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To be cited as: *ChemCatChem* 10.1002/cctc.201902364

Link to VoR: http://dx.doi.org/10.1002/cctc.201902364

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COMMUNICATION

Separate sets of mutations enhance activity and substrate scope of amine dehydrogenase

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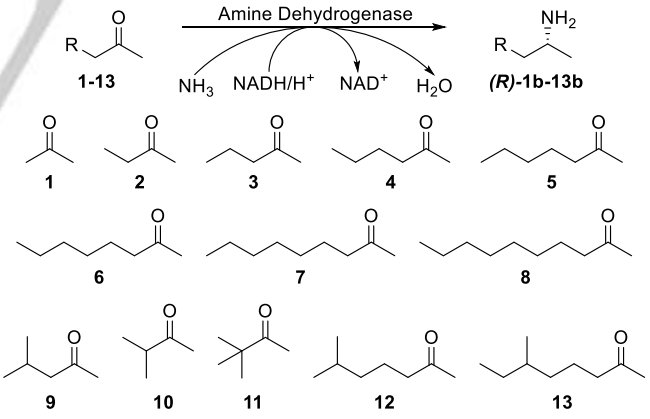
Abstract: Mutations were introduced into the leucine amine dehydrogenase (L-AmDH) derived from *G. stearothermophilus* leucine dehydrogenase (LeuDH) with the goals of increased activity and expanded substrate acceptance. A triple variant (L-AmDH-TV) including D32A, F101S, and C290V showed an average of 2.5-fold higher activity toward aliphatic ketones and an 8.0 °C increase in melting temperature. L-AmDH-TV did not show significant changes in relative activity for different substrates. In contrast, L39A, L39G, A112G, and T133G in varied combinations added to L-AmDH-TV changed the shape of the substrate binding pocket. L-AmDH-TV was not active on ketones larger than 2-hexanone. L39A and L39G enabled activity for straight-chain ketones as large as 2-decanone and in combination with A112G enabled activity toward longer branched ketones including 5-methyl-2-octanone.

For decades, chiral amine compounds have proven to be key intermediates for blockbuster drugs. Many of the top selling small molecule drugs today contain chiral amine groups.[1] Recently, there has been a growing interest in utilizing biocatalytic reductive amination of prochiral ketones to produce chiral amines. Biocatalytic production of chiral amines offers distinct advantages over classical heterogeneous catalysis. Key enzyme families in the field include ω-transaminases,[2] reductive aminases,[3] and amine dehydrogenases.[4] Amine dehydrogenases (AmDHs), first developed in 2012[5], catalyze the reductive amination of prochiral ketones to form chiral primary amines with the addition of aqueous ammonia. The reaction is dependent on a hydride transfer from NADH to form NAD^+ [Scheme 1]. To-date, all the AmDHs engineered from amino acid dehydrogenases produce (R)-amines. The first AmDH, called leucine amine dehydrogenase (L-AmDH) was developed through targeted libraries of mutations in the active site of the leucine dehydrogenase (LeuDH) from *Geobacillus stearothermophilus*. After multiple rounds of mutations, activity toward keto acids was removed, and new activity toward ketones was obtained. Since 2012, multiple groups have produced similar amine dehydrogenases from other amino acid dehydrogenase scaffolds[6] or have been identified from natural sources.[7] Others have introduced new mutations to increase activity for new substrates by altering the size of the ketone binding pocket.[6h] Still more work has been performed on enzyme immobilization,[6e, 8] rate law determination,[9] whole-cell biocatalysis,[6d, 10] and multi-enzyme cascades[8b, 11] to enable the use of amine dehydrogenases in the large-scale synthesis of chiral amines.



