Alteration of substrate specificity of leucine dehydrogenase by site-directed mutagenesis

Journal of Molecular Catalysis B: Enzymatic

(Regular paper)

Title:

Alteration of Substrate Specificity of Leucine Dehydrogenase by Site-directed Mutagenesis[1]

Authors:

Kunishige Kataoka[a,*] and Katsuyuki Tanizawa[b]

Affiliations:

[a] Department of Chemistry, Faculty of Science, Kanazawa University, Kakuma, Kanazawa, Ishikawa 920-1192, Japan

[b] Department of Structural Molecular Biology, Institute of Scientific and Industrial Research, Osaka University, Ibaraki, Osaka 567-0047, Japan

*Correspondence auther: (kataoka@cacheibm.s.kanazawa-u.ac.jp).

[1]Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday.

Abstract

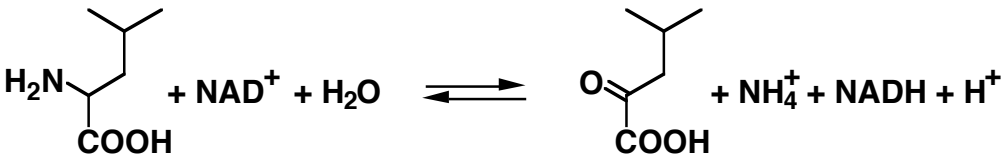
The residues L40, A113, V291, and V294, in leucine dehydrogenase (LeuDH), predicted to be involved in recognition of the substrate side chain, have been mutated on the basis of the molecular modeling to mimic the substrate specificities of phenylalanine (PheDH), glutamate (GluDH), and lysine dehydrogenases (LysDH). The A113G and A113G/V291L mutants, imitating the PheDH active site, displayed activities toward L-phenylalanine and phenylpyruvate with 1.6 and 7.8% of k_{cat} values of the wild-type enzyme for the preferred substrates, L-leucine and its keto-analog, respectively. Indeed, the residue A113, corresponding to G114 in PheDH, affects the volume of the side-chain binding pocket and has a critical role in discrimination of the bulkiness of the side chain. Another two sets of mutants, substituting L40 and V294 of LeuDH with the corresponding residues predicted in GluDH and LysDH, were also constructed and characterized. Emergence of GluDH and LysDH activities in L40K/V294S and L40D/V294S mutants, respectively, indicates that the two corresponding residues in the active site of amino acid dehydrogenases are important for discrimination of the hydrophobicity/polarity of the aliphatic substrate side chain. All these results demonstrate that the substrate specificities of the amino acid dehydrogenases can be altered by protein engineering. The engineered dehydrogenases are expected to be used for production and detection of natural and non-natural amino acids.

Key words:

leucine dehydrogenase, substrate specificity, protein engineering, enzymatic synthesis

1. Introduction

Leucine dehydrogenase (LeuDH) [EC 1.4.1.9] is an NAD^+- dependent oxidoreductase that catalyzes the reversible deamination of L-leucine and some other branched-chain L-amino acids to their keto analogs (Scheme 1). The enzyme occurs ubiquitously in *Bacillus* species [1] and functions catabolically in the bacterial metabolism of branched-chain L-amino acids [2]. It has been suggested that the enzyme plays an important role in spore germination in cooperation with alanine dehydrogenase [3, 4]. The amino acid dehydrogenase has considerable commercial potential for the production of novel non-proteinous amino acids in pharmaceutical industries [5, 6] and for the diagnosis of genetic diseases of amino acid metabolisms including phenylketonuria [7], maple syrup urine disease [8, 9], and homocystinuria [10].



Scheme 1

The thermostable LeuDH cloned from *Bacillus stearothermophilus* [11] shares considerable sequence similarities in the catalytic and coenzyme-binding domains with the enzymes acting on other amino acids, such as glutamate (GluDH) [12, 13], phenylalanine (PheDH) [14, 15], and valine (ValDH) [16, 17] dehydrogenases. Although the overall similarities among these enzymes are not high, sequence similarities between these dehydrogenases clearly indicate the existence of an enzyme superfamily related by divergent evolution [18]. However, the substrate specificities of this superfamily are different; GluDH

recognizes and binds glutamate in preference to all other amino acids [19], LeuDH and ValDH catalyze the oxidation of only branched-chain, aliphatic amino acids [1, 20, 21], and PheDH has a marked preference for aromatic amino acids as its substrate, although it also accepts smaller hydrophobic amino acids with reduced efficiency [22, 23].

Results of structural studies in the last decade have revealed that these enzymes share a very similar subunit structure, with each subunit composed of two domains separated by a cleft harboring the active site [24-26]. To study the structure and function of the domains of amino acid dehydrogenases before their crystal structures were available, we constructed and characterized the chimeric enzyme consisting of an N-terminal domain of thermostable PheDH from *Thermoactinomyces intermedius* and a C-terminal domain of LeuDH from *B. stearothermophilus* [27]. Furthermore, we constructed and expressed genes of fragmentary forms of *B. stearothermophilus* LeuDH to investigate the function of the enzyme domains [28]. The results of our domain manipulation have suggested that the substrate specificity is determined by structural interactions of the two domains, as corroborated from the presently known crystal structures.

On the basis of the molecular structures of GluDH [24], LeuDH [25], and PheDH [26], substrate specificity of amino acid dehydrogenases is explained by the types of residues comprising the substrate side-chain binding pocket. In GluDH of *Clostridium symbiosum*, the principal interactions that determine the specificity are between the γ-carboxyl group of the substrate glutamate and the amino group of K89 and the hydroxyl group of S380, with G90, A163, and V377 interacting with the hydrophobic component of the glutamate side chain [29]. In the *Bacillus sphaericus* LeuDH structure, the latter three residues are conserved (G41, A113, and V291, respectively), whereas K89 and S380 in GluDH are replaced by L40 and V294 in LeuDH (residue numbers refer to *B. sphaericus* LeuDH), making up a more

hydrophobic substrate side-chain binding pocket [25]. X-ray crystallographic and homology-based molecular modeling studies of PheDH revealed that this enzyme has a hydrophobic and large side-chain binding pocket enough to bind the phenylalanine benzene ring by replacing glycine for A113 of LeuDH [18, 30].

