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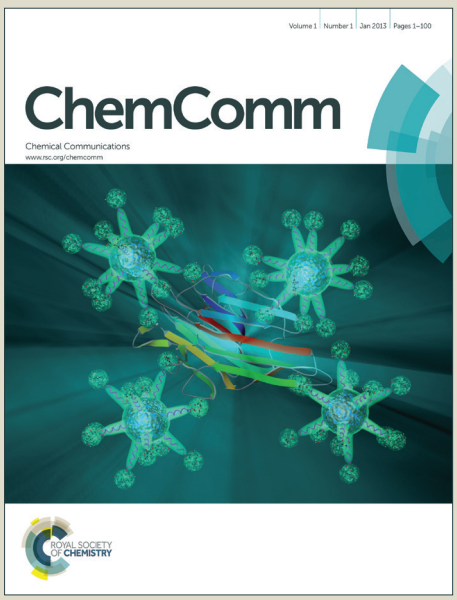

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A novel chimeric amine dehydrogenase shows altered substrate specificity compared to its parent enzymes

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We created a novel chimeric amine dehydrogenase (AmDH) via domain shuffling of two parent AmDHs ('L- and F-AmDH'), which in turn had been generated from leucine and phenylalanine DH, respectively. Unlike the parent proteins, the chimeric AmDH ('cFL-AmDH') catalyzes the amination of acetophenone to (R)-methylbenzylamine and adamantly-methylketone to adamantylethylamine.

Enantiomerically pure amines are sought-after building blocks of active pharmaceutical ingredients (APIs) in pharma, as exemplified by sitagliptin (Januvia®), rasagiline (Azilect®), and oseltamivir (Tamiflu®). Sitagliptin has recently been made accessible via transaminase catalysis (1). Even more recently, our lab developed direct amination of ketones with NH_3, catalyzed by amine dehydrogenases (AmDHs) with different substrate specificities from either leucine DH ('L-AmDH')(2) or phenylalanine DH ('F-AmDH') (3). However, neither L- nor F-AmDH can convert benzylic ketones with appreciable activity.

Based on similar results in amino acid dehydrogenases (AADHs) (4), we have employed domain shuffling to generate a new chimeric amine dehydrogenase,cFL1 -AmDH, from F-AmDH and L-AmDH using overlap PCR, which is described in detail in the Method section of the Supplementary Information. The previously described amine dehydrogenases from our lab served as the parental enzymes for this chimera. Generation of chimeric proteins via domain shuffling can lead to new enzymes with improved functionality or extended range of substrate specificity ((5); (6); (7); (8)). Residues 1-149 were contributed by F-AmDH (F-AmDH numbering) and 140 to the terminus 366 by the L-AmDH (L-AmDH numbering) (Figure 1). Thus, the cFL1-AmDH retains the ketone/amine binding pocket of F-AmDH and the cofactor binding domain from L-AmDH.

Even though the authentic ketone binding domain from F-AmDH is present in the new enzyme, the chimeric amine dehydrogenase now accepts new substrates such as benzylic carbonyl substrates, in addition to maintaining the substrate specificity of its parent enzyme measured so far, F-AmDH (Table 1). Chiral GC analysis of cFL1-AmDH substrates, such as p-F-phenylacetone (pFPA), with previously determined enantioselectivity with the parent enzyme F-AmDH (3) exhibited the same enantioselectivity towards (*R*)-amine.

Moreover, the chimeric enzyme features a shifted temperature profile towards higher temperatures with a temperature of optimum activity T_{opt} of > 60°C compared to a T_{opt} of 50°C for F-AmDH.

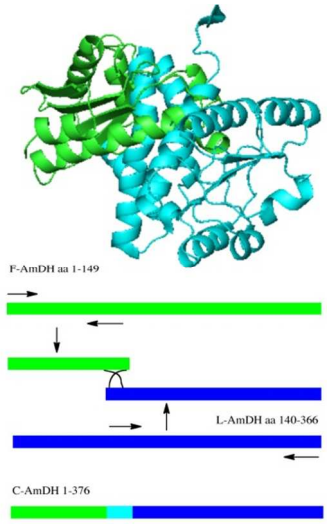

Figure 1: A. Model of the chimeric enzyme based on the structure of the related phenylalanine dehydrogenase from *Rhodococcus sp*. M4 (1BW9) (green) (9) and leucine dehydrogenase from *Bacillus sphaericus* (1LEH), (blue) (10).
B. Schematic outline of construction of the chimeric enzyme (green F-AmDH, dark blue L-AmDH, teal loop overlap). The amino acid sequence is listed in Figure S2.

Compared to its parent enzymes, this new chimera converts benzylic ketones when probed for substrate activity at 60°C. Specifically, we demonstrate the novel transformation of acetophenone to (*R*)-methylbenzylamine. In addition, conversion of the non-aromatic adamantylmethylketone to (*R*)-1-(1-adamantyl)ethylamine and the aliphatic methoxyacetone to (*R*)-methoxyisopropylamine (*R*)-MOIPA was observed (Suppl. Figure 3). Thus, we find that the cofactor binding domain in dehydrogenases can play a significant role in substrate specificity, reshaping and extending the substrate

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pocket to allow conversion of aliphatic as well as aromatic and bulky ketones.

cFL1-AmDH was characterized with respect to its kinetic properties (Table 2) as well as thermal behaviour (Suppl. Figure 4).

