Purification of an inducible L-valine dehydrogenase of *Streptomyces coelicolor* A3(2)

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Valine dehydrogenase (VDH) from *Streptomyces coelicolor* A3(2) was purified from cell-free extracts to apparent homogeneity. The enzyme had an M_r 41 000 in denaturing conditions and an M_r 70 000 by gel filtration chromatography, indicating that it is composed of two identical subunits. It oxidized L-valine and L- α -aminobutyric acid efficiently, L-isoleucine and L-leucine less efficiently, and did not act on D-valine. It required NAD⁺ as cofactor and could not use NADP⁺. Maximum dehydrogenase activity with valine was at pH 10.5 and the maximum reductive amination activity with 2-oxoisovaleric acid and NH₄Cl was at pH 9. The enzyme exhibited substrate inhibition in the forward direction and a kinetic pattern with NAD⁺ that was consistent with a sequential ordered mechanism with non-competitive inhibition by valine. The following Michaelis constants were calculated from these data: L-valine, 10.0 mM; NAD⁺, 0.17 mM; 2-oxoisovalerate, 0.6 mM; and NADH, 0.093 mM. In minimal medium, VDH activity was repressed in the presence of glucose and NH₄⁺, or glycerol and NH₄⁺ or asparagine, and was induced by D- and L-valine. The time required for full induction was about 24 h and the level of induction was 2- to 23-fold.

Introduction

Branched-chain amino acids are normally catabolized in bacteria by dehydrogenation to a 2-oxoacid, followed by its oxidative decarboxylation to the coenzyme A derivative of a branched-chain fatty acid by a 2-oxoacid dehydrogenase complex (Massey *et al.*, 1976; Matin *et al.*, 1973). The latter compound can be converted to the coenzyme A esters of simpler fatty acids by the pathway shown in Fig. 1, or used as the primer for the synthesis of branched-chain fatty acids (Massey *et al.*, 1976; Matin *et al.*, 1973). Several L-amino acid dehydrogenases have been purified from different bacterial sources (Asano *et al.*, 1987; Porumb *et al.*, 1987; Schutte *et al.*, 1985; Vancura *et al.*, 1989; Vancurova *et al.*, 1988b) and some of them are inducible by their normal substrates (Asano *et al.*, 1987; Omura *et al.*, 1984a). The L-alanine dehydrogenase from *Bacillus cereus* is thought to play a role in the sporulation of this organism (Porumb *et al.*, 1987), whereas the physiological role of the other

bacterial amino acid dehydrogenases is uncertain, except for their use in fatty acid synthesis.

In *Streptomyces* spp., the catabolic products of amino acids can be incorporated into the carbon skeleton of some antibiotics (Dotzlaef *et al.*, 1984; Madduri *et al.*, 1989; Omura & Tanaka, 1984) and esterified to macrolide antibiotics (Omura & Tanaka, 1984), as well as being used for the synthesis of branched-chain fatty acids (Vancura *et al.*, 1987). Valine degradation in these bacteria takes a somewhat different course (Sherman *et al.*, 1986) than in other bacteria (Massey *et al.*, 1976; Matin *et al.*, 1973), particularly the unusual formation of *n*-butyrate from isobutyrate by an intramolecular rearrangement (Fig. 1) (Brendelberger *et al.*, 1988; Reynolds *et al.*, 1988; Sherman *et al.*, 1986). The valine catabolites, butyrate and 2-methylmalonate, plus the acetate and propionate derived from them are incorporated into the aglycone structures of the macrolide antibiotics tylosin (Omura *et al.*, 1984a) and leucomycin (Omura *et al.*, 1983b), and into the polyether antibiotics monensin A (Pospisil *et al.*, 1983; Sood *et al.*, 1988) and lasalocid A (Sherman *et al.*, 1986). Furthermore, in studies of *Streptomyces fradiae* mutants, it has been shown that the production of tylactone (the aglycone of tylosin) is positively correlated with the level of a valine dehydrogenase (Omura *et al.*, 1983a).

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Abbreviation: VDH, valine dehydrogenase.

Fig. 1. The catabolic pathway of L-valine in *Streptomyces*. The fatty acids following 2-ketoisovaleric acid are shown as their coenzyme A esters, although this has not been established experimentally.

