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Purification and characterization of alanine dehydrogenase from a marine bacterium, *Vibrio proteolyticus*

Shin-ichiro Kato[a,1], Toshihisa Ohshima[b], Andrey Galkin[a,2], Ljudmila Kulakova[a,3], Tohru Yoshimura[a], Nobuyoshi Esaki[a,*]

[a] *Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan*
[b] *Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, Tokushima, Tokushima 770-8506, Japan*

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

Abstract

Alanine dehydrogenase of *Vibrio proteolyticus* DSM 30189 shows high activity toward β-hydroxypyruvate, and the enzyme is applicable to the production of L-serine. We have cloned the enzyme gene from the bacterium into *Escherichia coli* TG1 with a vector plasmid, pUC118. The enzyme was overproduced by the transformed cells and purified to homogeneity with a yield of 46%. The molecular mass of the enzyme was about 230 kDa and consisted of six identical subunits. The enzyme showed broad specificity toward α-keto acids in the reductive amination. The relative activities of the enzyme for pyruvate, β-fluoropyruvate, and β-hydroxypyruvate were 100%, 74%, and 54%, respectively. The enzyme retained more than 90% of the activity after incubation at 65 °C for 60 min in the presence of 2.0 M NaCl, but 98% of its original activity was lost in the absence of NaCl. RbCl, as well as NaCl, significantly stabilized the enzyme. On the other hand, LiCl and KCl were not as effective as stabilizers such as RbCl and NaCl.

Keywords: Alanine dehydrogenase; β-Hydroxypyruvate; L-Serine; Thermostability; Stabilization by salts

1. Introduction

Alanine dehydrogenase (L-alanine: NAD^+ oxidoreductase, deaminating, EC 1.4.1.1) (AlaDH) catalyzes the reversible deamination of L-alanine to pyruvate and ammonia in the presence of NAD^+, as shown in Scheme 1. The enzyme has been found in vegetative cells and spores of various bacilli [1,2] and in some other bacteria [3–7], and its enzymological properties have been elucidated. AlaDH plays an important role in carbon and nitrogen metabolism. In *Bacillus* strains, the enzyme is involved in the production of pyruvate as an energy source through the tricarboxylic acid cycle

* Corresponding author. Tel.: +81-774-38-3240; fax: +81-774-38-3248.
E-mail address: esaki@scl.kyoto-u.ac.jp (N. Esaki).
[1] Present address: Research Institute of Molecular Genetics, Kochi University, 200 Nankoku, Kochi 783-8502, Japan.
[2] Present address: Center for Advanced Research for Biotechnology, University of Maryland Biotechnology Institute, Rockville, MD 20850, USA.
[3] Present address: Laboratory of Parasitic Diseases, NIAID, National Institute of Health, Bethesda, MD 20892-0425, USA.

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Scheme 1.

during sporulation [8]. In nitrogen-fixing organisms, such as *Anabaena cylindorica* [9] and *Rhodobacter capsulatus* [6], AlaDH plays an important role in the incorporation of ammonia into organic compounds.

AlaDH is highly specific toward L-alanine and is used as a catalyst for the analysis of L-alanine [10]. AlaDH has been also used as a catalyst for membrane reactors to produce L-alanine and ^{15}N-labeled L-alanine [11]. Since NADH is readily regenerated with formate dehydrogenase, amino acid dehydrogenases including AlaDH are applicable to the synthesis of optically active amino acids [12]. Galkin et al. [13] have developed a system for the production of D-amino acids from the corresponding α-keto acids with recombinant *Escherichia coli* cells expressing four heterologous enzymes simultaneously: formate dehydrogenase, AlaDH, alanine racemase, and D-amino acid transferase. Galkin's method depends on the highly specific action of AlaDH on pyruvate. Otherwise, the optical purity of the D-amino acid produced in the system will be decreased by the unfavorable action of AlaDH on the substrate α-keto acids to be converted to antipodal L-amino acids. Thus, in this system, it is important that the AlaDH used have absolute specificity for pyruvate. On the other hand, in order to produce various L-amino acids, in particular, non-proteinous artificial L-amino acids, more versatile amino acid dehydrogenases with broad substrate specificities are desired. For example, L-tertiary-leucine is produced industrially from the corresponding α-keto acid with leucine dehydrogenase with a broad substrate specificity [14]. Ohshima et al. [15] developed an interesting method to produce L-β-fluoroalanine from β-fluoropyruvate with an AlaDH, which is capable of accepting this pyruvate analog as a substrate. L-β-[^{19}F]Fluoroalanine are useful as a radioactive substance for positron emission tomography imaging of breast tumors [16]. Since then, the development of new AlaDHs with broad substrate specificities has been desirable.

