The tylosin producer, *Streptomyces fradiae*, contains a second valine dehydrogenase

Lieu Thi Nguyen, Kien Trung Nguyen, Jaroslav Spížek and Vladislav Běhal

Author for correspondence: Vladislav Běhal. Tel: +42 2 4752350. Fax: +42 2 4715743.

Institute of Microbiology,
Academy of Sciences of the
Czech Republic, Vídeňská
1083, 142 20 Prague 4,
Czech Republic

A second NAD-dependent valine dehydrogenase (VDH) of *Streptomyces fradiae* was detected and purified to homogeneity by affinity chromatography on Reactive-Blue 2 Sepharose followed by gel filtration and Mono Q fast protein liquid chromatography. The relative molecular masses of the native enzyme and its subunits were determined to be 80 000 and 41 000, respectively, indicating that the enzyme is a homodimer. The enzyme was the only active VDH in *S. fradiae*; its activity was significantly induced by L-valine, but was repressed by ammonia. Among branched- and straight-chain amino acids that serve as enzyme substrates, L-2-aminobutyrate and L-valine are preferred. Significant activities were found with deamino-NAD⁺ and 3-pyridinealdehyde-NAD⁺. The molecular and catalytic properties of the enzyme distinguish it from the enzyme previously purified, and thus indirectly indicate the existence of two VDHs in *S. fradiae*.

Keywords: *Streptomyces fradiae*, valine dehydrogenase, affinity purification

INTRODUCTION

Valine metabolism in *Streptomyces* provides an alternative source of *n*-butyrate, 2-methylmalonate and propionate building units of the aglycones of the macrolide antibiotics leucomycin (Omura *et al.*, 1983b) and tylosin (Omura *et al.*, 1983a; Omura & Tanaka, 1986), and the polyether antibiotics monensin (Pospíšil *et al.*, 1983; Sood *et al.*, 1988) and lasalocid A (Sherman *et al.*, 1986). Valine dehydrogenase (VDH, EC 1.4.1.8), which catalyses the reversible oxidative decarboxylation of branched-chain amino acids yielding the corresponding 2-keto acids, is the first enzyme of the valine catabolic pathway (Massey *et al.*, 1976; Priestley & Robinson, 1989; Navarrete *et al.*, 1990). Thus, VDH may be important for the biosynthesis of these antibiotics. Suppression of VDH activity either by elevating ammonium concentration in the fermentation medium (Omura *et al.*, 1983a; Omura & Tanaka, 1986), or by disrupting the VDH-encoding *vdb* gene (Hutchinson, 1994), lowers tylosin production in *S. fradiae*. Disruption of *vdb* also results in a similar effect on spiramycin production in *S. ambofaciens* (Hutchinson, 1994). However, production is restored by adding lower fatty acids such as *n*-butyrate, propionate and acetate to

the fermentation medium (Omura & Tanaka, 1986; Hutchinson, 1994), or by introducing *vdb* into the VDH[−] mutant (Hutchinson, 1994). Since disruption of the *S. coelicolor vdb* gene does not affect production of actinorhodin, an acetate-derived antibiotic (Tang & Hutchinson, 1993), it has been suggested that valine metabolism is an important source of precursors only for antibiotics that are produced from propionate (Hutchinson, 1994).

VDH is assumed to be the main enzyme responsible for utilization of branched-chain amino acids by *Streptomyces* because *S. fradiae* mutants that lack VDH activity (Omura *et al.*, 1983a), and *S. coelicolor* mutants with a disrupted *vdb* gene (Tang & Hutchinson, 1993), lost the ability to grow with branched-chain amino acids as the sole N source. VDH activity was first detected in the tylosin-producing *S. fradiae* (Omura *et al.*, 1983a), and a VDH enzyme with 12 subunits, each of *M_r* 18 000, was isolated and characterized (Vančura *et al.*, 1988b). The fact that the *S. fradiae* VDH differs significantly from the tetrameric VDH from *S. aureofaciens* (Vančurová *et al.*, 1988), and the dimeric VDHs from *S. cinnamonensis* (Priestley & Robinson, 1989) and *S. coelicolor* (Navarrete *et al.*, 1990), prompted us to examine whether *S. fradiae* possessed additional VDHs. In the present work, we have purified a second VDH of *S. fradiae* by affinity chromatography on Reactive-Blue 2 Sepharose.

