Purification and catalytic properties of L-valine dehydrogenase from *Streptomyces cinnamonensis*

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NAD⁺-dependent L-valine dehydrogenase was purified 180-fold from *Streptomyces cinnamonensis*, and to homogeneity, as judged by gel electrophoresis. The enzyme has an M_r of 88 000, and appears to be composed of subunits of M_r 41 200. The enzyme catalyses the oxidative deamination of L-valine, L-leucine, L-2-aminobutyric acid, L-norvaline and L-isoleucine, as well as the reductive amination of their 2-oxo analogues. The enzyme requires NAD⁺ as the only cofactor, which cannot be replaced by NADP⁺. The enzyme activity is significantly decreased by thiol-reactive reagents, although purine and pyrimidine bases, and nucleotides, do not affect activity. Initial-velocity and product-inhibition studies show that the reductive amination proceeds through a sequential ordered ternary–binary mechanism; NADH binds to the enzyme first, followed by 2-oxoisovalerate and NH₃, and valine is released first, followed by NAD⁺. The Michaelis constants are as follows; L-valine, 1.3 mM; NAD⁺, 0.18 mM; NADH, 74 μ M; 2-oxoisovalerate, 0.81 mM; and NH₃, 55 mM. The *pro-S* hydrogen at C-4' of NADH is transferred to the substrate; the enzyme is B-stereospecific. It is proposed that the enzyme catalyses the first step of valine catabolism in this organism.

INTRODUCTION

Methylmalonyl-CoA is one of the key primary metabolites used by many strains of *Streptomyces* for the biosynthesis of polyketide antibiotics, including the well known and commercially important macrolides (erythromycin, tylosin etc.), polyethers (monensin, salinomycin etc.) and polyene macrolides (amphotericin) (Corcoran, 1981; Robinson, 1988). The source of methylmalonyl-CoA *in vivo* for secondary metabolism is currently of great interest, and one well-established route is from the tricarboxylic-acid-cycle intermediate succinyl-CoA, via the methylmalonyl-CoA mutase reaction (Hunaiti & Kolattukudy, 1984). However, a novel enzyme activity was discovered recently in streptomycetes (Brendelberger *et al.*, 1988) that catalyses the reversible rearrangement of isobutyryl-CoA into butyryl-CoA

(Reynolds *et al.*, 1988), thereby forging a direct link between the pathways of straight-chain- and branched-chain-fatty-acid metabolism in these organisms. This should allow the products of both fatty-acid catabolism and amino-acid catabolism to be channelled into methylmalonyl-CoA via the oxidation of isobutyryl-CoA (Scheme 1). The enzyme responsible for the rearrangement, a putative isobutyryl-CoA mutase, has been partially purified from *S. cinnamonensis*, and shown to be dependent upon coenzyme B₁₂ for activity (Brendelberger *et al.*, 1988). In an effort to elucidate some details of these pathways, at the level of individual enzymes and genes, we have initiated studies on the pathway of valine catabolism in *Streptomyces*. Apart from being an important source of isobutyryl-CoA, this pathway is of interest for the following additional reasons.

One of the important regulatory mechanisms

Scheme 1. Part of the pathway of valine catabolism in *Streptomyces cinnamonensis*

Enzymes: i, valine dehydrogenase; ii, branched-chain 2-oxo-acid dehydrogenase; iii, isobutyryl-CoA:n-butyryl-CoA mutase.

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; PMSF, phenylmethanesulphonyl fluoride; i.e.f., isoelectric focusing; g.p.c., gel-permeation chromatography; DTT, dithiothreitol; YADH, yeast alcohol dehydrogenase; BSA, bovine serum albumin.

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controlling secondary-metabolite formation in streptomycetes appears to involve nitrogen catabolite regulation (see Kleinkauf *et al.* 1986). In the case of tylosin production in cultures of *S. fradiae*, the titres of antibiotic, and of valine dehydrogenase activity in cell extracts, have both been shown to be dramatically lowered during growth in the presence of elevated levels of NH_3 (Omura *et al.*, 1983a, 1984; Omura & Tanaka, 1986; Vu-Trong & Gray, 1987), suggesting that antibiotic production is directly influenced by the flow of essential building blocks from this primary metabolic pathway. Secondly, the pathway of valine catabolism in these organisms must occur by a route that is different to that found in *Pseudomonas* (Massey *et al.*, 1976) and mammals (Wolf & Ackers, 1986). Thus results from stable-isotope labelling experiments require that $[2\text{-}^{13}\text{C}]\text{valine}$ (Omura *et al.*, 1983b) and $[1\text{-}^{13}\text{C}]\text{isobutyryl-CoA}$ (Pospisil *et al.*, 1983) should afford intact (Reynolds & Robinson, 1985) $(S)\text{-}[1\text{-}^{13}\text{C}]\text{methylmalonyl-CoA}$ in streptomycetes (Scheme 1). In these other organisms, not all the details of valine catabolism are known, but labelling studies indicate that C-2 of valine is lost as CO_2 during catabolism, and that the end product, propionate, formally arising by decarboxylation and hydrolysis of methylmalonyl-CoA, is derived from C-3, C-4 and C-4' of the amino acid.