These facts indicate a metabolic connection between valine catabolism and the biosynthesis of macrolide or polyether antibiotics in *Streptomyces*. Since the genetics of valine catabolism would best be studied in *Streptomyces coelicolor* A3(2) to capitalize on the vast amount of genetic information available about this organism (Hopwood, 1988; Hutchinson, 1988), we have purified and partially characterized an inducible L-valine dehydrogenase (VDH) from *S. coelicolor*. Many of the properties of this enzyme resemble those of the other bacterial L-amino acid dehydrogenases; however, the fact that it is a dimeric protein composed of M_r 40000 subunits and that, in minimal medium, its induction by L-valine is repressed by glucose + NH_4^+ , set it apart from the recently described VDH of *Streptomyces aureofaciens* (Vancurova *et al.*, 1988a). The induction of VDH in various actinomycetes, as noted herein, and the ensuing production of simple acyl-CoA esters explains why fermentation media optimized for the production of tylosin and monensin contain large amounts of valine.

Methods

Organism and growth conditions. *S. coelicolor* A3(2), obtained from David Hopwood (John Innes Institute, Norwich, UK), was grown for about 48 h in the liquid YEME medium of Hopwood *et al.* (1985), which contained (g l^{-1}): Difco yeast extract, 3; Difco malt extract, 3; Difco Bacto-peptone, 5; glucose, 10; sucrose, 340; and 2.5 M- $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2 ml. The cells were collected under sterile conditions, suspended in the minimal medium (MM) of Hopwood *et al.* (1985) [which normally contains, (g l^{-1}): L-asparagine, 0.5; K_2HPO_4 , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; and glucose, 10] without

glucose or a nitrogen source but containing L-valine (10 g l^{-1}), and incubated for 24 h more under the same conditions. The induced cells were collected by suction filtration and stored at -80°C until used, usually within less than 2 weeks.

Enzyme purification. All procedures were carried out at 4°C except for fast protein liquid chromatography, which was done at room temperature and the fractions collected kept on ice. A cell-free extract was prepared starting with 65 g of frozen cells that were thawed and suspended in 200 ml 50 mM-potassium phosphate pH 7.5, 10% (v/v) glycerol, 1 mM-EDTA, and 5 mM- β -mercaptoethanol (PGEM buffer). The suspension was sonicated in a Branson model 185 sonifier set at 40% scale intensity for six periods of 5 min each with 5 min intervals. The extract was centrifuged in a type 45Ti Beckman rotor at 36000 r.p.m. (100000 g) for 2 h and the supernatant was used as the source of enzyme.

The cell-free extract was passed through a Q-Sepharose (Pharmacia) column ($4 \times 5 \text{ cm}$) previously equilibrated with PGEM buffer. After applying the extract, the column was washed with 100 ml of this buffer and then with 300 ml of buffer containing 0.05 M-KCl, and both fractions were discarded. The enzyme was eluted from the column with 150 ml of PGEM buffer containing 0.1 M-KCl. The fractions were concentrated to 40 ml with a Spectra/Por ultrafilter type C (50000 M_r cutoff) using dilution with buffer twice to reduce the KCl concentration.

The previous fraction was loaded in two portions onto an HR5/20 Mono Q column (Pharmacia) previously equilibrated with the same buffer, the column was washed with the equilibrating buffer, and the following gradient was applied: buffer A was PGEM and buffer B was PGEM containing 2 M-KCl. At a flow rate of 2 ml min^{-1} , the column was washed with 100% buffer A for 5 min, buffer A with 0–9% buffer B over a period of 60 min, 100% of buffer B during 30 min, then re-equilibrated with buffer A for 40 min. The active fractions appearing at 6% buffer B, i.e. about 120 mM-KCl, were pooled and dialysed against 50 mM-Tris/HCl pH 7.5, 10% (v/v) glycerol, 1 mM-EDTA, and 5 mM- β -mercaptoethanol (TGEM) buffer.

The previous fraction was loaded onto the same Mono Q column previously equilibrated with TGEM buffer, the column was washed

Fig. 2. Alkyl-Superose chromatography of *S. coelicolor* A3(2) VDH. The conditions are described in Methods. Buffer A is PEM and buffer B is PEM with 2 M-(NH₄)₂SO₄. The peak with VDH activity is shaded, and the decrease in the base line is due to the decreasing concentration of (NH₄)₂SO₄.

with the equilibrating buffer, and the following gradient was applied (buffer A was TGEM and buffer B was TGEM containing 1 M-KCl) at a flow rate of 2 ml min⁻¹: 100% buffer A for 5 min, buffer A with 9–25% of buffer B over a period of 60 min, then column washing and re-equilibration as above. VDH activity appeared at 18% buffer B, i.e. about 180 mM-KCl.

