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Kinetic analysis of phenylalanine dehydrogenase mutants designed for aliphatic amino acid dehydrogenase activity with guidance from homology-based modelling

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Through comparison with the high-resolution structure of *Clostridium symbiosum* glutamate dehydrogenase, the different substrate specificities of the homologous enzymes phenylalanine dehydrogenase and leucine dehydrogenase were attributed to two residues, glycine 124 and leucine 307, in *Bacillus sphaericus* phenylalanine dehydrogenase, which are replaced with alanine and valine in leucine dehydrogenases [Britton, K.L., Baker, P.J., Engel, P.C., Rice, D.W. & Stillman, T.J. (1993) *J. Mol. Biol.* **234**, 938–945]. As predicted, making these substitutions in phenylalanine dehydrogenase decreased the specific activity towards aromatic substrates and enhanced the activity towards some aliphatic amino acids in standard assays with fixed concentrations of both substrates [Seah, S.Y.K., Britton, K.L., Baker, P.J., Rice, D.W., Asano, Y. & Engel, P.C. (1995) *FEBS Lett.* **370**, 93–96]. This study did not, however, distinguish effects on affinity from those on maximum catalytic rate. A fuller kinetic characterization of the single- and double-mutant enzymes now reveals that the extent of the shift in specificity was underestimated in the earlier study. The maximum catalytic rates for aromatic substrates are reduced for all the mutants, but, in addition, the apparent K_m values are higher for the single-mutant G124A and double-mutant G124A/L307V compared with the wild-type enzyme. Conversely, specificity constants (k_{cat}/K_m) for the nonpolar aliphatic amino acids and the corresponding 2-oxoacids for the mutants are all markedly higher than for the wild type, with up to a 40-fold increase for L-norvaline and a 100-fold increase for its 2-oxoacid in the double mutant. In some cases a favourable change in K_m was found to outweigh a smaller negative change in k_{cat}. These results emphasize the risk of misjudging the outcome of protein engineering experiments through too superficial an analysis. Overall, however, the success of the predictions from molecular modelling indicates the usefulness of this strategy for engineering new specificities, even in advance of more detailed 3D structural information.

Keywords: homology modelling; kinetic properties; phenylalanine dehydrogenase; site-directed mutagenesis; substrate specificity.

Solution of the first high-resolution structure for an amino acid dehydrogenase, glutamate dehydrogenase (GluDH) from *Clostridium symbiosum* [1,2], was followed soon after by the realization that this enzyme belongs to a wider family of homologous enzymes that catalyse the reversible oxidative deamination of various amino acids to the corresponding 2-oxoacids. Members of this family are categorized according to their preferred amino acid substrate. In general, GluDHs (EC 1.4.1.2–4) have narrow amino acid substrate specificity. GluDH from *C. symbiosum*, for example, accepts poorly the alternative substrates L-norvaline, which has the same number of carbon atoms as L-glutamate but lacks the terminal carboxyl group, or L-aspartate, one carbon shorter than L-glutamate but still possessing a terminal carboxyl group [3]. Leucine dehydrogenases (LeuDHs) have somewhat wider specificity [4,5] and react with various nonpolar aliphatic amino acids but not aromatic amino acids. Phenylalanine dehydrogenases (PheDHs) (EC 1.4.1.20), on the other hand, use aromatic amino acids as preferred substrates, although they also exhibit some activity with nonpolar aliphatic amino acids [6,7].

The sequence identity between PheDH(s) and LeuDH(s) is generally at the level of $\approx 50\%$, whilst the identity between either PheDH or LeuDH and GluDH is only $\approx 20\%$ [8]. Despite the latter low figure, structure-based sequence alignment and molecular modelling studies [8] strongly suggested that the three groups of enzymes share a similar folded structure. Key features of the active site were conserved in all three, encouraging the view [8] that, even

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Abbreviations: GluDH, glutamate dehydrogenase; LeuDH, leucine dehydrogenase; PheDH, phenylalanine dehydrogenase; ValDH, valine dehydrogenase.

Enzymes: glutamate dehydrogenase (EC 1.4.1.2); phenylalanine dehydrogenase (EC 1.4.1.20); leucine dehydrogenase (EC 1.4.1.9).