We have found that an alanine dehydrogenase of a marine bacterium, *Vibrio proteolyticus* DSM30189 (VprAlaDH), shows a high activity toward β-hydroxypyruvate and that the enzyme is applicable to the production of L-serine. We describe here the purification, catalytic properties, gene cloning, and overexpression of this new AlaDH.

2. Experimental

2.1. Materials

NAD^+, NADH, $NADP^+$, and NADPH were products of Kojin Co., Tokyo, Japan. Reactive Red 120 and marker proteins for molecular mass determination were purchased from Sigma Chemicals, St. Louis, MO; Sepharose 4B and Sephacryl S-300 from Amersham Biosciences, Uppsala, Sweden; TSKgel G3000SW$_{XL}$ from Tosoh, Tokyo, Japan; plasmid pUC118, all restriction enzymes, and Easytrap from Takara Shuzo, Kyoto, Japan; DIG DNA labeling mixture and Anti-DIG-AP conjugate from Roche, Basel, Switzerland; and the oligonucleotides from Biologica, Nagoya, Japan. All other chemicals were analytical grade products from Nacalai Tesque, Kyoto, Japan.

2.2. Enzyme and protein assay

AlaDH was assayed essentially according to the method of Ohshima et al. [17]. The reaction mixture was incubated at 25 °C in a cuvette with a 1.0 cm light path. One unit of the enzyme was defined as the amount of enzyme catalyzing the formation of 1 μmol of NADH per min at 25 °C. The ability of the enzyme to catalyze the oxidative deamination and reductive amination was examined in the presence of 50 mM of amino acids and 10 mM of α-keto acids, respectively. The inhibitory effects of various compounds were examined by measuring the activity of the enzyme in the deamination of L-alanine after incubation of the enzyme in the presence of 0.1 or 1 mM of an inhibitor at 25 °C for 20 min. Initial velocity and product inhibition experiments were carried out by varying the concentrations of one substrate at different fixed

concentrations of another substrate or product. The analysis of kinetic data was carried out as described previously [18,19]. K_m values were determined from the secondary plots of intercepts versus reciprocal concentrations of the substrate. Protein concentrations were measured by the spectrophotometric method of Kalb and Bernlohr [20].

2.3. Gene cloning and plasmid construction

Fragments of the chromosomal DNA of *V. proteolyticus*, which were obtained by partial digestion with *Sau*3AI, were ligated into the *Bam*HI site of pUC118. *E. coli* TG1 was used as the host for library construction. Positive clones carrying the AlaDH gene were selected from the gene library by colony hybridization with DNA probes labeled with digoxigenin. The plasmid isolated from the positive transformant and containing a 1.6 kb fragment was designated as pVprAlaDH. DNA sequencing was performed in both directions with universal and specific primers. The nucleotide sequence has been submitted to the DDBJ with the accession number AF070716.

2.4. Purification of VprAlaDH

All operations for the enzyme purification were performed at 4 °C, and a 10 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA and 0.01% 2-mercaptoethanol was used as the standard buffer throughout purification.