The active fraction was dialysed against 1.5 M-(NH₄)₂SO₄ in PEM buffer (50 mM-potassium phosphate pH 7.5, 1 mM-EDTA, 5 mM-β-mercaptoethanol) and loaded onto an HR5/5 Alkyl-Superose column (Pharmacia) that was developed with buffers A (PEM) and B [PEM containing 2 M-(NH₄)₂SO₄]. The column was equilibrated with buffer A, the sample was loaded (at 0.5 ml min⁻¹) with 75% of buffer B in buffer A, and the column was washed for 5 min. The column then was washed with 60% buffer B in buffer A for 5 min, 60–30% buffer B in buffer A over 35 min, and buffer A for 25 min. The active 3 ml fraction (see Fig. 2) was dialysed overnight against 1 l of PGEM buffer and stored at –80 °C. Some decrease in the specific activity of the purified enzyme was observed upon long periods of storage (>one month).

Enzyme assay. VDH activity was determined in the direction of oxidative deamination by measuring the appearance of NADH at 340 nm with a Varian 2200 or Cary 14 spectrophotometer, the latter fitted with an Olis 4000C update system (On-line Instrument Systems, Madison, Wisconsin, USA). Two 1 ml reaction mixtures were set up in cuvettes containing 0.5 mM-NAD⁺ in 50 mM-CAPS buffer, pH 9. Valine was added to one of them in concentrations varying from 2 to 80 mM and both cuvettes were preincubated at 30 °C for 2 min. The enzyme reaction was started by adding extract containing 0.5 or 1 μg of purified enzyme and recorded for 1–5 min. The initial velocity was determined from plots using a molar absorption coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ l}^{-1}$ for NADH and full-scale absorbances of 0.05 or 0.1. One unit of activity is defined as the amount of enzyme required to form 1 μmol of NADH in 1 min under the assay conditions.

The data from two determinations per sample were fitted by non-linear least squares regression to equation 1 or 2 using the BASIC

program of Duggleby (1984) on a NorthStar Horizon computer:

$$v = VA/(K + A + A^2/K_i) \quad (1)$$

$$v = VAB/[K_bA(1 + A/K_i) + K_aB + AB(1 + A/K_i) + K_{ia}K_b] \quad (2)$$

where v is the measured velocity, V the maximal velocity, K_a and K_b the Michaelis-Menten constants, K_i the substrate inhibition constant, K_{ia} the dissociation constant of A, and A and B the substrate concentrations. The data obtained with varying concentrations of NAD⁺ from 20 to 300 μM and fixed concentrations of valine (10, 20, 30 and 40 mM) were analysed in the same way.

The activity was determined in the direction of reductive amination by measuring the disappearance of NADH using varying concentrations of 2-oxoisovaleric acid or NADH at fixed, saturating concentrations of the non-variant substrate in a 0.75 M-NH₄Cl/NH₄OH buffer (pH 9) as described by Porumb *et al.* (1987). One unit of activity is defined as the amount of enzyme required to consume 1 μmol of NADH in 1 min under the assay conditions.

Chemicals, reagents and general methods. NAD⁺, NADH, all amino acids and specialized reagents were purchased from Sigma. All other chemicals were of reagent grade. Protein concentration was determined by the method of Bradford (1976). Cell densities were measured with a Bausch and Lomb Spectronic 20 spectrophotometer using fresh growth medium as the blank.

Results

Purification of VDH from *S. coelicolor*

VDH activity in cell extracts was initially found to be rather unstable and could not be preserved at low temperature. A preliminary study with crude extracts from induced cells established that the use of glycerol (10%) and to a lesser extent polyethylene glycol MW 1000 (10%, w/v) preserved VDH activity. The enzyme activity was equally stable in 50 mM-Tris or phosphate buffers at pH 7.5, and was more stable in the presence of 5 mM-β-mercaptoethanol than 0.5 mM-dithiothreitol. Addition of 1 mM-EDTA also helped preserve the activity. Neither valine nor *p*-methylphenylsulphonyl fluoride helped to preserve the activity, and the possible stabilizing effect of NAD⁺ or NADH was not determined.

