Valine Dehydrogenase from *Streptomyces fradiae*: Purification and Properties

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Valine dehydrogenase (VDH) was purified to homogeneity from cell-free extract of *Streptomyces fradiae*, which produces tylosin. The enzyme was purified 1508-fold in a 17.7% yield using a combination of hydrophobic chromatography and ion-exchange fast protein liquid chromatography. The Mr of the native enzyme was determined to be 218000 and 215000, by equilibrium ultracentrifugation and size-exclusion high-performance liquid chromatography, respectively. The enzyme is composed of 12 subunits of Mr 18000. Using analytical isoelectric focusing the isoelectric point of VDH was found to be 4.7. Oxidative deamination of L-valine was optimal at pH 10.6. Reductive amination of 2-oxoisovalerate was optimal at pH 8.8.

**INTRODUCTION**

Valine dehydrogenase (VDH; EC 1.4.1.8) activity was detected in a cell-free extract of *Streptomyces fradiae* in which it was thought to be a regulatory enzyme involved in biosynthesis of n-butyrate, a building unit of the oligoketide antibiotic tylosin (Omura et al., 1983). Inhibition of VDH synthesis and activity by NH₄⁺ was correlated with a decrease in branched-chain fatty acids (Vančura et al., 1987a). VDH belongs to the group of NAD(P)-dependent dehydrogenases of branched-chain amino acids catalysing reversible oxidative deamination of branched, and occasionally unbranched, amino acids to the corresponding oxo acid. Amongst these, NAD-dependent leucine dehydrogenase (LDH; EC 1.4.1.9) of *Bacillus* spp. (Sanwal & Zink, 1961; Zink & Sanwal, 1962) and NADP-dependent VDH of pea shoots (Kagan et al., 1968, 1969) have been recognized. LDH from *Bacillus sphaericus* (Soda et al., 1971; Ohshima et al., 1978), *B. cereus* (Schütte et al., 1985) and *B. stearothermophilus* (Ohshima et al., 1985) have been purified to homogeneity and characterized.

The VDH of *S. fradiae* has therefore been studied similarly on account of its involvement in oligoketide biosynthesis (Omura et al., 1983; Vančura et al., 1987a).

*Abbreviations:* ADH, alanine dehydrogenase; FPLC, fast protein liquid chromatography; GDH, glutamate dehydrogenase; LDH, leucine dehydrogenase; NGD⁺, nicotinamide guanine dinucleotide; VDH, valine dehydrogenase.
Materials. NAD⁺, NADP⁺ and NADH were obtained from Reanal, Budapest, Hungary. Tris, Bistris, amino acids, 2-oxo acids, NAD⁺ analogues and NADPH were purchased from Sigma. Phenyl-Sepharose CL-4B and the Mono Q HR 5/5 prepacked fast protein liquid chromatography (FPLC) column were from Pharmacia. All other chemicals were of the highest purity available.

Micro-organism and growth conditions. The micro-organism used was Streptomyces fradiae 30/3 obtained from the collection of the Research Institute of Biofactors and Veterinary Drugs in Kouim near Prague (VanEurony, 1987b). The first generation was cultivated for 48 h in a medium containing (% v/v): sucrose, 2-0; Casamino acids (Difco), 0-3; NaCl, 0-5; CaCO₃, 0-3; MgSO₄, 7H₂O, 0-05; K₂HPO₄, 0-1; L-valine, 0-2; yeast extract, 0-1; (NH₄)₂SO₄, 0-17. For the second generation, a medium containing (% v/v): sucrose, 2-0; Casamino acids, 0-3; NaCl, 0-5; CaCO₃, 0-3; MgSO₄, 7H₂O, 0-05; K₂HPO₄, 0-1; L-valine, 0-47; yeast extract, 0-05. Both media were adjusted to pH 7-3 with 1 M-HCl before thermal sterilization. Cultivation was done in 500 ml flasks containing 70 ml medium on a reciprocal shaker (1-7 Hz, 29°C).

Enzyme and protein assay. The ammonium-assimilating activity of VDH was measured as the decrease of NADH absorbance at 340 nm. The reaction mixture (1 ml) contained: 100 μmol Tris/HCl buffer, 10 μmol sodium 2-oxoisovalerate, 0-1 μmol NADH, 100 μmol NH₄Cl and 10 μmol 2-mercaptoethanol. The assay was done at pH 8-8 and 30°C.