Several attempts have been made to alter the substrate specificities of the amino acid dehydrogenase superfamily by molecular modeling. Wang *et al*. engineered a K89L/S380V double mutant of GluDH, expecting that it would show substrate specificity of LeuDH with a hydrophobic pocket [30], but the mutant had no activity because of steric clash of the pocket [31]. The substrate specificity of *B. sphaericus* PheDH was successfully shifted toward that of LeuDH by replacing G124 and L307 (corresponding to A163 and V377 in GluDH, respectively) to alanine and valine, respectively [32]. More recently, Seah *et al*. succeeded in modulating the specificity of *B. sphaericus* PheDH [33].

In the present study, aiming at altering the substrate specificity of *B. stearothermophilus* LeuDH by homology-based modeling, we chose L40, A113, V291, and V294 for a target of mutagenesis to mimic the substrate-binding pocket of PheDH, GluDH, and LysDH. The detailed kinetic analysis of the mutant enzymes suggests that these residues are important in the substrate side-chain recognition of the enzyme superfamily. The alteration of substrate specificity by protein engineering presented here is not only of scientific interest – understanding the structural basis for the difference in substrate discrimination of the amino acid dehydrogenases – but also of applied one, possibly providing an amino acid dehydrogenase with novel properties for production and detection of natural and non-natural amino acids.

2. Experimental

2.1. Materials

A site-directed mutagenesis kit (Mutan-K), *Taq* DNA polymerase, T4 DNA ligation kit, and DNA blunting kit were purchased from Takara Biochemicals (Japan). All reagents for DNA synthesis and DNA sequencing were purchased from Applied Biosystems (U.S.A.). The plasmid pICD2 carrying the leucine dehydrogenase gene of *B. stearothermophilus* and the plasmid pKPDH2 containing the phenylalanine dehydrogenase gene of *T. intermedius* were described previously [11, 14].

2.2. Mutagenesis

Substitutions of aspartate or lysine for L40, glycine for A113, leucine for V291, and serine for V294 of LeuDH were performed by the method of Kunkel *et al.* [34] using a commercial kit (Mutan-K), as described previously [35]. The following five oligonucleotide primers were synthesized with an Applied Biosystems DNA synthesizer model 381 to contain appropriate mismatched bases (indicated by asterisks) in the complementary codons for each residue (underlined):

L40D: 5’-ATACGCGTCCCGCC<u>TTT</u>CGCCGGGCCGAG-3’ (* * *)

L40K: 5’-ATACGCGTCCCGCC<u>GTC</u>CGCCGGGCCGAG-3’ (* *)

A113G: 5’-GACGTCTTC<u>CCC</u>CGTGATGTAG-3’ (*)

V291L: 5’-ACGTTGATG<u>AGG</u>CCGCCGGC-3’ (*)

V294S: 5’-TTCGTCCGCG<u>GAG</u>GTTGATGACG-3’ (* *)

After confirming the nucleotide sequence of the mutant gene, appropriate restriction fragments were replaced for the corresponding wild-type gene fragments in the LeuDH expression plasmid pICD212.

Substitution of alanine for G114 of PheDH was performed by the two-step PCR method as described previously [36] with two mutation primers shown below and pKPDH2 as a template (1st PCR):

G114A-sense: 5'-AACGGCCGTTTCTATACC*GCAACCGACATGGG-3'

G114A-antisense: 5'-CCATGTCGGT*TGCGGTATAGAAAC-3'

After the second PCR using universal primers, the amplified fragment was sequenced and digested with *Sac*II and *Spl*I. The digested fragment was replaced for the corresponding wild-type gene fragments in pKPDH2.

2.3. Purification of the mutant enzyme

The wild-type and mutant enzymes of LeuDH were purified to homogeneity from the crude extract of recombinant *Escherichia coli* cells grown at 37 °C for 12 h in Luria broth supplemented with 50 μg/ml ampicillin and 0.5 mM IPTG, as described previously [11]. The wild-type and G114A mutant of PheDH were also purified to homogeneity from the crude extract of recombinant *E. coli* cells, as described previously [14].

2.4. Steady-state kinetic analysis

The oxidative deamination of L-amino acids and the reductive amination of α-keto acids were measured by monitoring spectrophotometrically the appearance and disappearance,

respectively, of NADH under the conditions described previously [11]. The steady-state kinetic parameters were determined by varying systematically the concentrations of both substrate and coenzyme, except for ammonia, which was held at a constant, saturating concentration (1.0 M). Protein concentrations of the wild-type and mutant LeuDH and the wild-type and G114A PheDH were estimated using absorbencies ($A^{0.1\%}$) of 0.851 and 0.634 at 280 nm, respectively.

2.5. CD measurements

Circular dichroism (CD) spectra were measured at 25 °C in 10 mM potassium phosphate buffer (pH 7.2) with a Jasco spectropolarimeter model J-600. In the calculation of the mean residue ellipticity (s), the mean residue weight was taken to be 111 for the enzyme protein. The CD spectra were obtained at a protein concentration of 0.2 mg/ml in a 2.0-cm light path length cuvette for the measurements in the wavelength region above 250 nm and at a protein concentration of 0.1 mg/ml in a 0.1-cm light path length cuvette for measurements below 250 nm.