Abbreviations: FPLC, fast protein liquid chromatography; LDH, leucine dehydrogenase; VDH, valine dehydrogenase.

METHODS

Materials. Coenzymes, amino acids, 2-keto acids, buffers and Reactive-Blue 2 Sepharose were purchased from Sigma.

Micro-organism and cultivation conditions. The micro-organism used was a sporulating isolate of *S. fradiae* 30/3 (Vančura *et al.*, 1988b). A vegetative inoculum was cultivated for 48 h in a medium containing (% w/v): glucose, 2.0; enzymically hydrolysed casein (IMUNA, Czech Republic), 0.3; NaCl, 0.5; CaCO₃, 0.3; MgSO₄·7H₂O, 0.05; K₂HPO₄, 0.1; yeast extract, 0.1; (NH₄)₂SO₄, 0.17. The culture was used as a 5% (v/v) inoculum for a medium containing the following minerals (% w/v): NaCl, 0.5; CaCO₃, 0.3; MgSO₄·7H₂O, 0.05; K₂HPO₄, 0.1. One millilitre of a solution of trace elements containing FeSO₄·7H₂O, MnCl₂·4H₂O, ZnSO₄·7H₂O, CuSO₄·5H₂O and CoCl₂·6H₂O (each of 0.1%) was added to 1 l of medium. C and N sources were sterilized separately and added to the media before inoculation at the final concentrations indicated in the text. The pH of both media before autoclaving was about 7.0. Cultivation conditions were as previously described (Vančura *et al.*, 1988b).

Purification of VDH2. Except for fast protein liquid chromatography (FPLC), which was performed at 22 °C, all operations were done at 4 °C.

(i) **Preparation of crude extracts.** Mycelia grown for 72 h with 25 mM L-valine and 0.3% (w/v) enzymically hydrolysed casein as the N source were harvested by centrifugation at 4000 g at 4 °C (4 min), washed once with ice-cold 50 mM Tris/HCl buffer, pH 7.4, containing 5 mM 2-mercaptoethanol, 1 mM EDTA and 10% (v/v) glycerol (buffer A), centrifuged at 20000 g at 4 °C (5 min), and finally stored at -20 °C. The thawed mycelium (15 g, wet weight) was suspended in 50 ml 50 mM imidazole/HCl, pH 6.3, containing 5 mM 2-mercaptoethanol, 1 mM EDTA and 10% (v/v) glycerol (buffer B), and disrupted by sonication as described elsewhere (Navarrete *et al.*, 1990). Supernatants obtained after centrifugation at 100000 g at 4 °C (45 min) were used as crude extracts.

(ii) **Reactive-Blue 2 Sepharose chromatography.** The crude extract (70 ml) was recycled overnight at 20 ml h⁻¹ through a Reactive-Blue 2 Sepharose column (1.6 × 10 cm, bed volume 20 ml) pre-equilibrated with buffer B. The column was washed with 200 ml buffer B and the VDH activity was eluted with 150 ml buffer C (buffer B, pH 7.5) at 40 ml h⁻¹. Fractions of 3 ml were collected; the volume of the pooled active fractions (100 ml) was reduced to 5 ml by ultrafiltration using a CF25 Amicon membrane (25000 cut off).

(iii) **Gel filtration.** The concentrated enzyme solution from step 2 (5 ml) was loaded on a Sephacryl-S300 HR column (1.6 × 58 cm, void volume 41 ml) pre-equilibrated with buffer D (buffer A containing 0.2 M NaCl). Proteins were fractionated with the same buffer at 4.2 ml h⁻¹ and 0.5 ml fractions were collected.

(iv) **Mono Q FPLC.** The pooled active fractions from step 3 (6 ml) were transferred to 25 mM Bis-Tris/HCl buffer, pH 6.3, containing 5 mM 2-mercaptoethanol, 1 mM EDTA and 10% (v/v) glycerol (buffer E) by ultrafiltration as described in step (ii). Six 1 ml samples were loaded on a Mono Q HR5/5 column pre-equilibrated with buffer E. After the column had been washed with 5 ml of the same buffer, proteins were eluted with a linear gradient of 0–40% buffer F (buffer E containing 1 M NaCl) at 1 ml min⁻¹ in a total buffer volume of 22 ml. Fractions of 0.5 ml were collected. The active fractions were pooled, dialysed overnight against 1 l buffer A, and stored at 4 °C.

