Isolation and Characterization of Valine Dehydrogenase from *Streptomyces aureofaciens*

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Valine dehydrogenase (ValDH) (EC 1.4.1.8) belongs to the group of NAD(P)+-dependent dehydrogenases of branched-chain amino acids. Of this group of microbial enzymes, only leucine dehydrogenases (LeuDH) (EC 1.4.1.9) of the genus *Bacillus* have been described so far. Leucine dehydrogenase of *Bacillus cereus* was described first (12). It was also detected in a number of other *Bacillus* species, both in vegetative cells (3, 8, 10) and in spores (5, 7, 18). LeuDH catalyzes reversible oxidative deamination of L-leucine, L-isoleucine, L-valine, L-norvaline, and L-norleucine and, occasionally, other structurally related amino acids to their oxoacids. LeuDH of *B. sphaericus* (8, 14), *B. cereus* (13), and *B. stearothermophilus* (9) have a different preference for oxidative deamination and were also isolated from *Corynebacterium* species and in *Alcaligenes faecalis* (8).

In streptomycetes, ValDH has been demonstrated only in a crude cell extract of *Streptomyces fradiae* (11). The enzyme catalyzed oxidative deamination of the identical amino acids as did LeuDH, but with a different preference. We also found a relatively high activity of the enzyme in *S. aureofaciens*. In the present communication we describe the purification and characterization of ValDH from *S. aureofaciens*, a bacterium that produces the oligoketide antibiotic tetracycline.

**MATERIALS AND METHODS**

*Microorganism and growth conditions.* The microorganism used was *S. aureofaciens* 50/137, a UV mutant derived from the high-producing strain 84/25 obtained from the Research Institute of Antibiotics and Biotransformations, Roztoky, Czechoslovakia.

Complex medium used for the isolation of ValDH contained the following (in grams per liter): sucrose, 25; CaCO3, 3; NaCl, 5; K2HPO4, 0.5; MgSO4·7H2O, 0.5; Tris, 12.1. The pH was adjusted to 7.3 with 3 M HCl. Nitrogen sources were sterilized separately and used at the concentrations indicated in the text. The vegetative inoculum (5%, vol/vol; 24 h) was grown in the complex soybean meal medium. The cultivation was carried out in 500-ml Erlenmeyer flasks filled with 60 ml of medium on a reciprocal shaker (1.6 Hz, 28°C). Growth was determined gravimetrically as described by Erban et al. (2).

*Enzyme assay.* The ammonium-assimilating activity of ValDH was measured as a decrease of NADH A340. One milliliter of reaction mixture contained 100 μmol of Tris hydrochloride buffer, 10 μmol of sodium α-ketoisovalerate, 0.1 μmol of NADH, and 100 μmol of NH4Cl. The assay was performed at pH 9.0 and 30°C.

The oxidative deamination activity of ValDH was measured as an increase of NADH A340. One milliliter of reaction mixture contained 100 μmol of glycine-KCl-KOH buffer, 10 μmol of L-valine, and 1.5 μmol of NAD+; the final pH was 10.7, and the temperature was 30°C.

One enzyme activity unit was defined as the amount required to convert 1 mol of substrate per second (katal). Unless otherwise stated, the enzyme activity was measured in an oxidative deaminating system.

Proteins were determined by an absorbance method described by Whitaker and Granum (17) and by a spectrophotometric method described by Lowry et al. (4), with bovine serum albumin as a standard. All spectrophotometric measurements were performed with a Cary 118 C spectrophotometer (Varian, Palo Alto, Calif.).