The oxidative deamination activity of VDH was measured as the increase of NADH absorbance at 340 nm. The reaction mixture (1 ml) contained: 100 μmol glycine/KCl/KOH buffer, 10 μmol L-valine, 1-5 μmol NAD⁺ and 10 μmol 2-mercaptoethanol; the final pH was 10-6, temperature 30°C. One enzyme activity unit was defined as the amount required to convert 1 mol substrate per second (katal). Unless otherwise stated, the enzyme activity was measured in the oxidative deaminating system.

Proteins were determined by the absorbance method of Whitaker & Granum (1980) and by the Lowry method with bovine serum albumin (BSA) as standard. All spectrophotometric measurements were made by using a Cary 118 C (Varian) spectrophotometer.

Purification of VDH. Unless otherwise stated, all operations were done at 20°C.

Step 1. Preparation of crude extract. A 72 h mycelium was separated from the fermentation broth by centrifugation at 4000 g at +4°C (5 min), washed with ice-cold distilled water and centrifuged at 20000 g at 4°C for 30 min. The mycelium was disintegrated in a Biox X-Press at −25°C and at a pressure of 300 MPa. Broken cells were suspended in 0-1 M-Tris/HCl buffer, pH 7-4, and after 40 min the homogenate was centrifuged for 30 min at 22000 g at 4°C. The supernatant was designated as the crude extract.

Step 2. Hydrophobic interaction chromatography on Phenyl-Sepharose CL-4B. The crude extract (100 ml) was supplemented with KCl to 1 M concentration, adjusted to pH 7-4 with 0-5 M-acetic acid and the whole volume applied to a Phenyl-Sepharose CL-4B bed (2-6 × 8-0 cm) preequilibrated with 0-1 M-Tris/HCl buffer, pH 7-4, containing 1-0 M-KCl (buffer A). After the column had been washed with buffer A (170 ml), the adsorbed material was eluted with a linear gradient (from 30 to 100%) of buffer B (0-01 M-Tris/HCl, pH 7-4) in 300 ml at a flow rate of 3 ml min⁻¹ and 10 ml fractions were collected.

Step 3. First anionic chromatography on Mono Q HR 5/5 FPLC column. The solution of the combined fractions containing VDH activity was transferred into 0-02 M-Bistris/HCl buffer, pH 6-3, (buffer C) in an Amicon Model 52 UF cell, membrane PM-10. The effective volume was reduced from 70 ml to 20 ml. Two 10 ml samples of about 10 mg protein each) were each applied via a 10 ml Superloop to a Mono Q column preequilibrated with buffer C. After washing the column with buffer C the elution was continued with a linear gradient of 0-40% buffer D (1 M-NaCl in buffer C) in 22 ml. Fractions of 0-8 ml were collected at a flow rate of 1 ml min⁻¹.

Step 4. Second anionic chromatography on Mono Q HR 5/5 FPLC column. The active fractions from step 3 were combined (2-4 ml), desalted on a Pharmacia PD-10 column and subjected to repeated chromatography on a Mono Q column as for step 3 except that a linear gradient of 5-30% buffer D in 12 ml was used. The adsorbed material was eluted at a flow rate of 1 ml min⁻¹ and 0-5 ml fractions were collected.

Analytical methods. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing were done as described by Vančurová et al. (1988). The M₅ of the enzyme subunits was estimated, with BSA (M₅, 67000), egg albumin (M₅, 45000), chymotrypsinogen A (M₅, 25000) and lysozyme from chicken egg white (M₅, 14000) as standard proteins (Sera).

Size-exclusion high-performance liquid chromatography (HPLC) of the purified VDH was done with 0-1 M-sodium phosphate buffer, pH 7-0, on a TSK G 3000 SW column (7-5 × 300 mm) at a flow rate of 0-5 ml min⁻¹. M₅ standards (Kit MS II, Sera) were used to calibrate the column.

Equilibrium ultracentrifugation was done in a Beckman Spinco model E ultracentrifuge according to Chervenka (1970). Centrifugation was for 17 h at 20°C at a rotor velocity of 9945 r.p.m. The sample was dissolved in a solution of 0-2 M-NaCl in 0-02 M-Bistris/HCl, pH 6-3. The rotor An-H-Ti, interference optics and double sector cell were used. Partial specific volume was assumed to be 0-72 ml g⁻¹.
Valine dehydrogenase of *Streptomyces fradiae*

**Table 1. Purification of VDH**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (μkat)</th>
<th>Specific activity (μkat (mg protein)-1)</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>500.0</td>
<td>270.0</td>
<td>0.54</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>20.8</td>
<td>183.2</td>
<td>8.8</td>
<td>16.3</td>
<td>67.9</td>
</tr>
<tr>
<td>1. Mono Q</td>
<td>0.72</td>
<td>97.73</td>
<td>135.85</td>
<td>251.4</td>
<td>36.2</td>
</tr>
<tr>
<td>2. Mono Q</td>
<td>0.06</td>
<td>47.90</td>
<td>815.10*</td>
<td>1508.4</td>
<td>17.7</td>
</tr>
</tbody>
</table>

*The specific activity of VDH, 815.1 μkat (mg protein)-1, obtained in the oxidative deamination system corresponds to 3994 μkat (mg protein)-1 in the reductive amination system.

