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An engineered alcohol oxidase for the oxidation of primary alcohols

Rachel S. Heath,[a] William R. Birmingham,[a] Matthew P. Thompson,[a] Andreas Taglieber,[b] Laurent Daviet[b] and Nicholas J. Turner[a]*

Abstract: Structure guided directed evolution of choline oxidase has been carried out using the oxidation of 1-hexanol to hexanal as the target reaction. A six amino acid variant was identified with an increased k_{cat} of 20 fold compared to the wild-type enzyme. This variant enabled the oxidation of 10 mM hexanol to hexanal in less than 24 h with 100% conversion. Furthermore, this variant showed a marked increase in thermostability with a corresponding increase in T_m of 20 degrees. Improved solvent tolerance was demonstrated with organic solvents including ethyl acetate, heptane and cyclohexane, thereby enabling improved conversions to the aldehyde by up to 30% above conversion for the solvent free system. Despite evolution of choline oxidase towards 1-hexanol, this new variant also showed increased specific activities (by up to 100-fold) for ca. 50 primary aliphatic, unsaturated, branched, cyclic, benzylic and halogenated alcohols.

Introduction

Aldehydes are versatile intermediates in the synthesis of fine chemicals and pharmaceuticals and are components of flavours, fragrances and food ingredients. Oxidation of primary alcohols to aldehydes is commonly employed, although typical chemical reagents are toxic heavy metals or highly reactive oxidants and the process generates large amounts of waste. Increasingly there is a need for catalytic methods that use mild oxidizing agents, with minimal waste, especially using oxygen or peroxide as the primary oxidant. Examples include use of porphyrin derivatives with hydrogen peroxide or tungsten, vanadium or molybdate catalysts, nitroxyl radical based systems (TEMPO) and *N*-oxyl adamantane derivatives in the presence of sodium hypochlorite or air.[1,2] Supported nanoparticles of noble metals have been used to catalyze the oxidation of alcohols under an applied pressure of oxygen.[3]

Alternatively biocatalysts can be used: specifically three different classes of enzyme, namely laccases, alcohol dehydrogenases (ADHs), and alcohol oxidases. Laccases are Cu containing enzymes of largely fungal origin that reduce oxygen to water alongside oxidation of phenolic substrates but require TEMPO or other electron mediators as a cocatalyst.[4] ADHs have been used as biocatalysts but require a NAD(P)$^+$/cofactor recycling system and the equilibrium is unfavourable for preparation of the aldehyde. Use of an NAD(P)H oxidase (NOX) to recycle the cofactor alleviates these issues.[5,6] Alcohol oxidases meanwhile simply use oxygen (air) for cofactor recycling. Importantly, the reaction is irreversible, though hydrogen peroxide is generated as the by-product. If necessary, peroxide may be removed using catalase.

Alcohol oxidases contain either a Cu or flavin adenine dinucleotide (FAD) cofactor and although the family of enzymes covers a wide substrate scope,[7] each individual alcohol oxidase has a relatively narrow substrate range. Previously, enzyme engineering of alcohol oxidases has focused on the Cu containing galactose oxidase with examples of engineering for stability, and switching the activity away from galactose and towards benzylic alcohols.[8,9] Recently however, alcohol oxidases have begun to attract more attention: a fungal alcohol oxidase was engineered towards activity with glycerol,[10] and 5-hydroxymethylfurfural oxidase (HMF oxidase) has been engineered for enhanced enhanced catalytic activity, improved overoxidation to the acid, thermostability and activity (albeit low) towards a secondary alcohol.[11–13]

The aim of this work is to apply structure guided directed evolution to an alcohol oxidase in order to generate an alcohol oxidase with a wider substrate scope for the oxidation of primary alcohols (Figure 1).