In the present paper we report an efficient procedure for the purification of valine dehydrogenase, ostensibly the first enzyme acting in valine catabolism, from the monensin-producing organism *S. cinnamonensis*, as well as a characterization of its steady-state kinetic mechanism, specificity and stereospecificity. Amino acid dehydrogenases (e.g. for alanine, phenylalanine and leucine) have been isolated from other microorganisms, notably *Bacillus* (Porumb *et al.*, 1987; Asano *et al.*, 1987a,b; Ohashima *et al.*, 1978) and very recently the first reports of the isolation and characterization of valine dehydrogenases from other species of *Streptomyces* have been described (Vancura *et al.*, 1988; Vancurova *et al.*, 1988).

EXPERIMENTAL

Materials

Chromatography supports were from the following sources; DE 52 DEAE-cellulose from Whatman, Sephadex G-150 from Pharmacia, phenyl-Sepharose and Reactive Red-120-agarose from Sigma and hydroxyapatite from Bio-Rad. Fast-protein-liquid-chromatographic TSK-G3000-SWG and TSK-DEAE-5PW columns were from LKB. Molecular-mass standards for SDS/polyacrylamide-gel electrophoresis (PAGE) were supplied by Sigma, for i.e.f. by Pharmacia and for g.p.c. by Bio-Rad. Immobiline dry plates were obtained from Pharmacia. Yeast alcohol dehydrogenase was from Sigma, as was NAD^+ (grade III, used without purification). All other reagents used were purchased from Aldrich, Sigma or BDH.

General methods

U.v. measurements were recorded by using a Pye-Unicam SP 1800 spectrophotometer at 21 °C. N.m.r. spectra were recorded on either Bruker AM360 or JEOL GX-270 spectrometers. H.p.l.c. was carried out at room temperature on an LKB GTi system comprising an h.p.l.c. controller, a Ti pump, a Uvicord SD monitor, a SuperRac fraction collector and a recorder. All solvents

for h.p.l.c. were filtered through 0.22 μm -pore-size membranes and degassed with helium. Cells were disrupted at 4 °C by using a Heat Systems ultrasonic cell disruptor, model W-220F, at setting 7, using 30 s pulses with intermittent 30 s cooling periods, for a total of 20 min. PMSF was dissolved in propan-2-ol and added to buffer solutions immediately before use.

Cell culture

S. cinnamonensis strain A3823.5 was obtained from Eli Lilly and Co., whereas strain A.T.C.C. 15413 was from the American Type Culture Collection; both were stored, as a spore suspension in 20 % (v/v) glycerol, in liquid N_2 . Comparable yields were obtained from both strains. Cell paste was prepared in a two-stage fermentation. The spore suspension was added to a medium containing D-glucose (0.3 g), potato extract (1.2 g), soybean flour (Arkasoy 50, 0.9 g), yeast extract (Difco, 0.15 g) and CaCO_3 (60 mg) in distilled water (60 ml), and grown in a wide-necked 500 ml Erlenmeyer flask, at 32 °C, on an orbital shaker at 300 rev./min, for 20 h. Four such cultures provided the inoculum (5 %, v/v) for the second stage, comprising L-tyrosine (16.5 g), L-valine (33.0 g), L-lysine (5.0 g), CaCO_3 (5.0 g), FeSO_4 (0.85 g), KH_2PO_4 (0.85 g), KCl (0.25 g), MgSO_4 (3.35 g) and CoCl_2 (0.01 g), in distilled water (5 litres), adjusted to pH 6.5. After sterilization at 121 °C for 50 min, D-glucose (83.5 g in 200 ml of water, sterilized at 121 °C) was added, followed immediately before inoculation by biotin (0.125 g) and folic acid (0.125 g) (each filter-sterilized). The fermentation was performed on an LH 2000 series fermenter, in a 12-litre glass vessel, at 31 °C, with stirring (1000 rev./min) and aeration (1 vol. of air/min per vol.). Benzamidine (1 mM) and phenylmethanesulphonyl fluoride (PMSF, 1 mM) were added to the fermentation 5 min before harvesting. The cells (~ 100 g wet wt.) were harvested between 42 and 44 h after inoculation by centrifugation (Beckman J2-21, JA-10 rotor) and washed twice with 0.1 M-phosphate buffer, pH 7.4, containing 10 % glycerol, EDTA (5 mM), benzamidine (1 mM) and PMSF (1 mM). The cells could be stored at -70 °C for several weeks without appreciable loss in activity.

Enzyme purification

All manipulations, except h.p.l.c., were carried out at 4 °C.

Crude extract. The cells (100 g) in sonication buffer [300 ml; 50 mM-potassium phosphate/5 mM-EDTA/1 mM-benzamidine/1 mM-PMSF/0.1 % (v/v) 2-mercaptoethanol/1 mM-dithiothreitol (DTT)/10 % (w/v) poly(ethylene glycol) 8000, pH 7.4], with added charcoal (3.0 g), were sonicated, and the cell debris removed by centrifugation at 48000 g for 40 min.