**RESULTS**

**Purification of VDH**

A 72 h culture of *S. fradiae* cultivated in a medium containing L-valine as an inducer of VDH synthesis was used for the isolation of VDH. Crude extract exhibited VDH specific activity of 0.54 μkat (mg protein)-1. VDH was purified by means of a combination of methods of hydrophobic interaction on a Phenyl-Sepharose column and FPLC on a Mono Q column. During hydrophobic chromatography on a Phenyl-Sepharose column VDH was eluted together with alanine dehydrogenase (ADH); glutamate dehydrogenase (GDH) remained bound to the column and was eluted only later using a gradient of ethyleneglycol. VDH was separated from ADH only during the first ion-exchange chromatography on a Mono Q column. The overall purification achieved was 1508-fold with a total yield in enzyme activity of 17.7%. A typical purification procedure is summarized in Table 1. The purification was repeated three times.

Purified VDH was homogeneous according to SDS-PAGE, analytical isoelectric focusing, HPLC on a TSK G 3000 SW column and analytical ultracentrifugation. The fraction corresponding to the only protein peak after HPLC exhibited VDH activity.

Purified VDH was stored without losing activity for 24 h at 4 °C in 0.2 M-Tris/HCl buffer, pH 7.4, and for 3 months in the same buffer at -20 °C. It was also stable for 5 months in the form of a 70% (NH4)2SO4 suspension in 0.2 M-Tris/HCl at -20 °C.

**Mₐ subunit structure and isoelectric point**

The Mₐ of the purified VDH was determined to be 215000 and 218000, by size-exclusion HPLC and equilibrium ultracentrifugation (Chervenka 1970), respectively.

After SDS-PAGE the enzyme exhibited a single band corresponding to an Mₐ of about 18000. Thus, the enzyme appears to consist of 12 subunits of similar Mₐ.

Isoelectric focusing of the VDH in a polyacrylamide gel showed the pI value to be 4.7 (Fig. 1).

**Effect of pH and temperature on the enzyme activity**

The enzyme exhibited maximal activity in the pH range 8.7–8.9 for the reductive amination of 2-oxoisovalerate in the presence of 0.3 M-Tris/HCl buffer. The pH optimum for the oxidative deamination of L-valine in 0.3 M-glycine/KCl/KOH buffer was between 10.5 and 10.7. At the optimal pH, the amination rate was 4.9 times higher than the deamination rate.

The optimal temperature for VDH activity, determined under standard conditions, was 65 °C for both reductive amination and oxidative deamination.

**Substrate and coenzyme specificity**

Substrate specificity of VDH in the direction of oxidative deamination is illustrated in Table 2. The highest activity was observed with L-valine as substrate, but high activities were also found with L-norvaline, L-α-aminobutyrate and L-norleucine. As compared to L-valine, the reaction velocities with L-isoleucine and L-leucine were only 29.4 and 24.5%, respectively. The lowest Kₘ values were found with L-leucine (0.667 mM) and L-valine (1.00 mM); the highest values were obtained with L-norleucine (6.68 mM) and L-alanine (28.2 mM). Under standard assay conditions and with L-valine as substrate, the Kₘ for NAD⁺ was 0.029 mM (± 0.001 mM).
Fig. 1. Densitometric evaluation of analytical isoelectric focusing of purified VDH (---). pI marker proteins (Pharmacia) (----) were: amyloglucosidase (pI 3.50), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), horse myoglobin, acidic band (pI 6.85), horse myoglobin, basic band (pI 7.35), lentil lectin, acidic band (pI 8.15), lentil lectin, middle band (pI 8.45), lentil lectin, basic band (pI 8.65), and trypsinogen (pI 9.30).

