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Two-enzyme hydrogen-borrowing amination of alcohols enabled by a cofactor switched alcohol dehydrogenase

Matthew P. Thompson [a] and Nicholas J. Turner *[a]

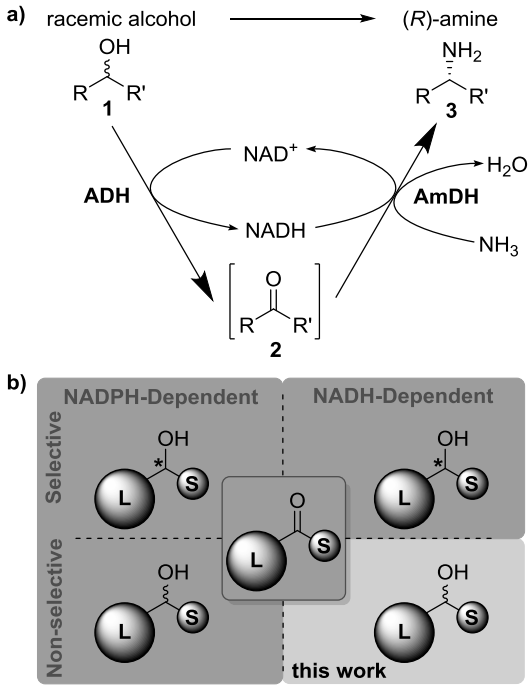
Abstract: The NADPH-dependent secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (TeSADH), displaying broad substrate specificity and low enantioselectivity, has been engineered to accept NADH as a cofactor. The engineered TeSADH shows a >10,000-fold switch from NADPH towards NADH compared to the wild type enzyme. This TeSADH variant has been applied to a biocatalytic hydrogen borrowing system that employs catalytic amounts of NAD^+, ammonia and an amine dehydrogenase (AmDH) thereby enabling the conversion a range of alcohols into chiral amines.

The activation of primary and secondary alcohols under mild conditions, to allow subsequent nucleophilic substitution, has been identified as a key area for method development in synthetic organic chemistry.[1] In this context the development of strategies based on hydrogen-borrowing is attractive because in principle the only by-product is water.[2] Consequently there has been considerable activity in this field and a number of elegant redox self-sufficient (hydrogen-borrowing) reactions, using ruthenium and iridium catalysts, have been developed.[3] Despite the impressive advances in terms of substrate scope and application, the requirement for high temperatures and high catalyst loading, as well as a general lack of chemo- and stereoselectivity, currently limit the application of these methods. Biocatalytic amination of alcohols through enzyme cascades employing ω-transaminases have been reported, although these systems typically require several equivalents of a sacrificial amine donor in order to driving the reactions to high conversion.[4–6] A recent report by Lavandera and co-workers described the use of stoichiometric diamines as the amine donor in an efficient laccase/TEMPO coupled reaction for amination of alcohols.[7]

Recently we reported a biocatalytic hydrogen-borrowing cascade in which a pair of enantio-complementary alcohol dehydrogenases (ADHs) were coupled with an amine dehydrogenase (AmDH) in order to achieve the conversion of racemic secondary alcohols to enantiomerically pure chiral amines on a preparative scale (Figure 1a).[8,9] This initial report established a key proof-of-concept but also highlighted some limitations, notably the requirement for a pair of enantiocomplementary ADHs to achieve the alcohol oxidation step. Following our first generation system, Xu and co-workers described a dual enzyme approach using a single non-selective ADH from *Streptomyces coelicolor* (ScCR) and a newly described AmDH from *Exiguobacterium sibiricum*.[10] Their approach addressed the limitation of using enantiocomplementary ADHs, but at the considerable expense of substrate scope, which was limited to simple aliphatic alcohols. Accordingly, there remains a need to further improve this biocatalytic hydrogen-borrowing method for the asymmetric amination of a broad range of alcohols with ammonia.

Our aim was to develop a second-generation hydrogen-borrowing system that combined the broad substrate scope of our first generation process with the elegance of a single, non-selective enzyme for alcohol oxidation. In order to realize this goal, a number of challenges needed to be simultaneously addressed (Figure 1b). The ADH employed must possess both low enantioselectivity and broad substrate scope for the oxidation of a wide range of alcohols. Furthermore the ADH needed to accept NADH as cofactor to be compatible with the cofactor dependence of engineered AmDHs. Finally, thermostability, low Michaelis constants towards substrate and cofactor, and ease of purification/handling would be highly advantageous for application in hydrogen-borrowing cascades.