We developed a rapid rather than a high-yield purification process, aimed at providing sufficient enzyme for partial characterization and amino acid sequence analysis. This was carried out as described in Methods, and the results of a typical purification are shown in Table 1. Our procedure resulted in homogeneously pure VDH with a 143-fold purification and a 4% recovery of the activity in the crude extract. The silver-stained gel also shows (Fig. 3, lane 3 versus 4) the considerable improvement in the enzyme purification resulting from the two different buffers used for the Mono Q chromatography steps. Nearly pure enzyme was obtained with a 43% recovery of initial activity after the

Table 1. Purification of VDH from *S. coelicolor* A3(2)

Fraction	Protein (mg)	Activity (U)	Specific activity [U (mg protein) ⁻¹]	Purification (-fold)	Yield (%)
Crude	550	75	0.14	1	(100)
Q-Sepharose	34	67	1.7	13	89
Mono Q (PO ₄)	13	34	2.6	19	46
Mono Q (Tris)	1.6	25	15	115	43
Alkyl-Superose	0.2	3.3	19	143	4

second Mono Q chromatography (Fig. 3, lane 4), but more than 90% of this activity was lost in the subsequent Alkyl-Superose chromatography step. This was due to the fact that, prior to this last step, the enzyme fraction had to be dialysed to remove glycerol and the (NH₄)₂SO₄ concentration adjusted to 1.5 M to promote the hydrophobic binding of the protein to the Alkyl-Superose column. The glycerol had to be removed because it has the opposite effect to (NH₄)₂SO₄ on protein binding, even though the enzyme was highly unstable in the absence of glycerol. Moreover, (NH₄)₂SO₄ caused a high amount of enzyme inactivation. Hydrophobic interaction chromatography was the only way tested to further purify the enzyme, since we were not successful in several attempts to purify it by affinity chromatography using Reactive-blue 2 Sepharose, -blue 4 Agarose, -brown 10 Agarose, -red 120 Agarose, or -yellow 86 Agarose, NAD-agarose, or valine-Agarose (Sigma). After the Alkyl-Superose step, the VDH activity was associated with a single protein band in SDS-PAGE (Fig. 3, lane 5). The highest specific activity found was 17–23 U (mg protein)⁻¹ when assayed as described in Methods (but at pH 10.5 rather than 9).

Under denaturing conditions (Fig. 3) the *M_r* of the protein was estimated to be 40000–42000. In an 8–25% gradient denaturing Phastgel (Pharmacia), the protein ran with an apparent *M_r* of 38000. The *M_r* of the native protein was determined by gel filtration through Superose 6 HR1/30 (Pharmacia) using the PGEM buffer containing 100 mM-KCl and bovine serum albumin (*M_r* 66200), ovalbumin (*M_r* 45000), carbonic anhydrase (*M_r* 31000) and lysozyme (*M_r* 14400) as standards. Under these conditions the purified protein ran as a single peak slightly ahead of bovine serum albumin; its *M_r* was calculated to be approximately 70000, suggesting that the VDH is composed of two identical subunits.

Substrate range and kinetic mechanism of *S. coelicolor* VDH

This enzyme oxidized L-valine using NAD⁺ as cofactor to produce 2-oxoisovaleric acid and presumably NH₄⁺; no activity was observed using NADP⁺. The pH for

maximum activity was 10.5 (no higher pH was assayed); at pH 10.0 the activity was 15% lower. As shown in Table 2, L-α-aminobutyric acid was a better substrate for the enzyme than L-valine, and four other L-amino acids were poorer substrates in the following order of decreasing reactivity: isoleucine, leucine, alanine and cysteine. D-Valine was not a substrate for the enzyme, and neither were the six other L-amino acids tested.

The enzyme was assayed in the reverse direction using 2-oxoisovaleric acid plus NH₄⁺ and NADH. NADPH could not replace NADH, and the pH for maximum activity was 9, with a 10% lower activity at pH 9.5 and a 40% lower activity at pH 8.5.