E. coli TG1 harboring pVprAlaDH was cultivated in a 500 ml flask containing 100 ml of 2 × YT medium at 37 °C. The harvested cells (0.3 g) were suspended in the standard buffer (5 ml) and sonicated. Unbroken cells and cell debris were removed by centrifugation at 12,000 × *g* for 10 min. Ammonium sulfate was added to the supernatant solution to a final concentration of 2.0 M. After centrifugation at 12,000 × *g* for 30 min, the supernatant solution was applied to a Phenyl-Toyopearl column (1.5 cm × 3.5 cm). The enzyme was eluted with a 2.0–0 M ammonium sulfate gradient. The active fractions were pooled, concentrated with an Amicon PM-10 membrane, and then applied to a Sephacryl S-300 column (2.2 cm × 65 cm) equilibrated with the standard buffer containing 0.20 M NaCl. The active fractions were eluted with the same buffer (400 ml) and concentrated with the same ultrafiltration membrane.

VprAlaDH was purified also from the cell-extract of *V. proteolyticus* DSM 30189, which was cultivated in a 2-l shaking flask containing 700 ml of medium containing 0.5% (w/v) polypeptone, 0.3% meat extract, 3% NaCl, and 20 mM DL-alanine at 26 °C for 20 h on a reciprocal shaker. The washed cells (about 12 g) were suspended in the standard buffer (50 ml) and disrupted by sonication. Unbroken cells and cell debris were removed by centrifugation (at 12,000 × *g*, for 10 min), and the supernatant solution was recovered. Sodium chloride was added to the supernatant solution at a final concentration of 2.0 M, and the resulting solution was incubated at 65 °C for 30 min. The precipitate formed was removed by centrifugation (at 12,000 × *g*, for 30 min). The supernatant solution was dialyzed against 1000 volumes of 10 mM potassium phosphate buffer (pH 6.0) containing 1 mM EDTA and 0.01% 2-mercaptoethanol. The enzyme solution (60 ml) was applied to a Reactive Red 120 column (1.8 cm × 25 cm) equilibrated with the same buffer (pH 6.0). After the column was washed with the same buffer (200 ml), the retained enzyme was eluted with a 0–2.0 M NaCl gradient. The active fractions were pooled, and the enzyme solution, which had been concentrated by ultrafiltration (Advantec UHP-43K), was dialyzed against 1000 volumes of the standard buffer. The enzyme solution (40 ml) was applied to a DEAE-Toyopearl column (1.8 cm × 25 cm) equilibrated with the buffer (pH 7.2). After the column was washed with 200 ml of the buffer, the retained enzyme was eluted with a 0–0.30 M NaCl gradient. The active fractions were concentrated by ultrafiltration and applied to a Sephacryl S-300 column (2.2 cm × 65 cm) equilibrated with the standard buffer containing 0.20 M NaCl. The active fractions eluted with the same buffer (400 ml) were pooled and concentrated by ultrafiltration.

2.5. Other analytical methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% gel, 1-mm thick) was performed by the procedure of Laemmli [21]. The molecular mass of the enzyme was determined by size-exclusion high performance liquid chromatography using a TSKgel G3000SW$_{XL}$ column (7.8 mm ×

300 mm) and SDS-PAGE in the same manner as described previously [22,23]. The N-terminal sequence of the purified enzyme (about 0.5 nmol) was analyzed by automated Edman degradation in the same manner as described previously [24].

3. Results and discussion

3.1. Purification of VprAlaDH

VprAlaDH shows a high activity toward β-hydroxypyruvate and is probably useful as a catalyst for the production of L-serine with the cofactor regeneration system we developed [13]. We constructed an overproducer of the enzyme by cloning its gene in order to facilitate easy preparation of the enzyme. The level of expression of VprAlaDH in the clone cells (*E. coli* TG1 carrying pVprAlaDH) was around 25% of the total soluble protein, as determined on the basis of the specific activity of AlaDH in the cell extract (38 unit/mg of protein). VprAlaDH was purified to the level of a single major protein band on SDS-PAGE with a satisfactory yield (Table 1). When the enzyme was purified from the cell-extract of *V. proteolyticus* DSM30189, a homogeneous preparation of AlaDH was obtained with an overall increment in specific activity of about 1400-fold and a yield of 55% (data not shown). Thus, overproduction of about 350-fold was attained by gene cloning.

