Valine Dehydrogenase from *Streptomyces hygroscopicus* CH-7: Purification and Properties

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(Received March 16, 1999 / Accepted July 19, 1999)

Valine dehydrogenase (VDH) from *Streptomyces hygroscopicus* CH-7 was purified 327-fold with 21% yield, using ion exchange and hydrophobic chromatography. The enzyme had an Mr 31000 in denaturing conditions and an Mr 63000 in gel filtration chromatography, indicating that it is homodimer. The most preferable substrates are L-valine in deamination reaction and 2-ketoisovalerate in amination reaction. The enzyme requires NAD⁺ as a cofactor. The VDH from *S. hygroscopicus* CH-7 shows maximum activity at approximately pH 10.7 and 9.7 for deamination and amination reactions, respectively. The enzyme was significantly inhibited by *p*-chloromercuri-benzoate, Hg²⁺ and other metal ions, which suggests that the presence of SH- groups are necessary for the catalytic reaction. The apparent Michaelis constants for L-valine, NAD⁺, 2-ketoisovalerate, NADH and NH₄⁺ were: 1.26 mM, 0.164 mM, 0.41 mM, 0.026 mM and 50.1 mM.

INTRODUCTION

Valine dehydrogenase (EC 1.4.1.8) belongs to the group of NAD(P)⁺-dependent amino acid dehydrogenases. It seems that valine catabolism is a vital source of important antibiotic precursors in actinomycetes¹⁾. The valine catabolites: *n*-butyrate, 2-methylmalonate and derivatives from them: acetate and propionate are incorporated in macrolide antibiotics tylosin and leucomycin^{2,3)} and into polyether antibiotics monensin⁴⁾ and lasalocid A⁵⁾. On the other hand, it was shown that the inactivation of the VDH gene in *Streptomyces cinnamonensis*, does not significantly affect the production of polyether antibiotic monensin, because valine transaminase activity provides an alternative route for converting valine into 2-ketoisovaleric acid⁶⁾. In attempt to determine the significance of VDH and its involvement in oligoketide biosynthesis, five different VDHs were purified from *Streptomyces*⁷⁻¹¹⁾.

Until now no VDHs have been detected and purified from *S. hygroscopicus*. Using the cell-free extract of *S. hygroscopicus* CH-7, a producer of

polyketide antibiotics: polyene hexaene, polyether nigericin and macrodiolide elaiophycin^{12,13)}, a valine dehydrogenase was isolated, purified and characterised.

MATERIAL AND METHODS

Materials: Chromatography materials: DEAE-Sephacel and Phenyl-Sepharose CL-4B were from Pharmacia; fractogel TSK Butyl-650 and electrophoresis chemicals were purchased from Merck, molecular mass standards supplied by Serva. NAD⁺, NADH, amino and keto acids from Sigma. All other reagents used were purchased from Merck. Chromatography columns and equipment were from Pharmacia and mini gel electrophoresis equipment SE260 from Hoefer.

Microorganism and growth conditions: The microorganism which produced VDH was *S. hygroscopicus* CH-7 from Culture Collection of the Faculty of Chemistry, Belgrade¹⁴⁾. It was cultivated for inoculum preparation during 48 hours on the horizontal shaker model No-B-82 (New Brunswick Co) at 28°C in complex medium¹⁴⁾

which contained (g/L): glucose 15, soybean meal (Soja-vita) 5, yeast extract (Torlak) 5, CaCO_3 2, NaCl 2.5, pH 6.8–7.0. VDH inducibility was determined using minimal medium, MM⁷⁾, and incubated for 40 hours in the same conditions.

Preparation of crude extract: The mycelium was separated from fermentation broth by centrifugation at 4000 g at 4°C (10 min), washed by ice-cold saline solution (0.9% NaCl, w/v) and centrifuged again, in the same conditions. The mycelium was suspended in 0.1 M TRIS-HCl buffer, pH 7.4 and disrupted by a Potter-homogeniser at 4°C. Homogenate was centrifuged for 30 min at 22000 g at 4°C with High Speed 18 (MSE) centrifuge. The supernatant was used as crude extract.