3. Results and Discussion

3.1. Molecular modeling and construction of mutant enzymes

Multiple sequence alignment of LeuDH, PheDH, and GluDH using structural information of *B. sphaericus* LeuDH and *C. symbiosum* GluDH pointed out important residues that are assumed to be the determinant of substrate side-chain binding (Fig. 1). This homology-based modeling suggests that the difference in substrate specificity between PheDH and LeuDH arises only from unacceptable steric interaction of the methyl group of A113 of LeuDH with the substrate phenylalanine benzene ring, which are relieved in PheDH by critical replacement of this residue by Gly114 [18]. To examine the validity of the models, two mutant enzymes of LeuDH, in which Ala113 is replaced by glycine (A113G), and A113 and V291 are both replaced by glycine and leucine, respectively (A113G/V291L), have been constructed. With the expectation of the opposite effect, we also prepared the mutant PheDH, in which G114 was changed to alanine (G114A-PheDH) by PCR mutagenesis.

Furthermore, to alter the specificities of LeuDH toward GluDH or LysDH, L40, corresponding to K89 in GluDH, whose ε-amino group interacts with the γ-carboxyl group of the substrate L-glutamate by hydrogen bonding, has been mutated to lysine (L40K) or aspartate (L40D). V294 of LeuDH, which is equivalent to S379 in GluDH that interacts with the substrate glutamate by hydrogen bonding, has been replaced as well by serine for making double mutants (L40K/V294S, L40D/V294S). In these variants, the hydrophilicity of the substrate side-chain binding pocket increases, and the interaction of the substrate with the polar side chain is expected.

All seven mutant enzymes were expressed and purified from the crude extract of recombinant *E. coli* cells as the wild-type enzymes. After the purification, all mutant enzymes

exhibited a single prominent band on the SDS-PAGE gel with molecular sizes equivalent to the wild-type LeuDH or PheDH (data not shown).

3.2. CD spectra of mutants

CD spectra of the wild-type and six mutant enzymes of LeuDH were measured to see whether the global conformations were changed by the mutation. All the mutant enzymes except A113G/V291L showed CD spectra practically identical with that of the wild-type enzyme in the 200-250 nm region (Fig. 2), indicating that the wild-type and these mutant enzymes contain very similar secondary structures. The decreased CD band of A113G/V291L in this region indicates the decreased secondary structure content and some slight conformational changes in the overall structure of this mutant.

On the other hand, the spectra of mutant enzymes in the 260-290 nm region, which reflects the environment of aromatic residues, were significantly different from that of the wild-type enzyme (Fig. 2A). The CD band of the wild-type LeuDH in this region is mainly due to the sole tryptophan (W46), which is predicted to be located in the vicinity of the active site [37]. The decreases in the near-UV CD bands of A113G and A113G/V291L indicate that the environment around W46 of these mutants became more hydrophobic than that of the wild-type enzyme, as in the case of mutations of the conserved glycine residues adjacent to the catalytic K80 [37]. On the contrary, the spectrum of the L40D/V294S mutant in this region, which is more negative than that of the wild-type, is indicative of a more hydrophilic environment around W46 than in the wild-type enzyme.

CD spectrum of the G114A mutant of PheDH appeared essentially identical to that of the wild-type enzyme (data not shown). Thus, the G114A mutant appears to have an identical conformation to that of the wild-type PheDH.

3.3. Catalytic properties of A113G and A113G/V291L mutants of LeuDH and G114A mutant of PheDH

Both single and double mutants of LeuDH prepared to mimic PheDH showed a marked decrease in activities towards the aliphatic substrates compared to the wild-type enzyme. The k_{cat} values of the single mutant A113G and the double mutant A113G/V291L decreased by about 20 and 60 folds for L-leucine, and by about 3 and 10 folds for α-keto-*iso*-caproate, respectively (Table 2). The substrate specificities of A113G and A113G/V291L were examined in the oxidative deamination with various amino acids and in the reductive amination with various α-keto acids as substrate at 10 mM. The results are compared with those of LeuDH and PheDH in Table 1. Both of the mutant enzymes have broader substrate specificities than the wild-type LeuDH. The mutants act on, in addition to the preferred substrate of LeuDH (short branched-chain amino acids including L-leucine, L-isoleucine, L-valine, and their keto-analogs), poor substrates of LeuDH such as L-norleucine, L-norvaline, L-methionine, L-ethionine, and α-keto-γ-methylthiobutyrate (long-chain aliphatic substrates). Furthermore, these two mutants also utilize L-phenylalanine and phenylpyruvate with over 10% relative activity for the preferred substrate L-leucine and its keto-analog, while the wild-type enzyme does not respond at all to the substrates with aromatic side chains. This could be attributed to the fact that the methyl side chain of A113 in LeuDH, which sterically interferes with binding of the substrate aromatic ring, was removed by substitution to glycine.

Table 2 summarizes the k_{cat} and K_m values of the mutant and wild-type enzymes of LeuDH and PheDH. Although the k_{cat} value for L-leucine and its keto analog of the LeuDH single mutant decreased considerably, the values for other aliphatic substrates did not change so much. In addition, the K_m values of A113G for aliphatic substrates are similar to the

corresponding values of the wild-type LeuDH. This result suggests that there is no significant conformational change in the active site of the mutant upon replacement of A113 with glycine. As expected from the molecular modeling, the single mutant showed PheDH activities, even though they were merely 4% and 11%, respectively, of those of the wild-type PheDH in the oxidative deamination of L-phenylalanine and reductive amination of phenylpyruvate. The K_m values of the A113G mutant for L-phenylalanine and its keto analog were also over 100 times larger than the values of the wild-type PheDH. The low affinities of A113G for both L-phenylalanine and phenylpyruvate lead to a conclusion that the side-chain methyl group of A113 in LeuDH is critical for discrimination between substrates with or without an aromatic side chain but its absence does not contribute for increasing the affinity for aromatic substrates.

