50 °C (○), 55 °C (●), or 60 °C (△) for various times. (B) The enzyme was stored at 30 °C in 50 mM potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol and 1 M (▲), 1.5 M (△), 2 M (●) or 2.5 M (○) NaCl, and the remaining activities were assayed for the oxidative deamination of L-leucine under the standard condition.

reductive amination of the enzyme is similar to those of other leucine dehydrogenases, the optimum pH for the oxidative deamination of the *N. magadii* enzyme is lower than those (pH 10.5–11.3) of leucine dehydrogenases from other sources [2–11].

### 3.6. Substrate specificity

The enzyme catalyzed the oxidative deamination of L-leucine (relative activity, 100%), L-isoleucine (72%), L-valine (59%), L-2-aminobutyrate (32%), *S*-mehtyl-L-cysteine (19%), and L-norvaline (7%). L-Leucine was the best substrate in the oxidative deamination. L-Alanine, L-serine, L-threonine, L-glutamate, L-aspartate, L-phenylalanine, L-lysine, L-tryptophan, L-arginine, D-leucine, D-isoleucine, D-valine, D-norvaline, and D-2-aminobutyrate were inert. 2-Ketoisocaproate (100%), 2-ketoisovalerate (150%), 2-ketovalerate (96%), 2-ketocaptorate (69%), and 2-ketobutyrate (70%) served as substrates for the

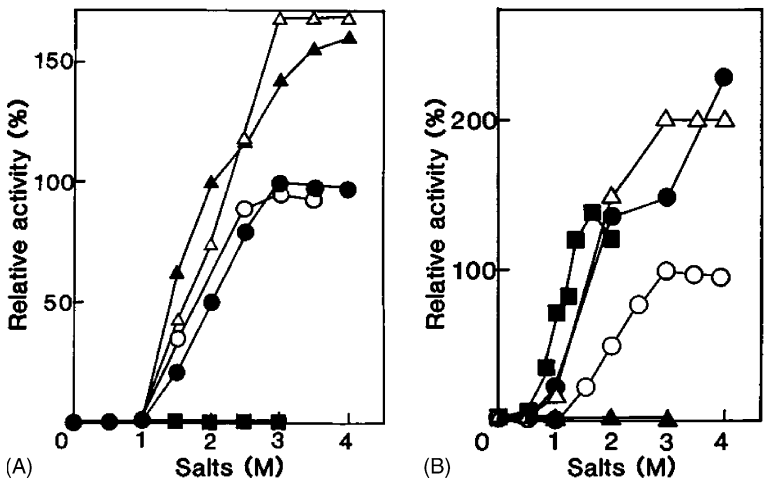


Fig. 3. Effect of various salts on the enzyme activity. (A) The enzyme activity was assayed for the oxidative deamination under the standard condition in the presence of various concentrations of LiCl (■), NaCl (●), KCl (○), RbCl (▲), or CsCl (△). (B) The enzyme activity was assayed for the oxidative deamination under the standard condition in the presence of various concentrations of one of the following sodium salts: NaCl (○); HCOONa (△); $CH_3COONa$ (●); $Na_2HPO_4$ (■); or NaBr (▲).

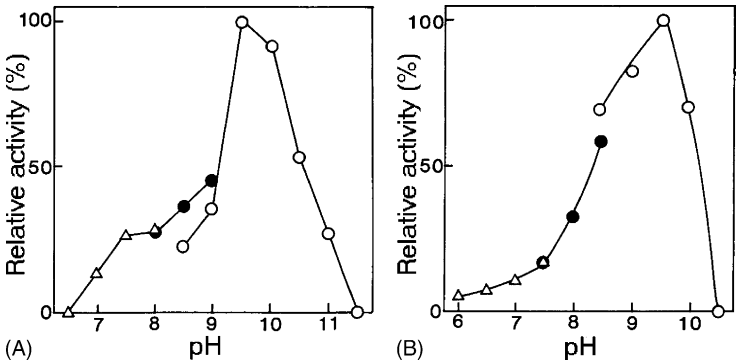


Fig. 4. Effect of pH on the enzyme activity. (A) The reaction mixture for the oxidative deamination contained 10 mM L-leucine, 5 mM $NAD^+$, 3 M NaCl, and 0.2 M potassium phosphate buffer (△), Tris–HCl buffer (●), or glycine–NaCl–NaOH buffer (○). (B) The reaction mixture for the reductive amination contained 10 mM sodium 2-ketoisocaproate, 0.2 mM NADH, 0.75 M $NH_4Cl$, 3 M NaCl, and 0.2 M potassium phosphate buffer (△), Tris–HCl buffer (●) or glycine–NaCl–NaOH buffer (○).

reductive amination. The enzyme required $NAD^+$ or NADH as a coenzyme, but $NADP^+$ and NADPH were inert. The enzyme is similar to other leucine dehydrogenases in cofactor specificity and substrate specificity.

