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Isolation and Characterization of Valine Dehydrogenase from *Streptomyces aureofaciens*

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Valine dehydrogenase was purified to homogeneity from the crude extracts of *Streptomyces aureofaciens*. The molecular weight of the native enzyme was 116,000 by equilibrium ultracentrifugation and 118,000 by size exclusion high-performance liquid chromatography. The enzyme was composed of four subunits with molecular weights of 29,000. The isoelectric point was 5.1. The enzyme required NAD^+ as a cofactor, which could not be replaced by $NADP^+$. Sulfhydryl reagents inhibited the enzyme activity. The pH optimum was 10.7 for oxidative deamination of L-valine and 9.0 for reductive amination of α-ketoisovalerate. The Michaelis constants were 2.5 mM for L-valine and 0.10 mM for NAD^+. For reductive amination the K_m values were 1.25 mM for α-ketoisovalerate, 0.023 mM for NADH, and 18.2 mM for NH_4Cl.

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 been purified to homogeneity and characterized. Low activity was detected in two *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, 30; soybean meal, 30; NaCl, 5; $CaCO_3$, 4; $(NH_4)_2SO_4$, 2; molasses, 2; corn steep, 5. The pH was 7.0. Soybean meal was used in the form of an extract.

Medium used for ValDH regulation study contained the following (in grams per liter): sucrose, 25; $CaCO_3$, 3; NaCl, 5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 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 A_{340}. 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 NH_4Cl. The assay was performed at pH 9.0 and 30°C.

The oxidative deamination activity of ValDH was measured as an increase of NADH A_{340}. 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.

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TABLE 1. Purification of ValDH from *S. aureofaciens*

Purification step	Total protein (mg)	Total activity (μkat)	Sp act (μkat/mg)[a]	Purification (fold)	Recovery (%)
Crude extract	1,590	200	0.13	1	100
Phenyl-Sepharose	21.6	114	5.3	40.8	57
Mono Q	0.46	46.8	102	785	23

[a] 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-ketoacids, 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 synthesis in *S. aureofaciens* was studied by using a minimal synthetic medium to which individual amino acids were added (Table 2). NH_4^+ in the range of 25 to 100 mM did not affect the specific activity of ValDH. Addition of L-valine (10 mM) and L-isoleucine (10 mM) to the medium with 25 mM NH_4^+ led to an increase in the specific activity of ValDH. Only slight growth was observed in the medium with the above amino acids (L-valine, L-isoleucine, and L-leucine) without NH_4^+ (maximal growth with 25 mM L-valine as the only N source was 1 g [dry cell weight] per liter, which is 25% of the value obtained in the medium with 25 mM NH_4^+). The specific activity of ValDH during growth in the medium with 25 mM NH_4^+ was nearly constant up to a 96-h cultivation (0.22 μkat/mg of protein) and then began to decrease gradually.

Homogeneity and stability of purified ValDH. The purified ValDH was homogeneous by sodium dodecyl sulfate-electrophoresis (Fig. 1), analytical isoelectric focusing (Fig. 2), size exclusion high-performance liquid chromatography, as well as in centrifugal fields generated at 9,945 rpm.

The purified ValDH did not lose activity when stored at 4°C in the form of a suspension in 70% $(NH_4)_2SO_4$ for 3 weeks or at −25°C in 0.2 M Tris hydrochloride buffer (pH 7.4) for 2 months.

Molecular weight, subunit structure, and isoelectric point of ValDH. The molecular weight of native ValDH determined by equilibrium ultracentrifugation was calculated to be 116,000. The molecular weight estimated by size exclusion high-performance liquid chromatography on a TSK G 3000 SW column was 118,000. The denaturated molecular weight of ValDH was measured on sodium dodecyl sulfate-polyacrylamide gels by using molecular weight standards and was found to be approximately 29,000 (Fig. 1). Thus, ValDH appears to be a tetrameric enzyme.

TABLE 2. Specific activities of ValDH from *S. aureofaciens* cultivated on different sources of nitrogen

Nitrogen source	Concn (mM)	Sp act of ValDH (μkat/mg of protein)[a]
NH_4^+[b]	25	0.22
NH_4^+[b]	50	0.25
NH_4^+[b]	100	0.27
L-Glutamate	25	0.04
L-Aspartate	25	0.03
L-Alanine	25	0.08
L-Glutamine	25	0.06
L-Asparagine	25	0.05
L-Valine	25	0.49
L-Isoleucine	25	0.41
L-Valine + 25 mM NH_4^+	10	0.43
L-Leucine + 25 mM NH_4^+	10	0.24
L-Isoleucine + 25 mM NH_4^+	10	0.45

[a] A 72-h culture.
[b] From $(NH_4)_2SO_4$.