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with only 20% sequence identity, it might be possible to use molecular modelling based on the GluDH structure to predict target residues for site-directed mutagenesis in PheDH and LeuDH in advance of definitive structure solutions for the individual enzymes. High-resolution structures have subsequently been solved for both a PheDH [9] and a LeuDH [10], confirming the prediction of a high degree of essentially conserved tertiary structure across the enzyme family, but it is important to bear in mind that these structures were not available to guide construction [11,12] of the mutants discussed in this report, which should therefore be seen as a test of the effectiveness of homology-based molecular modelling.

The sequence alignments [8] indicate that the glycine residues which shape the active site (G122 and G123), orient functional groups at the active site (G90 and G91) and lie close to the binding site for the coenzyme nicotinamide ring (G376) in *C. symbiosum* GluDH [1], are all conserved in the other two groups of enzymes. Likewise, the lysine residue that interacts with the α-carboxyl group of the substrate (K113), and the aspartate (D165) and lysine (K125), thought to be catalytic residues [2], are also conserved. On the other hand, crucially, Lys89 and Ser380, the residues in GluDH that recognize the side-chain γ-carboxyl group of the substrate glutamate [2], are replaced with the same two hydrophobic residues, leucine and valine, respectively, in both LeuDH and PheDH [8].

The key difference between the substrate binding sites of PheDH and LeuDH seems to lie in the residues corresponding to positions V377 and A163 of *C. symbiosum* GluDH [10]. These are unchanged in LeuDH, but in PheDH leucine replaces valine in the first position, and the replacement of an alanine residue with glycine in the second position, on the opposite side of the binding pocket, is thought to create a deeper binding pocket for access of substrates with large aromatic side-chains. More recent sequence determinations support this proposal, with LeuDH from *Thermoactinomyces intermedius* and *Bacillus cereus* [13,14], and valine dehydrogenase (ValDH) [15–17], another member of the family that accepts aliphatic amino acid substrates, all having valine and alanine at the respective positions, whereas leucine and glycine are present in the PheDH from *B. badius* [18,19] and *Sporosarcina ureae* [20] (Fig. 1). One exception is the PheDH from *Rhodococcus* sp. M4 [23], where alanine is found corresponding to position 377 of the *C. symbiosum* GluDH instead of leucine. This is the PheDH for which a solved structure is now available [9], and it is therefore relevant to note that the amino acid sequence identity between the *Rhodococcus* enzyme and PheDH from the other sources listed is only ≈ 30%, as opposed to sequence identities of ≥ 50% among the other PheDHs. Among the differences between the *Rhodococcus* enzyme and the other PheDHs are several other key residues which we predict would result in altered specificity [12,24].

The modelling predictions, discussed above, underpinned mutagenic experiments with PheDH of *B. sphaericus* [11,12] in which Gly124 and Leu307, corresponding to positions 163 and 377 in *C. symbiosum* GluDH, were replaced with alanine and valine. A preliminary study [12] showed that these substitutions do indeed shift substrate specificity towards that of LeuDH. However, this initial report was based only on a superficial analysis employing standard assays at single, fixed, high concentrations of both the amino acid substrate and the coenzyme, NAD^+. The protein engineering, although evidently at least partially successful, was based, as is the case for most similar exercises in the manipulation of substrate specificity, on the simplistic premise that improving the chances of binding the substrate should also improve the chances of catalysis. This assumption overlooks the facts that binding, even at the active site, may not necessarily be productive [25], and that the enzyme needs not only to bind the substrate, but also to release the cognate product. Simple assays under fixed conditions, with high concentrations of all substrates, are unlikely to reveal the full picture and certainly cannot show whether apparent success mainly reflects enhanced binding or improved catalysis in the substrate-saturated state. Conversely, an apparent failure, as measured at a high substrate concentration, may mask a major improvement in catalytic efficiency through improved binding apparent at low substrate concentrations. Accordingly, the roles of the two residues are now assessed in greater detail in the light of a detailed steady-state kinetic analysis, explaining some surprising aspects of the earlier findings [12].

Materials and methods

Purification of wild-type and mutant enzymes

The methods for construction of two single mutants of *B. sphaericus* PheDH (G124A and L307V) and the double mutant G124A/L307V, and also for the purification of wild-type and mutant enzymes, were as previously described [12] and provided all four proteins in a homogeneous state, as judged by SDS/PAGE.