Enzyme assay. VDH oxidative deamination and reductive amination activities at 30 °C were determined at pH 10.4 and

8.8, respectively, with a UV-160A Shimadzu spectrophotometer as previously described by Vančura *et al.* (1988b). Unless otherwise stated, the enzyme activity was measured in the deamination assay system with 10 mM L-valine as the substrate. Enzyme activity was expressed as nmol NADH formed or consumed per second (nkat). The Michaelis constant (K_m) of the purified enzyme was determined under standard assay conditions from double-reciprocal plots of reaction velocity versus substrate concentration. The plots were drawn using the least squares method from the results of nine measurements. For K_m determinations, concentrations of individual substrates were varied over the following ranges: L-valine, 0.2–10 mM; NAD⁺, 0.01–1.5 mM; 2-ketoisovalerate, 0.1–10 mM; NH₄⁺, 10–100 mM; NADH, 0.01–0.1 mM. While the concentration of one substrate was changed, the concentrations of the other substrates were maintained at saturation levels (maximum values in the tested concentration ranges).

Analytical methods. Native-PAGE was performed with 6.5% (w/v) acrylamide gels (Hames, 1981). VDH was detected on native-PAGE gels by the VDH activity staining method (Priestley & Robinson, 1989). SDS-PAGE was performed with 10% acrylamide gels (Laemmli, 1970) with the following standards: β -galactosidase (116000), phosphorylase (97400), BSA (66000), egg albumin (45000), L-lactate dehydrogenase (35000), carbonic anhydrase (29000), chymotrypsinogen (25000) and cytochrome c (12400). Gel filtration was done as described in step (iii) of the enzyme purification protocol; MS II kit proteins (Serva) were used as M_r standards. Protein concentrations were determined by the dye-binding method (Bradford, 1976) with BSA as the standard.

RESULTS

Detection and regulation of VDH2

A preliminary experiment with crude extracts of cells grown with L-valine as the sole N source showed that the deamination activity of the *S. fradiae* VDH with L-2-aminobutyrate was 200% of that with L-valine. In contrast, the corresponding value for the previously purified VDH (hereafter called VDH1) was only 68.9% (Vančura *et al.*, 1988b). In addition, the VDH activity in the crude extracts was eluted from a Sephacryl-S300 HR gel filtration column as a single activity peak with an M_r of 80000, whereas the M_r of VDH1 was 215000. We used VDH activity staining to detect isozymes on native-PAGE gels. Only one VDH was found in crude extracts of *S. fradiae* cells grown with several C and N sources (Fig. 1a). A NAD oxidase that oxidized NAD⁺ in the absence of L-valine was also detected. Since the VDH detected differed significantly in molecular and catalytic properties from VDH1 previously purified from *S. fradiae* (see below), it was called VDH2.

We measured VDH activity in 72 h *S. fradiae* cells, which were exponentially grown in chemically defined medium (Vančura *et al.*, 1989). The results showed that with glucose as the C source, the enzyme activity was induced by 25 mM L-valine (1.64 nkat mg⁻¹) but strongly repressed by 25, 50 or 100 mM ammonia (0.05–0.06 nkat mg⁻¹). However, in the presence of 25 mM valine, the repressive effect of 25 mM ammonium was partially reversed (1.27 nkat mg⁻¹). In cells grown with 100 mM valine as both the C and the N source, and in cells grown with 1% (v/v) glycerol (C source) and

Fig. 1. (a) Native-PAGE of extracted proteins from *S. fradiae*. Crude extract (25 μ l) was loaded in each lane. Cells were grown for 72 h with 2% (w/v) glucose (C source) and the following N sources: 25 mM valine (lanes 1 and 4); 25 mM valine + 25 mM ammonium (lane 2); 25 mM ammonium (lane 6); 50 mM ammonium (lane 7); 100 mM ammonium (lane 8). Cells were also grown with 1% (v/v) glycerol (C source) and 25 mM valine (N source) (lane 3), or with 100 mM valine (as both the C and the N source) (lane 5). The gel was stained for VDH activity with L-valine (lanes 2 to 8) or without L-valine as control (lane 1). Positions of VDH2, NAD oxidase (NADO) and the dye marker are indicated on the left. The basal VDH activity in lane 1 is probably due to the residual L-valine in the crude extract. Similar activity intensities of the NAD-oxidase were observed when the gels were stained with or without L-valine (lane 1, and data not shown). (b) SDS-PAGE of proteins after individual purification steps. Lane 1, crude extract; lane 2, Reactive Blue 2 fraction; lane 3, gel filtration fraction; lane 4, purified VDH2 after Mono Q FPLC; lane 5, standard M_r markers are indicated on the right. The gel was stained with Coomassie Blue R-250.