Figure 1. a) General scheme for the hydrogen-borrowing amination of an alcohol **1** to the corresponding primary amine **3** by employing an alcohol dehydrogenase and amine dehydrogenase cascade reaction. b) The first hydrogen-borrowing system relied on the use of enantiocomplementary ADHs to achieve the alcohol to ketone conversion. However, it is desirable to accomplish this with a single non-selective ADH. ADHs with broad substrate scope and low enantioselectivity are known but are exclusively NADPH-dependent. Protein engineering allows access to NADH-dependence by rational design.



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Although many ADHs have been previously characterised, only a limited number of wild-type enzymes have been reported to show the broad substrate scope and low selectivity that was required.[11,12] A single NADH dependent racemase has been described but the substrate scope and reaction parameters have not been explored.[13,14] Interestingly, Musa and co-workers recently reported engineered variants of the NAD(P)H dependent ADH from *Thermoanerobacter ethanolicus* (TeSADH) that showed low enantioselectivity in the reduction of phenylacetone.[15–17] TeSADH meets many of the required criteria with respect to selectivity, substrate scope, stability and ease of handling. However, TeSADH exhibits very high selectivity towards NADPH and hence it was envisaged that significant protein engineering would be required to improve the activity with NADH. Switching the cofactor specificity of oxidoreductases has been accomplished using a variety of approaches including using structural information to elucidate beneficial mutations, and the use of multiple sequence alignments of enzyme homologues that display differing cofactor specificity.[18–20] The strategies described above have been used to engineer the cofactor specificity in a number of enzymes; often with an overall loss in catalytic efficiency.[21–24] Recently, Arnold and co-workers reported an impressive general approach (CSR-SALAD) for engineering cofactor specificity and subsequent recovery of activity by screening targeted semi-rational libraries.[25] As a complementary approach, we considered taking advantage of the natural diversity in the cofactor-binding domain amongst structural homologues of TeSADH, to provide a basis for rational re-engineering of cofactor specificity. Herein we describe a structural and sequence guided approach to affect a switch in the cofactor specificity and subsequent application to a two-enzyme hydrogen-borrowing cascade.

Since crystal structures of the non-selective variants of TeSADH have not been reported we used the structure of the wild-type enzyme as the basis for engineering cofactor specificity. Examination of the crystal structure of TeSADH (PDB: 2NVB) revealed a number of residues that are potential points of contact for the ribose phosphate of NADP.[26] Figure 2 highlights residues (G198, S199, R200, and Y218) that are proposed to be important for NAD/NADP discrimination in TeSADH.

In the 3DM™ public database of ADHs, TeSADH belongs to the 3FPLA subfamily containing 455 protein sequences (see Supporting information). This allowed for expedient comparison of TeSADH sequence to the broader subfamily. Wild-type TeSADH possesses a tryptophan residue at position 110 and Phillips and co-workers have shown that mutations at this position confer low enantiospecificity towards a range of secondary alcohols.[15–17,27–29] Inspection of the data revealed approximately half of the sequences featured different residues at position 110. The alignments were therefore constrained to the 226 sequences that contained a conserved Trp-110. Using the automatically generated 3DM sequence alignments we examined the amino acid distribution at each of the four putative positions (G198, S199, R200 and Y218) proposed to be involved in NAD/NADP discrimination (Supporting Information, Figures S2–S5). From the data it was apparent that in the cases of R200 and G198, substitution for aspartate occurred with similar or greater frequency than the analogous amino acid in TeSADH. At position S199, the distribution of residues is broader, however mutation of hydrophobic residues such as leucine appeared in approximately 35 % of the sequences.

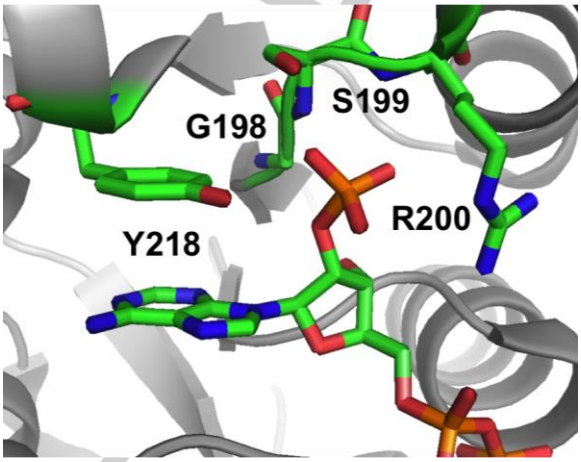


Figure 2. Ribose phosphate of NADP in TeSADH, labelled residues are proposed to play a role in cofactor discrimination.

The most commonly occurring single variants were generated using the non-stereoselective W110A variant as a template, and the relative activity with NAD vs. NADP was compared. (Table 1) Both the variants R200D and Y218F were inactive towards 4-methyl-2-pentanol.

Table 1. Specific activities of TeSADH variants for alcohol oxidations using both NAD and NADP.