In contrast with the single mutant, the k_{cat} values of the A113G/V291L double mutant of LeuDH greatly decreased with all the aliphatic substrates examined. Furthermore, the K_m values for all amino acid and α-keto acid substrates increased over 10 folds, although there was not such a large change in the K_m values for the coenzymes (NAD^+ and NADH). These results are consistent with the significantly altered CD properties of the double mutant in the UV region, as described above. It is plausible that the double mutation has caused some perturbation of local conformations in the substrate side-chain binding region.

The G114A mutant of PheDH showed broader substrate specificity than the wild-type enzyme, and the relative activity for the aliphatic substrates (L-isoleucine, L-norvaline, L-methionine, α-keto-β-methylvalerate, and α-ketobutyrate) increased markedly (Table 1). Despite the fact that the A113G mutant of LeuDH is considerably active with phenylalanine and phenylpyruvate likely due to the removal of the side-chain methyl group, the G114A mutant of PheDH still retains a low activity toward aromatic substrates (4.2% of k_{cat} of the

wild-type PheDH for L-phenylalanine, Table 2). The broad substrate specificity of this mutant is consistent with the specificities of the corresponding G124A and G124A/L307V mutants of *B. sphaericus* PheDH, which also retained about 5% of activities for substrates with an aromatic side chain [32]. Comparing the K_m values of the G114A mutant with those of the wild-type PheDH, we note that the values for aliphatic substrates are unchanged, although those for both L-phenylalanine and phenylpyruvate increased by 160 times. Therefore, we conclude that the affinity for aromatic substrates decreases specifically by introducing a mutation in this position. The greatly decreased activity and the affinity towards aromatic substrates with G114A indicate that G114 in PheDH is indeed critical for recognition of aromatic substrates.

3.4. Catalytic properties of L40K and L40K/V294S mutants of LeuDH

The substrate specificities of the mutants that were designed to mimic the active site of GluDH were also studied in both the oxidative deamination and reductive amination reactions (Table 3). The specificity of the single mutant L40K, in which L40 of LeuDH has been replaced by lysine, corresponding to K89 in GluDH that plays a role in anchoring the γ-carboxyl group of the substrate L-glutamate, did not change greatly from that of the wild-type LeuDH. It could react neither with L-glutamate nor with α-ketoglutarate as substrates. In contrast, the double mutant L40K/V294S with an additional mutation of V294 to serine (equivalent to S379 in GluDH, interacting with the substrate side chain) has a weak GluDH activity, as expected from the molecular modeling. The double mutant of LeuDH acts on polar amino acids such as L-asparagine, L-aspartate, L-glutamine, and L-glutamate, in addition to the preferred substrates of the wild-type enzyme.

The kinetic parameters of the single and double mutants of LeuDH in both deamination and amination reactions are presented in Table 4. The single mutant showed reduced k_{cat} values as compared to those of the double mutant having similar values with the wild-type enzyme. The K_m values of the single mutant for L-isoleucine, L-norleucine, α-keto-*iso*-caproate, and α-keto-β-methylvalerate increased by more than 10 folds as compared to the wild-type. The kinetic parameters of L40K are similar to those obtained with the K89L mutant of GluDH, prepared in a manner opposite to the present studies [31]. Both of the single mutants (L40K of LeuDH and K89L of GluDH) displayed large reductions in the original catalytic activities and failed to reverse the substrate specificities. In the L40K mutant, the positively charged lysine side chain has been introduced, but the hydrophobic valine remains in position 294. The K89L mutant of GluDH has oppositely the L89/S379 pair. Presumably, these incompatible pairings of side chains cannot be stabilized by binding of the normal or alternative substrates.

In marked contrast to the single mutant, the double mutant of LeuDH (L40K/V294S) showed k_{cat} values even similar to those of the wild-type enzyme (except for L-leucine). However, the double mutant had high K_m values for the preferred substrates of LeuDH, like the single mutant. Although the single and double mutants had slight activities toward L-glutamate and α-ketoglutarate, the catalytic rates with the two substrates were unsaturable with their concentrations (data not shown); thus the K_m values for L-glutamate and α-ketoglutarate could not be obtained, and the k_{cat} values, determined with the substrate at 50 mM, shown in Table 4 are a lower limit. The high K_m values of the double mutant for aliphatic substrates are indicative of the increased hydrophilicity of the pocket that is advantageous for binding an acidic substrate, but further fine tuning of the substrate-binding pocket is certainly required to realize the high affinity for L-glutamate.

3.5. Catalytic Properties of L40D and L40D/V294S mutants of LeuDH

We constructed another set of mutant enzymes of LeuDH in order to confer the activity for the substrates with a positively charged side chain (L-lysine and L-arginine). As shown in Table 3, the substrate specificities of the single (L40D) and double (L40D/V294S) mutants for aliphatic amino acids were virtually unaffected as compared with the wild-type enzyme. These mutants, however, displayed entirely new activities for L-lysine and L-arginine, even in the single mutant (L40D), unlike in the case of the L40K mutant described above.

However, the substitution of L40 with aspartate caused marked reductions of k_{cat} values for aliphatic substrates (lower by ~100 folds) compared with those of the wild-type LeuDH (Table 4). Moreover, the K_m values for these preferred substrates increased much; for example, the values for L-leucine, L-isoleucine, and L-norleucine were too high to determine. The large reduction of activities of the mutants for aliphatic substrates seems to be mainly due to the increased K_m values. The k_{cat} values of the single and double mutants for deamination of L-lysine were 0.029 and 0.45 s^{-1}, which were only 0.06 and 0.9%, respectively, of that for L-leucine of the wild-type LeuDH. Nevertheless, the K_m values of the mutants for L-lysine were only one order of magnitude higher than those for aliphatic substrates of the wild-type enzyme. These kinetic properties, together with the CD spectrum of the L40D/V294S mutant, suggest that the hydrophilicity of the substrate side-chain binding pocket, enhanced by the substitutions of L40 by aspartate and V294 by serine, is important in permitting the binding of a substrate with a positively charged side chain such as L-lysine.