The kinetic constants for valine, 2-oxoisovaleric acid and NAD(H) were determined at pH 9 (rather than pH 10.5 where the initial velocities were too imprecise) with 0.5 and 1.0 μg of purified enzyme as described in Methods. At valine concentrations higher than ~20 mM the enzyme exhibited substrate inactivation (Fig. 4a). The 1/*v* versus 1/[NAD⁺] plots, at four different valine concentrations, gave a set of lines intersecting to the left of the *y*-axis (Fig. 4b) whose pattern of first decreasing then increasing slopes and *y*-intercepts with increasing valine concentration is consistent with a sequential ordered mechanism with non-competitive inhibition by valine (Cleland, 1979). Normally, the kinetic constants can be extracted from patterns such as that in Fig. 4(b), but attempts to fit the data to a rate equation for a sequential, ordered, bisubstrate reaction with non-competitive inhibition by one substrate (equation 2, see Methods) (Cleland, 1979) did not yield convergence. Therefore, we fitted the data for valine to a simpler rate equation for substrate inhibition (equation 1, see Methods) (Cleland, 1979). The *K_m* for valine thus calculated was 10.0 ± 1.6 mM, the *K_i* 36.8 ± 5.8 mM, and the *V* 6.92 ± 0.7 U mg⁻¹. Using the data for 20 mM-valine, the corresponding values for NAD⁺ were *K_m* 0.17 ± 0.02 mM and *V* 6.25 ± 0.8 U mg⁻¹. For 2-oxoisovaleric acid, in assays run at pH 9 with an NADH concentration of 2 mM, an NH₄⁺ concentration of 750 mM, and 2-oxoacid concentrations from 0.25 to 5 mM, the *K_m* was calculated to be 0.6 ± 0.07 mM and the *V* 72.4 ± 5.7 U mg⁻¹. Similarly, for NADH, at a 2-

Fig. 3. Silver-stained SDS-PAGE electropherogram of the fractions listed in Table 1. Lanes: 1, crude extract (10 µg); 2, Q-Sepharose (6 µg); 3, Mono Q-phosphate (6 µg); 4, Mono Q-Tris (4 µg); 5, Alkyl-Superose (2 µg); 6, M_r standards: BSA (66200), ovalbumin (42000), carbonic anhydrase (30000), and lysozyme (14400). The acrylamide concentration was 10% and the proteins were stained with the kit from Bio-Rad.

Table 2. Activity of the VDH from *S. coelicolor* A3(2) with different substrates

The enzyme activity was determined at pH 10.5 with purified VDH, the amino acid (20 mM), and NAD⁺ (0.5 mM) as described in Methods. The activities found with D-valine, L-asparagine, L-glutamine, L-phenylalanine, L-serine, L-threonine and L-tyrosine were all lower than the limit of detection of the assay (0.7 mU mg⁻¹).

Substrate	Specific activity [U (mg protein) ⁻¹]	Relative activity (%)
L- α -Aminobutyric acid	19	130
L-Valine	14	100
L-Isoleucine	3.8	28
L-Leucine	1.2	8
L-Alanine	0.70	5
L-Cysteine	0.42	3

oxoisovaleric acid concentration of 10 mM, an NH₄⁺ concentration of 750 mM, and nucleotide concentrations from 50 to 300 µM, the values were K_m 0.093 $\pm$ 6.5 mM and V 68.8 $\pm$ 5.0 U mg⁻¹.

VDH activity in other micro-organisms

VDH activity, measured in cells grown in Luria broth (Hopwood *et al.*, 1985), YEME or the medium normally used for antibiotic production, was found in cell-free extracts of *Bacillus subtilis*, *Pseudomonas fluorescens* and *Saccharomyces cerevisiae*, but not in extracts of *Escherichia coli* (data not shown). Two of the actinomycetes tested, *Streptomyces peucetius* and *Saccharopolyspora erythraea*, exhibited very low VDH activity under the growth conditions tested (Table 3). *S. coelicolor* A3(2), *Streptomyces glaucescens* and *Streptomyces lividans* had about the same activity when grown in the YEME medium, whereas *Streptomyces cinnamonensis* and *S. fradiae* had no detectable activity. On the other hand, the VDH activity in *S. cinnamonensis* and *S. fradiae* was 129-fold and 43-fold greater, respectively, in optimized monensin or tylosin production media, which contain 0.1–0.6% valine.

Induction of VDH in *S. coelicolor*

The specific activity of VDH measured in cell-free extracts of *S. coelicolor* A3(2) grown in the YEME medium varied from 22 mU (mg protein)⁻¹ in the early exponential phase to 36 mU (mg protein)⁻¹ in the early stationary phase, then decreased to 26 mU (mg protein)⁻¹ at 77 h and 13 mU (mg protein)⁻¹ at 96 h (late stationary phase). Thus, in YEME medium, which contains about 260 mg valine l⁻¹ (Difco Laboratories Technical Bulletin), VDH activity was maximal in the early stationary phase of growth.

