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Identification of Essential Amino acid Residues in Valine Dehydrogenase from *Streptomyces albus*

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Cys-29 and Cys-251 of *Streptomyces albus* valine dehydrogenase (ValDH) were highly conserved in the corresponding region of NAD(P)$^+$-dependent amino acid dehydroganase sequences. To ascertain the functional role of these cysteine residues in *S. albus* ValDH, site-directed mutagenesis was performed to change each of the two residues to serine. Kinetic analyses of the enzymes mutated at Cys-29 and Cys-251 revealed that these residues are involved in catalysis. We also constructed mutant ValDH by substituting valine for leucine at 305 by site-directed mutagenesis. This residue was chosen, because it has been proposed to be important for substrate discrimination by phenylalanine dehydrogenase (PheDH) and leucine dehydrogenase (LeuDH). Kinetic anaysis of the V305L mutant enzyme revealed that it is involved in the substrate binding site. However it displayed less activity than the wild type enzyme toward all aliphatic and aromatic amino acids tested.

Keywords: *Streptomyces*, valine dehydrogenase, mutagenesis, kinetic analysis

Streptomyces albus has been used for the production of salinomycin, a commercially important polyether antibiotic. The biosynthesis of salinomycin and other polyethers requires the cells to produce and synthesize substancial 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 polyketide antibiotic biosynthesis. 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 activity, suggests that branched chain amino acid catabolism may be an important source of building blocks for macrolide biosynthesis (Hyun *et al.*, 2000b; Omura *et al.*, 1983; Sherman *et al.*, 1986).

Valine dehydrogenase (ValDH; EC 1.4.1.8) is a NAD(P)$^+$-dependent oxidoreductase that catalyzes reversible deamination of L-valine and some other branched chain L-amino acids to their keto analogs. This serves as the first catabolism step of these amino acids in bacteria. ValDH belongs to a family of branched -chain amino acid dehydrogenases, whose members include both leucine dehydrogenase (LeuDH; EC 1.4.1.9) and phenylalanine dehydrogenase (PheDH; EC 1.4.1.20). The kinetic and basic properties of the valine dehydrogenases from several strains of *Streptomyces* (Hyun *et al.*, 2000b; Nguyen *et al.*, 1995) and *Cytophaga* (Oikawa *et al.*, 2001) have been studied. ValDH is involved in branched-chain amino acids in these organisms, but little information is available about the molecular structure and detailed properties of the enzyme. This is in striking contrast to extensive studies on LeuDH (EC 1.4.1.9), which has been minutely characterized among NAD$^+$-dependent amino acid dehydrogenases (Nagata *et al.*, 1988; Ohshima *et al.*, 1994). Both amino acid dehydrogenases catalyze similar reactions, but are considerably different from each other in substrate specificity, quaternary structure, and other properties (Fig. 1).

In order to elucidate the molecular mechanism of ValDH catalysis, we have cloned the gene and identified the active site (Lys residues) of *S. albus* ATCC21838 ValDH (Hyun *et al.*, 2000b). We have also identified an another active site, Ala-124, involved in substrate discrimination (Hyun *et al.*, 2000a). Furthermore, we have reported that ValDH was reversibly inactivated by *p*-hydroxymercuribenzoate (Hyun *et al.*, 2000a). This observation indicates that ValDH

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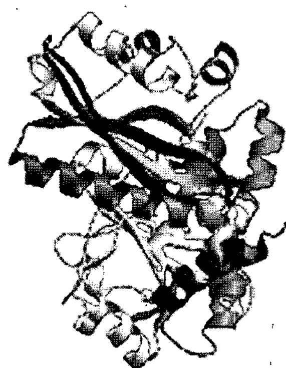

Fig. 1. The 3D Structure of ValDH constructed by molecular modeling. The SWISS-MODEL REPOSITORY program (Kopp and Schwede, 2004) was used for modeling with the representative 3D structures of leucine dehydrogenase from *Bacillus sphaericus* (Turnbull et al., 1994b)

appears to require at least one cysteine sulfhydryl for enzymatic activity. The ValDH enzyme contains six cysteine residues located at positions 29, 125, 141, 251, 171, and 320. In this study, we describe the mutational modification of cysteine residues of *S. albus* ValDH to investigate the role of these residues in the enzymatic activity. To identify the essential amino acid for substrate discrimination in valine dehydrogenase, we also used site-directed mutagenesis to construct ValDH mutant by substituting valine for leucine at 305.