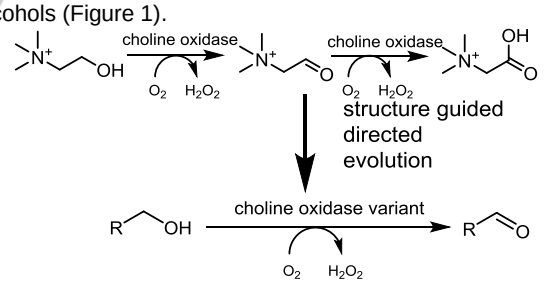

Figure 1. Aim of this work: use structure guided mutagenesis to evolve choline oxidase into a broader spectrum alcohol oxidase.

The FAD containing choline oxidase from *Arthrobacter cholorphenolicus* (AcCO) was chosen as our target enzyme for protein engineering (Figure 1) because: i) initial screening of the wildtype (WT) enzyme showed activity (albeit low in many cases) towards a panel of primary alcohols including hexanol and benzyl alcohol (Table 3, **1** and **37**) (previous reports showed that the substrate scope was limited to choline and analogues)[14,15], ii) the enzyme can be heterologously expressed in *E. coli* and is thus easily manipulated (many other alcohol oxidases require eukaryotic expression systems) and iii) the crystal structure of choline oxidase from *Arthrobacter globiformis*

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Supporting information for this article is given via a link at the end of the document.

(AgCO) (to which AcCO has a high sequence identity) is known, thus allowing structure guided evolution.

Our chosen target substrate for evolution of choline oxidase was 1-hexanol. Previous examples of hexanol oxidation by wild-type alcohol oxidases are limited. Aliphatic alcohols are oxidized by long chain alcohol oxidases and short chain alcohol oxidases but either activity towards hexanol is low, or the substrate scope is small, or these enzymes require the use of eukaryotic expression systems.[16–20] For example, the alcohol oxidase from *Pichia pastoris* has high activity towards short chain aliphatic alcohols (methanol, ethanol) but activity decreases with increasing chain length (though oxidation of longer chains is possible in biphasic systems).[21,22] A galactose oxidase homologue from *Colletotrichum graminicola* demonstrated excellent kinetic parameters for the oxidation of C3-C7 primary alcohols but conversions in biotransformations did not exceed <30%.[23]

Results and Discussion

Activity towards 1-hexanol: The active site and access channel of AgCO were identified from the crystal structure as key areas for mutagenesis to alter the substrate scope (Figure 2).[24] Saturation libraries (ie. covering all possible natural amino acids) were thus made at these sites. (AcCO has 91% sequence identity to AgCO thus the AgCO structure was used as a homology model for AcCO). To allow for cooperativity, libraries contained pairs of proximal residues; three or more positions increases screening effort above our current feasibility. A colony-based solid-phase screen in which the by-product hydrogen peroxide is detected using a horse-radish peroxidase (HRP) and a dye was used to screen libraries W61/M62, S101, V355/F357, D358/M359 and Y465/H466.[25] Colonies which changed colour were picked and the DNA sequenced in order to determine the causative mutation(s).

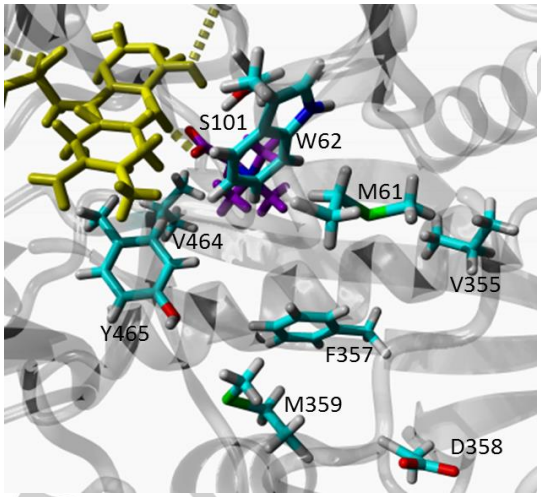


Figure 2. Structure of AgCO active site and entrance channel. (PDB 4mJW) The FAD cofactor is in yellow and natural product glycine betaine is shown in purple. Positions of libraries made in active site and access channel of AcCO (for which residue number also applies) are indicated.

