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Development of an engineered thermostable amine dehydrogenase for the synthesis of structurally diverse chiral amines

Using directed evolution, we have engineered a thermostable amine dehydrogenase based on a phenylalanine dehydrogenase that catalyses direct reductive amination of ketones and functionalized hydroxy ketones with ammonia or primary amines to diverse chiral primary amines, secondary amines and amino alcohols.

Development of an engineered thermostable amine dehydrogenase for the synthesis of structurally diverse chiral amines†

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Amine dehydrogenases (AmDHs) are emerging as a class of attractive biocatalysts for synthesizing chiral amines via asymmetric reductive amination of ketones with inexpensive ammonia as an amino donor. However, the AmDHs developed to date exhibit limited substrate scope. Here, using directed evolution, we engineered a GkAmDH based on a thermostable phenylalanine dehydrogenase from Geobacillus kaustophilus. The newly developed AmDH is able to catalyze reductive amination of a diverse set of ketones and functionalized hydroxy ketones with ammonia or primary amines with up to >99% conversion, thus accessing structurally diverse chiral primary and secondary amines and chiral vicinal amino alcohols, with excellent enantioselectivity (up to >99% ee) and releasing water as the sole by-product.

In particular, a recently developed class of engineered enzymes, amine dehydrogenases (AmDHs), are now appreciated as attractive biocatalysts for the synthesis of chiral primary amines using simple and green approaches; they can use inexpensive free ammonia as an amino donor for the reductive amination of ketones. Moreover, only water is generated as a by-product in this process. Three AmDHs were initially created by Bommarius and co-workers through directed evolution of naturally occurring leucine dehydrogenases and phenylalanine dehydrogenases.32,42 Using the same approach, Li and co-workers subsequently developed an AmDH based on Rhodococcus phenylalanine dehydrogenase, which displayed detectable activity towards the bulky 4-phenyl-2-butanone, a challenging substrate for these enzymes.31 More recently, Schell and co-workers also developed a thermostable CtAmDH by directed evolution of a Caldalkalibacillus thermarum phenylalanine dehydrogenase, and demonstrated an efficient biphasic system for the amination of phenoxy-2-propanone.30 In addition, Vergne-Vaxelaire and Grogan identified a family of native AmDHs for reduction amination of ketones.35

Notably, by coupling the engineered AmDHs with alcohol dehydrogenases (ADHs), both the Turner group and our group constructed elegant dual-enzyme ‘hydrogen-borrowing’ cascade systems for the production of chiral amines from available racemic alcohols in a green manner.41,43 Mutti and coworkers performed a detailed investigation of AmDH-catalyzed reductive amination that considered substrate acceptance, optimal reaction conditions, and stereoselectivity.44 Very recently, we further engineered AmDHs through mutation of two key residues surrounding
the substrate-binding pocket which affect the binding of bulky aliphatic ketones.\textsuperscript{29} The resultant AmDH mutants displayed broad substrate scopes, and are capable of accepting previously inaccessible bulky substrate ketones like 2-heptanone and 2-octanone.

Despite recent progress with AmDHs, the enzymes were mainly used for the synthesis of chiral primary amines via reductive amination of ketones with ammonia. Until recently, Mutti and co-workers reported the synthesis of chiral secondary amines from ketones and achiral organic amine donors using AmDHs.\textsuperscript{28,45} However, few examples showed that AmDHs could have been utilized for asymmetric reductive amination of functionalized aromatic ketones (e.g., aromatic hydroxy ketones).\textsuperscript{46} Herein, we performed directed evolution of a thermostable phenylalanine dehydrogenase from Geobacillus kaustophilus to develop an AmDH that possesses a broad substrate scope. The resulting GkAmDH, phenylalanine dehydrogenase (bBk), displayed the highest amination activity (6.0 U mg\textsuperscript{-1} protein) towards 2-heptanone and 2-octanone.\textsuperscript{29} The resultant AmDH mutants displayed broad substrate scopes, and are capable of accepting previously inaccessible bulky substrate ketones like 4-phenyl-2-butanone (8). GkAmDH exhibited higher activities towards 1–4, which bear electron-withdrawing substituents on their benzene rings, than towards 6 and 7 with electron-donating substituents, clearly suggesting that electron-withdrawing substituents may activate the carbonyl carbon. Moreover, GkAmDH displayed similar activities towards 1–3, suggesting that the substituent’s position on the benzene ring had no apparent effect on the enzyme’s activity.