Protamine sulphate. Protamine sulphate (0.3 g) was dissolved in sonication buffer (25 ml) and added over 15 min to the sonication supernatant. After 1 h the precipitate was removed by centrifugation at 48000 g for 40 min.

DEAE-cellulose DE 52 chromatography. The supernatant was applied at a flow rate of 70 $\text{ml} \cdot \text{h}^{-1}$ to a DEAE-cellulose DE 52 column (2.5 cm $\times$ 15 cm) previously equilibrated with buffer A (50 mM-potassium phosphate/5 mM-EDTA/1 mM-benzamidine and 1 mM-

DTT, pH 7.4). After the column had been washed with 2 bed volumes of the buffer A, the protein was eluted with a linear salt gradient (0–0.4 M-KCl in equilibration buffer) over 16 h (flow rate 40 ml·h⁻¹), and 8.5 ml fractions were collected. The active fractions were combined for the next stage.

Phenyl-Sepharose column. KCl was added to this solution, to a final concentration of 2.0 M, and this was applied to a phenyl-Sepharose column (2.5 cm × 10 cm) previously equilibrated in the buffer A used in the previous step, containing also 1.5 M-KCl. After washing the column with 2 bed volumes of this high-salt buffer, the protein was eluted with a linear salt gradient (1.5–0 M-KCl in buffer A) over 6 h at a flow rate of 40 ml·h⁻¹. The active fractions were combined and concentrated by ultrafiltration to about 2 ml. The protein was then desalted (Bio-Gel P6D-G column, 1 cm × 15 cm) into buffer B (5 mM-potassium phosphate/5 mM-EDTA/1 mM-benzamidine/1 mM-DTT, pH 7.4).

Hydroxyapatite column. The protein was applied to a hydroxyapatite column (1 cm × 7 cm) equilibrated with buffer B. The active protein was eluted with the void fractions and was concentrated to less than 1.5 ml by ultrafiltration (Amicon Ym 30 membrane).

H.p.l.c. columns. The protein solution was filtered through a 0.2 µm-pore-size filter (Sartorius) and applied to a TSK-G3000-SWG column (21.5 mm × 600 mm) previously equilibrated with buffer C (100 mM-sodium phosphate/5 mM-EDTA/1 mM-DTT/1 mM-benzamidine, pH 7.0). The fractions showing a high specific activity were combined and then applied directly to a TSK-DEAE-5PW column (21.5 mm × 150 mm) previously equilibrated with buffer D (100 mM-sodium phosphate/5 mM-EDTA/1 mM-DTT/1 mM-benzamidine, pH 7.4). After washing the column with 2 bed volumes of buffer D, the active protein was eluted with a linear salt gradient (0–0.3 M-NaCl in buffer D) over 50 min at a flow rate of 1 ml·min⁻¹.

Reactive Red 120-agarose. The fractions containing activity were combined and filtered through a Reactive Red 120-agarose column (1 cm × 3 cm) previously equilibrated in buffer E (25 mM-sodium phosphate, pH 7.0). The eluate was diluted with an equal volume of glycerol for storage at -70 °C. In this form the activity is completely stable for over 3 months.

Enzyme assays

Valine dehydrogenase activity in the direction of oxidative deamination was assayed by measuring the initial rates of NAD⁺ reduction, as shown by the increase in absorbance at 340 nm, assuming an absorption coefficient of 6220 M⁻¹·cm⁻¹ for NADH. The assay mixture contained glycine (50 mM), L-valine (10 mM), NAD⁺ (2.56 mM), bovine serum albumin (1 mg/ml) and enzyme solution (20–150 µl), in a final volume of 1 ml of distilled water, pH 10. Enzyme activity in the direction of reductive amination was measured in a similar way, by monitoring the rate of NADH oxidation. The assay mixture contained, in a final volume of 1 ml, NH₃/NH₄Cl (500 mM), pH 9.5, NADH (0.27 mM), 2-oxoisovaleric acid (10 mM), bovine serum albumin (1 mg/ml) and enzyme preparation (20–150 µl). NH₃ solutions were

standardized by pH titration against 0.1 M-HCl. NADH solutions were standardized by their u.v. absorptions at 340 nm. NAD⁺ solutions were standardized by their absorption at 260 nm ($\epsilon = 17600 \text{ M}^{-1} \cdot \text{cm}^{-1}$, or by complete enzymic reduction to NADH, using yeast alcohol dehydrogenase (YADH), hydrazine and ethanol, with determination of the NADH at 340 nm. One unit of activity is defined as the amount of enzyme which catalyses the appearance of 1 µmol of NADH/min in the oxidative deamination reaction. All assays were conducted at 21 °C. Transaminase activity was assayed as follows: an assay cocktail contained 2.5 µmol of L-[1-¹⁴C]-valine (0.18 µCi), 1.0 µmol of 2-oxoglutarate, 50 nmol of pyridoxal phosphate, in sodium phosphate buffer (50 mM) at pH 7.0, in a final volume of 0.7 ml. The reaction was initiated with the addition of protein (~0.7 mg). Samples (100 µl) were withdrawn at 5 min intervals, quenched with concentrated HCl (150 µl) and extracted with diethyl ether (3 × 0.5 ml). The organic extracts were combined and subjected to liquid-scintillation counting. Crude cell extracts, containing typically 0.1 unit of valine dehydrogenase·mg⁻¹, contained approx. 1 munit of valine transaminase·mg⁻¹ (i.e. 1.0 nmol·min⁻¹·mg⁻¹).