4. Conclusions

Enzymes belonging to the amino acid dehydrogenase superfamily have already been used to produce various amino acids in commercially feasible quantities. Understanding the molecular mechanisms for the distinct substrate specificities displayed by these enzymes would enable us to engineer the substrate specificities by site-directed mutagenesis and further facilitate their applications to the enzymatic production or detection of novel amino acids.

In the studies herein reported, we constructed three types of mutant enzymes of LeuDH to mimic the substrate specificities of PheDH, GluDH, and LysDH by protein engineering. The kinetic parameters obtained with these mutants clearly showed that A113, V294, L40, and V294 in LeuDH are the key residues involved in recognition of the substrate side chain. A113 in LeuDH and the corresponding G114 in PheDH control the volume of the side-chain binding pocket and play a critical role in discrimination of the bulkiness of the side chain. Emergence of GluDH and LysDH activities in the L40K/V294S and L40D/V294S mutants, respectively, indicates that the two residues in the active sites of the amino acid dehydrogenases are important for discrimination of the hydrophobicity/polarity/charge of aliphatic substrate side chains.

Acknowledgements

This work was supported by a Grant-in-Aid for Encouragement of Young Scientists (to K.K., No. 07780537) from the Ministry of Education, Science, Sports and Culture of Japan.

References

[1] T. Ohshima, H. Misono and K. Soda, J. Biol. Chem., 253 (1978) 5719

[2] M.W. Zink and B.D. Sanwal, Arch. Biochem. Biophys., 99 (1962) 72

[3] J. Hermier, J.M. Lebeault and C. Zevako, Bull. Soc. Chim. Biol., 52 (1970) 1089

[4] J. Hermier, M. Rosseau and C. Zevako, Ann. Inst. Pasteur Paris, 118 (1970) 611

[5] R.L. Hanson, J. Singh, T.P. Kissick, R.N. Patel, L.J. Szarka and R.H. Mueller, Bioorg. Chem., 18 (1990) 371

[6] A.S. Bommarius, M. Schwarn and K. Drauz, J. Mol. Catal. B: Enzymatic, 5 (1998) 1

[7] K.C. Dooley, Clin. Biochem., 25 (1992) 271

[8] G. Livesey and P. Lund, Methods Enzymol., 166 (1988) 3

[9] A. Yamaguchi, Y. Mizushima, M. Fukushi, Y. Shimizu, Y. Kikuchi and N. Takasugi, Screening, 1 (1992) 49

[10] S.H. Mudd, H.L. Levy and F. Skovby, in C.R. Sciver, A.L. Beudet, W.S.Sly and D. Valle (Ed.), The Metabolic Basis of Inherited Disease, McGraw-Hill, New York, 1989, p. 693

[11] S. Nagata, K. Tanizawa, N. Esaki, Y. Sakamoto, T. Ohshima, H. Tanaka and K. Soda, Biochemistry, 27 (1989) 9056

[12] J.K. Teller, R.J. Smith, M.J. McPherson, P.C. Engel and J.R. Guest, Eur. J. Biochem., 206 (1992) 151

[13] M.J. McPherson and J.C. Wootton, Nucleic Acid Res., 11 (1983) 5257

[14] H. Takada, T. Yoshimura, T. Ohshima, N. Esaki and K. Soda, J. Biochem., 109 (1991) 371

[15] N. Okazaki, Y. Hibino, Y. Asano, M. Ohmori, N. Numao and K. Kondo, Gene, 63 (1988) 337

[16] A. Leiser, A. Birch and J.A. Robinson, Gene (Amst.), 177 (1996) 217

[17] L. Tang and C.R. Hutchinson, J. Bacteriol., 175 (1993) 4176

[18] K.L. Britton, P.J. Baker, P.C. Engel, D.W. Rice and T.J. Stillman, J. Mol. Biol., 234 (1993) 938

[19] S.E.H. Syed, P.C. Engel and D.M. Parker, Biochim. Biophys. Acta, 1115 (1991) 123

[20] T. Ohshima, N. Nishida, S. Bakthavatsalam, K. Kataoka, H. Takada, T. Yoshimura, N. Esaki and K. Soda, Eur. J. Biochem., 222 (1994) 305

[21] N.D. Priestley and J.A. Robinson, Biochem. J., 261 (1989) 853

[22] Y. Asano, A. Nakazawa and K. Endo, J. Biol. Chem., 262 (1987) 10346

[23] T. Ohshima, H. Takada, T. Yoshimura, N. Esaki and K. Soda, J. Bacteriol., 173 (1991) 3943

[24] P.J. Baker, K.L. Britton, P.C. Engel, G.W. Farrants, K.S. Lilley, D.W. Rice and T.J. Stillman, Proteins, 12 (1992) 75

[25] P.J. Baker, A.P. Turnbull, S.E. Sedelnikova, T.J. Stillman and D.W. Rice, Structure, 3 (1995) 693

[26] J.L. Vanhooke, J.B. Thoden, N.M.W. Brunhuber, J.S. Blanchard and H.M. Holden, Biochemistry, 38 (1999) 2326

[27] K. Kataoka, H. Takada, K. Tanizawa, T. Yoshimura, N. Esaki, T. Ohshima and K. Soda, J. Biochem., 116 (1994) 931

[28] T. Oikawa, K. Kataoka, Y. Jin, S. Suzuki and K. Soda, Biochem. Biophys. Res. Commun., 280 (2001) 1177

[29] T.J. Stillman, P.J. Baker, K.L. Britton and D.W. Rice, J. Mol. Biol., 234 (1993) 1131