A time-course analysis of VDH activity in MM + L-valine or MM + glucose and (NH₄)₂SO₄ showed that the enzyme activity appeared within 4 h after transfer of washed mycelium to fresh growth medium. VDH activity almost disappeared by 6 h after transfer to MM with glucose + (NH₄)₂SO₄, whereas it increased approximately linearly up to 24 h in medium containing L-valine as the only carbon (C) and nitrogen (N) source. These results (not shown) suggested that the induction of this enzyme by L-valine is repressed in the presence of a more easily available C (glucose) or N [(NH₄)₂SO₄] source.

The inducibility of VDH was then measured under different conditions of growth to assess how its activity was affected by alternative C and N sources, particularly the ones that have been used in standard *S. coelicolor* A3(2) growth media (Hopwood *et al.*, 1985) or in studies of C and N assimilation in this organism (Fisher, 1988; Hodgson, 1982). Representative data from these experiments are listed in Table 4. In the YEME medium, the specific activity of VDH varied less than twofold in the presence or absence of valine or glucose. The amount of valine in YEME ($\geq$ 2 mM) must be sufficient to induce

Fig. 4. (a) Double reciprocal plot of valine concentration versus *S. coelicolor* A3(2) VDH activity. The straight line was obtained by fitting the data to equation 1 (Methods) using valine concentrations from 2 to 80 mM and 0.5 mM-NAD⁺. (b) Double reciprocal plot of NAD⁺ concentration versus *S. coelicolor* A3(2) VDH activity. The data obtained at 10 (○), 20 (●), 30 (□) and 40 (■) mM valine concentrations were analysed as described in Methods.

Table 3. VDH activity in different actinomycetes

Strain	Specific activity* (mU mg ⁻¹)
<i>S. coelicolor</i> A3(2)	22
(Culture supernatant)	<0.7)
<i>S. glaucescens</i> GLA.O	26
<i>S. lividans</i> 1326	24
<i>S. cinnamonensis</i> WMH585	<0.7 (129†)
<i>S. fradiae</i> GS14	<0.7 (43‡)
<i>S. peucetius</i> AD2-40	<0.7 (<0.7§)
<i>Sac. erythraea</i> WMH22	<0.7

* The enzyme activity was determined at pH 10.5 with crude cell-free supernatant prepared from cells grown in YEME or the medium specified, L-valine (20 mM) and NAD⁺ (0.5 mM) as described in Methods.

† Grown in (g l⁻¹): glucose, 16.7; L-tyrosine, 3.3; DL-valine, 6.6; L-lysine, 1.0; CaCO₃, 1; FeSO₄.7H₂O, 0.17; K₂HPO₄, 0.05; MgSO₄, 0.67; biotin, 0.025; and folic acid, 0.025.

‡ Grown in (g l⁻¹): trypticase soy broth, 20; L-valine, 1; CoCl₂, 1; and soybean oil, 30.

§ Grown in (g l⁻¹): glucose, 60; brewers yeast, 25; NaCl, 2; KH₂PO₄, 1; CaCO₃, 2; MgSO₄, 0.1; FeSO₄.7H₂O, 0.01; and ZnSO₄.7H₂O, 0.01.

|| Grown in (g l⁻¹): glucose, 5; brown sugar, 10; tryptone, 5; yeast extract, 2.5; EDTA, 0.036; betaine, 1.29; and sodium propionate, 0.11.

VDH activity under these growth conditions. In MM with glucose + NH₄⁺, glycerol + NH₄⁺ or glycerol + asparagine as the respective C and N sources, the level of VDH activity was below the limit of detection of the assay. Mycelium grown in MM with glucose + L-histidine showed about the same VDH activity as that grown in YEME. VDH activity was induced 13-fold (relative to the value for MM with glucose + histidine)

Table 4. Induction of VDH activity in *S. coelicolor* A3(2) in different growth media

YEME broth (500 ml in a 2 l flask) was inoculated with a dense *S. coelicolor* A3(2) spore suspension and the culture was incubated at 300 r.p.m. at 30 °C. The entire culture was collected at 48 h, a portion of the cells was removed and assayed for VDH activity, while the remaining cells were washed with MM. A portion of this mycelium was resuspended in MM containing the specified ingredient + L-isoleucine (0.05 g l⁻¹) unless stated otherwise. The concentrations of the different C and N sources were, g l⁻¹: glucose, 10; glycerol, 10; L-valine, 10; D-valine, 10; DL-valine, 10; (NH₄)₂SO₄, 2; L-asparagine, 5; and L-histidine, 5. The culture was then grown at 30 °C and 300 r.p.m. for 24 h. No significant difference was observed in the amount of cells obtained.