Protein assays

Protein concentrations were determined by the dye-binding method of Bradford (1976), using crystalline bovine serum albumin as a standard.

Electrophoresis

PAGE was performed by the discontinuous buffer system of Laemmli (1970). Proteins were stained after electrophoresis, either with Coomassie Brilliant Blue R250, or by the silver-stain method of Goldman *et al.* (1981). Valine dehydrogenase activity could be detected in gels by using a modification of the method of Bellion & Tan (1987). The gel was soaked in a solution containing Nitroblue Tetrazolium chloride (5 mg), phenazine methosulphate (2.5 mg) and NAD⁺ (50 mg) in L-valine buffer (100 mM, pH 10). Blue bands of insoluble formazan precipitate appeared within 15 min, and the reaction was terminated by washing the gel and fixing in 7% acetic acid. No bands were seen when NAD⁺ was omitted from the buffer, nor when valine was replaced by glycine. Only one band was seen in electrophoretograms of crude protein extracts.

Molecular-mass and subunit determination

The native molecular mass was determined by g.p.c. using a TSK-G3000-SW (0.75 cm × 60 cm) column equilibrated and run in buffer (100 mM-sodium phosphate/100 mM-NaCl, pH 7.0) at a flow rate of 0.5 ml·min⁻¹. The column was calibrated by using protein standards supplied by Bio-Rad (molecular masses 670, 158, 44, 17 and 1.35 kDa), with and without added valine dehydrogenase. The molecular mass was 88 ± 2 kDa, estimated from a plot of log(molecular mass) against retention volume. The subunit molecular mass was estimated by SDS/PAGE from enzyme run in 12.5%-acrylamide gels containing 0.1% SDS. The protein standards were supplied by Sigma and were of molecular mass 66, 45, 36, 29, 24 and 14.1 kDa. The molecular mass, determined as an average of seven separate measurements, was 41 200 ± 700 Da.

Kinetic measurements

Initial-velocity and product-inhibition experiments were carried out by varying the concentration of one substrate at several different fixed concentrations of the other substrate(s) or the product. The analysis of kinetic data was carried out according to the methods proposed by Cleland (1971). Michaelis constants were determined in the direction of oxidative deamination by non-linear least-squares regression fitting to the following rate equation:

$$v = V_{AB}[A][B]/(K_{ia}K_b + K_a[B] + K_b[A] + [A][B]) \quad (1)$$

where A and B are substrates, v is the initial velocity, V_{AB} is the V_{max} for oxidative deamination, K_{ia} is inhibition constant for substrate A, and K_a and K_b are Michaelis constants for substrates A and B [the nomenclature used is that of Cleland (1963a,b, 1971)]. In the direction of reductive amination, apparent K_m values were determined by varying the concentration of one substrate at fixed saturating concentrations of the other two; the kinetic data were fitted, as above, to eqn. (2):

$$v = V[S]/(K_{app} + [S]) \quad (2)$$

Stereospecificity of the hydride transfer

The stereospecificity of the enzyme with respect of NADH was determined by a modification of the procedure of Arnold & You (1978). (*R*)-[4-²H₁]NADH was prepared by using YADH. YADH (300 units) was added to NAD⁺ (170 mg, 256 μmol), glycine (150 mg) and hydrazine hydrate (0.2 g) in distilled water (20 ml) at pH 8, and the reaction was initiated by the addition of [²H₆]ethanol (60 μl, 99.8 atom %). After 15 h in the dark at ambient temperature, the solution was applied to a column (2 cm × 25 cm) of DEAE-cellulose DE 52 previously equilibrated with NH₄HCO₃ (0.01 M). After washing with 1 column volume of 0.01 M-NH₄HCO₃, the coenzyme was eluted with 250 mM-NH₄HCO₃. Fractions with a substantial absorption at 340 nm were combined and freeze-dried to afford (*R*)-[4-²H₁]NADH; the 360 MHz ¹H-n.m.r. spectrum of this product in ²H₂O showed a > 98 % incorporation of deuterium only at the 4-*pro-R* position (Oppenheimer, 1987). The reductive amination using this deuterated coenzyme was performed as follows: 2-oxoisovaleric acid (110 mg) and (4'*R*)-[4'-²H₁]NADH (297 μmol; containing also NH₄HCO₃, 1.18 g) were dissolved in phosphate buffer (50 mM, pH 8). The reaction was initiated with 0.22 unit of valine dehydrogenase and incubated in the dark for 24 h at 20 °C. The solution was then applied to a column

of DEAE-cellulose DE52 to remove unchanged NADH. The fractions containing valine and NAD⁺ were collected and evaporated, and exchanged twice in ²H₂O, for analysis by n.m.r. The spectrum of NAD⁺ showed the absence of a chemical-shift signal at 8.8 p.p.m. due to the presence of deuterium at C-4'.