Table 1. Purification of VDH2 from *S. fradiae*

Purification step*	Total protein (mg)	Total act. (nkat)	Sp. act.† (nkat mg ⁻¹)	Recovery (%)	Purification (-fold)
(i) Crude extract	185.0	174.0	0.94	100	1
(ii) Reactive Blue 2	6.2	149.0	23.9	85.5	25.5
(iii) Gel filtration	1.6	132.0	83.2	75.7	88.5
(iv) Mono Q FPLC	0.2	68.4	326.0	39.3	346.0

* See Methods for details; purification was repeated three times with similar results.

† The specific activity of 326 nkat (mg protein)⁻¹ obtained in the oxidative deamination assay corresponds to 682 nkat (mg protein)⁻¹ obtained in the reductive amination assay.

25 mM valine (N source), the enzyme activities were 0.27 and 0.53 nkat mg⁻¹, respectively. In these media, growth of *S. fradiae*, defined as mg cell protein per ml culture broth, was about four- to fivefold lower than that in glucose-valine medium.

Purification of VDH2

Mycelium grown for 72 h with 25 mM L-valine plus 0.3% (w/v) enzymically hydrolysed casein as the N source was used as a source of VDH2 for purification. Preliminary experiments showed that the enzyme activity and stability in buffer B (pH 6.3) were similar to those in buffer A

(pH 7.4). However, the enzyme lost 90% of its activity when stored in either buffer for 24 h at 4 °C without 2-mercaptoethanol and glycerol. VDH2 bound to Phenyl-Sepharose in the presence of 1.2 M KCl but unlike VDH1, it could not be eluted by decreasing the salt concentration (Vančura *et al.*, 1988b). Affinity chromatography on a Reactive-Blue 2 Sepharose column could be used as the first purification step. The enzyme weakly bound to the affinity matrix at pH 6.3 and was eluted by increasing the pH of the buffer to 7.5, resulting in a 25.5-fold purification (Table 1). Other contaminant proteins were separated from VDH2 by subsequent purification steps, including gel filtration on a Sephacryl-S300 HR column and Mono Q FPLC (Fig. 1b). Overall, the enzyme was purified 346-

Table 2. Substrate specificity for oxidative deamination by VDH2 and VDH1 from *S. fradiae*

Substrate (10 mM)*	Relative activity (%)†	
	VDH2	VDH1‡
Valine	100	100
Norvaline	96.0	98.0
2-Aminobutyrate	158.0	68.9
Leucine	24.0	24.5
Norleucine	34.5	51.5
Isoleucine	22.0	29.4
Alanine	8.3	14.7

* All amino acids were L-isomers; no activity was observed with D-valine, or with glycine, threonine, serine, cysteine, glutamate, aspartate, asparagine, arginine, DL-phenylalanine, histidine or tryptophan.

† VDH2 relative activity of 100 % corresponds to a specific activity of 326 nkat (mg protein)⁻¹ obtained in the oxidative deamination assay. The data are the means of three measurements; standard deviations were all within 5 % of the means.

‡ Data from Vančura *et al.* (1988b).

fold with a yield of 39.3 % (Table 1) to homogeneity as judged by SDS-PAGE (Fig. 1b).

The purified enzyme was stable when stored in buffer A at 4 °C for 48 h, but it lost 90 % of its activity within 24 h when stored at -20 °C in buffer A containing 30 % (v/v) glycerol.

Molecular mass and structure

The M_r of the purified VDH2 was determined to be 80 000 by gel filtration and 41 000 under denaturing conditions by SDS-PAGE (Fig. 1b). These data suggest that the enzyme is composed of two identical subunits.

pH and temperature optima

VDH2 exhibited maximum oxidative deamination of L-valine at pH 10.4 in 0.3 M glycine/KCl/KOH buffer. The pH optimum for the reductive amination of 2-ketoisovalerate in 0.3 M Tris/HCl buffer was pH 8.8. At the pH optima, the amination rate was twice the deamination rate. The optimum temperature for VDH2 activity was 65 °C for both oxidative deamination and reductive amination.