### 3.7. Effects of inhibitors and metal ions

The enzyme was not inhibited by 1 mM EDTA and metal ions such as $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Fe^{2+}$, $Zn^{2+}$, and $Al^{3+}$. The enzyme, however, was completely inhibited by 1 mM $HgCl_2$ and $AgNO_3$.

### 3.8. Kinetic mechanism

Steady-state kinetic analyses were carried out to obtain $K_m$ values for the substrates. The initial velocity of the oxidative deamination was determined with $NAD^+$ as a variable substrate in the presence of 4 M NaCl and several fixed concentrations of L-leucine. Double reciprocal plots of initial velocity versus the $NAD^+$ concentration gave intersecting straight lines. These results indicate that the reaction proceeds by a sequential mechanism via the formation of a ternary complex of the enzyme with $NAD^+$ and L-leucine. The $K_m$ values for L-leucine and $NAD^+$ were calculated to be 0.69 and 0.48 mM, respectively, from the secondary plots of the intercept versus the reciprocal concentration of the other substrate.

A kinetic analysis of reductive amination was performed to investigate several possible reaction mechanisms. At a high concentration of NADH, the double reciprocal plots of velocities versus 2-ketoisocaproate concentrations at several fixed concentrations of ammonia gave straight lines intersecting on the abscissa. The double reciprocal plots of velocities versus the NADH concentration at several concentrations of ammonia and a constant concentration of 2-ketoisocaproate also gave straight lines intersecting on the abscissa. However, with ammonia at a saturating concentration, the double reciprocal plots of velocities versus the NADH concentration at several different concentrations of 2-ketoisocaproate gave parallel lines. These observed kinetic patterns indicate a sequential ordered mechanism, where ammonia binds to the enzyme between NADH and 2-ketoisocaproate [24]. $K_m$ values for NADH, 2-ketoisocaproate, and ammonia were calculated to be 0.02, 4.0, and 220 mM, respectively.

Product-inhibition studies were carried out to determine the order of substrate binding and product release. With NADH as an inhibitor in the oxidative deamination, the double reciprocal plots of velocities versus $NAD^+$ concentrations at a high level of L-leucine showed competitive inhibition, and NADH showed noncompetitive inhibition with respect to L-leucine with $NAD^+$ at a saturating concentration. Thus, $NAD^+$ binds first to the enzyme, followed by L-leucine; 2-ketoisocaproate, ammonia, and NADH

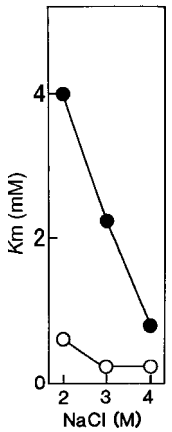


Fig. 5. Effect of NaCl on the kinetic parameters of the enzyme in the oxidative deamination. The $K_m$ values for L-leucine (●) and $NAD^+$ (○) were measured in the presence of various concentrations of NaCl.

are released in this order from the enzyme. The product inhibition patterns for the reductive amination also indicated a sequential ordered ternary–binary kinetic mechanism, in which NADH, ammonia, and 2-ketoisocaproate bind to the enzyme in this order, and L-leucine and then $NAD^+$ are released from the enzyme.

$K_m$ values for L-leucine and $NAD^+$ were measured in the presence of several concentrations of NaCl. The $K_m$ value for L-leucine decreased under high-salt conditions (Fig. 5). This result suggests that a high concentration of salt is required for the proper conformation of the enzyme, since halophilic enzymes contain many acidic amino acid residues. Such a change of $K_m$ values for substrates upon salt concentrations is also observed in the 2-aminobutyrate dehydrogenase from an extreme halophile, *H. saccharovorum* [25]. $K_m$ values for L-isoleucine and L-valine were 0.66 and 0.71 mM, respectively, in the presence of 4 M NaCl. The sequential ordered ternary–binary mechanism of the *N. magadii* enzyme is similar to the *B. sphaericus* and *C. pseudodiphtheriticum* enzymes [2,5], but the sequence of the substrate binding for the reductive amination is different from those of these enzymes.

The *T. intermedius* enzyme is stabilized by high concentrations of salts, such as NaCl and KCl, but the enzyme did not require high concentrations of salts for its activity [11]. The *N. magadii* enzyme is unique in its halophilicity among leucine dehydrogenases studied so far. This is the first report on the halophilic leucine dehydrogenase. The *N. magadii* enzyme is also stable in the presence of high concentrations of ammonium acetate or ammonium formate (data not shown). Thus, the enzyme is useful for the enzymatic syntheses of branched-chain L-amino acids from their 2-keto analogs in the presence of a high concentration of ammonium formate.

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