RESULTS

Purification of valine dehydrogenase

A summary of the purification procedure used for the dehydrogenase is shown in Table 1. The enzyme was purified to apparent homogeneity about 180-fold from crude extracts of *S. cinnamomensis*, in 24 % overall yield. Preliminary screening of the fermentation cycle showed that valine dehydrogenase activity in cell-free extracts peaked at around 42 h, corresponding to the end of the exponential phase of cell growth, and declined rapidly beyond 45 h. When L-valine was omitted from the growth medium, valine dehydrogenase activity in crude cell extracts was lower by a factor of 150-fold, although the biomass reached 70 % of the value attained in the complete medium. The activity in partially purified forms was almost completely, and irreversibly, inactivated by (NH₄)₂SO₄ precipitation. The final step of the purification involves a simple filtration through Reactive-Red-agarose to remove a single contaminating protein; the valine dehydrogenase is eluted with the void volume to afford material that, by SDS/PAGE, migrates as a single polypeptide of molecular mass 41.2 kDa (Fig. 1). At the same time, the elution volume from a calibrated TSK-G3000-SW gel-filtration column gives a molecular mass for the native enzyme of 88 kDa, indicating it to be a dimer with subunits of the same mass. Immobiline i.e.f. gave only a single band of protein, indicating a pI of 6.2. The pH optima for valine dehydrogenase activity, in the reductive amination and oxidative deamination, as shown in Fig. 2, were 9.5 and 10.0 respectively; both rates also fall off dramatically above pH 10.5. The enzyme is most stable at pH 7.4, and becomes markedly unstable below pH 7.0. The equilibrium constant, K ($= [NAD^+] \cdot [L\text{-valine}] / [NADH][2\text{-oxoisovalerate}][NH_3]$) was determined to be $2.6 \pm 0.8 \times 10^4$ mmol⁻¹ at 21 °C and pH 10 under standard assay conditions.

Kinetic mechanism

The mechanism of the oxidative deamination and reductive amination reactions was explored through initial-velocity studies and product-inhibition patterns (Cleland, 1963a). A double-reciprocal plot of $1/v$ against

Table 1. Summary of the purification procedure for valine dehydrogenase

Step	Volume (ml)	[Protein] (mg/ml)	Specific activity (units/mg)	Total activity (units)	Recovery (%)	Relative purity
Crude extract	300	3.44	0.11	113	100	1.0
Protamine sulphate	340	3.26	0.097	108	96	0.88
DEAE-cellulose DE 52 chromatography	140	1.69	0.39	93	82	3.57
Phenyl-Sepharose	110	0.38	2.43	102	90	22.1
Hydroxyapatite	18.5	1.34	3.03	75.1	66	27.5
TSK-G3000-SWG	6.0	0.93	7.49	41.8	37	68.1
TSK-DEAE-5PW	3.0	0.6	15.9	28.6	25	144.5
Reactive Red 120-agarose	10.5	0.13	19.6	26.9	24	179

Fig. 1. Analysis of valine dehydrogenase by gel electrophoresis

(a) Dehydrogenase in SDS/PAGE before Reactive Red 120-agarose; (b) valine dehydrogenase in SDS/PAGE after Reactive Red 120-agarose; (c) dehydrogenase in native gel.

$1/[\text{valine}]$ at several fixed concentrations of $[\text{NAD}^+]$ produced a set of converging lines that intersected to the left of the y -axis (see Fig. 3), a pattern consistent with a sequential mechanism proceeding through a ternary substrate-coenzyme-enzyme complex (Cleland, 1963*b*, *c*). In order to determine the kinetic constants, the data were fitted by non-linear least-squares regression (Cleland, 1979) to the equations described by Cleland (1971) for the sequential mechanism. This gave a K_m for L-valine of 1.3 ± 0.2 mM, and of 0.18 ± 0.06 mM for NAD^+ , where $K_i^{\text{NAD}^+} \cdot K_m^{\text{val}} = 1.07 \pm 0.3$ mM.

A kinetic analysis of the reductive amination was performed in a similar manner. The double-reciprocal plots for: (a) velocity versus [2-oxoisovalerate], at several concentrations of NH_3 and saturating $[\text{NADH}]$; (b) velocity versus [2-oxoisovalerate], at high levels of NH_3 and several fixed concentrations of $[\text{NADH}]$; and (c) velocity versus $[\text{NADH}]$ at several concentrations of NH_3 , always at saturating concentrations of 2-oxoisovalerate, are shown in Fig. 4. These patterns rule out a random mechanism and are consistent with a sequential mechanism, where 2-oxoisovalerate binds to the enzyme between NADH and NH_3 . The apparent K_m values for NADH , 2-oxoisovalerate and NH_3 were determined to be $74 \pm 7 \mu\text{M}$, 0.81 ± 0.08 mM, and 55 ± 3 mM respectively. No activity was seen with NADP^+ or NADPH . The order of substrate addition in each direction was next established through product-inhibition studies. The theoretical predictions were derived by algebraic manipulation of the full rate equation for an ordered Ter-Bi mechanism elucidated by Cleland (1963*a*, *b*). These predictions, together with the experimentally determined inhibition patterns, are summarized in Table 2.