Medium	Activity* [mU (mg protein) ⁻¹]	Induction (fold)
YEME	16.3	ND
YEME + valine	25.9	ND
YEME + DL-valine	24.4	ND
YEME - glucose + valine	22.4	ND
YEME - glucose + DL-valine	25.4	ND
MM + glucose + NH ₄ ⁺	<0.7†	—
MM + glycerol + NH ₄ ⁺	<0.7	—
MM + glycerol + asparagine	<0.7	—
MM + glucose + histidine	13.6	1
MM + glucose + valine	25.9	1.9
MM + glycerol + valine	90.5	6.6
MM + valine	182	13
MM + valine - isoleucine	177	13
MM + D-valine	296	23
MM + DL-valine	193	14

ND, Not determined. —, Complete repression of VDH activity.

* Equal amounts of cells as determined by packed cell volume were used for each assay and the enzyme assays were done twice for each sample as described in Methods, except at pH 10.5 instead of pH 9.

† The activity was lower than the limit of detection of the assay (0.7 mU mg⁻¹).

when L-valine was used as the sole C and N source in MM, 23-fold by D-valine and 14-fold by DL-valine. Smaller increases in VDH activity were seen in MM with glucose + valine or glycerol + valine.

Although these data do not show the possible effect(s) of cellular metabolites on enzyme activity, the trends in VDH activity clearly show that this enzyme is strongly induced by D-, L- or DL-valine when they are used as the sole C and N source. When valine is the sole N source, this strong induction of VDH is partially repressed by a more easily available C source (glucose or glycerol). Among the alternative N sources tested, NH_4^+ had the most repressive effect on the induction of VDH.

Discussion

We chose the VDH from *S. coelicolor* A3(2) for study because a subsequent investigation of the genetics of valine catabolism would lead to data comparable with the properties of the other catabolic enzymes and their genes that have been studied in this genetically well-characterized organism (reviewed in Hutchinson, 1988). Since VDH is the first enzyme of the valine catabolic pathway (Fig. 1), the regulation of its level and activity could be the predominant factor in regulating valine catabolism. Knowledge about this will be important later to examine the relationship between valine catabolism and antibiotic production in commercially important *Streptomyces* species.

Pseudomonas species degrade L-valine by transamination as well as by dehydrogenation (Massey *et al.*, 1976; Matin *et al.*, 1973), but we could not detect any transamination activity with L-valine and 2-oxoglutaric acid in cell-free extracts of *S. coelicolor* A3(2). Thus, this organism appears to metabolize L-valine and possibly the other common branched-chain amino acids by an initial dehydrogenation step as has been reported for the majority of bacteria (Asano *et al.*, 1987; Porumb *et al.*, 1987; Schutte *et al.*, 1985; Vancura *et al.*, 1989; Vancurova *et al.*, 1988b).

The *S. coelicolor* A3(2) VDH was expediently purified by exploiting the different behaviour of the protein on anion-exchange chromatography under two different (Tris/HCl or potassium phosphate) buffer conditions, a method we have used previously for an enzyme from *Sac. erythraea* (Vara & Hutchinson, 1988). Our purification scheme did not result in a high recovery of active VDH, however, due to the instability of the enzyme under the conditions used for hydrophobic interaction chromatography, and to the fact that we were not successful in our attempts to purify it by affinity chromatography. Priestly & Robinson (1989) have reported that the VDH of *S.*

cinnamonomensis has a similar instability and insignificant binding to such affinity chromatography media.

Four L-valine dehydrogenases have now been purified from streptomycetes. The *S. coelicolor* A3(2) and *S. cinnamonomensis* enzymes are dimeric and consist of two, apparently identical subunits with similar M_r , while the *S. aureofaciens* enzyme is a tetramer, and the *S. fradiae* enzyme an oligomer composed of smaller-sized subunits. Three of the enzymes have similar K_m values for valine, whereas the *S. coelicolor* A3(2) VDH, which exhibited substrate inhibition *in vitro* (Fig. 4a), has an approximately five-fold higher value. All four enzymes have similar K_m values for 2-oxoisovaleric acid and NAD(H), pH optima in the oxidative and reductive directions [the two pH maxima may reflect the differing state of protonation of the coenzyme and reaction intermediates in the forward and reverse directions, as shown for L-glutamate dehydrogenase from different sources (Smith *et al.*, 1975)], and abilities to oxidize valine, leucine, isoleucine and α -aminobutyrate, although at different relative rates (Priestly & Robinson, 1989; Vancura *et al.*, 1988; Vancurova *et al.*, 1988a).