The most active variants resulting from screening these initial libraries with 1-hexanol were S101A, M359R and V355T/F357R. Kinetic parameters of these variants, and those in which the mutations were combined (from here on abbreviated by the number of mutations *e.g.* S101A/V355T/F357R = $AcCO_3$), were determined and compared to the wildtype (WT) enzyme for both the natural substrate choline and 1-hexanol. With the exception of M359R, the mutations were found to decrease the k_{cat} and k_{cat}/K_M towards choline compared to WT whilst simultaneously enhancing activity towards 1-hexanol (Tables 1 & 2). Biotransformations performed with 10 mM hexanol correlated with the kinetic data: WT enzyme gave <50% conversion after 24h whereas $AcCO_4$ gave 100% conversion, with the other variants in between (Figure SI3). The k_{cat}/K_M for 1-hexanol was not significantly different (see SI for calculations) for V355T/F357R, $AcCO_3$, $AcCO_4$ and $AcCO_6$ (see below) but it seems that k_{cat} is the major factor influencing conversion.

Table 1. Kinetic parameters of choline oxidase variants with choline.

Variant	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})
wt	872 ± 15	0.61 ± 0.04	1430 ± 92
M359R	541 ± 16	2.01 ± 0.18	269 ± 25
S101A	271 ± 8	3.3 ± 0.3	82.2 ±6.7
V355T/F357R	11.5 ± 0.9	5.8 ± 1.2	2.0 ± 5.8
S101A/V355T/F357R ($AcCO_3$)	10.2 ± 0.3	3.41 ± 1.8	3.0 ± 1.6
S101A/V355T/F357R/M359R ($AcCO_4$)	0.9 ± 0.3	1.8 ± 0.6	0.5 ± 0.2
S101A/D250G/F253R/V355T/ F357R/M359R ($AcCO_6$)	1.2 ± 0.1	4.7 ± 1.4	0.3 ± 0.1

Table 2. Kinetic parameters of choline oxidase variants with choline.

Variant	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})
wt	2.8 ± 0.6	6.14 ± 1.80	0.5 ± 0.2
M359R	2.2 ± 0.1	3.49 ± 0.50	0.6 ± 0.1
S101A	5.9 ± 0.1	0.67 ± 0.04	8.8 ± 0.5
V355T/F357R	9.9 ± 0.4	0.39 ± 0.06	25.4 ± 0.4
S101A/V355T/F357R ($AcCO_3$)	10.1 ± 0.5	0.43 ± 0.04	23.5 ± 2.5
S101A/V355T/F357R/M359R ($AcCO_4$)	20.6 ± 0.8	0.84 ± 0.09	24.5 ± 2.8
S101A/D250G/F253R/V355T/ F357R/M359R ($AcCO_6$)	59.2 ± 2.4	3.16 ± 0.40	18.7 ± 2.5

Assay conditions: 30 °C, 0.1 mg ml^{-1} HRP, 0.7 mg mL^{-1} ABTS, air-saturated 100 mM potassium phosphate buffer, pH 8.0. The increase in absorbance at 420 nm was followed. Detailed method can be found in the Supporting Information.

Stability: The stability of the WT enzyme was measured at different temperatures (by incubating purified enzyme at a defined temperature for 3 hours before measuring initial rates). Up to 30 °C the enzyme remained stable, however at >40 °C, the remaining activity was negligible (Figure SI1). A further experiment determined the T_m to be 32 °C (Figure 3 and SI2). $AcCO_4$ performed marginally better after incubation at 40 °C, retaining 10% activity (Figure SI1). In an attempt to improve the thermostability we targeted residues that had high B factors for mutatagenesis. Examination of the B factor (which reflects the flexibility) of each residue using BFITTER[26] identified five with high B factors. Saturated libraries at these positions (F253, R241/R242 and D238/A239) were constructed in $AcCO_4$ as well as a random (error prone) library over residues 225-265. The libraries were screened after incubation at 50 °C for three hours using the previously described colony based screen. The positive hits D250G and F253R were combined with $AcCO_4$ to give AcCO S101A/D250G/F253R/V355T/F357R/M359R ($AcCO_6$). $AcCO_6$ maintained close to 50% activity after incubation for three hours at 40 °C, a five-fold improvement over $AcCO_4$ (Figure SI1). We then measured the T_m of $AcCO_6$ to be 52 °C (Figure 3 and SI2), thus showing a twenty degree increase in the thermostability of the six-point variant above the WT. $AcCO_6$ also displayed higher k_{cat} towards hexanol, 3-fold greater than $AcCO_4$ (Table 2) and in biotransformations showed a 20% higher conversion after 1h (Figure SI4).

