Valine dehydrogenase from *Streptomyces albus*: gene cloning, heterologous expression and identification of active site by site-directed mutagenesis

Chang-Gu Hyun, Sang Suk Kim, Kwan-Hyung Park, Joo-Won Suh *

Department of Biological Science, Institute of Bioscience and Biotechnology, Myong Ji University, Yongin 449-728, South Korea

Received 30 September 1999; received in revised form 25 October 1999; accepted 25 October 1999

Abstract

A gene encoding valine dehydrogenase (Vdh) has been cloned from *Streptomyces albus*, a salinomycin producer, and expressed in *Escherichia coli*. The *S. albus* Vdh is composed of 364 amino acids that showed high homology with several other amino acid dehydrogenases as well as Vdh from *Streptomyces* spp. and leucine and phenylalanine dehydrogenases (Ldh and Pdh) from *Bacillus* spp. A protein of 38 kDa, corresponding to the approximate mass of the predicted *S. albus* Vdh product (38.4 kDa) exhibiting specific Vdh activity, was observed when the *S. albus vdh* gene was overexpressed in *E. coli* under the controlled T7 promoter and was subsequently purified to homogeneity. Among branched- and straight-chain amino acids, L-valine and L-α-aminobutyrate were the preferred substrates for the enzyme. Lys-79 and Lys-91 of *S. albus* Vdh were highly conserved in the corresponding region of NAD(P)⁺-dependent amino acid dehydrogenase sequences. To elucidate the functional roles of the lysyl residues, the Lys residues have individually been replaced with Ala by site-directed mutagenesis. Kinetic analyses of the Lys-79 and Lys-91-mutated enzymes revealed that they are involved in the substrate binding site and catalysis, respectively, analogous to the corresponding residues in the homologous Ldh and Pdh. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Active site; Cloning; Site-directed mutagenesis; Valine dehydrogenase; *Streptomyces albus*

1. Introduction

*Streptomyces albus* has been used for the production of the commercially important polyether antibiotic salinomycin [1]. The biosynthesis of salinomycin and other polyethers requires the cells to produce and synthesize substantial amounts of methylmalonyl-CoA for the assembly of polyketide carbon backbones. Isotope labelling experiments with both polyether and macrolide producing streptomycetes have shown that valine may be efficiently catabolized to provide isobutyryl-CoA and subsequently methylmalonyl-CoA for the assembly of polyketide carbon backbones. Isotope labelling experiments with both polyether and macrolide producing streptomycetes have shown that valine may be efficiently catabolized to provide isobutyryl-CoA and subsequently methylmalonyl-CoA for the assembly of polyketide carbon backbones. Isotope labelling experiments with both polyether and macrolide producing streptomycetes have shown that valine may be efficiently catabolized to provide isobutyryl-CoA and subsequently methylmalonyl-CoA for the assembly of polyketide carbon backbones. Isotope labelling experiments with both polyether and macrolide producing streptomycetes have shown that valine may be efficiently catabolized to provide isobutyryl-CoA and subsequently methylmalonyl-CoA for the assembly of polyketide carbon backbones. Isotope labelling experiments with both polyether and macrolide producing streptomycetes have shown that valine may be efficiently catabolized to provide isobutyryl-CoA and subsequently methylmalonyl-CoA for the assembly of polyketide carbon backbones.

The observation that media supplemented with valine and isoleucine can stimulate macrolide production, but with the negative effect of increasing ammonium ion concentrations on both macrolide production and valine dehydrogenase (Vdh) activity, suggests that branched-chain amino acid catabolism may be an important source of building blocks for macrolide biosynthesis.

Vdh (EC 1.4.1.8) belongs to the group of NAD(P)⁺-dependent dehydrogenases of branched-chain amino acids catalyzing reversible oxidative deamination of branched-and occasionally unbranched-amino acids to the corresponding oxo acid. Vdh is assumed to be the main enzyme responsible for utilization of branched-chain amino acids by *Streptomyces*, because *Streptomyces fradiae* mutants that lack Vdh activity [2] and *Streptomyces coelicolor* mutants with a disrupted *vdh* gene lost the ability to grow with branched-chain amino acids as the sole nitrogen source [4]. The *vdh* gene has been cloned recently from the actinorhodin producer *S. coelicolor* [4], the monensin producer *Streptomyces cinnamomensis* [5], the spiramycin producer *Streptomyces aureofaciens* and the tylosin producer *S. fradiae* [6]. These genes share a high sequence homology with each other and also with other known amino acid dehydrogenases, especially leucine dehydrogenase (Ldh) and phenylalanine dehydrogenase (Pdh) from *Bacillus* species [4,7]. The other dehydrogenases, such as Ldh, glutamate dehydrogenase and Pdh, all share