Scheme 1. Reductive amination of prochiral ketones to form chiral amines with an amine dehydrogenase

In the present work, we sought to improve the published L-AmDH (referred to here as the base case) through two separate sets of mutations. The first group was selected to increase activity and stability without necessarily impacting substrate specificity. Position V291 was shown to be important for substrate binding in LeuDH.[12] In the base case enzyme, this residue was mutated from valine to cysteine. It was speculated that mutating back to valine [Figure 1A] could positively impact activity. Two residues

farther away from the active site were also shown to be promising candidates in a 2004 patent on mutations in LeuDHs.[13] Based on the reported results, F101S and D32A were incorporated into the L-AmDH base case.

In the second group of mutations, three residues close to the substrate side chain were identified as potential targets for altering the L-AmDH substrate scope. Kataoka and Tanizawa found that for *G. stearothermophilus* LeuDH, L39K and A112G mutations both caused major changes in the relative specific activities for different amino acid and keto acid substrates.[12] Recently, Chen *et al.* showed mutations homologous to A112G and T133G enhanced activity toward larger and bulkier ketones in multiple engineered L-AmDHs.[6h] Replacing the residues at positions L39, A112, and T133 with smaller residues was hypothesized to enable activity toward larger ketones in our L-AmDH as well. The positions of the proposed mutations relative to the substrate binding pocket can be seen in Figure 1B.

Combinations of the six proposed mutations were introduced into L-AmDH and the resulting variants were screened for activity on a variety of straight and branched methyl ketones of different sizes. Other mutations were tested but showed poor results (see Supplementary Information).