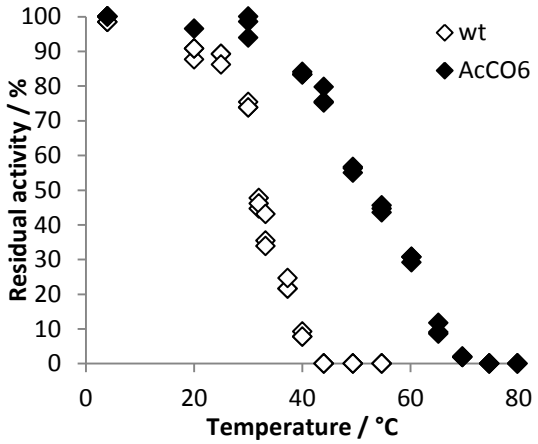


Figure 3. Shift in temperature stability of $AcCO_6$ compared to WT. Enzyme was incubated for 15 minutes at the given temperature before the initial rate was measured at 420 nm. Conditions: 5 mM hexanol, 30 °C, 0.1 mg ml^{-1} HRP, 0.7 mg mL^{-1} ABTS, air-saturated 100 mM potassium phosphate buffer, pH 8.0.

The effect of organic solvent was also examined using WT and $AcCO_6$ with 50 mM hexanol (Figure 4). Remarkably, three solvents increased the conversion achieved by $AcCO_6$ by up to 30%. For the WT, all solvents tested inhibited conversion; in many cases conversion was negligible. Thus the mutations responsible for improving temperature stability also enhanced tolerance for organic solvents, a phenomenon that has been observed with other enzymes.[27]

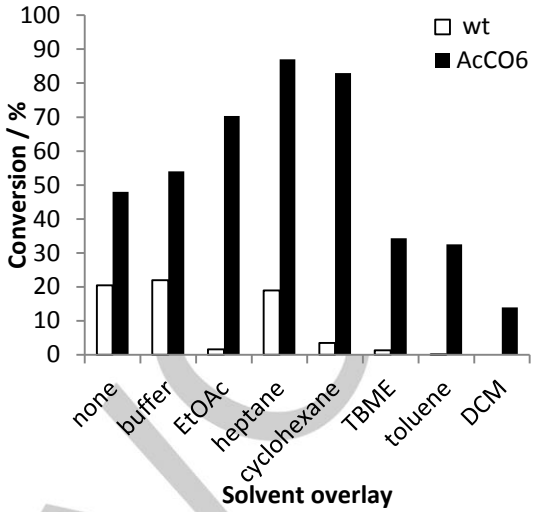
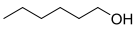
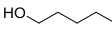
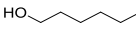
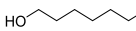
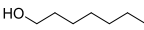
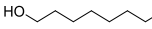
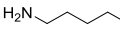
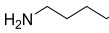
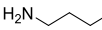
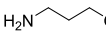
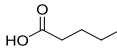
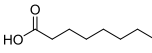
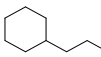
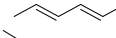
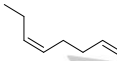
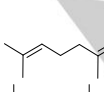
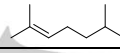


Figure 4. Effect of solvent overlays on conversion of 1-hexanol to hexanal using WT and $AcCO_6$. Conditions: 50 mM 1-hexanol, 1 mg mL^{-1} enzyme, 50% v/v air-saturated 100 mM potassium phosphate buffer, pH 8.0, 50% solvent v/v, 30 °C, 200 rpm, 24h. Conversion measured by GC-FID. Full experimental details can be found in the Supporting Information.