[30] P.J. Baker, M.L. Waugh, X.-G. Wang, T.J. Stillman, A.P. Turnbull, P.C. Engel and D.W. Rice, Biochemistry, 36 (1997) 16109

[31] X.-G. Wang, K.L. Britton, P.J. Baker, S. Martin, D.W. Rice and P.C. Engel, Protein Eng., 8 (1995) 147

[32] Y.K. Seah, K.L. Britton, P.J. Baker, D.W. Rice, Y. Asano and P.C. Engel, FEBS Lett., 370 (1995) 93

[33] Y.K. Seah, K.L. Britton, D.W. Rice, Y. Asano and P.C. Engel, Biochemistry, 41 (2002) 11390

[34] T.A. Kunkel, J.D. Roberts and R.A. Zakour, Methods Enzymol., 154 (1987) 367

[35] K. Kataoka, K. Tanizawa, T. Fukui, H. Ueno, T. Yoshimura, N. Esaki and K. Soda, J. Biochem., 116 (1994) 1370

[36] K. Kataoka, M. Nakai, K. Yamaguchi and S. Suzuki, Biochem. Biophys. Res. Commun., 250 (1998) 409

[37] T. Sekimoto, T. Fukui and K. Tanizawa, J. Biochem., 116 (1994) 176

Figure legends

Fig. 1. Sequence alignment of amino acid dehydrogenases using structural information from *B. sphaericus* LeuDH and *C. symbiosum* GluDH. Residues L40, A113, V291, and V294 of *B. stearothermophilus* LeuDH and their corresponding residues are shown with white letters in black background.

Fig. 2. CD spectra of the wild-type and mutant enzymes of LeuDH. The CD spectra were recorded at 25 ºC in 10 mM potassium phosphate buffer (pH 7.2). (A) Spectra of the wild-type (solid line), A113G (broken line), and A113G/V291L (dotted line) mutant enzymes. (B) Spectra of the wild-type (solid line), L40K (broken line), and L40K/V294S (dotted line) mutant enzymes. (C) Spectra of the wild-type (solid line), L40D (broken line), and L40D/V294S (dotted line) mutant enzymes. The unit of [θ] is $\mathrm{mdeg \cdot cm^2 \cdot dmol^{-1}}$.

Table 1. Substrate specificities of wild-type and mutant enzymes

Substrate (10 mM)	Relative activity (%)[a]				
	LeuDH			PheDH	
	Wild type	A113G	AG/V291L	Wild type	G114A
Deamination					
L-Leucine	100	100	100	3.9	42
L-Isoleucine	54	550	150	0.4	90
L-Valine	39	62	13	1.3	39
L-Norleucine	14	530	110	6.3	66
L-Norvaline	56	110	23	2.1	72
L-Methionine	0.7	81	19	2.2	72
L-Ethionine	0	35	12	4.0	11
L-Alanine	0	0	0	0.4	3.5
L-Phenylalanine	0	15	11	100	100
L-Tyrosine	0	0	0	40	5.4
L-Tryptophan	0	0	0	1.2	0
L-Histidine	0	0.4	0	0.2	1.3
L-Glutamate	0	0	0	0	0
Amination					
α-Keto-*iso*-caproate	100	100	100	47	151
α-Keto-β-methylvalerate	100	180	67	16	174
α-Ketovalerate	86	102	19	37	224
α-Keto-γ-methylthiobutyrate	15	130	36	55	102
α-Ketobutyrate	47	5.1	0.6	5.5	104
Phenylpyruvate	0	17	11	100	100
p-Hydroxyphenylpyruvate	0	0	0	80	0

[a] Specific activities of the wild-type and mutant enzymes of LeuDH and PheDH for various substrates are shown as % activities relative to the values for L-leucine and L-phenylalanine, respectively, taken as 100.

Table 2. Steady-state kinetic parameters of wild-type and mutant enzymes[a]

	LeuDH	A113G	A113G/V291L	PheDH	G114A
k_{cat} (s^{-1})					
L-Leucine	50 ± 3.0	2.8 ± 0.08	0.74 ± 0.010	0.20 ± 0.006	0.12 ± 0.002
L-Isoleucine	28 ± 0.77	23 ± 0.53	1.4 ± 0.037	0.24 ± 0.02	0.22 ± 0.006
L-Norleucine	1.3 ± 0.03	18 ± 0.04	0.60 ± 0.018	1.9 ± 0.005	0.16 ± 0.004
L-Norvaline	13 ± 0.15	7.7 ± 0.19	0.29 ± 0.008	0.59 ± 0.006	0.21 ± 0.004
L-Phenylalanine	0	0.80 ± 0.004	1.4 ± 0.049	23 ± 0.27	0.97 ± 0.012
α-Keto-*iso*-caproate	280 ± 22	94 ± 1.9	29 ± 0.35	36 ± 0.49	37 ± 0.48
α-Keto-β-methyl valerate	280 ± 4.1	235 ± 5.2	37 ± 0.52	26 ± 0.22	46 ± 0.24
α-Ketocaproate	110 ± 2.4	140 ± 2.8	24 ± 0.91	79 ± 1.1	61 ± 0.49
Phenylpyruvate	0	22 ± 0.70	9.9 ± 1.2	200 ± 1.3	36 ± 0.46
K_m (mM)					
L-Leucine	5.1 ± 0.50	1.4 ± 0.10	35 ± 2.5	0.12 ±0.006	0.082 ± 0.002
L-Isoleucine	2.4 ± 0.15	7.8 ± 0.49	33 ± 1.8	0.081 ± 0.007	0.082 ± 0.003
L-Norleucine	4.4 ± 0.21	4.1 ± 0.026	24 ± 1.6	0.19 ± 0.002	0.17 ± 0.008
L-Norvaline	7.8 ± 0.29	27 ± 1.8	70 ± 8.6	0.50 ± 0.016	0.18 ± 0.006
L-Phenylalanine	—	31 ± 1.2	66 ± 7.1	0.10 ± 0.003	16 ± 0.42
α-Keto-*iso*-caproate	0.88 ± 0.15	1.7 ± 0.11	30 ± 2.7	4.4 ± 0.19	4.1 ± 0.16
α-Keto-β-methyl valerate	3.8 ± 0.16	9.4 ± 0.59	69 ± 5.1	12 ± 0.45	5.2 ± 0.067
α-Ketocaproate	4.7 ± 0.34	2.0 ± 0.10	28 ± 2.9	2.5 ± 0.11	4.4 ± 0.11
Phenylpyruvate	—	7.1 ± 1.1	9.9 ± 1.2	0.065 ± 0.003	11 ± 0.47
NAD^+ (μM)	63 ± 7	76 ± 1	280 ± 13	170 ± 10	50 ± 20
NADH (μM)	35 ± 6	42 ± 2	17 ± 7	83 ± 1	27 ± 7