Substrate specificity

VDH2 catalysed oxidative deamination of a number of branched- and straight-chain amino acids (Table 2). L-2-aminobutyrate (relative activity 158 %) was the preferred substrate, followed by L-valine (100 %) and L-norvaline (96 %), while the corresponding values of VDH1 were 68.9, 100, and 98 % (Table 2). Lower activities were

Table 3. Substrate specificity for reductive amination by VDH2 and VDH1 from *S. fradiae*

Substrate (10 mM)	Relative activity (%)*	
	VDH2	VDH1†
2-Ketoisovalerate	100	100
2-Ketobutyrate	100	78.0
2-Ketoisocaproate	20.0	20.0
2-Keto-3-methyl- <i>n</i> -valerate	16.6	23.2
Pyruvate	23.3	31.6

* No activity was observed with 2-ketoglutarate or oxalacetate. VDH2 relative activity of 100 % corresponds to a specific activity of 682 nkat (mg protein)⁻¹ obtained in the reductive amination assay. The data are the means of three measurements. Standard deviations were all within 5 % of the means.

† Data from Vančura *et al.* (1988b).

obtained with L-norleucine, L-isoleucine, L-leucine and L-alanine.

The reductive amination activity with 2-ketobutyrate was similar to that with 2-ketoisovalerate. Lower activities were found with other keto acids (Table 3). No amination activity was observed when NH₄⁺ was replaced with Tris/HCl, hydroxylamine or ethylamine.

The K_m values of the purified VDH2 were as follows: L-valine, 0.43 mM; NAD⁺, 0.04 mM; NH₄⁺, 25.6 mM; 2-ketoisovalerate, 0.31 mM; and NADH, 0.028 mM. Catalytic properties of VDHs from various *Streptomyces* species are shown in Table 4. The K_{cat}/K_m values of the oligomeric VDHs are much higher than those of the dimeric enzymes (Table 4).

Coenzyme specificity

VDH2 required NAD⁺ as a specific natural cofactor for the oxidative deamination of L-valine; no activity was observed with NADP⁺. The enzyme activities with deamino-NAD⁺ and 3-pyridinealdehyde-NAD⁺ were 124 and 47.2 % of those with NAD⁺, respectively, whereas the corresponding values of VDH1 were 63.9 and 0 %, although both enzymes exhibited similar activities with other NAD⁺ analogues (Table 5). No reductive amination activity was found when NADH was replaced with NADPH.

Inhibitors

VDH2 activity was reduced to 0 and 30 % by the -SH group inhibitors, iodoacetamide (1 mM) and *p*-hydroxymercuribenzoate (0.01 mM), respectively, indicating that sulfhydryl groups are necessary. However, the enzyme activity was not significantly affected by 1 mM ATP, ADP, AMP, Mg²⁺, Ca²⁺, Mn²⁺, Zn²⁺ and citric acid cycle intermediates. Cu²⁺ (1 mM) exerted a 35.5 % inhibition. When 20 or 50 mM L-valine was used instead of

Table 4. Catalytic properties of VDHs from various *Streptomyces* species

Species	pH optimum		K_m (mM)					K_{cat}/K_m (s ⁻¹ mmol ⁻¹ l ⁻¹)*	Reference
	Deamination	Amination	Valine	NAD ⁺	NH ₄ ⁺	2-KIV	NADH		
<i>S. coelicolor</i>	10.5	9.0	10.0	0.17	—	0.6	0.093	2.2	Navarrete <i>et al.</i> (1990)
<i>S. cinnamonensis</i>	10.0	9.5	1.3	0.18	55.0	0.8	0.074	21.8	Priestley & Robinson (1989)
<i>S. aureofaciens</i>	10.7	9.0	2.5	0.1	18.2	1.25	0.023	4814	Vančurová <i>et al.</i> (1988)
<i>S. fradiae</i> VDH1	10.6	8.8	1.0	0.029	22.0	0.8	0.05	175 246	Vančura <i>et al.</i> (1988b)
VDH2	10.4	8.8	0.43	0.04	25.6	0.31	0.028	60.6	This paper

* Data were calculated from published specific activities and K_m with L-valine, and the enzyme M_r values determined by gel filtration. 2-KIV, 2-ketoisovalerate; —, not reported.