* Corresponding author. Tel.: +82 (335) 330-6190; Fax: +82 (335) 335-8249; E-mail: jwsuh@wh.myongji.ac.kr

0378-1097/00/$20.00 © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

PII: S0378-1097(99)00561-3
2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

S. albus ATCC21838 was used as the source of chromosomal DNA for a gene sublibrary. S. albus was grown at 28°C on R2YE plates [12]. Escherichia coli DH5α F' was used as host strain for subcloning and sequencing work and E. coli BL21(DE3) was used for the expression of wild-type and mutant vdh genes. These strains were grown on Luria-Bertani (LB) media containing ampicillin (50 Wg/l). S. albus was grown at 28°C on R2YE plates [12]. Escherichia coli DH5α F' was used as host strain for subcloning and sequencing work and E. coli BL21(DE3) was used for the expression of wild-type and mutant vdh genes. These strains were grown on Luria-Bertani (LB) media containing ampicillin (50 Wg/l). S. albus was grown at 28°C on R2YE plates [12].

2.2. DNA manipulations and hybridization

All DNA manipulations and E. coli transformations were performed by standard methods [13] and the inserted DNA was digested with restriction endonucleases in single or double digests for the construction of a restriction map. For a DNA hybridization, DNA samples were run in a 0.8% agarose gel and blotted on to positively charged Hybond-N membranes by capillary transfer. The digoxigenin (DIG)-AP labelling, hybridization and detection were done with the Genus kit (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer’s protocols. Hybridization was performed at 68°C overnight.

2.3. DNA sequencing and computer analysis

For DNA sequencing work, nested deletions were constructed with the Erase-a-Base system (Promega Biotech, Madison, WI, USA) according to the manufacturer’s instructions. Restriction fragment or deletion mutants were subcloned into M13mp18 or M13mp19 [14]. M13 single-stranded DNA templates were sequenced with either commercially available ‘Universal’ primer or specific synthetic oligonucleotide primers using Sequenase 2.0 (U.S. Biochemical, Cleveland, OH, USA) or an ABI 373 automatic sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were done with the Genus kit (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer’s protocols. Hybridization was performed at 68°C overnight.

2.4. Expression and purification of Vdh

To express Vdh protein, a PCR with plasmid template pWHM1068, primers designed to introduce the BamHI (Val-F; 5'-ACACAGGATCCACCAACGGTA-3') and HindIII (Val-R; 5'-AAGCTTCACCGCGGGGCGGT-3') restriction sites encompassing vdh, was performed by using a final volume of 50 µl that contained 100 pmol of each primer, 50 ng of DNA from plasmid pWHM1068, 0.25 mM dNTP, 4 pmol of each oligonucleotide and 2 U Taq polymerase and 1×buffer (Takara, Japan). Amplification was performed in a thermal cycler (model 480, Perkin-Elmer Cetus, Norwalk, CT, USA) by denaturing the samples at 94°C for 3 min, subjecting them to 30 cycles of denaturing (98°C, 20 s), annealing (68°C, 1 min) and then elongating at 72°C for 10 min.

An expected PCR product of 1.1 kb containing BamHI and HindIII was recovered by 1% agarose gel electrophoresis and ligated into pT7Blue (Novagen, Madison, WI, USA) to give pHCG547. This supercoiled recombinant vector was digested with BamHI and HindIII and ligated into the restriction-digested pET28a expression vector to

Fig. 1. Restriction map of the vdh locus in S. albus. The thick bold arrows indicate the direction of transcription for the vdh gene and putative ORF1. Restriction enzyme abbreviations: A, Apal; B, BamHI; Bg, BglII; K, KpnI; N, NotI; P, PstI; S, SalI; Sm, Smal; Sc, SacI; X, XhoI.
The T7 expression vector pET28a was used for overproduction of the wild and mutant Vdh proteins fused to a His6 tag at their N-terminus. E. coli BL21(DE3) carrying pHCG548 was grown at 37°C in LB medium containing kanamycin. When the culture reached an A<sub>600</sub> value of 0.7, isopropyl-<i>L</i>-D-thiogalactopyranoside (IPTG) was added to yield a final concentration of 1 mM. After an additional 3-h incubation, cells were lysed by lysozyme treatment followed by sonication. Vdh was purified from the sonicated cell extracts using the His-bind resin column according to the manufacturer's protocol (Novagen, Madison, WI, USA). Fractions collected from the His-bind affinity column were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. Site-directed mutagenesis

To obtain mutant plasmids, oligonucleotide-directed mutagenesis was performed by multiple rounds of PCR. Plasmid pHCG131 specifying the K79A mutant Vdh protein in which Lys-79 was replaced with Ala was constructed as follows. Plasmid pHCG548 was used as a template for the first round of PCR. By using two sets of primers, K79A-F (5'<i>P</i>-ATGTCGTACCACACCCGCAAAATCCGTCGTATCCCGC-<i>3'</i>P) plus Val-R and K79A-R (5'<i>P</i>-CATGCGTATCCCGCAAAATCCGTCGTATCCCGC-<i>3'</i>P), plasmid pHCG131 was constructed.