A detailed kinetic analysis of the *S. cinnamonomensis* enzyme by Priestly & Robinson (1989) showed that the reductive amination of 2-oxoisovalerate proceeds through a sequential ordered ternary-binary mechanism. NADH binds to the enzyme first, followed by 2-oxoisovalerate and then ammonia, and L-valine is released first followed by NAD^+ . An apparent K_i of 158 ± 8 mM was estimated for NH_4^+ in the oxidative deamination reaction (Priestly & Robinson, 1989). Because of this high value, we believe that the VDH activity seen in the presence of valine for the four streptomycetes is not due to a significant alteration of its activity by NH_4^+ . For *S. coelicolor* A3(2), valine may have some effect on the VDH activity *in vivo* since it inhibits the activity *in vitro* at concentrations > 20 mM.

Most of the L-amino acid dehydrogenases of other bacterial genera (Asano *et al.*, 1987; Porumb *et al.*, 1987; Schutte *et al.*, 1985) have larger native M_r values (255000–310000) and are composed of 6–8 subunits each. Their properties are otherwise similar to the VDHs of the *Streptomyces* spp. studied, which indicates that the latter enzymes should also be classified as branched-chain L-amino acid dehydrogenases.

VDH activity in *S. coelicolor* A3(2) was found only intracellularly and at all periods of growth in the rich (YEME) medium. Under these conditions, maximum enzyme activity was reached at the early stationary stage, the time of antibiotic production in this and many other streptomycetes. The level of VDH activity in MM with glucose + histidine was similar to that in the YEME medium (Table 4). It was induced from 2- to 23-fold in MM with valine as the sole C and N, or N source, but

was repressed by glucose + NH_4^+ , glycerol + NH_4^+ or glycerol + asparagine (Table 4). These results show that the enzyme activity is highly inducible by the normal substrate as well as by its non-metabolizable enantiomer, but strongly repressed by more readily utilizable C and N sources. The high inducibility of this enzyme in some other streptomycetes (Table 3) explains why valine is so efficiently incorporated, via its catabolism to butyrate, 2-methylmalonate and propionate, into macrolide and polyether antibiotics (Dotzlaef *et al.*, 1984; Omura *et al.*, 1983*b*; Pospisil *et al.*, 1983; Reynolds *et al.*, 1988; Sherman *et al.*, 1986). It may be that the acyl-CoA esters used for antibiotic biosynthesis can be provided more efficiently by valine catabolism than by the direct esterification of acetate, butyrate and propionate added to the fermentation medium.

The data in Table 4 suggest that the catabolism of valine in *S. coelicolor* A3(2) might be controlled in the same way as histidine and proline utilization in enteric bacteria (Magasanik & Neidhardt, 1987). Since glucose or glycerol in the presence of NH_4^+ repress VDH activity while valine induces it, formation of this enzyme could be controlled by global carbon and nitrogen utilization regulons. On the other hand, valine catabolism differs considerably from histidine catabolism in *S. coelicolor* (Kendrick & Wheelis, 1982) and *S. griseus* (Kroening & Kendrick, 1987, 1989). In these two cases, the induction of histidine ammonia lyase, the first enzyme of the histidine catabolic pathway, is not repressed by glucose or NH_4^+ . The urocanic acid produced by histidase has been postulated as the inducer of the *S. coelicolor* hut system (Kendrick & Wheelis, 1982), whereas in *S. griseus*, histidase activity is regulated post-translationally (Kroening & Kendrick, 1987, 1989).

The knowledge gained in this study has laid the foundation for an investigation of the genetics of valine catabolism in *S. coelicolor* A3(2). Towards this end we have isolated *S. coelicolor* A3(2) Vut^- mutants that are unable to utilize valine as the sole C and N source (unpublished results). VDH activity could not be induced above the basal level in one of them. Further study of these and *S. coelicolor* A3(2) VDH^- mutants should lead to an understanding of the genetic regulation of VDH in this organism.

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