Fig. 2. Effect of pH on oxidative deamination (●) and reductive amination (○)

Oxidative deamination was in 50 mM-glycine buffer, NAD^+ (2.56 mM), L-valine (10 mM) and BSA (1 mg/ml); reductive amination was in NH_3 (350 mM), NADH (0.24 mM), 2-oxoisovalerate (10 mM) and BSA (1 mg/ml).

Fig. 3. Kinetic analysis of the oxidative deamination reaction

Initial-velocity patterns are shown, with L-valine as the variable substrate at the following fixed concentrations of NAD^+ : A, 0.254 mM; B, 0.388 mM; C, 0.516 mM; D, 0.904 mM; E, 1.290 mM; F, 2.580 mM.

These data indicate that the reaction follows an ordered sequential Ter-Bi mechanism with substrates binding in the order NAD^+ , and L-valine, and products dissociating in the order NH_3 , 2-oxoisovalerate and finally NADH . Of the known amino acid dehydrogenases, most appear to operate via a sequential ordered Ter-Bi mechanism, with the cofactor binding before the amino acid; this includes phenylalanine dehydrogenase (Asano *et al.*, 1987*a*), leucine dehydrogenase (Ohashima *et al.*, 1978), *meso*- α , ϵ -diaminopimelate dehydrogenase (Misono & Soda, 1980) and alanine dehydrogenase (Ohashima &

Fig. 4. Initial-velocity patterns for reductive amination

(a) Double-reciprocal plot of velocity against 2-oxoisovalerate concentration at several fixed concentrations of NH_3 , with saturating NADH; the NH_3 concentrations used were: A, 35.7 mM; B, 50.0 mM; C, 83.3 mM; D, 250 mM. (b) Double-reciprocal plot of velocity against 2-oxoisovalerate concentration at saturating levels of NH_3 and the following fixed levels of NADH: A, 13.4 mM; B, 26.8 mM; C, 40.2 mM; D, 93.8 mM. (c) Double-reciprocal plot of velocity against NADH concentration with saturating levels of 2-oxoisovalerate at the following fixed levels of NH_3 : A, 50 mM; B, 100 mM; C, 500 mM.

Soda, 1979); with some glutamate dehydrogenases, however, other mechanisms have been noted (LeJohn *et al.*, 1968; Engel & Dalziel, 1970; Shio & Ozaki, 1970; King & Wu, 1971; Stevenson & LeJohn, 1971; Van Laere, 1988). At this stage it would be reasonable to assume that the chemical mechanism of valine dehydrogenase is essentially analogous to that for glutamate dehydrogenase, as described by Rife & Cleland (1980).

Substrate specificity and stereospecificity

The dehydrogenase requires only NAD^+ as a coenzyme for the oxidative deamination; NADP^+ is inert and has no effect on the NAD^+ -dependent process. The specificity of the dehydrogenase with respect to the oxidative deamination of various amino acids was examined, and relevant data are in Table 3. In addition to L-valine, L-leucine and L-isoleucine, L-valine being the preferred substrate, several straight-chain aliphatic L-amino acids, including L-norvaline, L-norleucine and L-2-aminobutyric acid, are also effectively deaminated by the enzyme. Those other amino acids tested that were not found to be effective substrates under the assay conditions are indicated in Table 3.

The substrate specificity of the enzyme for the reductive amination is given in Table 4. All of the oxo analogues of the substrates for the oxidative deamination serve as good substrates for the amination reaction. In addition several other 2-oxo acids showed appreciable rates under the assay conditions. A good correlation was evident between chain length and amination rate for a series of straight-chain 2-oxo acids, the optimum chain length being that of 2-oxovalerate. NH_3 appears to be the sole substrate as an amino donor; Tris/HCl, hydroxylamine, methylamine and hydrazine are unreactive.

When 2-oxoisovalerate was allowed to react in the presence of NH_3 and $[4R\text{-}^2\text{H}_1]\text{NADH}$, the NAD^+ formed was recovered by ion-exchange chromatography and examined by ^1H -n.m.r. spectroscopy (Fig. 5). The presence of deuterium at C-4' was indicated by the absence of a doublet at 8.8 p.p.m. assigned to the C-4' proton of NAD^+ and the loss of coupling to the C-3' resonance at 8.2 p.p.m. These data indicate that the 4'-*pro-S* hydrogen atom of NADH is transferred to the substrate in the reductive-amination reaction, and the deuterium atom is retained in the NAD^+ . In support of this, the L-valine recovered from the same enzymic reaction was shown by n.m.r. to contain protium at C-2. This valine dehydrogenase should therefore be classified as *B*-stereospecific.