3.2. Subunit structure of VprAlaDH

The open reading frame of the gene encoding VprAlaDH comprised 1122 bp encoding 374 amino acid residues with a putative molecular mass of 39,807 Da, which was in agreement with the value of the molecular mass (40 kDa) of the purified enzyme determined by SDS-PAGE. The molecular mass of VprAlaDH was determined to be about 230 kDa by gel filtration. These results suggest that VprAlaDH consists of six identical subunits in the same manner as other AlaDHs thus far purified and characterized [3,4,6,17,25–28]. Recently, the hexameric structure of the AlaDH from *Phormidium lapideum* (PlaAlaDH) was demonstrated unequivocally by X-ray crystallography [29]. VprAlaDH has an amino acid sequence that is quite similar to that of PlaAlaDH throughout the whole range from the N-terminus to the C-terminus (data not shown). Therefore, VprAlaDH probably has a hexameric structure in the same manner as PlaAlaDH.

3.3. Catalytic properties of VprAlaDH

VprAlaDH showed the maximum reactivity in the range of pH 10.0–10.3 in the oxidative deamination of L-alanine and pH 8.0 in the reductive amination of pyruvate. The enzyme required NAD^+ as a coenzyme for the oxidative deamination and NADH for reductive amination. $NADP^+$ and NADPH were inert. The substrate specificities of the enzyme in the oxidative deamination of amino acids and the reductive amination of α-keto acids are summarized in Table 2. VprAlaDH specifically acted on L-alanine. In contrast, the enzyme showed low substrate specificity in reductive amination of α-keto acids; not only pyruvate but also β-hydroxypyruvate and β-fluoropyruvate served as good substrates. The reaction equilibrium favors the direction toward the synthesis of L-serine, and this is a great advantage for VprAlaDH as a catalyst in the production of L-serine.

3.4. Kinetics of reactions catalyzed by VprAlaDH

The K_m values for the substrates were: NAD^+, 0.18 mM; NADH, 0.090 mM; L-alanine, 30 mM; pyruvate, 0.61 mM; and ammonia, 110 mM. The kinetic

Table 1
Summary of the purification procedure for VprAlaDH from *E. coli* TG1 carrying pVprAlaDH

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Crude extract	32	1200	38	100
Ammonium sulfate fractionation	26	1100	42	92
Phenyl-TOYOPEARL	8.2	630	77	53
Sephacryl S-300	3.6	550	150	46

Table 2
Substrate specificity of VprAlaDH

Oxidative deamination[a]		Reductive amination[b]	
Substrates	Relative activity (%)	Substrates	Relative activity (%)
L-Alanine	100	Pyruvate	100
L-α-Aminobutyrate	0.7	β-Fluoropyruvate	74
L-Serine	0.5	β-Hydroxypyruvate	54
D-Alanine	0	α-Ketobutyrate	4.0

[a] Inert: β-alanine, glycine, L-valine, L-norvaline, L-leucine, L-norleucine, L-isoleucine, L-methionine, L-proline, L-aspartate, L-glutamate, L-cysteine, L-threonine, L-phenylalanine, L-tyrosine, L-tryptophan, L-asparagine, L-glutamine, L-lysine, L-arginine, L-histidine, L-ornithine, L-citrulline, 3-(2-thienyl)-DL-alanine, D-β-fluoroalanine, D-serine, and DL-homoserine.

[b] Inert: glyoxylate, oxaloacetate, α-ketoglutamate, α-ketoisobutyrate, α-ketomalonate, β-bromopyruvate, α-ketovalerate, α-ketoisovalerate, α-ketocaproate, and α-ketoisocaproate.

studies on VprAlaDH indicated that the oxidative deamination proceeds through a sequentially ordered binary–ternary mechanism: NAD^+ binds first to the enzyme, followed by the binding of L-alanine, and products are released in the order of ammonia, pyruvate, and NADH. The mechanism is the same as that of AlaDH from *B. subtilis* [30,31]. VprAlaDH was strongly inhibited by *p*-chloromercuribenzoic acid (97% inhibition at 0.1 mM), $HgCl_2$ (100% inhibition at 1 mM), and methyl *p*-nitrobenzenesulfonate (100% inhibition at 0.1 mM). Some other ions such as Cu^{2+}, Zn^{2+}, Sn^{2+}, Pb^{2+}, and SO_4^{2-} were inhibitory.