Molecular weight determination

The molecular weights of the native enzymes were determined by FPLC gel filtration (LCC-500; Pharmacia) on a Superose 6 HR 10/30 column (Pharmacia) calibrated with apoferritin (M_r = 443 000), *C. symbiosum* GluDH (M_r = 295 000), sweet potato β-amylase (M_r = 200 000), rabbit muscle aldolase (M_r = 158 000), bovine albumin (M_r = 68 000) and hen egg albumin (M_r = 45 000) and eluted at a flow rate of 0.5 mL·min^{-1} with 50 mM phosphate buffer, pH 7.9, containing 0.15 M NaCl.

Enzyme assay

L-amino acids and 2-oxoacids were obtained from Sigma. NAD^+ (Grade II, free acid) and NADH (Grade I, disodium salt) were from Boehringer Mannheim.

Oxidative deamination was assayed at 25 °C by measuring the reduction of NAD^+ spectrophotometrically at 340 nm in a 1-cm cuvette. The reaction mixture [6] contained, in a total volume of 1 mL, 50 μmol glycine/KOH buffer, pH 10.4, 100 μmol KCl, 2.5 μmol NAD^+, varying amounts of L-amino acid substrate and the appropriate concentration of enzymes. For reductive amination reactions, also at 25 °C, the disappearance of NADH was monitored at 340 nm in a 1-mL mixture containing