Substrate scope: To determine the substrate scope of $AcCO_6$ we examined the activity with a panel of 50 primary alcohols and compared the results to the WT enzyme (Table 3). For most substrates the activity of $AcCO_6$ was higher than the WT with improvements ranging from *ca*. two-fold (**9**) to >100 fold (**44**, **45**). Amino alcohols were the only substrates where $AcCO_6$ showed marked lower activities compared to the WT, perhaps this is not surprising since we have intentionally mutated activity away from the natural substrate – the amino alcohol choline. Biotransformations with $AcCO_6$ were then applied to alcohols **1**, **7**, **8**, **9**, **10**, **11, 28**, **29, 30**, **32**, **34**, **35**, **36**, **38**, **39**, **40**, and **42** on a 10 mM scale (Figure 5), thus covering a range of saturated, unsaturated, aliphatic, cyclic and benzylic alcohols. For all alcohols tested, $AcCO_6$ showed improved activity relative to the WT. Conversions were improved by >50% compared to WT for **1**, **28**, **29**, **30**, **35**, **40** and **42**. Saturated alcohols with C6-C10 chain lengths and unsaturated alcohols were excellent substrates. For cyclic alcohols, $AcCO_6$ gave some of the most improved conversions compared to WT.

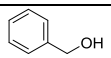
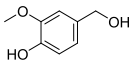
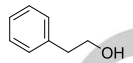
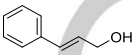
Preparative oxidation of hexanol (100 mg) with $AcCO_6$ gave 100% conversion to hexanal with an isolated yield of 72%. WT choline oxidase catalyzes over oxidation to the carboxylic acid and hence this side-activity was checked for $AcCO_6$ (see SI). Hexanol over-oxidation was negligible (<1%) but with cinnamyl alcohol (**40**), 20% conversion to carboxylic acid was observed. Due to the solvent tolerance of the six-point variant, further oxidation could be minimized by use of a biphasic system in which the aldehyde partitions to the organic phase preventing gem-diol formation necessary for over-oxidation.[28]

Table 3. Specific activities of WT and $AcCO_6$

	Structure	WT mU/mg	$AcCO_6$ mU/mg	[a]fold increase
1		20	571	29
2		0	0.53	N/A
3		0	1.2	N/A
4		0.15	4.4	29
5		16	246	15
6		31	558	18
7		42	234	5.6
8		18	38	2.1
9		16	26	1.6
10		17	15	0.88
11		2.5	2.4	0.96
12		104	803	7.7
13		69	384	5.6
14		46	118	2.6
15		28	100	3.6
16		8.6	16	1.9
17		0	15	N/A
18		318	52	0.16
19		151	16	0.1
20		98	2.6	0.03
21		16	0	N/A
22		10623	91	0.009
23		34	0	N/A
24		1.1	6.8	6.2
25		1.3	76	58
26		1.6	47	29
27		14	597	43
28		0.74	39	53
29		3	91	30
30		n.d.[b]	n.d.	N/A
31		119	536	4.5
32		23	643	28
33		10	523	52
34		36	118	3.3
35		45	84	1.9
36		96	85	0.9

[a] in SA from WT to $AcCO_6$, [b] n.d. = not determined (color change pre enzyme), n.t. = not tested, N/A =not applicable

Table 3 cont.

	Structure	WT mU/mg	$AcCO_6$ mU/mg	[b]fold increase
37		1.7	115	68
38		n.t.	1.7	N/A
39		45	636	14
40		1.5	8.2	5.5
41		3.7	56	15
42		0.25	6.2	25
43		0.07	5.8	82.9
44		2.8	351	125
45		2.4	319	133
46		0	2.4	N/A
47		0.64	51	80
48		17	122	7
49		62	655	10.6
50		33	549	16.6

Assay conditions: 30 °C, 0.1 mg ml^{-1} HRP, 0.7 mg mL^{-1} ABTS, 5 mM substrate, air-saturated 100 mM potassium phosphate buffer, pH 8.0. The increase in absorbance at 420 nm was followed. Further details can be found in the Supporting Information.