We next attempted the reductive amination of ketones 1–12 in 5 M NH\textsubscript{4}H\textsubscript{2}O/NH\textsubscript{4}Cl buffer (pH 9.0) using GkAmDH combined with glucose dehydrogenase (GDH) used for the regeneration of NADH. However, we found that high concentrations of NH\textsubscript{4}\textsuperscript{+} caused poor GDH activity (Fig. 2a). We therefore examined another system based on formate dehydrogenase (FDH) with NH\textsubscript{3}/H\textsubscript{2}O/NH\textsubscript{4}COOH buffer (Fig. 2b). Consequently, use of a high concentration of NH\textsubscript{3}, H\textsubscript{2}O/NH\textsubscript{4}COOH buffer had no apparent deleterious effects on FDH activity. We therefore used an optimized FDH-based regeneration system (5 M, pH 9.0) in subsequent reductive amination reactions.

Using the optimized reaction system, analytical scale reductive amination reactions of various ketones 1–12 with ammonia were carried out using the GkAmDH/FDH coupling system in NH\textsubscript{3}/H\textsubscript{2}O/NH\textsubscript{4}COOH buffer (5 M, pH 9.0); GkAmDH exhibited great reactivity towards ketones 1–6 (Table 1), findings consistent with our results for specific activity (Fig. 1). 50 mM substrates 1–6 were converted with up to 99% conversions with low enzyme loading. For the challenging substrates 7–11, good-to-excellent conversions were also achieved using 10 g L\textsuperscript{-1} GkAmDH. Only for substrate 12, GkAmDH exhibited poor conversion (<5%), suggesting that excessively long alkyl chains can hinder substrate access to...
strong potential of \(Gk\)AmDH as a biocatalyst for the synthesis of chiral primary amines.

Recently, a reductive aminase AspRedAm and several imine reductases have been described for the synthesis of chiral secondary amines via reductive amination of ketones with primary amines.\(^{19,26,47,48}\) To explore the possibility of using AmDHs for the synthesis of chiral secondary amines, reductive amination of the model substrate 1 with four primary amines \(b-e\) was attempted with \(Gk\)AmDH. Consequently, \(Gk\)AmDH can reduce the imines formed with primary amines \(b\) and \(c\) with 1 at 97% conversion and 88% conversion, respectively. In contrast, \(Gk\)AmDH only provided 15% and <5% conversions in the reduction of 1 with ethylamine \(c\) and allylamine \(d\), highlighting the substantial impact of the amino donors on \(Gk\)AmDH’s activity (Scheme 2). The corresponding chiral secondary amines were produced with good stereoselectivity (78–90% \(ee\)). Interestingly, a reversed stereoselectivity was observed for 1d, perhaps owing to the rotation of the carbon–carbon bond connecting the phenyl and carbon of the carbonyl group.

Recently, Mutti and co-workers reported AmDH-catalyzed coupling amination of ketones with primary amines in which up to 43% of the substrate was converted into the \((R)\)-selective secondary amines with up to 72% \(ee\).\(^{45}\) Taken together, these results demonstrate that AmDHs possess the unique ability to reduce the imines formed from both inorganic ammonia and organic amines with ketones for the preparation of optically pure primary and secondary amines.

Encouragingly, we observed that \(Gk\)AmDH showed amination activity towards aromatic hydroxy ketones 13 and 14 (3.50 U mg\(^{-1}\) and 2.06 U mg\(^{-1}\), respectively). Docking analysis showed that the hydroxy ketone 14 is accommodated into the binding pocket of \(Gk\)AmDH in a reasonable conformation in which the terminal hydroxyl group points towards the binding residues S78 and L276, while the carbonyl group points to the two catalytic residues K90 and D125, thereby generating satisfactory amination activity (Fig. 3).