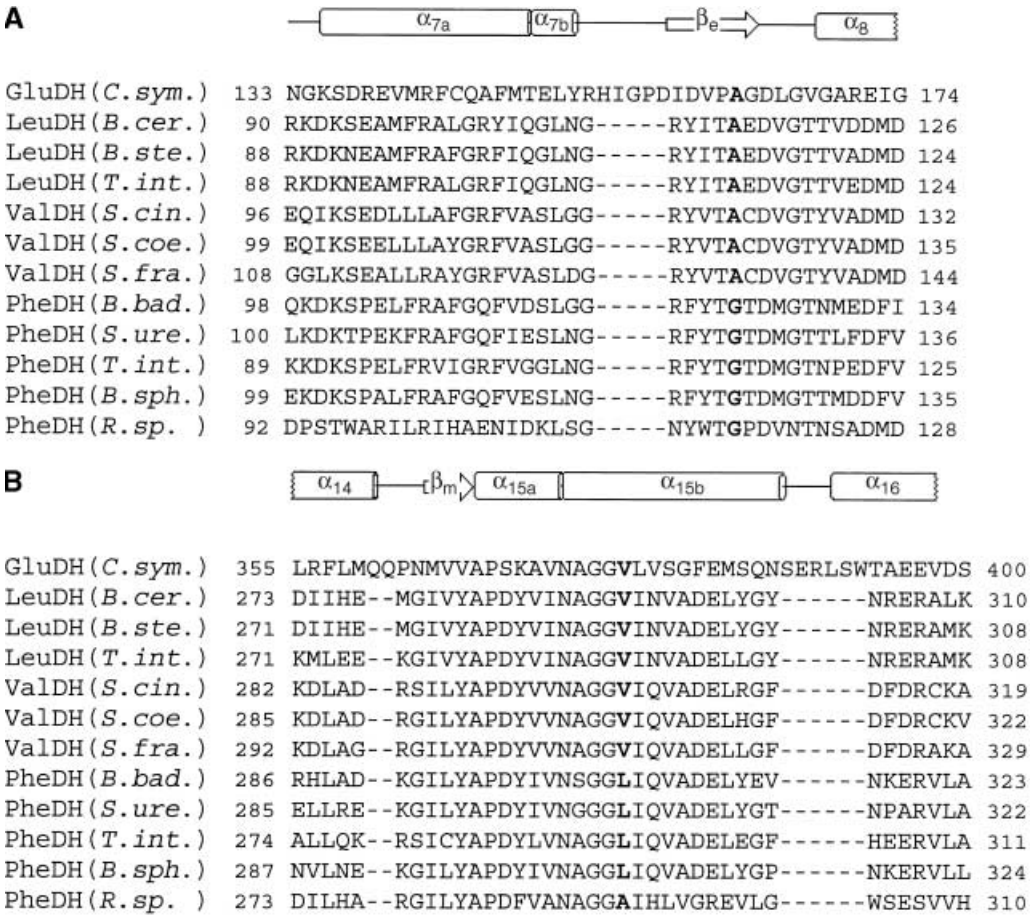

Fig. 1. Sequence comparisons of amino acid dehydrogenases belonging to the glutamate dehydrogenase (GluDH) superfamily. Residues corresponding to positions 163 and 377 of *Clostridium symbiosum* GluDH, thought to be responsible for the differences in substrate specificity between phenylalanine dehydrogenase (PheDH) and leucine dehydrogenase (LeuDH), are shown in bold in A and B, respectively. The secondary structure elements from the 3D structure of *C. symbiosum* GluDH [1,2] are shown above the aligned sequences, with helices represented as cylinders and strands as arrows. Residue numbers are shown at the beginning and end of each stretch of sequence. Sequences shown are GluDH, LeuDH, valine dehydrogenase (ValDH) and PheDH. The sources of the enzymes from which the sequences were obtained are given in parenthesis, with *C. sym.*, *B.cer.*, *B.ste.*, *T.int.*, *S.cin.*, *S.coe.*, *S.fra.*, *B.bad.*, *S.ure.*, *B.sph.* and *R.sp.* indicating *C. symbiosum* [21], *Bacillus cereus* [14], *B. stearothermophilus* [5], *Thermoactinomyces intermedius* [13], *Streptomyces cinnamonensis* [22], *S. coelicolor* [16], *S. fradiae* [17], *B. badius* [18], *S. ureae* [20], *B. sphaericus* [7] and *Rhodococcus* sp. M4 [23], respectively.

50 μmol glycine/KOH, 100 μmol KCl, 0.1 μmol NADH, 400 μmol ammonium chloride, varying amounts of 2-oxoacids and appropriate amounts of enzyme at a pH of 8.6. At least a 10-fold range of concentrations of amino acid or oxoacid substrates was used, with a minimum of three different concentrations both above and below the K_m value. One unit of enzyme was the amount that catalysed the formation or disappearance of 1 μmol NADH min^{-1}. The steady-state kinetic parameters, K_m and k_{cat}, were determined by the method of Wilkinson [26] using the program ENZPACK version 3.0 (Biosoft).

Results and Discussion

The wild-type and mutant polypeptides (G124A, L307V and G124A/L307V) displayed identical mobilities on SDS/PAGE, indicating an M_r of 39 000. All four proteins also gave the same retention time on the gel-filtration column, corresponding to an M_r of 380 000, as determined from the standard curve. The substitution of residues in the mutant enzymes therefore did not disrupt the native oligomeric structure.

Wild-type PheDH and the three mutants all showed simple Michaelis-Menten behaviour in both directions of reaction, allowing determination of apparent k_{cat} and K_m values for each amino or oxoacid substrate at close to saturating concentrations of the other substrate(s). Detailed studies of the oxidative deamination reaction [12], with systematic variation of the concentrations of both substrates to allow evaluation of the constants in the full initial-rate equation, suggest that this enzyme follows a rapid-equilibrium random-order mechanism under the conditions used in this study. For such a mechanism, the extrapolated K_m for the amino acid substrate at saturating concentrations of coenzyme is equal to the dissociation constant for the amino acid leaving the ternary enzyme–coenzyme–amino acid

complex. In what follows therefore, the K_m values for amino acids, although not extrapolated to infinite coenzyme concentration, have been taken as approximate indicators of the affinity of the enzyme–coenzyme complex for the amino acid substrate.

Under standard assay conditions, the specific activities of the three mutant PheDHs towards the aromatic substrates L-phenylalanine and L-tyrosine were previously shown to be substantially diminished compared with the wild-type enzyme [12]. From Tables 1 and 2, these reduced activities can now be seen to reflect lower k_{cat} values in all the mutants (four- to sixteenfold lower for L-phenylalanine), and, in the case of the G124A and double mutant, an increased K_m (six- to eightfold for L-phenylalanine) for these substrates also. Specificity constants (k_{cat}/K_m) for the aromatic substrates L-phenylalanine, L-tyrosine and the corresponding substrates for the reverse reaction, phenylpyruvate and hydroxyphenylpyruvate, were decreased for all mutants. A 120-fold reduction for L-phenylalanine was observed for the G124A mutant and an 87-fold reduction in the case of the double mutant. These factors are much larger than the changes in specific activity reported previously [12], because the standard assay, deliberately employing high concentrations of both substrates, is insensitive to changes in K_m. The K_m values for L-tyrosine and its oxoacid are too high to be measured for either the G124A mutant or the double mutant. However, the specificity constant can be determined and is found to be lowered 450- to 700-fold compared with the wild-type enzyme.