*Preparation of cell extract.* A 24-h mycelium grown in a complex medium was separated from the fermentation broth by centrifugation at 4,000 × g at 4°C for 5 min, washed with ice-cold distilled water, and centrifuged at 20,000 × g for 30 min at 4°C. The mycelium was disintegrated in a Biox X-Press at −25°C and a pressure of 300 MPa. Broken mycelium (wet weight, 75 g) was suspended in 100 ml of 0.1 M Tris hydrochloride buffer (pH 7.4), and after 40 min the homogenate was centrifuged for 40 min at 22,000 × g at 4°C.
TABLE 1. Purification of ValDH from S. aureofaciens

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (μkat)</th>
<th>Sp act (μkat/mg)*</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1,590</td>
<td>200</td>
<td>0.13</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>21.6</td>
<td>114</td>
<td>5.3</td>
<td>40.8</td>
<td>57</td>
</tr>
<tr>
<td>Mono Q</td>
<td>0.46</td>
<td>46.8</td>
<td>102</td>
<td>785</td>
<td>23</td>
</tr>
</tbody>
</table>

* The specific activity of ValDH (102 μkat/mg of protein) obtained in the oxidative deamination system is equal to 510 μkat/mg of protein for the reductive amination reaction.

Purification of ValDH. The cell extract (150 ml) was supplemented with KCl to a 0.8 M concentration and adjusted to pH 7.4 with 0.5 M acetic acid, and the whole volume was applied to a phenyl-Sepharose CL-4B bed (2.6 by 8.0 cm) preequilibrated with 0.1 M Tris hydrochloride buffer containing 0.8 M KCl (pH 7.4) (buffer A). After the column was washed with the same buffer (400 ml), the absorbed material was eluted with a linear gradient (300 ml) of 0 to 100% buffer B (0.02 M Tris hydrochloride [pH 7.4]) at a flow rate of 3 ml/min, and 10-ml fractions were collected.

Fractions containing ValDH activity were pooled (90 ml), concentrated (20 ml), and transferred into buffer C (0.02 M piperazine hydrochloride [pH 6.0]) in a model 52 UF cell, Membrane PM-10 (Amicon Corp., Lexington, Mass.). Two 10-ml samples (of about 11 mg of protein each) were each applied via a 10-ml Superloop to a Mono Q HR 5/5 column equilibrated with buffer C. After the column had been washed with the same buffer (10 ml), the elution was continued with a linear gradient of 0 to 40% buffer D (1 M NaCl in buffer C) in 21 ml. Fractions of 0.8 ml were collected at a flow rate of 1 ml/min. All operations were performed at 20°C.

The overall purification achieved was 785-fold, with a 23% recovery of the enzyme activity. A summary of the typical purification scheme is shown in Table 1.

Analytical methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing were performed as described previously (16).

Size exclusion high-performance liquid chromatography of the purified ValDH was performed with 0.1 M phosphate buffer (pH 7.0) on a TSK G 3000 SW column (7.5 by 300 mm) at a flow rate of 0.5 ml/min. Protein molecular weight standards (kit MS II; Serva, Heidelberg, Federal Republic of Germany) were used to calibrate the column.

Equilibrium ultracentrifugation was performed with a Spinco model E ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.) by the method of Chervenka (1). Centrifugation was carried out for 17 h at 20°C at a rotor velocity of 9,945 rpm. The sample was dissolved in a solution of 0.3 M NaCl in 0.02 M piperazine hydrochloride buffer (pH 6.0). Rotor An-H-Ti, interference optics, a double-sector cell, and a value of 0.72 ml/g for the partial specific volume were used.

Materials. NAD⁺, NADP⁺, and NADH were obtained from Reanal, Budapest, Hungary. Tris, piperazine, amino acids, α-keto acids, NADPH, and NAD⁺ analogs were purchased from Sigma Chemical Co., St. Louis, Mo. Phenyl-Sepharose CL-4B and Mono Q HR 5/5 high-performance liquid chromatography prepacked column were from Pharmacia, Uppsala, Sweden. All other chemicals were of the highest purity available.

RESULTS

Influence of various nitrogen sources on ValDH synthesis. The influence of various nitrogen sources on ValDH synthe-
Analytical isoelectric focusing in polyacrylamide gel with Ampholine (pH 3.5 to 10; LKB Sverige AB, Bromma, Sweden) showed that the enzyme has a pI of 5.1 (Fig. 2).

**pH and temperature optima.** The optimum pH for the reductive amination reaction in Tris hydrochloride and oxidative deamination reaction in glycine-KCl-KOH buffer was found to be 9.0 and 10.7, respectively.