Enzyme assay: Both VDH activities were determined by the method described previously⁷⁾, using 0.1 M TRIS-HCl buffer pH 8.8 for reductive amination activity, and 0.1 M glycine/KCl/KOH buffer, pH 10.6, for oxidative deamination activity, by UV spectrophotometer Gilford G-250. One enzyme activity unit (katal) was defined as the amount of enzyme which catalyses 1 mol of substrate per second.

Purification of VDH: All operations were done at room temperature.

Step 1. DEAE-Sephacel batch separation. DEAE-Sephacel was previously equilibrated with 50mM TRIS-HCl buffer, pH 7.4 containing 5 mM EDTA and 0.1 mM dithiothreitol-(DTT), (buffer A). 50 mL of equilibrated gel was added in 100 mL crude extract, suspension was gently mixed for 30 min. The active substance was collected by resuspending the gel in 100 mL buffer A with 1M KCl and mixing for 30 min, and centrifugation at 4000g for 5 min.

Step 2. First Phenyl-Sepharose CL-4B column chromatography. To the active fraction, obtained in the previous stage, KCl was added to a final concentration of 1.5 M and this was applied to a Phenyl-Sepharose CL-4B column (2 × 16 cm), previously equilibrated with buffer A containing 1.5 M KCl. After the column has been washed with two volume of high salt buffer, the adsorbed material was eluted with linear gradient (1.5–0 M KCl in buffer) at flow rate 3 mL/min, and 5 mL frac-

tions were collected. The active fractions were pooled (75 mL) and desalted by Ultrafree 15 (Millipore) using buffer A.

Step 3. Second Phenyl-Sepharose CL-4B column chromatography. VDH active solution was subjected to repeated chromatography on Phenyl-Sepharose CL-4B as in the previous step of purification. The active fraction was desalted and concentrated by Ultrafree -15.

Step 4. Fractogel TSK Butyl-650 batch separation. The desalted active fraction was supplemented with KCl to 3M concentration and added to 20 mL Fractogel TSK Butyl-650 preequilibrated with buffer A containing 3M KCl. Gel was mixed for 30 min, after that active fraction was desorbed by resuspending gel in buffer A with 2M KCl.

Effect of pH and temperature on VDH activity: The buffers used for determining the pH optima were the same as those used in enzyme assay test. VDH samples were preincubated for 15 min and initial velocity was measured. The optimal temperature for VDH activity was determined under standard conditions after preincubation for 15 min at different temperatures.

Effect of inhibitors on VDH activity: The remaining activity was assayed under the standard conditions after preincubation with inhibitor for 15 min.

Analytical Methods: Electrophoresis. SDS-PAGE was performed using 10% acrylamide gels¹⁶⁾ with protein test mixture (Test Mixture 4): carbonic anhydrase (29000), egg albumin (45000), bovine serum albumin (67000) and phosphorylase (97400).

Gel filtration. Molecular mass was estimated by the gel filtration¹⁷⁾ on Sephadex G-100 column (1.6 × 70 cm), previously standardized with standard protein mixture: myoglobin (17900), peroxidase (40000) and creatine-kinase (81000). The molecular mass was estimated from a plot of log(molecular mass) against elution volume.

Protein determination. Protein concentrations were determined by the Bradford method¹⁸⁾ using crystalline bovine serum albumin (BSA) as a standard.

Kinetic Measurements: Initial velocity was determined (under standard conditions) by varying

the concentration of one substrate at several fixed, saturating concentrations of other substrates. The analysis of kinetic data was done according to the method proposed by Cleland¹⁹⁻²¹. The data from two determinations were fitted using EZ-FIT, the curve fitting program for the analysis of enzyme kinetic data by F. Perella²². Linear double reciprocal plots were fitted to equation:

$$v = \frac{VA}{Km + A} \quad (1)$$

Non-linear concave up intercept replots indicated uncompetitive substrate inhibition²¹, and were fitted to equation:

$$v = \frac{VA}{Km + A + A^2/Ki} \quad (2)$$

where v : measured velocity, V : maximal velocity, Km : Michaelis-Menten constants, Ki : substrate inhibition constant, A : substrate concentration.