In the L307V mutant, by contrast, K_m values for L-phenylalanine (0.53 mM) and phenylpyruvate (0.22 mM) are actually lower than for the wild-type enzyme (1.1 mM for L-phenylalanine and 0.37 mM for phenylpyruvate), although the k_{cat} values of this mutant for these substrates are also lower. It seems that the extra space created by the Leu → Val substitution results in readier binding of the aromatic substrate, but that this binding is not productive and therefore is not translated into a higher maximum catalytic rate.

All the mutants had higher specificity constants (k_{cat}/K_m) than the wild type for aliphatic substrates, the most dramatic result being for the double mutant, which showed a 40-fold increase for the substrate norvaline and almost a 100-fold increase for the oxoacid in the reverse reaction. It was previously reported that the L307V mutant showed increased specific activities only for L-valine and L-isoleucine [12]. This reflects the fact that, of the amino acids tested, these are the only two for which there is an increase in k_{cat}. However, the present, more detailed kinetic analysis also reveals that in this mutant the specificity constants (k_{cat}/K_m) for all the other aliphatic amino acids tested are higher than the wild-type values. The unfavourable changes in the catalytic rate constant are more than outweighed by favourable changes in K_m. Clearly, again, the previous analysis of specific activities alone, using a fixed high concentration of substrate, fails to take into account these decreases in K_m, which are in some cases 10–20-fold. Thus, the k_{cat} for L-leucine and its oxoacid analogue, α-ketoisocaproate, L-norleucine and its oxoacid analogue, α-ketocaproate, and L-norvaline, are all lower than with the wild-type enzyme, but the K_m values for these substrates are also substantially lowered, resulting in overall specificity constants considerably higher than for the wild type.

For L-norleucine and L-methionine, which have long straight side-chains, and for their oxoacid analogues, the K_m in the case of the G124A mutant is higher than with the L307V or the double mutant. The presence of alanine at the bottom of the active site cleft in the G124A mutant may cause steric repulsion with the straight chain compounds. In the double mutant, substitution of leucine with the shorter valine at the side of the active site might relieve this steric strain by allowing the substrate to move laterally in the binding pocket.

Unsurprisingly, there are differences in the kinetic constants for the other aliphatic substrates with each of the mutant enzymes, these differences residing most obviously in the K_m values for the amino acid rather than the k_{cat}. In an analogous manner, native LeuDH from *B. sphaericus* [27], *T. intermedius* [13], *B. cereus* [28] and *Corynebacterium pseudodiphtheriticum* [29] show similar differences in specificity for leucine, isoleucine and other aliphatic amino acid substrates. In theory, these differences must all be ultimately explicable in terms of the protein 3D structure. However, differential substrate specificity may well involve a second shell of residues surrounding the surface of the active site, and these in the present case would interact with residues 124 and 307 in determining the overall shape of the substrate-binding pocket [30]. Such subtle modulation of the substrate-binding site cannot be reliably predicted by homology modelling.

Inspection of the K_m values in the wild-type enzyme for the aliphatic amino acid substrates reveals, surprisingly, that K_m values for L-leucine (0.55 mM) and L-isoleucine (0.14 mM) are lower than for L-phenylalanine, which is assumed to be the major natural substrate (1.1 mM). It seems that PheDHs have greater affinity for L-leucine and L-isoleucine than for L-phenylalanine, but binding is less productive, so that the k_{cat} is ≈ 100 times lower for leucine than for phenylalanine, and a further factor of five lower for isoleucine. The K_m values for the corresponding oxoacids, α-ketoisocaproate and α-keto-β-methyl-n-valerate were, however, higher than for phenylpyruvate. These differences in trends between the forward and reverse reactions have also been observed for PheDH from *T. intermedius* [13]. Although the side-chains of the amino acid and its oxoacid analogue are the same, the difference in substitution of the α-carbon, with a positively charged amino group in one case and an oxo group in the other, must account for these differences.