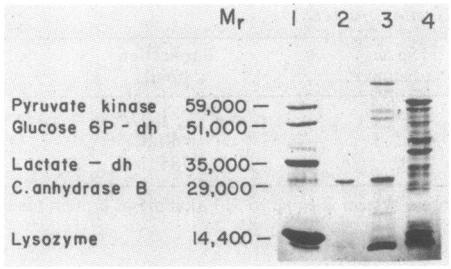

FIG. 1. SDS-polyacrylamide gel electrophoresis of ValDH from *S. aureofaciens*. Gels (10%) were stained for protein with Coomassie blue R-250. Lane 1 shows marker proteins: rabbit muscle pyruvate kinase (M_r 59,000), yeast glucose 6-phosphate dehydrogenase (M_r 51,000), rabbit muscle lactate dehydrogenase (M_r 35,000), carbonic anhydrase B (M_r 29,000), and lysozyme from chicken egg white (M_r 14,400). Lane 2 contains purified ValDH (10 μg); lane 3 contains the phenyl-Sepharose fraction; lane 4 contains crude cell extract.

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-aminobutyrate. 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.

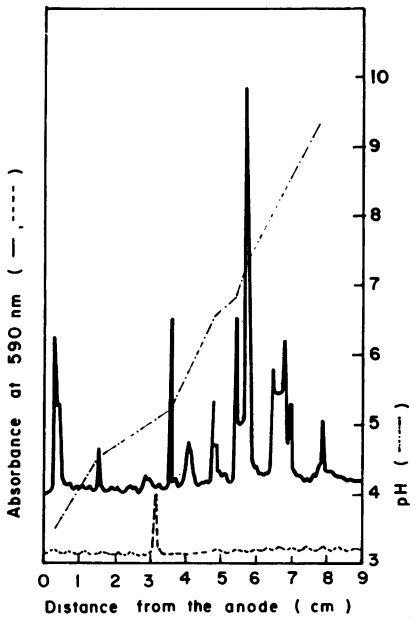

FIG. 2. Densitometric evaluation of analytical isoelectric focusing of purified ValDH (----). pI marker proteins (——) were amyloglucosidase (pI 3.50), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), horse myoglobin (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) from Pharmacia.

TABLE 3. Substrate specificity for oxidative deamination

Substrate (10 mM)[a]	Relative activity (%)[b]	K_m (mM)[c]
L-Valine	100	2.5
L-Isoleucine	46.5	5.0
L-Norvaline	43.0	5.7
L-Leucine	36.0	6.3
L-α-Aminobutyrate	16.5	14.8
L-Norleucine	10.5	15.6
L-Alanine	1.9	333.6

[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.

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, hydroxylamine, ethylamine, 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 $NADP^+$ was only 8.4%; however, high reaction rates were obtained with a

TABLE 4. Substrate specificity for reductive amination

Substrate (10 mM)[a]	Relative activity (%)[b]	K_m (mM)[c]
α-Ketoisovalerate	100	1.3
α-Ketoisocaproate	38.1	0.8
α-Keto-β-methyl-*n*-valerate	22.3	6.7
α-Ketobutyrate	48.2	1.5
Pyruvate	1.3	15.3

[a] No activity was observed with α-ketoglutarate, phenylpyruvate, oxalacetate, and glyoxalate.

[b] The 100% level of relative activity corresponds to a specific activity of 510 μkat/mg of protein in reductive amination system.

[c] The K_m values were determined by using Lineweaver-Burk plots.

TABLE 5. Coenzyme specificity

Coenzyme (2.5 mM)[a]	Relative activity (%)[b]
NAD^+	100
$NADP^+$	8.4
1-N^6-Etheno-NAD^+	58.4
3-Acetylpyridine-NAD^+	100
Thionicotinamide-NAD^+	0
Deamino-NAD^+	83.7
α-NAD^+	0
3-Pyridinealdehyde-NAD^+	37.4
NGD^+	72.3
Deamido-NAD^+	0

[a] The assay with coenzyme analogs was conducted by measuring an increase in the absorbance at the following wavelengths: 3-acetylpyridine-NAD^+, 363 nm (molar absorption coefficient, (ε) = 9.1×10^3); thionicotinamide-NAD^+, 395 nm (ε = 11.3×10^3); deamino-NAD^+, 338 nm (ε = 6.2×10^3); 3-pyridinealdehyde-NAD^+; 358 nm (ε = 9.3×10^3) (8). The increase in A_{340} of the other NAD^+ analogs was measured. The reaction was carried out at pH 9.5 to avoid degradation of NAD^+ analogs at higher pHs.

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

number of NAD^+ analogs (Table 5): 1-N^6-etheno-NAD^+, 3-acetylpyridine-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 $HgCl_2$ [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 α-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 NH_4^+ K_m values of ValDH and LeuDHs from *Bacillus* spp. may be physiologically important. In *B. cereus* (13) the K_m value for NH_4^+ 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 NH_4^+, NADH, and branched-chain α-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.

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