The data were obtained with varying concentrations of: L-valine, 0.1–20 mM; NAD^+ , 0.1–7.5 mM; 2-ketoisovalerate, 0.1–10 mM; NADH, 0.01–0.4 mM; and NH_4^+ , 50–400 mM.

RESULTS AND DISCUSSION

Valine dehydrogenase activity was found only intracellularly at all periods of fermentation in the rich complex medium. Maximum VDH activity was observed at the early stationary stage. The level of VDH activity after 40 hours in the complex medium was 0.255 μ kat/mg. When L-valine was added in the complex medium, the activity increased 2-fold. Also, when *S. hygroscopicus* CH-7 was cultivated in MM with the addition of different concentrations of L-val and NH_4^+ , the enzyme activity was induced 2-fold by 20 mM L-

val and 50 mM and 100 mM NH_4^+ , respectively. The inducible nature of VDH produced by *S. hygroscopicus* CH-7 is similar to what has been observed for other VDH enzymes produced by other species of *Streptomyces*⁷⁻¹¹.

VDH was purified using a combination of ion exchange and hydrophobic chromatography. A summary of the purification procedure is shown in

The enzyme was purified 327-fold from crude extract, with yield of 21%. After repeated hydrophobic chromatography on Phenyl Sepharose, the protein solution contained VDH and four minor and one major contaminating proteins, which were successfully removed by fractogel TSK Butyl-650, as it can be seen in Fig.1. Purified VDH was homogeneous according to SDS-PAGE.

Initial attempts to purify the enzyme by hydroxyapatite were not successful possibly because the enzyme was unstable at low ionic strength. Precipitation of VDH by ammonium sulphate inactivated it completely and irreversibly. The enzyme prefers a high ionic strength, and therefore hydrophobic chromatography is the best way of purification.

Purified VDH retained its activity for a year in 1.5 M KCl, at 4°C. The enzyme was stable up to 70°C when heated for 15 min. About 10–15% of the VDH activity remained after heating VDH sample in the boiling water bath for 90 seconds in the presence of 1% DTT (for this reason samples for SDS PAGE were heated for 5 min).

The native molecular weight was 63000 as determined by gel filtration using Sephadex G-100. The subunit molecular weight of 31000 was estimated by SDS-PAGE. Thus, the VDH from *S.*

Table1. Purification of VDH from *S. hygroscopicus*^a

Purification step	Total protein (mg)	Total activity (Mkat)	Specific activity (Mkat/mg)	Yield (%)	Purification (fold)
Crude extract	370.00	18.10	0.05	100	1.0
DEAE-Sepharcel	192.50	13.80	0.07	76	1.5
First Phenyl Sepharose CL-4B	14.70	5.73	0.39	32	8.0
Second Phenyl Sepharose CL-4B	0.50	3.52	7.04	20	144
Fractogel TSK Butyl-650	0.23	3.69	16.02	21	327

^aPurification was repeated three times with similar results

Fig.1. SDS-PAGE of samples during purification: Mr- marker proteins: carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine serum albumin (67 kDa), and phosphorylase (97.4 kDa), 1-crude extract, 2-second Phenyl Sepharose CL-4B, 3-Fractogel TSK-Butyl-650.

hygroscopicus CH-7 appeared to exist as a homodimer. Similar homodimeric structures were observed in VDHs from *S. coelicolor* A3(2), *S. cinamonensis* and VDH-2 from *S. fradiae*⁹⁻¹¹.

The optimal pH for amination reaction in TRIS-HCl buffer was 9.7 and for the deamination reaction in glycine-KCl-KOH buffer was 10.7.

Under standard test conditions, the optimal temperature for oxidative deamination activity of VDH was 50°C, but the amination activity did not significantly change in the range of 22–70°C. Similarly, high optima temperatures for the enzyme activity have been observed for VDHs purified from *S. fradiae*⁷ and *S. aureofaciens*⁸.