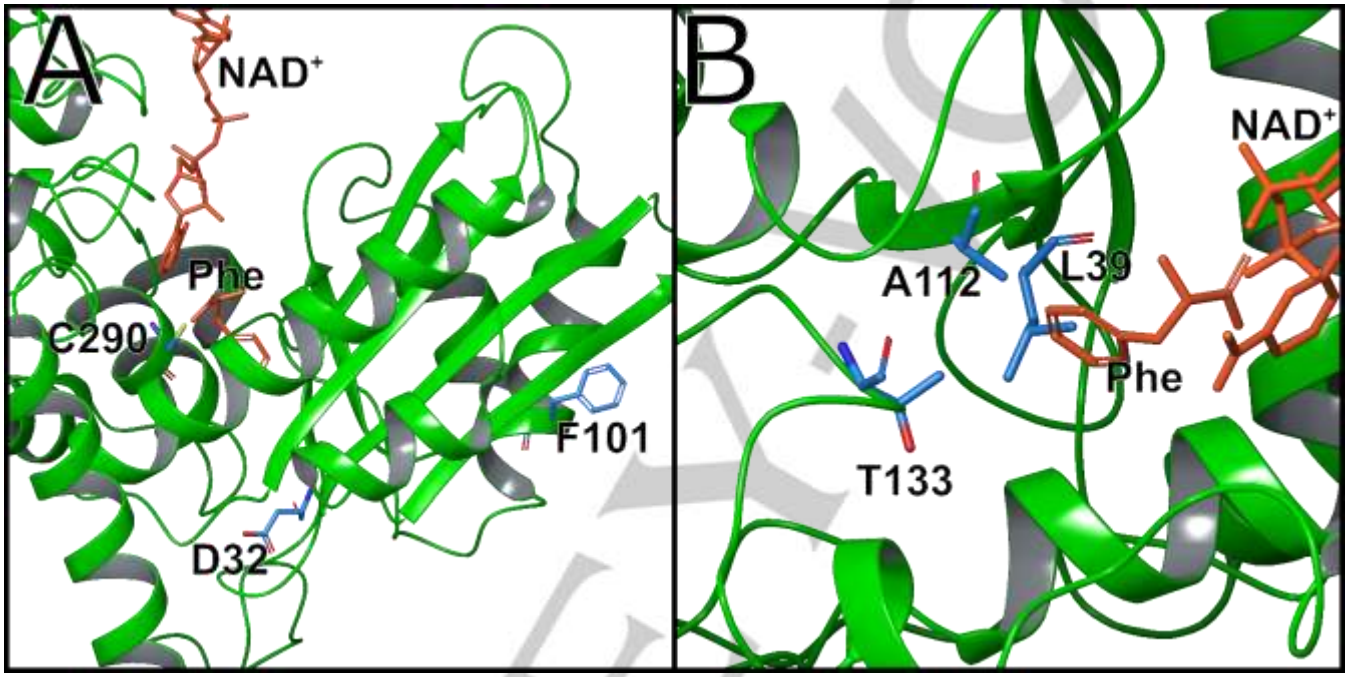


Figure 1. A homology model (constructed with SWISS-MODEL[14]) of the engineered L-AmDH sequence mapped onto a *B. stearothermophilus* LeuDH cryo-EM structure (PDB ID: 6ACF).[15] Because this structure is in the apo form, the holo-structure the PheDH from *Rhodococcus sp.* (PDB ID: 1C1D)[16] was aligned to the homology model, the phenylalanine substrate and NAD^+ cofactor position from this alignment can give a good idea of how the substrate and cofactor would be positioned in L-AmDH. Subfigure A shows the positions of D32, F101, and C290 relative to the active site. Subfigure B shows the positions of L39, A112, and T133 in the active site.

The L-AmDH mutations D32A, F101S, and C290V were generated sequentially using overlap extension PCR, followed by cloning and protein expression. Specific activity toward various aliphatic ketones was measured in 1 mL batch reactions by following the decrease in concentration of NADH with UV-visible spectrophotometry and is recorded in milliunits per milligram of enzyme, with one unit defined as the amount of enzyme required to catalyze the conversion of one micromole of substrate in one minute. The results of these assays are recorded in Table 1. Across the six substrates for which activity could be quantified, the combined D32A/F101S/C290V mutant (L-AmDH-TV) showed an average of a 2.5-fold increase in activity compared to the base case enzyme. The largest improvement was seen for 4-methyl-2-pentanone where L-AmDH-TV had 3.4-fold higher activity than the base case. Interestingly, the relative activity of the enzyme toward different substrates did not change due to the mutations, but rather the mutations increased activity for all substrates in roughly equal proportions. Additionally, differential scanning fluorimetry (DSF) experiments showed the melting point of L-AmDH-TV to be 73.6 °C, an increase of 8.0 °C compared to L-AmDH at 65.6 °C.

Table 1. Activity enhancement of L-AmDH toward aliphatic ketones and the generation of L-AmDH-TV

	Specific Activity (mU/mg)			
Substrate	L-AmDH	D32A	D32A/F101S	D32A/F101S/ C290V (“TV”)
1	n.d.[a]	n.d.	n.d.	n.d.
2	87.9	61.4	181.7	225.5
3	430.5	888.3	1363.9	1303.6
4	144.8	172.2	278.6	266.4
5	n.d.	n.d.	n.d.	n.d.
6	n.d.	n.d.	n.d.	n.d.

7	n.d.	n.d.	n.d.	n.d.
8	n.d.	n.d.	n.d.	n.d.
9	453.4	945.3	959.2	1146.5
10	531.1	1406.5	1661.4	1808.5
11	549.6	1083.3	1145.1	1056.2

Reaction conditions: 0.4-1.0 μM enzyme, 20 mM substrate, 200 μM NADH, 4M NH_4Cl/NH_4OH, pH 9.6, 25 °C, 1 mL reaction volume. [a] n.d. denotes an activity level below the limit of detection.

Sequence alignments of the active sites of multiple amino acid dehydrogenases suggested L-AmDH position L39 as a potential target for expansion of the substrate binding pocket to accommodate larger substrates.[12] Leucine is conserved at this position for leucine dehydrogenases, but a lysine at the homologous position in glutamate dehydrogenases is likely responsible for binding the acid group on the glutamate substrate sidechain. The residue is positioned with its side chain pointing in toward the substrate binding site. The L-AmDH-TV scaffold presented in the previous section formed the basis for further mutations. L-AmDH-TV/L39A and L39G mutants were produced. Figure 2 shows a comparison of specific activities toward straight-chain ketones of varying lengths. For L-AmDH-TV, 2-pentanone shows the highest activity at 1.31 U/mg, while zero activity was observed for ketones longer than 2-hexanone. When the leucine at position L39 is replaced with the smaller alanine, activity toward 2-butanone and 2-pentanone are greatly reduced, while activity was more than doubled for 2-hexanone. More interestingly, TV/L39A showed activity for ketones as large as 2-decanone, with the highest activity found for 2-heptanone at 644 mU/mg. When the residue was further mutated to glycine, the n-ketone with the highest activity shifted to 2-nonanone. While TV/L49G showed decreased activity for 2-butanone through 2-heptanone, activity was increased compared to TV/L39A for 2-octanone through 2-decanone.