Table 5. Coenzyme specificity of VDH2 and VDH1 from *S. fradiae*

Coenzyme (2.5 mM)*	Relative activity (%)†	
	VDH2	VDH1‡
NAD ⁺	100	100
NADP ⁺	0	10.8
1,N ⁶ -Etheno-NAD ⁺	70.5	64.4
Thionicotinamide-NAD ⁺	0	0
Deamino-NAD ⁺	124.0	63.9
α-NAD ⁺	5.8	3.6
3-Pyridinealdehyde-NAD ⁺	47.2	0
Deamido-NAD ⁺	0	0

* The assay with coenzyme analogues measured an increase in absorbance at the following wavelengths: thionicotinamide-NAD⁺, 395 nm (ϵ 11.3 × 10³ M⁻¹ cm⁻¹); deamino-NAD⁺, 338 nm (ϵ 6.2 × 10³ M⁻¹ cm⁻¹); 3-pyridinealdehyde-NAD⁺, 358 nm (ϵ 9.3 × 10³ M⁻¹ cm⁻¹) (Ohshima *et al.*, 1978). With other NAD⁺ analogues, the increase of absorbance at 340 nm was measured. The reaction was performed at pH 9.5 to avoid degradation of NAD⁺ analogues at higher values.

† VDH2 relative activity of 100% was 208 nkat (mg protein)⁻¹ obtained in the oxidative deamination assay. The data are the means of three measurements; standard deviations were all within 5% of the means.

‡ Data from Vančura *et al.* (1988b).

10 mM L-valine in the standard reaction mixture, the enzyme activity was reduced by 22 or 40%, respectively.

DISCUSSION

VDH activity in the tylosin-producing *S. fradiae* was previously thought to be associated with the 12 subunit VDH1, purified from strain 30/3 (Vančura *et al.*, 1988b).

Our attempts to detect the enzyme from a sporulating isolate of this strain grown in similar media unexpectedly failed. Inactivation of VDH1 during native-PAGE is unlikely because the enzyme is relatively stable (Vančura *et al.*, 1988b). The absence of VDH1 is probably not caused by a change in the composition of the medium since we could not detect the enzyme in cells grown with several C and N sources. It is also unlikely that mycelium from mixed bald and white strains, defective in aerial mycelium formation and sporulation, respectively, was used in the previous purification of VDH1 (Vančura *et al.*, 1988b). Therefore, it appears that the expression of VDH1 in *S. fradiae* is unstable.

Instead of VDH1, we found the dimeric enzyme VDH2 as the only active VDH in *S. fradiae* (Fig. 1a). The result strongly suggests that VDH2 rather than VDH1 plays a role in metabolism of branched-chain amino acids and thus in tylosin biosynthesis. Ammonium is a strong repressor but L-valine acts as an inducer of VDH2 even in the presence of ammonium ions, suggesting a catabolic function for the enzyme. This is supported by the isolation of prototrophic *S. fradiae* mutants lacking VDH activity (Omura *et al.*, 1983a); also, valine transaminase (transaminase B) activity was detected (Vančura *et al.*, 1988a, 1989). The *S. coelicolor* VDH activity is also strongly induced by valine and repressed by ammonia and glucose (Navarrete *et al.*, 1990). However, the *S. aureofaciens* enzyme is not significantly repressed by ammonia (Vančurová *et al.*, 1988).

Whether the *S. fradiae* VDH activity is regulated by glucose, as is the *S. coelicolor* enzyme (Navarrete *et al.*, 1990), is unclear because VDH activity in *S. fradiae* cells grown in medium containing 100 mM L-valine as both the C and N source, or in glycerol-valine medium was three- to fivefold lower than in cells grown in glucose-valine. Poor growth of *S. fradiae* in these media, probably due to the absence of a readily assimilable C source, may lower the synthesis of VDH2, even though transcription of *vdh* is derepressed (Navarrete *et al.*, 1990).

Affinity chromatography on Reactive-Blue 2 Sepharose is the essential step in the 3-step purification of the dimeric VDH2 from *S. fradiae*. Previous attempts to purify dimeric VDHs from *S. cinnamomensis* (Priestley & Robinson, 1989) and *S. coelicolor* (Navarrete *et al.*, 1990) by affinity chromatography were unsuccessful, probably due to the instability of the enzymes at low pH values. The *S. fradiae* enzyme was stable and bound efficiently to Reactive-Blue 2 Sepharose at pH 6.3 and it was readily eluted by increasing the pH of the buffer to 7.5. The simple and effective purification procedure described here makes it possible to rapidly obtain large quantities of pure VDH, which could be used for enzyme crystallization and three-dimensional structure analysis.