Figure 5. Conversions of 10 mM alcohol to aldehyde by WT choline oxidase and $AcCO_6$. Substrates as in Table 3. Columns represent an average of a minimum of three replicates. Conversion analysed by GC-FID Conditions: 24 hrs, 30 °C, 200

rpm, 1 mg mL^{-1} enzyme, 100 mM pH 8.0 air-saturated phosphate buffer.

Mutations identified from screening libraries of residues thought to be important for substrate scope led to the identification of $AcCO_4$, a variant with 50 fold improvement in k_{cat}/K_M toward hexanol compared to WT. This variant also has much reduced activity towards choline. Analagous to the results here, the S101A mutation in AgCO had also previously been shown to reduce activity towards choline.[29]

Mutations remote from the active site have previously been shown to increase the activity of enzymes by lowering the free energy of the catalytic conformation of the enzyme, as well as increasing thermostability and/or solvent tolerance.[30,31] Two mutations at D250 and F253 combined with $AcCO_4$ to form the variant $AcCO_6$ which possessed improved stability at higher temperatures including a 20 degree higher T_m than the WT and also showed a 3-fold increase in activity (k_{cat}) compared to $AcCO_4$. Residues D250 and F253 form part of a loop comprising amino acids 250-255 which by X-ray crystallography appear in two different conformations ("open" and "closed").[24] In the closed conformation, the side chain of F253 (from the other subunit) sterically hinders access to the channel to the active site. Mutation of these residues may affect the position of the loop and hence substrate access to the active site. Further increase in activity will likely require more mutations that are distal to the active site and will be predicted computationally.[32]

The solvent tolerance of $AcCO_6$ was also significantly enhanced compared to WT and in several cases the conversion to product was increased compared to no solvent overlay. Such was the solvent tolerance of the six-point variant that it enabled us to perform (continuous) flow reactions (using immobilized enzyme) for the oxidation of 1-hexanol in pure organic solvent.[33] For aldehydes that are sensitive to over-oxidation to the acid, this route, with its absence of water, provides a solution.

Although $AcCO_6$ was identified by screening with 1-hexanol, pleasingly it demonstrates a broader substrate scope with oxidation of other primary alcohols including **31**, **37**, **38** and **50** which are natural substrates of other alcohol oxidases.[11,34–36]

Conclusions

By examination of the crystal structure of choline oxidase, key sites for mutagenesis to both alter substrate scope and increase thermostability were determined. A six-point variant was evolved which was more active on our substrates of interest as well as demonstrating markedly improved thermostability and solvent tolerance compared to the wildtype. The enzyme could be used in biotransformations for the oxidation of primary alcohols to aldehydes with full conversion in many cases.

Experimental Section

Full experimental details can be found in the Supporting Information.

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Keywords: enzyme engineering • oxidase • thermostability • alcohol • aldehyde

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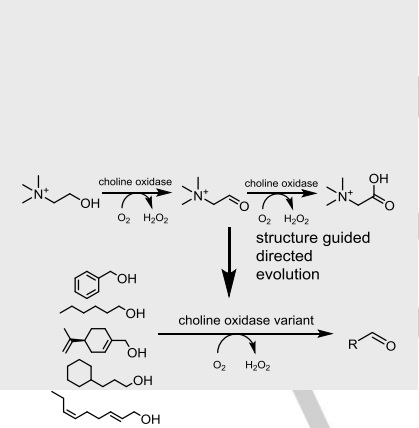
Alcohol oxidases are advantageous in that they require no additional cofactor or cofactor recycling systems, simply requiring molecular oxygen for catalysis. Their use is currently limited by activity and substrate scope. An alcohol oxidase was evolved to produce an enzyme with a broad scope for primary alcohol oxidations. Further improvements were made to enzyme stability and demonstrated by its increased thermostability and solvent tolerance.



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