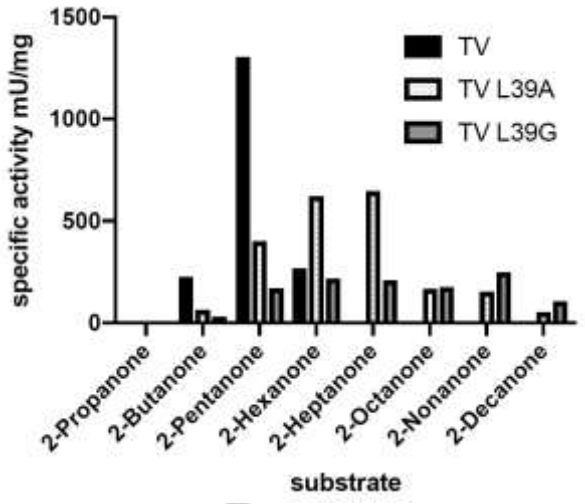


Figure 2. Expansion of the L-AmDH binding pocket to accommodate ketones of larger sizes due to mutations at position L39. Reaction conditions: 0.4-1.0 μM enzyme, 20 mM substrate, 200 μM NADH, 4M NH_4Cl/NH_4OH, pH 9.6, 25 °C, 1 mL reaction volume.

Based on results reported for a different set of engineered L-AmDHs,[6h] A112G and T133G were investigated as additional sites for binding pocket expansion. The TV/A112G variant shows new activity for 2-heptanone, and a new optimal substrate length of six carbons rather than five, as was found for TV [Table 2]. The further addition of T133G increased activity for 2-pentanone through 2-heptanone, while enabling new activity for 2-octanone and 2-nonanone. Finally, the addition of L39A to TV/A112G and TV/A112G/T133G shifted the optimal substrate length to 7 carbons and enabled activity toward 2-decanone. The addition of T133G to TV/L39A/A112G did not have the same synergistic effects as were found when TV/A112G, suggesting that the benefits of L39A and T133G are not additive.

Table 2. Effect of A112G and T133G on activity toward n-ketones

	Specific Activity (mU/mg)				
Substrate	TV	TV/ A112G	TV/ A112G/ T133G	TV/ L39A/ A112G	TV/ L39A/ A112G/ T133G
1	n.d.[a]	n.d.	n.d.	n.d.	n.d.
2	225.5	n.d.	n.d.	n.d.	n.d.
3	1303.6	80.5	565.1	35.1	19.0
4	266.4	166.6	777.3	308.9	177.7
5	n.d.	31.9	596.9	446.1	431.5
6	n.d.	n.d.	159.2	268.6	237.4
7	n.d.	n.d.	145.9	268.8	171.2
8	n.d.	n.d.	n.d.	140.5	92.9

Reaction conditions: 0.4-1.0 μM enzyme, 20 mM substrate, 200 μM NADH, 4M NH_4Cl/NH_4OH, pH 9.6, 25 °C, 1 mL reaction volume.

The reported variants were also tested for their ability to convert longer branched ketones, **12** and **13** [Table 3]. The combination of L39A and A112G allowed for the conversion of these bulky ketones, while each mutation on its own was insufficient. TV/L39G also showed low but measurable activity toward **13** at 39.4 mU/mg. As seen with the straight-chain ketones, T133G does not increase activity for larger substrates when in combination with L39A.