[a] Steady-state kinetic parameters were determined by varying the concentration of the substrate to be measured in the presence of fixed concentrations of the cofactor and co-substrate (1.25 mM NAD^+ in the oxidative deamination and 0.1 mM NADH and 1 M ammonia in the reductive amination).

Table 3. Substrate specificities of wild-type and mutant enzymes of LeuDH

Substrate (10 mM)	Relative activity (%)[a]				
	Wild type	L40K	L40K/V294S	L40D	L40D/V291S
Deamination					
L-Leucine	100	100	100	100	100
L-Isoleucine	54	273	187	212	358
L-Valine	39	81	26	64	45
L-Norleucine	14	28	17	35	27
L-Norvaline	56	29	24	37	33
L-Methionine	0.7	2.6	3.6	3.0	4.2
L-Ethionine	0	0.5	0.2	0	0
L-Alanine	0	0.04	0	0	0
L-Phenylalanine	0	0	0	0	0
L-Histidine	0	0.02	0	0	0
L-Asparagine	0	0	0.1	0	0
L-Glutamine	0	0	0.4	0	0
L-Aspartate	0	0	0.04	0	0
L-Glutamate	0	0	0.1	0	0
L-Lysine	0	0	0	9.2	38
L-Arginine	0	0	0	1.8	1.1
Amination					
α-Keto-*iso*-caproate	100	100	100	100	100
α-Keto-β-methylvalerate	100	86	58	92	63
α-Ketovalerate	86	ND[b]	30	52	ND[b]
α-Ketobutyrate	47	ND[b]	2.7	6.1	ND[b]

[a] Specific activities of the wild-type and mutant enzymes of LeuDH for various substrates are shown as % activities relative to the values for L-leucine, taken as 100.

[b] Not determined.

Table 4. Steady-state kinetic parameters of wild-type and mutant enzymes of LeuDH[a]

	Wild type	L40K	L40K/V294S	L40D	L40D/V291S
k_{cat} (s^{-1})					
L-Leucine	50 ± 3.0	3.8 ± 0.038	9.8 ± 0.054	0.21[b]	0.80[b]
L-Isoleucine	28 ± 0.77	1.8 ± 0.41	17 ± 0.46	0.49[b]	2.8[b]
L-Norleucine	1.3 ± 0.03	1.9 ± 0.04	1.7 ± 0.03	0.070[b]	0.22[b]
L-Glutamate	0	0	0.02[b]	0	0
L-Lysine	0	0	0	0.029 ± 0.001	0.45 ± 0.03
α-Keto-*iso*-caproate	280 ± 22	95 ± 1.7	270 ± 5.9	9.9 ± 0.051	47 ± 0.50
α-Keto-β-methyl valerate	280 ± 4.1	82 ± 1.6	280 ± 3.5	1.9 ± 0.024	30 ± 0.47
α-Ketoglutarate	0	0	0.57[b]	0	0
K_m (mM)					
L-Leucine	5.1 ± 0.50	5.2 ± 3.7	26 ± 0.52	ND[c]	ND[c]
L-Isoleucine	2.4 ± 0.15	64 ± 1.8	24 ± 1.5	ND[c]	ND[c]
L-Norleucine	4.4 ± 0.21	110 ± 3.0	27 ± 1.1	ND[c]	ND[c]
L-Glutamate	–	–	ND[c]	–	–
L-Lysine	–	–	–	64 ± 3.9	72 ± 5.0
α-Keto-*iso*-caproate	0.88 ± 0.15	46 ± 3.2	42 ± 3.3	260 ± 22	120 ± 11
α-Keto-β-methyl valerate	3.8 ± 0.16	49 ± 3.8	84 ± 6.7	47 ± 2.4	110 ± 14
α-Ketoglutarate	–	–	ND[c]	–	–

[a] Steady-state kinetic parameters were determined by varying the concentration of the substrate to be measured in the presence of fixed concentrations of the cofactor and co-substrate (1.25 mM NAD^+ in the oxidative deamination and 0.1 mM NADH and 1 M ammonia in the reductive amination).

[b] Determined for the substrate at 50 mM.

[c] Not determined due to the catalytic rates,being unsaturable with the substrate concentrations.