Entry	Variant	Specific activity **NADP**[a]	Specific activity **NAD**[b]	Specific for **NA** vs. **NAD**
1	W110A (parent)	237.06 ± 7.96	1.79 ± 0.07	1
2	W110A/R200D	n.d.	n.d.	
3	W110A/R200V	14.30 ± 2.77	2.11 ± 0.11	19
4	W110A/Y218F	n.d	n.d.	
5	W110A/S199L	48.66 ± 2.77	2.73 ± 0.03	7
6	W110A/G198D	1.01 ± 0.09	100.31 ± 3.31	13153
7	W110A/G198D/S 199L	23.71 ± 1.18	50.50 ± 0.02	282

[a] Specific activity in mU mg^{-1} protein measured at 25 °C with 20 mM methyl isobutyl ketone, 1 mM NAD. [b] As for NAD except using 1 mM NADP+ [b] Not determined – specific activity < 0.5 mU/mg.

Remarkably however, the single point mutation G198D simultaneously gave a more than 50-fold improvement in activity towards NAD together with a *ca*. 200-fold reduction in activity towards NADP, equating to >10^4-fold switch in specificity towards NAD compared to the parent (Table 1, Entry 6).

Despite a single mutation in TeSADH affording high activity with NAD, we considered whether it would be possible to further improve the activity this variant. It has been shown that mutations around the adenine moiety of the cofactor can play an important role in improving activity and even altering substrate specificity.[30,31] Given the previous observation that the mutation Y218F rendered TeSADH inactive, this site was excluded and residues G244, N245, I248 were selected as candidates for single-site saturation mutagenesis (Figure 3). The libraries were generated using TeSADH W110A/G198D as a template, and screened by monitoring the formation of NADH spectrophotometrically for the oxidation of racemic 4-methyl-2-pentanol (Supporting Information Figure S6) resulting in the identification of a number of clones with increased activity.

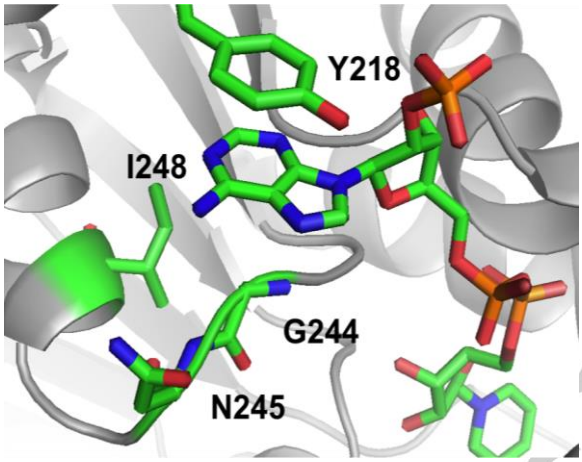


Figure 3. Residues proposed to play a role in stabilising the Adenine moiety of NADP in TeSADH.

The parent (TeSADH W110A/G198D) was the best performing variants in the G244NNK pool. The N245NNK and I248NNK pools revealed mutations to valine in the most active clones. (Supporting Information Table S1). The newly identified triple variants (TeSADH W110A/G198D/N245V and TeSADH W110A/G198D/I248V) were overexpressed and purified. Their kinetic parameters were determined and compared to both previous TeSADH variants. (Table 2)

The triple mutant TeSADH W110A/G198D/N245V displayed an improvement in K_m with NAD of more than 20-fold over the parent TeSADH W110A, however, this was accompanied by a decrease in the $k_{cat,}$ leading to a lower second-order rate constant overall. (Table 2). Therefore the best variant was the W110A/G198D that showed a more than 90-fold improvement in second-order rate constant compared to the parent.

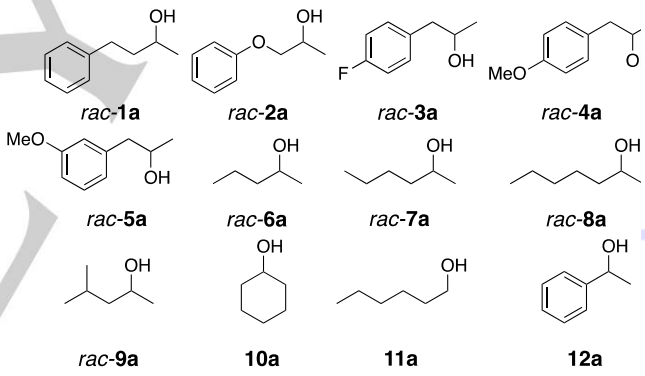
Table 2. Kinetic parameters of TeSADH variants for alcohol oxidation using NAD