Materials and Methods

Plasmid construction and site-directed mutagenesis

To obtain mutant plasmids, oligonucleotide-directed mutagenesis was performed by multiple rounds of PCR. Plasmid pC29S specifying the C29S mutant ValDH protein in which Cys29 was replaced with Ser was constructed as follows. Plasmid pC29S was used as a template for the first round of PCR. By using two sets of primers, C29S-F (5'-caagtcgtgctctcccag-gaccgggcc-3') plus Val-R (Hyun *et al.,* 2000b) and C29S-R (5'-ggcccggtcctgggagagcacgacttg-3') plus Val-F (Hyun *et al.,* 2000b), 0.85- and 0.24-kb DNA fragments which correspond to N-terminal and C-terminal region of ValDH, 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 *Bam*HI and *Hind*III and inserted into the corresponding site of pET28a to yield pC29S. Plasmids pC251S and pV305L specified the C251S and V305L mutant ValDH proteins in which Cys-251 and Val-305 were replaced with Ser and Leu. These plasmids were constructed in the same way as above, except that the primers C29S-F and C29S-R, used for the construction of pC29S in the first round of PCR, were replaced with primers C251S-F (5'-atctacgccccgtccgcg ctcggcggc-3') and C251S-R (5'-gccgccgagcgcggacgggg cgtagat-3'). In the case of pV305L, the primers were replaced with V305L-F (5'-gccggcgggctcatccaggtc-3') and V305L-R (5'-gacctggatgagcccgccggc-3').

Purification of ValDH and its mutated enzymes

For overexpression of ValDH and its mutated enzymes, a 1.1-kb *Bam*HI-*Hind*III DNA fragments were cloned into pET28a to yield pC29S, pC251S and pV305L. The resulting plasmids were introduced into *E. coli* BL21(DE3). Expression of the mutated gene was induced in exponentially growing cells (optical density at 600 nm, 0.6) by the addition of 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG). After a further 3 hours of cultivation, cells were harvested by centrifugation at 12,000 *g* for 10 minutes, resuspended in 3 ml of a binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl; pH 7.9) and disrupted by sonication. Cell debris was removed by centrifugation at 12,000 *g* for 10 minutes. The resulting supernatants were mixed with nickel nitrilotriacetate (Ni/NTA) resin (Novagen, Madison, WI, USA) pre-equilibrated with the afore mentioned binding buffer. The adherent proteins were eluted from the Ni/NTA resin with a buffer containing 1 M imidazole, 0.5 M NaCl, and 20 mM Tris-HCl at pH 7.9.

Enzyme assay and determination of protein concentration

The oxidative deamination of L-valine and other amino acids was measured spectrophotometrically by monitoring for the appearance of NADH at 340 nm. The reaction mixture (1 ml) contained 100 mM glycine-NaOH buffer (pH 10.5), 1 mM NAD^+, and 10 mM substrate. The reaction was initiated by adding the enzyme. The steady-state kinetic parameters were determined by varying the concentration of a substrate in the presence of a constant saturating concentration of coenzyme. The initial velocity was determined from plots using a molar absorption coefficient of $6.22 \times 10^3\ M^{-1}L^{-1}$ for NADH under assay conditions. Protein concentration was determined by the Bradford methods using a Bio-Rad protein assay reagent with bovine serum albumin as the standard (Bradford, 1976). All spectrophotometric measurements were performed with a Ultrospec II (Pharmacia).