We also detected the activity of \(Gk\)AmDH for aliphatic hydroxy ketones 15 and 16 (0.39 U mg\(^{-1}\) and 0.04 U mg\(^{-1}\), respectively) (Table 2). The results indicate that AmDHs are able to catalyze the reduction of imines formed from hydroxy ketones with inexpensive ammonia to access vicinal amino alcohols. These amino alcohols are extensively exploited as privileged scaffolds in pharmacologically active molecules and natural products,\(^{49}\) such as norephedrine and norephedrine, which are used as amphetamine pharmaceuticals in most countries.\(^{50}\) In addition, they also serve as important chiral auxiliaries or ligands in asymmetric synthesis reactions.\(^{51,52}\)

Seeking to develop AmDHs with higher activity towards hydroxy ketones, we undertook double site combinational mutagenesis at two key residues S78 and L276 affecting substrate specificity using \(Gk\)AmDH as the template. After screening separately using hydroxy ketones 13–16, the best mutants displayed further increased activity towards the corresponding substrate (Table 2). To investigate whether

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**Table 1 Reductive amination of ketones 1–12 with ammonia using a \(Gk\)AmDH/FDH coupling system in 5 M NH\(_3\)/NH\(_4\)-Cl buffer**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Substrate concn. (mM)</th>
<th>(Gk)AmDH (mg mL(^{-1}))</th>
<th>Conv. (%)</th>
<th>ee (%)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>1</td>
<td>99</td>
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<td>10</td>
<td>10</td>
<td>&lt;5</td>
<td>&gt;99</td>
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</table>

* Reaction conditions: 0.5 mL reaction mixture containing NH\(_3\)/H\(_2\)O/NH\(_4\)-COOH (5 M NH\(_3\), pH 9.0), 10–50 mM substrate, 1 mM NAD\(^{+}\), 10 mg mL\(^{-1}\) FDH (lyophilized cell-free extract) and \(Gk\)AmDH (lyophilized cell-free extract) shaken at 1000 rpm at 40 °C for 24 h. Conversions and enantiomeric excess were determined via GC and chiral GC, respectively.

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**Fig. 2** The relative activity of \(Gk\)AmDH and GDH in NH\(_3\)/H\(_2\)O/NH\(_4\)-Cl buffer (a) and \(Gk\)AmDH and FDH in NH\(_3\)/H\(_2\)O/NH\(_4\)-COOH buffer (b). The activity of the enzymes in buffer containing 2 M NH\(_3\)/NH\(_4\)^+ was normalized as 100%.

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**Scheme 2** Reductive amination of the model substrate 1 with ammonia using a \(Gk\)AmDH/FDH coupling system in 5 M NH\(_3\)/NH\(_4\)-COOH buffer.

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**Catalysis Science & Technology**

**Paper**

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Published on 12 February 2020. Published on 12/6/2023 7:58:29 PM. [View Article Online]
chiral vicinal amino alcohols could be achieved using the newly developed AmDHs, we next performed the reductive amination of 14 on a preparative scale as a representative example. Using the best AmDH K78S/N276T, 35 mM 14 was smoothly converted to (S)-2-amino-3-phenylpropanol ((S)-14a) in 41% isolated yield and >99% ee. This result showcases the potential of the newly developed AmDH enzymes for the preparation of chiral vicinal amino alcohols, especially for aromatic chiral vicinal amino alcohols.

Finally, ketone 7 was aminated with ammonia on a 100 mL scale (Scheme 3). The preparative reductive amination of 7 (200 mM, 5% (v/v) DMSO) was performed in NH₃·H₂O/NH₄COOH buffer (5 M, pH 9.0) and the pH was maintained at 9.0 by titrating 5 M NH₃·H₂O during the reaction course. Consequently, 99% of the substrate was converted into the corresponding chiral primary amine product ((R)-4-methoxyamphetamine ((R)-7a)) within 6 h, corresponding to a calculated space–time yield of 130.9 g L⁻¹ d⁻¹. After normal workup, the product was prepared with 43% isolated yield and >99% ee.

We also performed the reductive coupling of ketone 1 (10 mM, 1% (v/v) DMSO) with methylamine b on a 50 mL scale.
Notes and references