3.5. Stability of VprAlaDH

The stability of VprAlaDH at various pHs was examined. The activity was not affected by incubation at pH 6.0–9.5 and 25 °C for 10 min. The enzyme retained its full activity after incubation at pH 7.2 and 50 °C for 60 min but lost about 70% of its original activity when the temperature was shifted to 55 °C (Fig. 1A). The thermostability of VprAlaDH was significantly increased in the presence of NaCl. More than 90% of the activity was retained even after incubation at 65 °C for 60 min in the standard buffer supplemented with 2.0 M NaCl, but 98% of the activity was lost without NaCl (Fig. 1B). The enzyme was also stabilized by LiCl and KCl, but the degrees of stabilization by these salts were lower than those

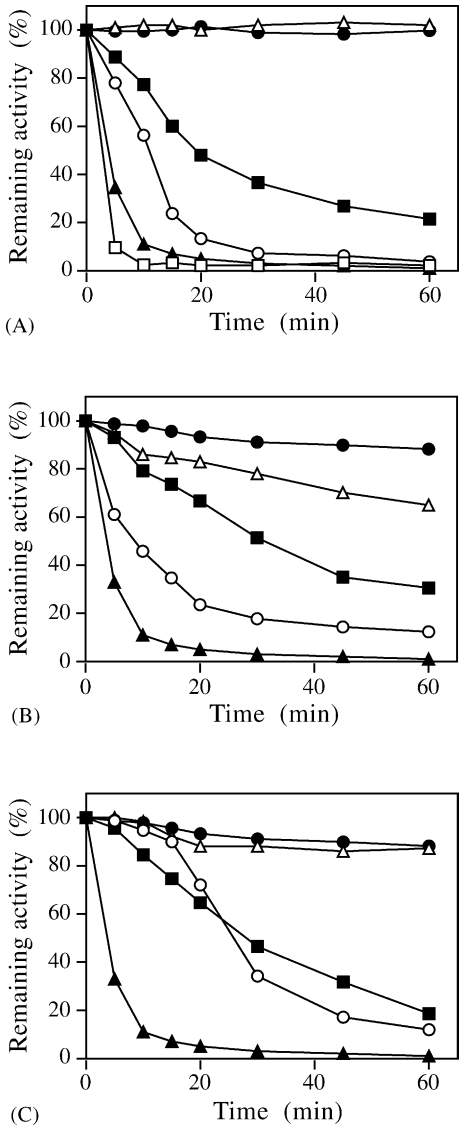

Fig. 1. Thermostability of VprAlaDH. (A) Time course of change in remaining activity after incubation in the buffer containing 20 mM potassium phosphate (pH 7.2) at 45 °C (●), 50 °C (△), 55 °C (■), 60 °C (○), 65 °C (▲), and 70 °C (□). (B) Time course of change in remaining activity after incubation in the presence of various concentrations of NaCl at 65 °C. NaCl concentrations were 0 M (▲), 0.2 M (○), 0.5 M (■), 1.0 M (△), and 2.0 M (●). (C) Time course of change in remaining activity after incubation at 65 °C in the absence (▲) or presence of NaCl (●), RbCl (△), LiCl (■), and KCl (○). The salt concentrations were 2.0 M.

by NaCl and RbCl (Fig. 1C). It could be speculated that the stabilization depends on the hydration effect by these alkali metal ions. However, the hydration potentials of the ions are in the order of Hofmeister's series: $Li^+ > Na^+ > K^+ > Rb^+$. This is inconsistent with the order of the stabilizing effect observed: $Na^+ > Rb^+ > Li^+, K^+$. Therefore, it seems likely that the stabilization of VprAlaDH is not only a result of the hydration effects but also depends on the direct interactions with charged groups in VprAlaDH.

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