The optimum temperature of ValDH under standard conditions was 65°C, for both the reductive amination and the oxidative deamination.

**Substrate and coenzyme specificity.** The ability of the enzyme to catalyze oxidative deamination of different amino acids is shown in Table 3. In addition to branched-chain amino acids (L-valine, L-isoleucine, and L-leucine), which are preferred as substrates, a relatively high reaction rate was also detected with the straight-chain aliphatic l-amino acids L-norvaline and L-α-amino butyrate. The reaction rate with L-alanine was only 1.9% of the maximal reaction rate; other l-amino acids and d-amino acids were not deaminated. The lowest value of the Michaelis constant was found with L-valine (2.5 mM); the $K_m$ for L-alanine was higher by more than 2 orders of magnitude. Under standard conditions and with L-valine as a substrate, the $K_m$ for NAD$^+$ was 0.10 mM.

The substrate specificity of ValDH in reductive amination is illustrated in Table 4. The highest reaction rate was detected with α-ketoisovalerate, a keto analog of L-valine. However, all other keto analogs of substrates of oxidative deamination were reductively aminated as well; the lowest reaction rate was detected with pyruvate as a substrate. $K_m$ values for α-keto acids were within the range of 0.8 to 1.5 mM; only with α-keto-β-methyl-n-valerate and pyruvate were $K_m$ values higher by an order of magnitude. The lowest $K_m$ was determined with α-ketoisocaproate, a keto analog of L-leucine.

Under standard assay conditions with α-ketoisovalerate, $K_m$ values of 18.2 and 0.023 mM were determined for NH$_4^+$ and NADH, respectively. NH$_4^+$ was the only substrate for reductive amination; no other compound could substitute it (Tris hydrochloride, hydroxyamine, ethyamine, methylamine, or ethylene diamine). Also, NADH could not be replaced with NADPH.

ValDH requires NAD$^+$ as a natural cofactor of the oxidative deamination. The reaction rate with NAD$^+$ was only 8.4%; however, high reaction rates were obtained with a

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**TABLE 3. Substrate specificity for oxidative deamination**

<table>
<thead>
<tr>
<th>Substrate (10 mM)$^a$</th>
<th>Relative activity (%)$^b$</th>
<th>$K_m$ (mM)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Valine</td>
<td>100</td>
<td>2.5</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>46.5</td>
<td>5.0</td>
</tr>
<tr>
<td>L-Norvaline</td>
<td>43.0</td>
<td>5.7</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>36.0</td>
<td>6.3</td>
</tr>
<tr>
<td>L-α-Aminobutyrate</td>
<td>16.5</td>
<td>14.8</td>
</tr>
<tr>
<td>L-Norleucine</td>
<td>10.5</td>
<td>15.6</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>1.9</td>
<td>333.6</td>
</tr>
</tbody>
</table>

$^a$ No activity was observed with d-valine, d-leucine, d-isoleucine, glycine, L-threonine, β-alanine, L-serine, l-cysteine, L-methionine, l-glutamic acid, l-aspartic acid, l-asparagine, l-glutamine, l-lysine, l-phenylalanine, l-tyrosine, l-hystidine, or l-tryptophan.

$^b$ The 100% level of relative activity corresponds to a specific activity of 102 μkat/mg of protein in the oxidative deamination system.

$^c$ The $K_m$ values were determined by using Lineweaver-Burk plots.

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**TABLE 4. Substrate specificity for reductive amination**

<table>
<thead>
<tr>
<th>Substrate (10 mM)$^a$</th>
<th>Relative activity (%)$^b$</th>
<th>$K_m$ (mM)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Ketoisovalerate</td>
<td>100</td>
<td>1.3</td>
</tr>
<tr>
<td>α-Ketoisocaproate</td>
<td>38.1</td>
<td>0.8</td>
</tr>
<tr>
<td>α-Keto-β-methyl-n-valerate</td>
<td>22.3</td>
<td>6.7</td>
</tr>
<tr>
<td>α-Ketobutyrate</td>
<td>48.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>1.3</td>
<td>15.3</td>
</tr>
</tbody>
</table>

$^a$ No activity was observed with α-ketoglutarate, phenylpyruvate, oxaloacetate, and glycolate.