Entry	Variant [a]	k_{cat} (s^{-1})	K_M (mM)
1	W110A (parent) [b]	1.60 ± 0.15	19.22 ± 3.09
2	W110A/G198D	7.60 ± 0.37	1.03 ± 0.11
3	W110A/G198D/N245V	1.93 ± 0.35	0.44 ± 0.21
4	W100A/G198D/I248V	3.65 ± 0.46	1.41 ± 0.34

Next, we examined the performance of these engineered variants of TeSADH in our hydrogen-borrowing cascade (Figure 1). Using the ChiAmDH[28] under conditions similar to those previously reported,[4] we performed the hydrogen-borrowing amination of 4-phenyl-2-butanol at [S] = 20 mM resulting initially in conversions that were modest but encouraging (*ca.* 30 %). In order to rapidly optimize conversions we took advantage of surface response methods to probe a large space of reaction conditions with a relatively low number of experiments. [32] (Supporting Information Table S2–S3 and Figure S7). The reactions were optimized for reaction temperature, cofactor concentration and relative loadings of TeSADH and AmDH. Under these conditions conversions up to 86 % were obtained employing the W110A/G198D variant. Despite their lower catalytic efficiency similar results could also be obtained with the remaining variants (Supporting Information Table S4).

Using the optimized reaction conditions the cascade was performed using TeSADH W110A/G198D against a panel of alcohols **1a–12a** (20 mM) representative of the broad substrate scope of our first generation system (Table 3). The substrates were aminated with excellent conversions of up to 90 %.

Table 3. Substrates, conversions and *ee* for the alcohols used in the hydrogen-borrowing amination with newly engineered, NADH-dependent TeSADH W110A/G198D and an AmDH.



rac-**1a** *rac*-**2a** *rac*-**3a** *rac*-**4a**

rac-**5a** *rac*-**6a** *rac*-**7a** *rac*-**8a**

rac-**9a** **10a** **11a** **12a**

Entry	Substrate [a]	Product distribution (%)[b]			*ee* (%)[b]
		Alcohol	Ketone	Amine	
1	*rac*-**1a**	8	7	85	87 (*R*)
2	*rac*-**2a**	33	1	66	>99 (*R*)
3	*rac*-**3a**	19	6	75	>99 (*R*)
4	*rac*-**4a**	18	10	72	>99 (*R*)
5	*rac*-**5a**	29	1	70	>99 (*R*)
6	*rac*-**6a**	16	13	71	>99 (*R*)
7	*rac*-**7a**	3	7	90	>99 (*R*)
8	*rac*-**8a**	49	4	47	99 (*R*)
9	*rac*-**9a**	47	5	48	94 (*R*)
10	**10a**	4	7	89	n.a.
11	**11a**	4	15	81	n.a.
12	*rac*-**12a**	82	7	11	>99 (*R*)

[a] Analytical scale reactions (500 µL) performed in NH_4Cl (1M, pH 9) with substrates (20 mM), NAD+ (3 mM), ChiAmDH (2 mg/mL) and TeSADH W110A/G198D (0.6 mg/mL). [b] Conversion and enantiomeric excess determined by GC analysis – see Supporting Information

Finally, to demonstrate the applicability of our second-generation system, the hydrogen-borrowing cascade with TeSADH W110A/G198D was performed on semi-preparative scale (100 mg). Substrates *rac*-**1a** and *rac*-**4a** were chosen owing to the potential use of the amine products as synthetic intermediates for (*R,R)*-dilevalol and (*R,R)*-formoterol respectively. The asymmetric amination of *rac*-**1a** and *rac*-**4a** was achieved with isolated yields of 69 and 84% respectively.

In summary, using a non-selective NADPH-dependent ADH (TeSADH) as a template, we have successfully applied semi-rational redesign, based on natural sequence diversity, to engineer this ADH to accept NADH as cofactor with a 10,000-fold switch in selectivity. The availability of this NADH-dependent ADH has subsequently allowed us to develop a second generation hydrogen-borrowing system for enantioselective amination of alcohols that combines the broad substrate scope of the first iteration with the benefits of a single, highly stable, non-selective ADH.

Acknowledgements

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Keywords: biocatalysis • enzyme cascades • hydrogen borrowing • protein engineering

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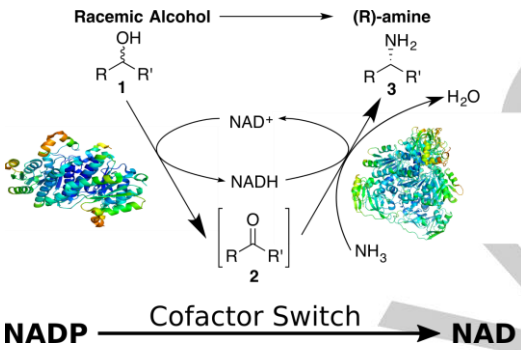

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