**Fig. 2.** Comparison of the deduced amino acid sequences of Vdh from <i>S. albus</i> and other branched-amino acid dehydrogenases. Amino acid sequences identical to those of <i>S. albus</i> Vdh are dotted using asterisks. Gaps introduced to maximize the fit are shown as dashed lines. Active sites are shown as vertical arrows. The amino acid sequences were taken from the following sources: SAVDH, <i>S. albus</i> Vdh; BCLDH, <i>Bacillus cereus</i> Ldh (U51099); BSDLD, <i>B. stearothermophilus</i> Ldh [8]; BBPDH, <i>Bacillus badius</i> Pdh [22] and BSDPDH, <i>B. sphaericus</i> Pdh [21].

give pHCG548. The T7 expression vector pET28a was used for overproduction of the wild and mutant Vdh proteins fused to a His6 tag at their N-terminus.

<i>E. coli</i> BL21(DE3) carrying pHCG548 was grown at 37°C in LB medium containing kanamycin. When the culture reached an A<sub>600</sub> value of 0.7, isopropyl-<i>L</i>-D-thiogalactopyranoside (IPTG) was added to yield a final concentration of 1 mM. After an additional 3-h incubation, cells were lysed by lysozyme treatment followed by sonication. Vdh was purified from the sonicated cell extracts using the His-bind resin column according to the manufacturer's protocol (Novagen, Madison, WI, USA). Fractions collected from the His-bind affinity column were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
GGCGTACGACAT-3’ plus Val-F, 0.24 and 0.85-kb DNA fragments which correspond to N-terminal and C-terminal regions of Vdh, respectively, were amplified. These two fragments were mixed and used as templates for the second round of PCR with the Val-F and Val-R primers. The amplified 1.1-kb fragment was digested with BamHI and HindIII and inserted into the corresponding site of pET28a to yield pHCG131. Plasmid pHCG132 specifying the K91A mutant Vdh protein in which Lys-91 was replaced with Ala was constructed in the same way as above, except that the primers K79A-F and K79A-R, used for the construction of pHCG131 in the first round of PCR, were replaced with primers K91A-F (5’-GGCGGCGGGCGCCGTTGATC-3’) and K91A-R (5’-GATCACGCGGCGCCCGC-3’).

2.6. Protein and enzyme assay

The oxidative deamination of L-valine and the reductive amination of α-ketosovaleric acid were measured by monitoring spectrophotometrically the appearance and disappearance of NADH under the conditions described by Navarrete et al. [17]. The steady-state kinetic parameters were determined by varying the concentration of a substrate (coenzyme) in the presence of constant saturating concentrations of an other substrate (coenzyme). The initial velocity was determined from plots using a molar absorption coefficient of 6.22 × 10³ M⁻¹ l⁻¹ for NADH in 2 min under the assay conditions.

The protein concentration was determined colorimetrically by the methods of Bradford using the Bio-Rad protein assay reagent with bovine serum albumin as the standard [18]. All spectrophotometric measurements were performed with an Ultrospec II (Pharmacia).

3. Results and discussion

3.1. Isolation and sequence analysis of the S. albus vdh gene

To isolate the S. albus vdh gene, the 0.35-kb SphI-KpnI or 1.6-kb BamHI fragments of pWHM1051 containing the S. coelicolor vdh gene were DIG-AP-labelled and used to identify cross-hybridizing bands in a Southern blotting experiment with S. albus ATCC21838 genomic DNA digested with several restriction enzymes. We found that the 8.0-kb fragment of SacI-PstI hybridized with these heterologous probes under high-stringency conditions. Based on these data, S. albus chromosomal DNA was digested with SacI/PstI. Fragments of 6–8 kb in length were isolated and ligated into pUC18 previously digested with the same restriction enzymes. The resulting ligation products were used to transform E. coli and colonies containing DNA that hybridized to the 0.35 or 1.6-kb fragments of pWHM1051 probes (S. coelicolor vdh gene) were identified by colony hybridization. Screening of plasmid DNA isolated from a sublibrary consisting of 6.0–8.0-kb PstI-SacI DNA fragments of S. albus genomic DNA yielded one positive clone, pWHM1068, which was shown to contain a 7.5-kb DNA fragment. By subcloning and further hybridizations, the 2.1 and 1.5-kb SmaI fragments from pWHM1068 that hybridized with the labelled probe were subcloned into the SmaI site of pBluescript II SK(+) to give plasmid pHCG544 and pHCG545, respectively. A restriction map of the cloned region is shown in Fig. 1. This region surrounding the vdh gene has been shown to have the same genetic organization in S. albus as in S. coelicolor and others [4,5,6].