$^b$ The 100% level of relative activity corresponds to a specific activity of 510 μkat/mg of protein in reductive deamination system.

$^c$ The $K_m$ values were determined by using Lineweaver-Burk plots.
number of NAD⁺ analogs (Table 5): 1-6-etheno-NAD⁺, 3-acetylpuridine-NAD⁺, deamino-NAD⁺, 3-pyridinealdehyde-NAD⁺, and nicotinamide guanine dinucleotide (NGD⁺). Inhibitors. The enzyme was inhibited by inhibitors of SH groups (p-chloromercuribenzoate [0.01 mM] and HgCl₂ [0.01 mM]) to 17 and 9% of the original enzyme activity, respectively. Metal ions influenced ValDH activity only insignificantly. None of the following compounds at 1 mM exhibited a significant effect on ValDH activity: AMP, ADP, ATP, adenine, adenosine, GMP, GTP, guanosine, cytosine, thymine, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), coenzyme A, acetyl coenzyme A, and EDTA.

### DISCUSSION

In cell extracts of *S. aureofaciens*, a dehydrogenase was detected that oxidatively deaminated a number of amino acids and exhibited the highest activity with l-valine as a substrate. In a chemically defined medium this enzyme, ValDH, was induced by l-valine and l-isoleucine; l-leucine was ineffective as the enzyme inducer. This finding is in agreement with the fact that *B. subtilis* LeuDH is also induced by l-valine and l-isoleucine, whereas l-leucine does not act as an inducer (7). It was found that ValDH in *S. fradiae* is significantly repressed by ammonium ions (11, 15), whereas we found in this work that in *S. aureofaciens* the effect of ammonia on ValDH synthesis was negligible.

Since ValDH of *S. aureofaciens* cell extract exhibited substrate specificity differing from that of other dehydrogenases of branched-chain amino acids described so far, we studied properties of the purified enzyme in more detail. Although LeuDHs of *B. cereus* (13), *B. stearothermophilus* (9), or *B. sphaericus* (8) consisted of eight, six, and six subunits, respectively, and the molecular weight of the enzymes was within the range of 245,000 to 310,000, ValDH of *S. aureofaciens* consisted only of four subunits, and the molecular weight of the enzyme was 116,000.

In the direction of oxidative deamination, the preferred substrate for ValDH of *S. aureofaciens* was l-valine, whereas for LeuDHs of bacilli (8, 9, 13) it was l-leucine. Surprisingly, in the direction of reductive amination, both enzymes exhibited the highest activity with ß-ketoisovalerate, a keto analog of l-valine. All LeuDHs described so far were NAD⁺ dependent. ValDH of *S. fradiae* could utilize both NAD⁺ and NADP⁺ as cofactors (11), whereas ValDH of *S. aureofaciens* was NAD⁺ specific; the reaction velocity with NADP⁺ was only 8.4% of that with NAD⁺.

The observed differences between the NH₄⁺/Kₘ values of ValDH and LeuDHs from *Bacillus* spp. may be physiologically important. In *B. cereus* (13) the Kₘ value for NH₄⁺ is 220 mM, and in *B. sphaericus* (8) it is 200 mM, whereas in *S. aureofaciens* it is 18.2 mM. The LeuDH of the *Bacillus* spp. apparently has a catabolic function, i.e., release of NH₄⁺, NAD⁺, and branched-chain ß-keto acids, and this function is particularly important in spore germination (6, 7). The physiological role of dehydrogenases of branched-chain amino acids in streptomycetes has not yet been studied. Only the regulation of ValDH synthesis in connection with tylosin synthesis (11) and branched-chain iso and anteiso fatty acid production (15) in *S. fradiae* has been described. A more-detailed clarification of the physiological role of ValDH in streptomycetes will require a thorough biochemical characterization of mutants defective in ValDH synthesis.

### ACKNOWLEDGMENTS

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### LITERATURE CITED