Inhibitors

The effects of a number of chemicals upon the rate of reductive amination have been studied. The enzyme was strongly inhibited by the presence of low concentrations (10 μM) of *p*-chloromercuribenzoate, mercuric, mercurous, silver(I) and cupric ions in the assay buffer, suggesting the presence in the protein of a thiol group essential for activity, whereas Zn^{2+} , Mg^{2+} and Ca^{2+} caused partial inhibition at 1 mM. The following chemicals caused no inhibition; EDTA, benzamidine, PMSF (all at 5 mM); citrate, cobalt(II), azide, Li^+ and Mn^{2+} (all at 1 mM). All of the free bases, the monophosphates, the diphosphates, and the triphosphates, of adenine, cytosine, thymine, guanine and uracil were tested at 1 mM concentration in the oxidative-deamination reaction, using substrates at their K_m concentrations; none of these materials showed significant inhibitory effects upon the reaction. Urea at 3.5 M caused a complete and irreversible inactivation of the enzyme.

DISCUSSION

The valine dehydrogenase described here should catalyse the first step in valine catabolism in *S.*

Table 2. Predicted and observed product inhibition patterns for a sequential ordered Ter-Bi mechanism

Predicted product-inhibition patterns were obtained from the steady-state rate equation for the sequential ordered Ter-Bi mechanism proposed by Cleland (1963a). Abbreviations. Com, competitive; Un, uncompetitive; Non, non-competitive; 2-OIV, 2-oxoisovalerate.

(a) Oxidative deamination

Varied substrate*	Inhibitors and inhibition patterns					
	NADH		2-OIV		NH ₃	
	Predicted	Observed	Predicted	Observed	Predicted	Observed
NAD ⁺	Com	Com	Un	Un	Non	Non
Valine	Non	Non	Un	Un	Non	Non

(b) Reductive amination

Varied substrate*	Inhibitors and inhibition patterns			
	NAD ⁺		L-Valine	
	Predicted	Observed	Predicted	Observed
NADH	Com	Com	Un	Un
2-OIV	Un	Un	Un	Non†
NH ₃	Un	Un	Non	Non

* Rates were measured at saturating concentrations of the other substrate(s), with one exception.

† This measurement was made with non-saturating concentrations of NH₃.

Table 3. Substrate specificity for oxidative deamination

The enzymic reactions were allowed to proceed under the standard conditions. For the K_m of valine, see the text. The other K_m values were calculated from data obtained by varying the concentration of substrate at one fixed saturating concentration of NAD⁺ (2.56 mM) at pH 10.0. The following amino acids gave no detectable activity under the conditions of the assay: L-alanine, β -alanine, L-serine, L-threonine, L-cysteine, L-methionine, L-arginine, L-tyrosine, L-tryptophan, L-phenylalanine, L-proline, L-lysine, L-histidine, L-aspartate, D-valine, D-leucine, D-isoleucine and D-alanine; all measurements were made at 10 mM amino acid in 50 mM-glycine, pH 10, containing NAD⁺ (2.56 mM) and bovine serum albumin (1 mg/ml).

Substrate	K_m (mM)	K_{cat} (s ⁻¹)	K_{cat}/K_m (s ⁻¹ ·mmol ⁻¹ ·dm ³)
L-Valine	1.3 ± 0.2	28.3 ± 0.6	21.8 ± 0.3
L-Norvaline	3.2 ± 0.2	18.0 ± 0.6	5.6 ± 0.4
L-2-Amino-butyr- ate	19.6 ± 4.9	104 ± 15	5.3 ± 1.5
L-Leucine	4.0 ± 0.7	11.9 ± 2.8	3.0 ± 0.9
L-Isoleucine	10.8 ± 1.5	5.75 ± 0.5	0.53 ± 0.09
L-Norleucine	30 ± 10	17.9 ± 1.5	0.60 ± 0.21

cinnamomensis; the crude cell extracts contained about 100-fold lower levels of valine transaminase (see the Experimental section), which could provide an alternative means of oxidizing L-valine to 2-oxoisovalerate, and the

Table 4 Substrate specificity in the reductive amination

All measurements were made under the standard conditions at pH 9.5, with 500 mM-NH₃ and 249 μ M-NADH.

Substrate	K_m (mM)	K_{cat} (s ⁻¹)	K_{cat}/K_m (s ⁻¹ ·mmol ⁻¹ ·dm ³)
2-Oxobutyrate	0.13 ± 0.02	99.0 ± 7	740 ± 130
2-Oxovalerate	0.48 ± 0.06	164 ± 12	340 ± 47
2-Oxoisovalerate	0.81 ± 0.08	226 ± 13	279 ± 35
2-Oxohexanoate	0.50 ± 0.065	71.9 ± 4.4	143 ± 21
2-Oxohexanoate	0.81 ± 0.09	107 ± 4.4	131 ± 16
Pyruvate	12.3 ± 1.3	114 ± 6	9.3 ± 1.2

dehydrogenase is induced over 100-fold by L-valine in the defined medium used in the present work. It is of interest that appreciable levels of tyrosine and lysine dehydrogenases were also absent in the extract, although substantial amounts of tyrosine and lysine are present in the culture medium. Amino acid dehydrogenases specific for L-leucine (Ohashima *et al.*, 1978), L-alanine (Porumb *et al.*, 1987; Ohashima & Soda, 1979), L-phenylalanine (Asano *et al.*, 1987a,b) and *meso*-a,e-diaminopimelate (Misono & Soda, 1980) have been purified from species of *Bacillus* and characterized; those for alanine and leucine appear to play an important role in sporulation. The normal metabolic role(s) of valine dehydrogenase in