There have been two previous attempts at switching the substrate specificity of PheDH to LeuDH [31,32]. In the first case the codons encoding a hexapeptide in PheDH of *T. intermedius* were replaced with the codons encoding the corresponding sequence of LeuDH from *B. stearothermophilus*. In a second case, a chimaeric enzyme containing the N-terminus of *T. intermedius* PheDH and the C-terminus of *B. stearothermophilus* PheDH was constructed. In both cases the substrate specificity profile was indeed altered. However, in the first example the catalytic efficiency of the resultant mutant enzyme was reduced for all amino acid substrates. In both studies, moreover, the native hexameric structure was disrupted, giving, in one instance, a dimeric enzyme and, in the other, an equilibrium mixture between

Table 1. Apparent K_m and k_{cat} values of wild-type and mutant phenylalanine dehydrogenase (PheDH) for various amino acid substrates. The concentration of the coenzyme NAD^+ was fixed at 2.5 mM. ND, K_m too large to be determined.

Substrate	Wild type			L307V			G124A			G124A/L307V		
	K_m ± SEM (mM)	k_{cat} ± SEM (s^{-1})	k_{cat}/K_m ($s^{-1}·mM^{-1}$)	K_m ± SEM (mM)	k_{cat} ± SEM (s^{-1})	k_{cat}/K_m ($s^{-1}·mM^{-1}$)	K_m ± SEM (mM)	k_{cat} ± SEM (s^{-1})	k_{cat}/K_m ($s^{-1}·mM^{-1}$)	K_m ± SEM (mM)	k_{cat} ± SEM (s^{-1})	k_{cat}/K_m ($s^{-1}·mM^{-1}$)
L-Phenylalanine	1.1 ± 0.070	68 ± 1.4	61.8	0.53 ± 0.039	18 ± 0.45	34	8.1 ± 0.48	4.2 ± 0.11	0.52	7.2 ± 0.81	5.1 ± 0.25	0.71
L-Tyrosine	1.3 ± 0.10	68 ± 2.1	52.3	0.49 ± 0.014	21 ± 0.17	43	ND	ND	0.11[a]	ND	ND	0.072[a]
L-Leucine	0.55 ± 0.066	0.70 ± 0.026	1.3	0.025 ± 0.0011	0.12 ± 0.0013	4.8	0.057 ± 0.0039	1.0 ± 0.021	18	0.046 ± 0.0033	1.1 ± 0.023	24
L-Isoleucine	0.14 ± 0.023	0.14 ± 0.009	1.0	0.19 ± 0.024	0.27 ± 0.016	1.4	0.11 ± 0.014	1.1 ± 0.042	10	0.11 ± 0.0080	2.3 ± 0.063	21
L-Norleucine	0.79 ± 0.069	3.2 ± 0.054	4.0	0.077 ± 0.0052	1.3 ± 0.029	17	0.096 ± 0.010	1.6 ± 0.063	17	0.088 ± 0.0087	2.2 ± 0.072	25
L-Valine	2.0 ± 0.10	0.91 ± 0.020	0.46	2.2 ± 0.19	1.4 ± 0.048	0.64	0.13 ± 0.012	0.97 ± 0.030	7.5	0.27 ± 0.019	1.9 ± 0.044	7.0
L-Norvaline	3.3 ± 0.22	1.4 ± 0.038	0.42	0.48 ± 0.036	0.91 ± 0.020	1.9	0.19 ± 0.017	1.5 ± 0.041	7.9	0.081 ± 0.0067	1.4 ± 0.041	17
L-Methionine	2.7 ± 0.19	1.2 ± 0.034	0.44	0.29 ± 0.018	1.2 ± 0.023	4.1	0.73 ± 0.042	1.4 ± 0.027	1.9	0.16 ± 0.012	1.0 ± 0.028	6.3

[a] Determined from the gradient of the linear part of the curve from a plot of activity against substrate concentrations.

Table 2. Apparent K_m and k_{cat} values of wild-type and mutant phenylalanine dehydrogenase (PheDH) for various ketoacid substrates. The concentration of coenzyme NADH was fixed at 0.1 mM. ND, K_m too large to be determined.