Observed inhibition of amination and deamination reactions, by 0.01mM *p*-chloromercuribenzoate and low concentration (0.1–1mM) of Hg²⁺, Cu²⁺, Zn²⁺, as it is shown in Table 2, suggests that the presence of thiol group is important for the activity. VDH is also inhibited by PMSF which reacts with the serine hydroxyl group in the active site of serine protease in general. The inhibition by PMSF suggests possibility of the involvement of a serine residue in catalytic action. No inhibition was observed by EDTA, adenine, AMP and ATP, suggesting that metal ions, free base, mono- and triphosphate are not required for the enzyme action.

The ability of VDH to catalyze oxidative deamination and reductive amination of various oxo and amino acids is presented in Table 3. Clearly, VDH has a relatively low substrate specificity for both reactions.

The mechanism of oxidative deamination and reductive amination was investigated by initial velocity studies and by comparing AIC (Akaike's Information Criterion) for several proposed models, using EZ-FIT software²². Reaction mechanism was determined to be sequential ordered, as it was shown for *S. cinamonensis*⁹ and *S. coelicolor*¹⁰ with substrate inhibition by 2-ketoisovalerate. Substrate inhibition by 2-ketoisovalerate is shown in Fig.2.

Equation (2) was used when substrate inhibition was observed and kinetic constant for 2-ketoisovalerate was calculated to be: Km = 0.41 mM and Ki = 4.79 mM. Km for L-valine was calculated by

Table 2. The effect of inhibitors on VDH activity

Inhibitor*	Concentration (mM)	Relative amination activity(%)	Relative deamination activity(%)
PMSF	0.7	10	10
EDTA	2	48	100
Hg ²⁺	0.01	30	10
Cu ²⁺	1	15	25
Zn ²⁺	1	14	31
p-CMB	0.01	23	21
Adenine	1	100	100
AMP	1	100	100
ATP	1	100	100

* PMSF- phenylmethanesulfonyl fluoride, p-CMB-*p*-chloromercuribenzoate

Table 3. Substrate specificity for oxidative deamination and reductive amination reaction

Substrate	Relative activity (%)
L-Valine	100
D, L-Norleucine	63
L-2-Aminobutyrate	61
L-Leucine	51
D-Valine	45
L-Isoleucine	38
D,L-Norvaline	10
2-Ketoisovalerate	100
2-Ketobutyrate	81
2-Ketovalerate	80
2-Ketoisocaproate	58

Fig. 2. Non-linear concave up intercept replots indicating substrate inhibition of VDH by 2-ketoisovalerate. 2-ketoisovalerate concentrations ranged from 0.1-10 mM. The standard deviation was 0.037.

linear last squares regression of data obtained by secondary plot of the intercepts against the fixed L-valine concentration¹⁹⁾, and it was to be 1.26 mM. The K_m values for NADH, NAD⁺ and NH₄⁺ were determined to be: 0.026 mM, 0.164mM and 50.1mM, respectively.

Similar substrate inhibition pattern was observed in VDH from *S. coelicolor* A3(2),¹⁰⁾ and in alanine-dehydrogenase from *R. capsulatum*²³⁾ and from soybean nodule bacterioids²⁴⁾.

The activity and properties of valine dehydroge-

nase, the first enzyme of valine catabolic pathway, could be important for examining the relationship between valine catabolism and antibiotic production in *S. hygroscopicus* CH-7, as a potential source of commercial antibiotics. The purification procedure yielded a highly purified preparation of VDH which was observed as a single band on a silver stained SDS PAGE. VDH from *S. hygroscopicus* CH-7 is a dimer consisting of two apparently identical subunits, similar to VDH from *S. coelicolor* A3(2) and *S. cinnamomensis* and VDH2 from *S. fradiae*^{17,10,11)}. All five purified VDHs have similar pH optima for reductive amination and oxidative deamination reactions. Observed inhibition by *p*-chloromercuribenzoate and metallic ions suggests that the presence of SH-group is important for the activity. The reaction mechanism was determined to be sequential ordered with substrate inhibition by 2-ketoisovalerate.

ACKNOWLEDGEMENTS

This research work was supported by the Ministry of Science and Technology of the Republic of Serbia (Grant 02M22PT3), Yugoslavia.

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