Table 1: Substrate specificity of cFL1-AmDH
(a) Substrate concentration = 20 mM, (b) 5 M NH_4Cl buffer pH 9.5, (c) measured at 60°C, (d) measured at 25°C, n.d. = not determined

Substrates [(a)]	**Activity (mU/mg)** [(b)]		
	cFL1[(c)]	F[(d)]	L[(d)]
ketones			
p-fluorophenylacetone	1725	4000	0
acetophenone	301	< 0.1	59
1-tetralone	107	0	n.d.
adamantylmethylketone	69	0	n.d.
3-methyl-1-phenylbutanone	30	0	n.d.
pinacolone	133	0	n.d,
2-tetralone	0	0	n.d.
benzophenone	0	n.d.	n.d.
amines			
(*R*)-methylbenzylamine	19	n.d.	476
(*R,S*) methylbenzylamine[#]	21	0.5	484
(*R,S*)-MOIPA	40	0	130

Table 2: Kinetic properties of cFL1-AmDH

Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
pFPA	$1.1^{+/-0.05}$	$1.24^{+/-0.02}$	1127
Acetophenone	$5.2^{+/-1.03}$	$0.24^{+/-0.01}$	48
NH_3	$350^{+/-133}$	$1.09^{+/-0.01}$	3
NADH	$0.04^{+/-0.004}$	$0.92^{+/-0.03}$	2 x 10^4

pH 9.6, T = 60°C; data for NH_3 and NADH measured with 15 mM p-FPA, 5 M formate buffer; data for p-FPA and NH_3 measured with 200 µM NADH, all values apparent.

Kinetic analysis revealed a reduced apparent K_M value for pFPA and ammonia compared to its parent enzyme, F-AmDH (3), but also reduced k_{cat} values.

Figure 2: amino acid DH positions 270-280

Sequence comparison of the amino acid DHs revealed two adjacent asparagines N270 and N271 (cFL numbering)(Figure 2),.Upon analysis of the protein structure model we concluded that the 2nd asparagine might have additional influence on amination. This finding lead to the creation of cFL2-AmDH in which both asparagines are mutated to leucine (N270L/N271L).

Table 3: Kinetic properties of cFL2-AmDH

Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
pFPA	$3.4^{+/-0.17}$	$2.52^{+/-0.07}$	741
Acetophenone	$2^{+/-0.24}$	$0.1^{+/-0.002}$	50
NH_3	$1000^{+/-28}$	$2.82^{+/-0.04}$	2.82

pH 9.6, T = 60°C; data for NH_3 and NADH measured with 15 mM p-FPA, 5 M formate buffer; data for p-FPA and NH_3 measured with 200 µM NADH, all values apparent.

While the apparent k_{cat} value for pFPA was indeed increased in cFL2- compared to cFL1-AmDH (Table 3), the value for acetophenone was decreased, indicating that N270/N271 both play a role in specific activity dependent on the substrate within the cFL2-AmDH.

Table 4: Thermal optima of different amine dehydrogenases
a: (2), b: (3)

AmDH	L-	F-	cFL1-	cFL2-
T_{opt} (°C)	50[a]	50[b]	>80	70

As mentioned before, domain shuffling of two related amine dehydrogenases L- and F-AmDH resulted in an altered thermal profile (Table 4). cFL1-AmDH is hardly active at 30°C, starts to exhibit good activity at 60°C and stays active beyond 70°C, (at > 70°C, cofactor stability starts to be impaired and our UV-VIS instrument reaches its limitations (Suppl. Figure 4, for details see Method section of Supplementary Information as well)), whereas cFL2-AmDH reaches its temperature of maximum activity T_{opt} at 70°C. The apparent activation energies $E_{a,app}$ for acetophenone and p-F-phenylacetone with cFL1-AmDH were determined to be 32.7 and 48.7 kJ/mol, respectively, (35 kJ/mol with cFL2-AmDH for p-F-phenylacetone), corresponding to 16.5, 10.9, and 15.4°C temperature increases, respectively, for doubling activity. cFL1-AmDH at 45 and 55°C (Figure S5 and Table S6) was found to be very stable ($t_{1/2}$ > 500 min); only at 70°C does half-life decrease to 40 min.

Conclusions

We find that the cofactor binding domain in amine dehydrogenases can play a significant role in ketone specificity and that domain shuffling can i) alter substrate specificity at comparable kinetic properties to the parents and ii) strongly improve thermal activity.

Notes and references

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† Standard molecular biology protocols were applied to generate the chimeric enzyme. Fusion of the domains was achieved using overlap PCR within a common loop region of the 2 parent enzymes. Enzymatic properties were determined using UV-spectroscopy monitoring the change in NADH absorption at 340 nm as well as GC and HPLC. The 3D model of the structure was generated using Pymol.

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Electronic Supplementary Information (ESI) available:
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