Substrate	Wild type			L307V			G124A			G124A/L307V		
	K_m ± SEM (mM)	k_{cat} ± SEM (s^{-1})	k_{cat}/K_m ($s^{-1}·mM^{-1}$)	K_m ± SEM (mM)	k_{cat} ± SEM (s^{-1})	k_{cat}/K_m ($s^{-1}·mM^{-1}$)	K_m ± SEM (mM)	k_{cat} ± SEM (s^{-1})	k_{cat}/K_m ($s^{-1}·mM^{-1}$)	K_m ± SEM (mM)	k_{cat} ± SEM (s^{-1})	k_{cat}/K_m ($s^{-1}·mM^{-1}$)
Phenylpyruvate	0.37 ± 0.035	113 ± 5.3	305	0.22 ± 0.019	49 ± 1.6	223	7.3 ± 1.2	31 ± 2.3	4.2	4.6 ± 0.65	46 ± 2.8	10
Hydroxyphenylpyruvate	0.19 ± 0.016	90 ± 2.8	474	0.23 ± 0.021	55 ± 2.0	239	ND	ND	0.91[a]	ND	ND	0.91[a]
α-Ketoisocaproate	20 ± 2.65	27 ± 2.0	1.4	1.4 ± 0.059	7.8 ± 0.11	5.6	0.63 ± 0.071	25 ± 1.1	40	0.36 ± 0.035	24 ± 0.98	67
DL-α-Keto-β-methyl-n-valerate	13 ± 1.8	7.2 ± 0.40	0.55	8.5 ± 0.70	10 ± 0.46	1.2	1.3 ± 0.15	16 ± 0.91	12	0.42 ± 0.041	13 ± 00.57	31
α-Ketocaproate	13 ± 0.70	51 ± 1.3	3.9	0.69 ± 0.061	17 ± 0.51	25	2.1 ± 0.16	64 ± 2.1	30	0.94 ± 0.11	66 ± 3.8	70
α-Ketoisovalerate	ND	ND	0.55[a]	14 ± 1.3	25 ± 1.0	1.8	3.0 ± 0.25	51 ± 2.0	17	2.0 ± 0.15	53 ± 2.0	27
α-Ketovalerate	52 ± 4.4	33 ± 1.8	0.63	11 ± 0.53	33 ± 0.78	3.0	2.5 ± 0.18	53 ± 2.0	21	0.82 ± 0.076	51 ± 2.1	62
α-Keto-γ-methiobutyrate	11 ± 0.73	27 ± 0.78	3.7	1.5 ± 0.096	34 ± 0.88	23	2.4 ± 0.27	38 ± 2.0	16	1.1 ± 0.13	52 ± 2.7	47

[a] Determined from the gradient of the linear part of the curve from a plot of activity against substrate concentrations.

monomer and dimer. These results reflect changes that go beyond the intended local alteration, making a detailed interpretation difficult. It is certainly now clear that quaternary interactions among amino acid dehydrogenase subunits have an important bearing on substrate specificity. Comparison between the structure of the hexameric *C. symbiosum* GluDH [1,2] and the recently solved crystal structure of the octameric *B. sphaericus* LeuDH [10] has revealed that, besides the nature of the residues corresponding to positions 89 and 380 of *C. symbiosum* GluDH, subtle changes in the shape of the substrate-binding pocket, owing to differences in quaternary structure between the two enzymes, make additional contributions to their respective substrate specificities [10,33].

In contrast, the observed functional changes in the site-directed mutants described in this article are broadly in line with the initial predictions [8] and clearly do not involve any secondary contributions resulting from changes in quaternary structure. However, the strategy employed here, to alter the specificity of PheDH, was expected to exclude large substrates, such as L-phenylalanine, from the active site on steric grounds. In the mutants actually obtained, unlike natural LeuDH, L-phenylalanine remains as a substrate and therefore other factors must exist to account for this difference. In general, proteins are deformable and will relax when there is a poor steric interaction [34]. Possibly the enzyme's inherent flexibility still allows it to accommodate large substrates, but at an energetic cost manifested by a poor specificity constant.

The basis of substrate specificity in the amino acid dehydrogenases is of practical interest because of their potential use in the biotechnology industries. The stereospecific reactions catalysed by these enzymes can be exploited to produce enantiomerically pure amino acids for nutritional, synthetic or therapeutic purposes. PheDH is used, for example, for chiral synthesis of L-phenylalanine, currently in demand as a precursor for the artificial sweetener, aspartame [35]. All the amino acid dehydrogenases also have the potential to be used in the production of unnatural amino acids for the pharmaceutical industry. Understanding the basis of their specificity now offers the potential to engineer much more efficient biocatalysts for such purposes [8,36]. The present study confirms the accuracy of target prediction for mutagenesis on the basis of structural homology. Although the authentic structure of a protein will always be the best guide in such work, and authentic structures have now been solved for both PheDH and LeuDH, this exercise stands as a case study to encourage the use of homology-based methods when an authentic structure is not immediately available. Furthermore, it also underlines the need for adequate kinetic analysis if important outcomes of mutagenesis are not to be missed.

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