VDH2, with two subunits each of M_r 41 000, is similar to the enzymes from *S. cinnamomensis* (Priestley & Robinson, 1989), *S. coelicolor* (Navarrete *et al.*, 1990) and *Alcaligenes faecalis* (Ohshima & Soda, 1993). However, it is significantly different from the *S. fradiae* VDH1, composed of twelve subunits, each of M_r 18 000 (Vančura *et al.*, 1988b), and from the *S. aureofaciens* enzyme, composed of four subunits, each of M_r 29 000 (Vančurová *et al.*, 1988). Navarrete *et al.* (1990) reported that the *S. fradiae* crude extract contained a protein that cross-reacted with polyvalent antibodies raised against *S. coelicolor* VDH. This protein was probably the VDH2 described here.

In the absence of a reducing substance such as 2-mercaptoethanol, the oligomeric VDHs are relatively stable (Vančura *et al.*, 1988b; Vančurová *et al.*, 1988), whereas dimeric VDHs are inactivated (Priestley & Robinson, 1989; Navarrete *et al.*, 1990; this paper). Therefore, it is likely that the oligomeric structures provide better protection of the -SH groups from oxidation.

VDH2 differs significantly from the *Bacillus* leucine dehydrogenases (LDHs, EC 1.4.1.9) composed of six to eight subunits, each of M_r ranging from 39 000 to 49 000 (Ohshima *et al.*, 1978; Kärst *et al.*, 1989). Although the amino acid sequence of the VDH polypeptide of M_r 41 000 shows a 55–60% identity with that of LDHs from *Bacillus* (Tang & Hutchinson, 1993), L-2-aminobutyrate and L-valine are the preferred substrates for the dimeric VDHs, whereas L-leucine is the preferred substrate for LDHs. Moreover, the dimeric VDHs differ from LDHs in coenzyme specificity (Ohshima *et al.*, 1978; this paper), amino acid composition and immunochemical properties (Ohshima & Soda, 1993).

Despite the differences in structure between VDH2 and the other dehydrogenases of *Streptomyces*, there is no marked difference in pH and temperature optima for enzyme activity, and K_m values for the main substrates. However, there is a fivefold higher K_m for L-valine in *S. coelicolor* and a twofold higher K_m for ammonia in *S. cinnamomensis* (Table 4). VDHs with more subunits have markedly increased catalytic efficiencies (K_{cat}/K_m values) (Table 4). Like the other dimeric VDHs, the *S. fradiae* VDH2 exhibited high activity with L-2-aminobutyrate (158%). This property clearly distinguishes the dimeric

enzymes from the oligomeric enzymes (Vančura *et al.*, 1988b; Vančurová *et al.*, 1988). Among several metabolites tested, only L-valine at concentrations higher than 20 mM partially inhibited VDH2 activity; hence, it may be involved in regulating the enzyme activity *in vivo*, as suggested for *S. coelicolor* VDH (Navarrete *et al.*, 1990). The increased activity of VDH2 with deamino-NAD⁺ suggests that the amino group in the adenine moiety of NAD⁺ is not essential, and can partially interfere with binding of NAD⁺ to the enzyme.

The results of the enzyme characterization show that VDH2 is different from VDH1 in enzyme stability, subunit size, native enzyme structure, and substrate and coenzyme specificities. The isolation of dimeric VDHs from *S. fradiae* as well as *S. cinnamomensis* and *S. coelicolor* indicate that these enzymes are widely distributed in *Streptomyces*. However, factors controlling expression of the dodecameric VDH1 remain to be defined. To address this question, we are currently looking for *S. fradiae* mutants exhibiting detectable VDH1 activities.

After this manuscript had been submitted, we learned that Tang *et al.* (1994) had cloned and sequenced the *vdh* gene encoding VDH2 of *S. fradiae*. The deduced products of this gene and the partial *vdh* gene of *S. ambofaciens* are very similar in their amino acid sequences to the deduced product of the *S. coelicolor* *vdh* gene. Disruption of *vdh* inhibits the biosynthesis of tylosin in *S. fradiae* and spiramycin in *S. ambofaciens*. The data suggest that *S. fradiae* and *S. ambofaciens* have only one *vdh* gene responsible for VDH activity, consistent with our evidence that VDH2 is the only active VDH in *S. fradiae*.

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