Table 2. Substrate specificity for oxidative deamination

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>Relative activity†</th>
<th>Km‡ (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Valine</td>
<td>100</td>
<td>1-00 (0-03)</td>
</tr>
<tr>
<td>L-Norvaline</td>
<td>98.0</td>
<td>1.33 (0-03)</td>
</tr>
<tr>
<td>L-a-Aminobutyrate</td>
<td>68.9</td>
<td>3.07 (0.07)</td>
</tr>
<tr>
<td>L-Norleucine</td>
<td>51.5</td>
<td>6.68 (0.16)</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>29.4</td>
<td>1.18 (0.03)</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>24.5</td>
<td>0.67 (0.02)</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>14.7</td>
<td>28.20 (0.75)</td>
</tr>
</tbody>
</table>

*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-histidine and L-tryptophan.
†The activity with valine (100%) corresponds to a specific activity of 815 μkat (mg protein)⁻¹ in the oxidative deamination system. The data represent the mean of three experiments.
‡The K_m values were determined from Lineweaver–Burk plots. (sd values are given in parenthesis). The lines were calculated on the basis of eight points by the method of the least squares. Except for concentrations of amino acids, which varied from 0.28 mm to 20 mm, the conditions were as described in Methods.
Valine dehydrogenase of Streptomyces fradiae

Table 3. Substrate specificity for reductive amination

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity† (%)</th>
<th>$K_m$ (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Oxoisovalerate</td>
<td>100</td>
<td>0.80 (0.02)</td>
</tr>
<tr>
<td>2-Oxovalerate</td>
<td>79.5</td>
<td>1.94 (0.05)</td>
</tr>
<tr>
<td>2-Oxobutyrate</td>
<td>78.0</td>
<td>1.00 (0.03)</td>
</tr>
<tr>
<td>2-Oxocaproate pyruvate</td>
<td>43.8</td>
<td>2.23 (0.06)</td>
</tr>
<tr>
<td>2-Oxo-β-methyl-n-valerate</td>
<td>23.2</td>
<td>3.33 (0.07)</td>
</tr>
<tr>
<td>2-Oxocaproatre</td>
<td>20.0</td>
<td>0.82 (0.02)</td>
</tr>
</tbody>
</table>

* No activity was observed with: 2-oxoglutarate, oxalacetate, phenylpyruvate and glyoxalate.
† The activity with 2-oxoisovalerate (100%) corresponds to a specific activity of 3994 μkat (mg protein)$^{-1}$ in the reductive amination system. The data represent the mean of three experiments.
‡ The $K_m$ values were determined by Lineweaver–Burk plots. (SD values are given in parenthesis). The lines were calculated on the basis of eight points by the method of the least squares.

Table 4. Coenzyme specificity

<table>
<thead>
<tr>
<th>Coenzyme*</th>
<th>Relative activity† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD+</td>
<td>100</td>
</tr>
<tr>
<td>NADP+</td>
<td>10.8</td>
</tr>
<tr>
<td>1,N6-Etheno-NAD+</td>
<td>64.4</td>
</tr>
<tr>
<td>3-Acetylpyridine-NAD+</td>
<td>64.5</td>
</tr>
<tr>
<td>Thionicotinamide-NAD+</td>
<td>0</td>
</tr>
<tr>
<td>Deamino-NAD+</td>
<td>93.9</td>
</tr>
<tr>
<td>α-NAD+</td>
<td>3.6</td>
</tr>
<tr>
<td>3-Pyridinealdehyde-NAD+</td>
<td>0</td>
</tr>
<tr>
<td>NGD+</td>
<td>92.9</td>
</tr>
<tr>
<td>Deamido-NAD+</td>
<td>0</td>
</tr>
</tbody>
</table>

* The assay with coenzyme analogues was conducted by measuring an increase in the absorbance at the following wavelengths: 3-acetylpyridine-NAD+, 363 nm ($e = 9.1 \times 10^4$ M$^{-1}$ cm$^{-1}$); thionicotinamide-NAD+, 395 nm ($e = 1.3 \times 10^3$ M$^{-1}$ cm$^{-1}$); deamino-NAD+, 338 nm ($e = 2 \times 10^4$ M$^{-1}$ cm$^{-1}$); 3-pyridinealdehyde-NAD+, 358 nm ($e = 9.3 \times 10^3$ M$^{-1}$ cm$^{-1}$) (Ohshima et al., 1978). The absorbance increase of the other NAD* analogues was measured at 340 nm. The reaction was done at pH 9.5 in order to avoid degradation of NAD* analogues at more alkaline pH.
† The activity with NAD* (100%) corresponds to a specific activity of 815 μkat (mg protein)$^{-1}$ in the oxidative deamination system. The data represent the mean of three experiments.

The substrate specificity of VDH in the reductive amination direction is illustrated in Table 3. As for oxidative deamination, an unambiguous preference for branched-chain 2-oxo acids could not be demonstrated. The highest reaction velocity was observed with 2-oxoisovalerate, an oxo analogue of L-valine, and high reaction velocities were also measured with 2-oxovalerate (79.5%) and 2-oxobutyrate (78%). However, compared to 2-oxoisovalerate, the reaction velocities were only 20-0 and 23-2% with 2-oxocaproate and 2-oxo-β-methyl-n-valerate, respectively. The lowest $K_m$ values were found with 2-oxoisovalerate (0.80 mm) and 2-oxoisocaproate (0.82 mm) and the highest values were observed with 2-oxo-β-methyl-n-valerate (3.33 mm) and pyruvate (25.00 mm).