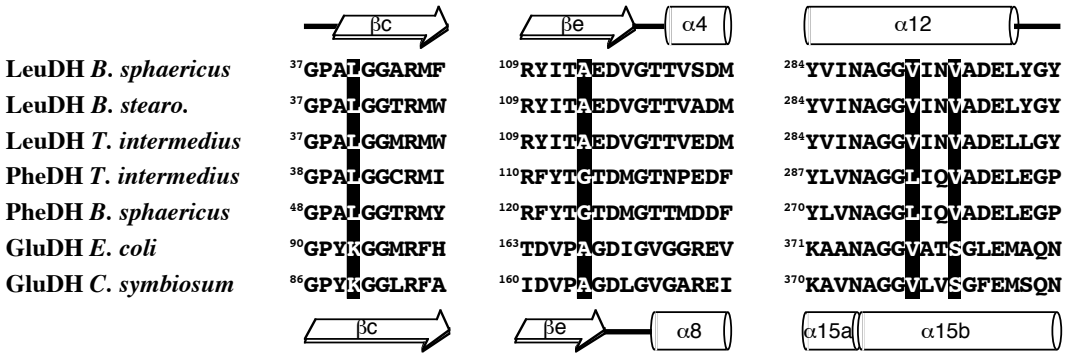
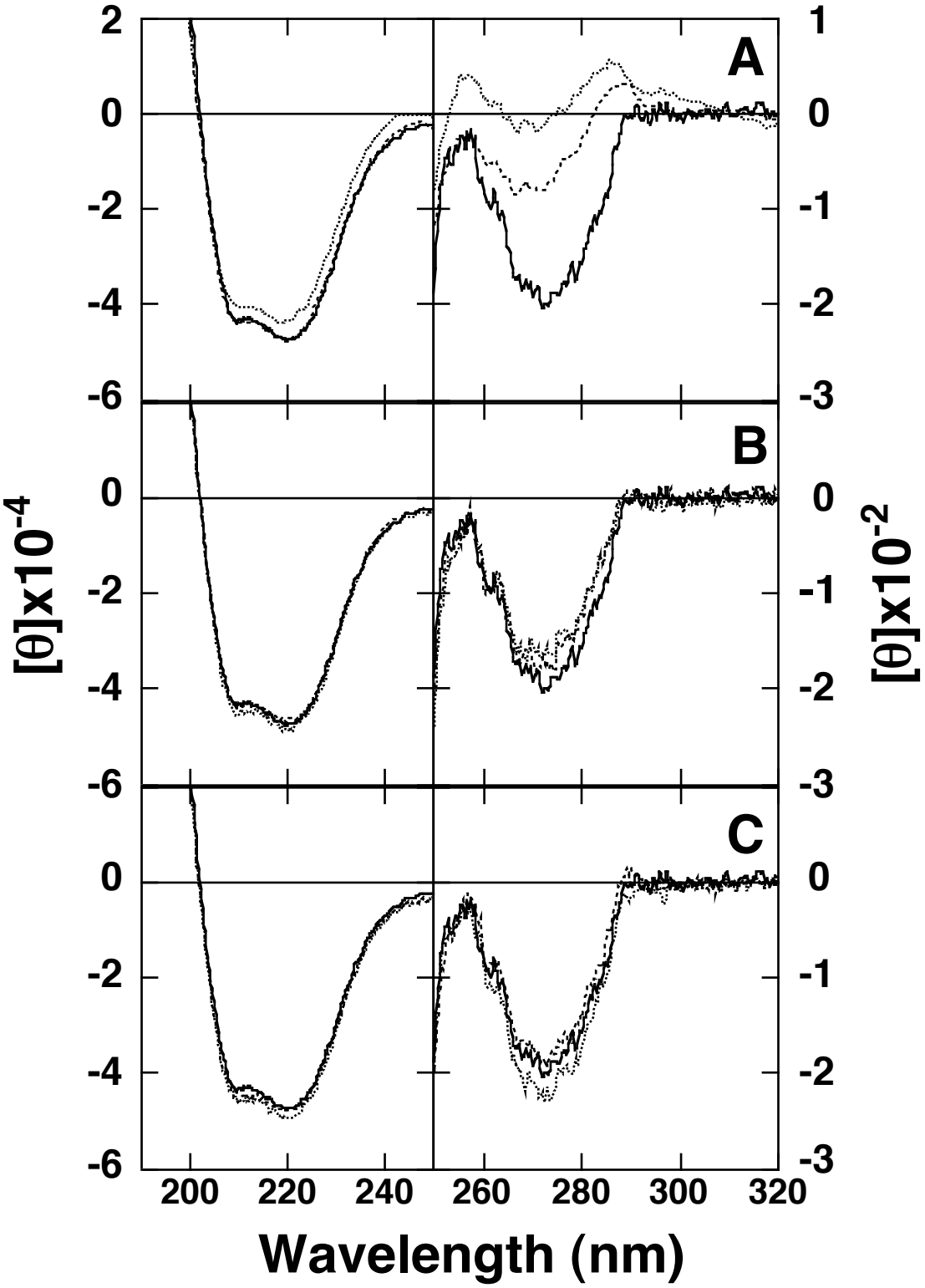

βc
βe
α4
α12
LeuDH B. sphaericus 37GPALGGARMF 109RYITAEDVGTTVSDM 284YVINAGGVINVADELYGY
LeuDH B. stearo. 37GPALGGTRMW 109RYITAEDVGTTVADM 284YVINAGGVINVADELYGY
LeuDH T. intermedius 37GPALGGMRMW 109RYITAEDVGTTVEDM 284YVINAGGVINVADELLGY
PheDH T. intermedius 38GPALGGCRMI 110RFYTGTDMGTNPEDF 287YLVNAGGLIQVADELEGP
PheDH B. sphaericus 48GPALGGTRMY 120RFYTGTDMGTTMDDF 270YLVNAGGLIQVADELEGP
GluDH E. coli 90GPYKGGMRFH 163TDVPAGDIGVGGREV 371KAANAGGVATSGLEMAQN
GluDH C. symbiosum 86GPYKGGLRFA 160IDVPAGDLGVGAREI 370KAVNAGGVLVSGFEMSQN
βc
βe
α8
α15a
α15b

Fig.1


A
B
C
[θ]x10-4
[θ]x10-2
Wavelength (nm)
2
0
-2
-4
-6
1
0
-1
-2
-3
200
220
240
260
280
300
320