Results and Discussion

Functional roles of Cys-29 and Cys-251

The ValDH enzyme was completely inhibited by ρ-chloromercuribenzoate, indicating that sulfyhydryl

groups are necessary for enzyme activity (Hyun *et al.*, 2000a). The cloned *S. albus* ValDH had 6 cysteine residues. Comparison of the sequences for LeuDH, PheDH, and ValDH revealed that Cys-29 and Cys-251 residues were conserved. This indicated that they may share an identical catalytic function. To test the importance of Cys-29 and Cys-251, they were mutated. Steady-state kinetic analyses were performed with these purified mutant enzymes in order to elucidate the functional roles of Cys-29, and Cys-251 in ValDH (Table 1). Steady-state kinetic parameters of the wild type, C29S mutant and C251S mutant enzymes were measured at 25℃ under variable concentrations of substrate and coenzyme. The catalytic rate constants of the C29S and C251S mutant enzymes compared to the wild type enzye were only 13.3 and 13.2% for the oxidative deamination of L-valine. These results indicate that the two cysteine residues are essential for catalytic activity. However, the Km value for NAD^+ and NADH were essentially unchanged in the wild type and mutant enzymes, suggesting that the replacement of Cys-29 and Cys-251 with other residues had little effect on the affinity for the nicotinamide coenzyme. Also, Km values of C29S and C251S mutant enzymes for L-valine were essentially unchanged. These results demonstrated that Cys-29 and Cys-251 of ValDH are likely to be involved in both catalyses.

Table 1. Steady-state kinetic parameters of the wild type and mutant enzymes

Sample	Kcat (sec^{-1})	Km (mM)	
	Deamination	L-valine	NAD^+
Wild type	46±0.2	1.686±0.002	0.15±0.01
C30S	6.12±0.97	0.8±0.16	0.091±0.009
C251S	6.09±0.12	1.6±0.12	0.159±0.002
V305L	0.0478±0.0024	100±15	0.37±0.03

Table 2. Relative activities of wild-type and V305L mutant enzyme on various aromatic and aliphatic substrates

Amino acids	Wild type	V305L
L-Valine	100	0.014
L-Norvaline	100.5	0
L-leucine	15	0
L-Norleucine	15	0.014
L-Isoleucine	27.5	0
L-Phenylalanine	1.25	0
L-Cysteine	3.75	0.066
L-Methionine	0.95	0.035
L-Alanine	0.37	0
L-Tyrosine	0	0

Construction and characterization of V305L mutants
Recently, an active site structure model has been proposed for ValDH based on the three-dimensional structures of *Clostridium symbiosum* GDH and *Bacillus sphaericus* LeuDH (Turnbull *et al.*, 1997a; Baker *et al.*, 1995). In this model, several amino acid residues are proposed to be important for recognition of the substrate side chain. However, this theory has not yet been experimentally verified. In an earlier attempt to shift the substrate specificity of valine dehydrogenase toward aromatic amino acid substrates, the active site residue A124 was replaced by glycine, which occupies the corresponding position in phenylalanine dehydrogenase (Hyun *et al.*, 2000a). We replaced Val-305 of *S. albus* ValDH with leucine, the corresponding residue in PheDH, in order to examine the important role played by Val-305. This residue was chosen, because it has been proposed to be important for substrate discrimination by PheDH and LeuDH (Seah *et al.* 1995; Seah *et al.*, 2002; Seah *et al.*, 2003)

Steady-state kinetic analysis was performed with the purified mutant enzyme in order to elucidate the functional role of Val-305 in ValDH (Table 1). Steady-state kinetic parameters of wild type and mutant enzymes were measured at room temperature under variable substrate and coenzyme concentrations. The catalytic rate constant of the V305L mutant enzyme was only 0.001% that of the wild type for the oxidative deamination of L-valine. These results indicate that Val-305 is as essential for the catalytic activity as the Lys-79 and Lys-91 previously identified. However, the Km values for NAD+ were essentially unchanged in the wild type and mutant enzymes, suggesting that the replacement of Val-305 with other residues has little effect on the affinity for the nicotinamide coenzyme. The V305L mutant enzymes showed 66.7-fold higher Km value for L-valine than the wild type enzyme. These results demonstrated that the Val-305 of ValDH is likely to be involved in substrate binding. To examine alteration of substrate specificity in the mutant enzymes, the V305L mutant enzyme was assayed with a variety of substrates. The specific activities expressed as a percentage of the activity with L-valine are shown in Table 2. However, activity on aromatic and aliphatic amino acid substrates was lower in the V305L mutant than in wild-type enzyme. Therefore, amino acid substitution at this position was insufficient to transform ValDH with narrow substrate specificity into an enzyme with broad preference for

several amino acid substrates under the above conditions.

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