Table 3. Activity of L-AmDH variants toward long branched ketones

Enzyme Variant	Activity Toward **12** (mU/mg)	Activity Toward **13** (mU/mg)
TV	n.d.[a]	n.d.
TV/L39A	n.d.	n.d.
TV/L39G	n.d.	39.4
TV/A112G	n.d.	n.d.
TV/A112G/T133G	n.d.	n.d.
TV/L39A/A112G	258.8	186.4
TV/L39A/A112G/T133G	177.9	161.5

Reaction conditions: 0.4-1.0 μM enzyme, 20 mM substrate, 200 μM NADH, 4M NH_4Cl/NH_4OH, pH 9.6, 25 °C, 1 mL reaction volume.

To further demonstrate the improvements to the applicability of L-AmDH to convert larger ketones, selected substrates were converted at a 50 mL preparative scale for 24 hours with both L-AmDH and TV/L39A. As shown in Table 4, relative conversion, (measured after derivatization with benzoyl chloride[7a]) between the two enzymes was in line with their relative specific activities. Additionally, the already exquisite enantioselectivity of the L-AmDH[5] was not impacted by the mutations, as measured after diastereomeric derivatization[7b] [Figures S4, S5, S8]. Aggregation occurred in all samples occurred over the course of the reaction and likely limited overall conversion. In the future, this could be mitigated through immobilization.

Table 4. Conversion values for preparative-scale reactions

		Conversion after 24 hours	
Substrate	Concentration	L-AmDH	TV/L39A
3	10 mM	65.4 ± 2.2%	48.5 ± 1.6%
5	10 mM	1.7 ± 0.1%	56.3 ± 4.4%
7	2 mM	n.d.	51.9 ± 0.5%

Reaction conditions: 2 mg each of AmDH and cbFDH, 1 mM NAD^+, ketone concentration as listed, 2M NH_4COOH/NH_4OH, pH 8.5, 21 °C, 50 mL reaction volume, rotating at 20 rpm for 24 hours.

In conclusion, we found two sets of mutations to improve L-AmDH which result in either increased specific activity and thermodynamic stability, or in an altered substrate specificity towards longer or branched methylketones. While the first set of mutations acts synergistically to increase L-AmDH activity and stability, the second set of three mutations for change substrate specificity does not. Instead, that second set enables picking a desired specificity trait, such as a long side chain of a methylketone or branched alkylmethylketone, with a specific mutation. Thus, the current work is an important step towards a differentiated family of sufficiently stable amine dehydrogenases.

Acknowledgements

Funding for the reported work by the National Science Foundation through NSF CBET grant 1512848 and NSF I/UCRC grant 1540017 to the Center for Pharmaceutical Development is gratefully acknowledged. We also wish to acknowledge the Biopolymer Characterization (BPC) core facility at the Parker H. Petit Institute for Bioengineering and Bioscience at the Georgia Institute of Technology for the use of its shared equipment, services and expertise.

Keywords: amine dehydrogenases • biocatalysis • enzyme catalysis • protein engineering • reductive amination

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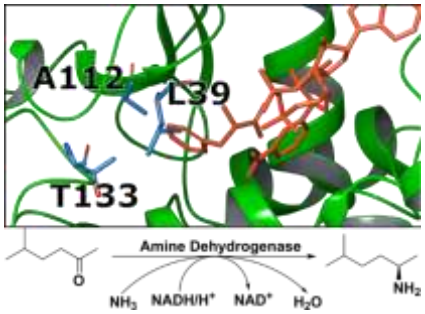

Mutations at six locations in an engineered leucine amine dehydrogenase (L-AmDH) greatly improved activity, stability, and substrate scope. A triple variant enzyme showed 1.8 to 3.4-fold increase in specific activity compared to parent and an 8.0 °C increase in melting temperature. A second set of mutations reshaped the binding pocket to enable new activity for straight-chain ketones up to ten carbons long, compared to six for the parent.