A 1.48-kb DNA fragment containing the region hybridizing with the S. coelicolor vdh gene was sequenced and analyzed. CODON PREFERENCE analysis showed that there are one partial open reading frame (ORF) and one complete ORF in this region, having the characteristic codon usage pattern for Streptomyces DNA [19]. The vdh gene is transcribed in the opposite direction to the incomplete ORF. The 1092-nucleotide (nt) vdh ORF started with a GTG at position 385 and was terminated with a TGA at position 1479. This ORF should encode a protein of 364 amino acid residues with a calculated molecular mass of 38.4 kDa. A region centered about nine nt from 5’ to the first codon of the vdh gene showed a high degree of complementarity to the 3’ end of 16S rRNA of S. coelicolor (5’-GAUCACCCUUUCU-3’) and should serve as the ribosomal binding site [20].

The amino acid sequences deduced from vdh gene have significantly high similarities to many NAD(P)⁺-dependent vdh genes, particularly to those derived from S. coelicolor (97.2%), S. fradiae (80.4%) and S. cinnamonensis (87.2%).

Fig. 3. SDS-PAGE of purified wild-type and mutant Vdh enzymes. Lane 1 indicates molecular mass markers: phosphorylase b (97.4 kDa), bovine albumin (66.2 kDa), aldolase (39.2 kDa), triose phosphate isomerase (26.6 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). Lane 2, wild-type Vdh. Lane 3, K81A. Lane 4, K91A.
Table 3
Steady-state kinetic parameters of the wild-type and mutant enzymes of Vdh

<table>
<thead>
<tr>
<th>Sample</th>
<th>$K_{cat}$ (s⁻¹)</th>
<th>$K_m$ (mM)</th>
<th>NAD⁺ (mM)</th>
<th>Ketosovaleric acid (mM)</th>
<th>NADH (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>47.47 ± 5.2</td>
<td>314.52 ± 18.55</td>
<td>1.5 ± 0.2</td>
<td>0.133 ± 0.01</td>
<td>0.438 ± 0.018</td>
</tr>
<tr>
<td>K79A</td>
<td>0.000476 ± 0.000081</td>
<td>7.224 ± 0.228</td>
<td>714 ± 74</td>
<td>0.2 ± 0.32</td>
<td>50 ± 5.5</td>
</tr>
<tr>
<td>K91A</td>
<td>0.0251 ± 0.0048</td>
<td>13.03 ± 1.441</td>
<td>2.08 ± 0.25</td>
<td>0.19 ± 0.02</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>K79A mutant</td>
<td>0.022 ± 0.0026</td>
<td>13.03 ± 1.441</td>
<td>2.08 ± 0.25</td>
<td>0.19 ± 0.02</td>
<td>4.5 ± 0.5</td>
</tr>
</tbody>
</table>
two lysyl residues are essential for the catalytic activity. However, the $K_m$ values for NAD$^+$ and NADH were essentially unchanged in the wild-type and mutant enzymes, suggesting that the replacement of Lys-79 and Lys-91 with other residues had little effect on the affinity for the nicotinamide coenzyme. However, K79A mutant enzyme showed $K_m$ values for $L$-valine and $\alpha$-ketoisovaleric acid 476 and 114.2-fold larger than the wild-type enzyme, respectively, and K91A mutant enzyme showed a $K_m$ value for $\alpha$-ketoisovaleric acid 10.3-fold larger than the wild-type enzyme. These results demonstrated that Lys-79 and Lys-91 of Vdh are likely to be involved in both substrate binding and catalysis, as was observed for the corresponding residues in the case of Ldh (Lys-68 and Lys-80) and Pdh (Lys-69 and Lys-81).

Acknowledgements

We warmly thank C.R. Hutchinson for the positive clone of S. albus vdh and his critical reading of the manuscript. This study was supported by a Grant from the Science and Technology Policy Institute (Project No. 98-N1-01-02-A-07) in 1999.

References