Under standard assay conditions with 2-oxoisovalerate, the $K_m$ values for NH$_2$ and NADH were 22.0 mm ($\pm 0.5$ mm) and 0.050 mm ($\pm 0.002$ mm), respectively. NH$_2$ was the only substrate for the reductive amination; no other substance (Tris/HCl, hydroxylamine, ethylamine, ethylenediamine) could replace it. Similarly, NADH could not be replaced with NADPH.

VDH requires NAD* as a natural cofactor for the oxidative deamination. The reaction velocity with NADP* was only 10-8%, although higher reaction velocities were observed with a number of NAD* analogues (Table 4).
The presence of either 2-mercaptoethanol or dithiothreitol at 10 mM concentration was required for maximal enzyme activity; in their absence the activity of the homogeneous enzyme in the reaction mixture was decreased by 36%. However, the enzyme stability was not substantially influenced by the above compounds. The enzyme was strongly inhibited by reagents that act on -SH groups, i.e. p-chloromercuribenzoate and HgCl₂, and their inhibitory effect could be partially abolished in the presence of 2-mercaptoethanol. Metal ions did not significantly affect VDH activity. AMP, ADP, ATP, adenine, adenosine, GMP, GTP, guanosine, cytosine, thymine, FAD, FMN, CoA, acetyl-CoA, thiamin pyrophosphate and EDTA at 1 mM concentrations did not influence VDH activity.

**DISCUSSION**

The structure of VDH from *S. fradiae* differs significantly from that of some LDHs. Whereas LDHs from *B. cereus* (Schütte et al., 1985), *B. stearothermophilus* (Ohshima et al., 1985) and *B. sphaericus* (Ohshima et al., 1978) consist of 8, 6, and 6 subunits, respectively, and have M₅ values of 310000, 300000 and 245000, respectively, VDH from *S. fradiue* consists of 12 subunits and the M₅ of the active enzyme is approximately 218000.

The homogeneous VDH isolated from *S. fradiue* 30/3 was NAD⁺ specific; the reaction velocity with NADP⁺ was only 10-8% of that with NAD⁺. This cofactor specificity is not in agreement with the finding of Ōmura et al. (1983), viz. that VDH in the crude cell-free extract of *S. fradiue* could utilize NAD⁺ and NADP⁺ with the same efficiency. This contradiction might be due to differences between the *S. fradiue* strains and could be explained by assuming that another NADP-dependent dehydrogenase, exhibiting a low substrate specificity, was present in the cell-free extract. Whereas LDH from *B. sphaericus* (Ohshima et al., 1978) cannot use NADP⁺, but can utilize 3-pyridinealdehyde-NAD⁺ and thionicotinamide-NAD⁺ as coenzymes with 19 and 21% efficiency, respectively, VDH from *S. fradiue* could not utilize these analogues.

The differences observed between the Kₐ values for NH₂ of VDH and LDH from *Bacillus* spp. may be physiologically important. Whereas in *B. cereus* (Schütte et al., 1985) the Kₐ value of LDH for NH₂ is 220 mM and in *B. sphaericus* (Ohshima et al., 1978) it is 200 mM for VDH. LDH in the *Bacillus* spp. apparently has a catabolic function, i.e. the release of NH₂. In addition, the formation of NADH and branched-chain 2-oxo acids is particularly important in spore germination (Obiermeier & Poralla, 1976, 1979). The physiological role of dehydrogenases of branched-chain amino acids has not yet been studied in streptomyces. Only the regulation of VDH in connection with tylosin biosynthesis (Ōmura et al., 1983) and branched iso- and anteiso-fatty acid production (Vančura et al., 1987a) has been described in *S. fradiue*. Since transaminase of branched-chain amino acids has been detected in *S. fradiue* (Vančura et al., 1988) and prototrophic strains of *S. fradiue* that do not synthesize VDH have been described (Ōmura et al., 1983), it may be assumed that VDH is not directly involved in the biosynthesis of branched-chain amino acids. The physiological significance of VDH in *S. fradiue* concerns a series of processes related to the biosynthesis and degradation of branched-chain amino and oxo acids. A thorough biochemical characterization of mutants defective in VDH synthesis will be required for a more detailed understanding of the physiological role of this enzyme.

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