Fig. 5. Aromatic region of the 360 MHz ^1H -n.m.r. spectra of (a) purified NAD^+ formed after oxidation of (R)-[4- ^2H] NADH by valine dehydrogenase and (b) authentic NAD^+

Both spectra were measured in $^2\text{H}_2\text{O}$ at pH 8.1. Chemical shifts are relative to residual $^1\text{HO}^2\text{H}$ at 4.8 p.p.m.

streptomycetes has not yet been established, although valine catabolism can afford important building blocks (in particular propionate and butyrate) needed for polyketide antibiotic biosynthesis which, with other data, suggests that this catabolic pathway may be important to support secondary metabolism in the cell.

Valine dehydrogenase isolated from *S. cinnamomensis* appears, from SDS/PAGE and from gel filtration on a TSK-G3000-SW column, to have a native molecular mass of 88 kDa and to be a dimer of subunits each of 41.2 kDa. The enzyme binds weakly to Cibacron Blue-agarose only below pH 6.5, a pH at which the activity is unstable. The dehydrogenase shows no allosteric effects, and is not inhibited by any of the common nucleotide mono-, di- or tri-phosphates, nor by the free bases, suggesting that these are not concerned in the control of enzyme action. The subunit structure is of special interest, since very recently valine dehydrogenase has been isolated from three other species of *Streptomyces*; a valine dehydrogenase from *S. aureofaciens* (Vancurova *et al.*, 1988) has a molecular mass of 116 kDa and is reported to exist as a tetramer with subunits each of 29 kDa; that from *S. fradiae* (Vancura *et al.*, 1988) has a native molecular mass of 215 kDa and is composed of 12 subunits each of 18 kDa; and that from *S. coelicolor* (Navarrete *et al.*, 1989) is about 70 kDa and is a dimer with subunits of 40 kDa. The last-named enzyme, from *S. coelicolor*, most closely resembles that from *S. cinna-*

monensis, described here, with a similar molecular mass, subunit structure and sensitivity to high concentrations of $(\text{NH}_4)_2\text{SO}_4$. However, the overall variation in quaternary structure is striking, and is paralleled by remarkably large differences in specific activities (each in the direction of oxidative deamination) reported for the pure proteins; 1.3-, 312- and 2495-fold higher for the enzymes from *S. coelicolor*, *S. aureofaciens* and *S. fradiae* respectively than the value reported here, when assayed under comparable conditions. On the other hand, such dramatic variations are not seen in the Michaelis constants for the substrates and cofactors in the forward and reverse reactions. It will now be of interest to discover how closely related these proteins are in their primary structures. Thus xylose isomerase (see Glasfeld *et al.*, 1988) has been isolated from *S. olivochromogenes* as a dimer and from *S. violaceoniger* as a tetramer, and yet the sequence identity between the two forms is greater than 90%. What significance for the physiology of the organisms these different forms of valine dehydrogenase may have is, at the present time, unclear.

Like the alanine, leucine and phenylalanine dehydrogenases from *Bacillus* (mentioned above), the kinetic mechanism for the *S. cinnamomensis* valine dehydrogenase should involve an ordered sequential Bi-Ter process, with valine binding after NAD^+ , and with products released in the order NH_3 , 2-oxoisovalerate and NADH . The inhibition by NH_3 is of interest since, in crude extracts of *S. fradiae*, decreasing levels of valine dehydrogenase activity have been correlated with increasing concentrations of NH_3 in the fermentation medium during cell growth; at the same time, production titres of the macrolide tylosin decreased as the NH_3 levels increased (Omura *et al.*, 1983a, 1984; Omura & Tanaka, 1986; Vu-Trong & Gray, 1987). On the other hand, alanine dehydrogenase activity did not correlate with the tylosin titres (Omura & Tanaka, 1986), suggesting that the variations in valine dehydrogenase are not a general response to NH_3 . In this work an apparent K_i of 158 ± 8 mM for NH_3 in the oxidative deamination was estimated from the product-inhibition data by non-linear least-squares fitting of the data to the appropriate rate equation for non-competitive inhibition. The control of valine dehydrogenase activity in the cell, when it is mediated through NH_3 concentration during cell growth, is likely to be exercised at the level of gene transcription.

The stereochemical course of the valine dehydrogenase reaction involves transfer of the *pro-S* hydrogen atom at C-4' in NADH (i.e. *B*-specific); it is of interest that the leucine dehydrogenase from *B. sphaericus* is *B*-stereospecific (Ohashima *et al.*, 1978), whereas the alanine dehydrogenase also from *B. sphaericus* is *A*-stereospecific (Ohashima & Soda, 1979). The availability of pure protein should now allow the cloning of the structural and regulatory genes controlling valine dehydrogenase, and assuming the genes for the entire pathway are clustered, also the other DNA involved in valine catabolism in streptomycetes.

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