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Chemo-promiscuity of alcohol dehydrogenases: reduction of phenylacetaldoxime to the alcohol

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ABSTRACT

The reduction of phenylacetaldoxime was catalysed by alcohol dehydrogenases in the presence of NAD(P)H yielding finally the primary alcohol via the imine and aldehyde intermediates. This suggests that the hydride of the cofactor NAD(P)H is transferred to the N-atom of the oxime moiety and not to the carbon atom, as usual stated. This reaction represents the first example of a catalytic chemo-promiscuity of alcohol dehydrogenases.

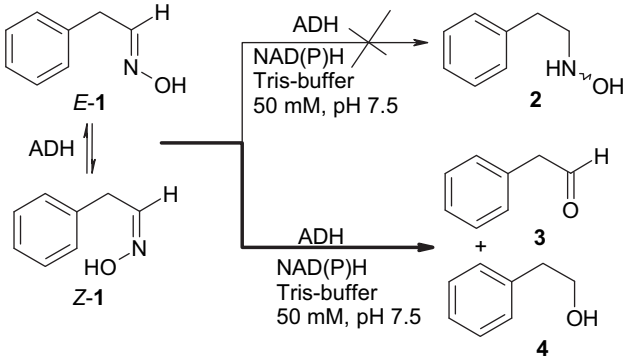
1. Introduction

Alcohol dehydrogenases (ADHs) are enzymes, which are responsible to transform ketone/aldehydes to the corresponding alcohols and vice versa at the expense of a nicotinamide cofactor that acts as hydride donor and acceptor, respectively.[1] Baker's yeast, which is known to possess many ADHs,[2] has been reported to transform oximes to carbonyl compounds or alcohols;[3] however, contradicting reports indicated that Baker's yeast catalysed the reduction of oximes to afford the corresponding hydroxylamines and amines.[4] Since amines are valuable intermediates and products,[5] we envisioned that ADHs might act as suitable catalysts to reduce the C=N double bond of oximes yielding hydroxylamines, which could easily be transformed to the corresponding amines.[6] Overall, this would represent a first example of a promiscuous activity[7] of ADHs.

2. Results and discussion

2.1. Testing ADHs

Using phenylacetaldoxime **1** (*Z*/*E*=95:5) as a test substrate the desired reduction to hydroxylamine **2** was investigated employing various ADH preparations and NAD(P)H recycling systems (Scheme 1, Table 1). Unfortunately, no formation of hydroxylamine or amine could be detected. The control experiment showed that the substrate was stable under the reaction conditions employed. Employing the ADH from *Rhodococcus erythropolis* (RE-ADH) no transformation of **1** was detected at all. The other five ADHs showed *E*/*Z*-isomerisation [e.g., ADH from *Paracoccus pantotrophus* DSM 11072 (PpADH)].[8] Unexpectedly four ADH preparations (HLADH, ADH-A, RasADH, LK-ADH) led to the formation of alcohol **4**, whereby in the case of LK-ADH and RasADH phenylacetaldehyde **3** was detected, too. For instance ADH-A led to the formation of 16% of alcohol **4** within 24 h and 38% within 48 h.



Scheme 1. Biotransformation of oxime **1** employing alcohol dehydrogenases.

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Table 1
Transformation of aldoxime **1** (*Z*/*E*=95:5) by various ADH preparations[a]

Entry	ADH	NAD(P)H recycling	*Z*-**1**[k] (%)	*E*-**1**[k] (%)	Aldehyde **3**[k] (%)	Alcohol **4**[k] (%)
1	—[b]	—	95	5	<0.1	<0.1
2	RE-ADH[c]	2-PrOH	95	5	<0.1	<0.1
3	PpADH[d,e]	2-PrOH	58	42	<0.1	<0.1
4	HLADH[f]	Ethanol	84	15	<0.1	1
5	ADH-A[e,g]	2-PrOH	49 (28)[l]	35 (34)[l]	<0.1	16 (38)[l]
6	RasADH[e,h]	GDH[j]	47	46	1	6[m]
7	LK-ADH[i]	GDH[j]	73	24	1	2

[a] Reaction conditions: Tris/HCl buffer (pH 7.5, 50 mM), 1 mM NAD(P)H, 6 g/L substrate, ADH preparation and cofactor recycling systems, 30 °C, 24 h.
[b] Control in the absence of enzyme.
[c] RE-ADH: ADH from *Rhodococcus erythropolis*, NADH.
[d] PpADH: ADH from *Paracoccus pantotrophus* DSM 11072, NADH, see Ref. 8.
[e] Overexpressed in *E. coli*, used as lyophilised *E. coli* powder.
[f] HLADH: horse liver alcohol dehydrogenase, NADH.
[g] ADH-A: ADH from *Rhodococcus ruber* DSM 44541, NADH, see Ref. 9.
[h] RasADH: ADH from *Ralstonia* sp. DSM 6428, NADPH, see Ref. 10.
[i] LK-ADH: ADH from *Lactobacillus kefir*, NADPH.
[j] Glucose dehydrogenase employing glucose.
[k] Measured by GC–MS.
[l] 48 h reaction time.
[m] In the presence of 5% v/v DMSO; without DMSO: 3% alcohol **4**.

2.2. Isomerisation

These unexpected biocatalytic activities—namely oxime isomerisation as well as reduction of the oxime to the alcohol—prompted us to investigate further this system. The chemical mechanism for (acid- and base-catalysed) oxime isomerisation has been elucidated in literature.[11] In general, oximes are stable at neutral pH towards isomerisation and hydrolysis[12] as confirmed in our blank experiment. In order to elucidate whether the observed *E*/*Z*-isomerisation of **1** was a genuine catalytic activity of the ADHs (except RE-ADH) or mediated in a non-specific fashion, a set of well-known protein preparations were tested (Table 2). The results showed that isomerisation of oxime **1** was not specific for alcohol dehydrogenases, since every preparation tested led to isomerisation. Therefore we did not investigate this unspecific reaction in more detail.

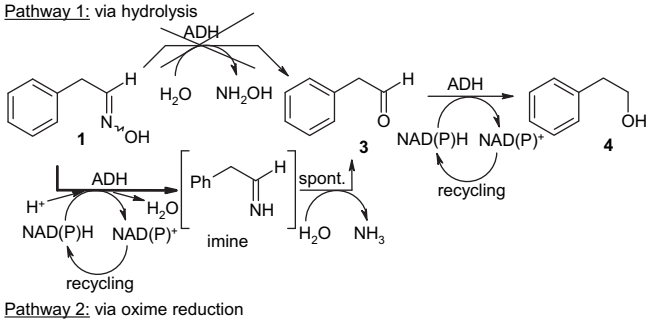
Table 2
E/*Z*-isomerisation of aldoxime **1** (*Z*/*E*=95:5) catalysed by protein preparations[a]

Entry	Protein preparation	*Z*-**1**[c] (%)	*E*-**1**[c] (%)
1	—[b]	95	5
2	Lipase *Aspergillus niger*	90	10
3	Esterase BS3 *Bacillus stearothermophilus*	90	10
4	Bovine serum albumine	76	24
5	CAL-B *Candida antarctica* lipase B	51	49
6	*E. coli*, lyophilised cells	55	45

[a] Reaction conditions: Tris/HCl buffer (pH 7.5, 50 mM), 1 mM NAD(P)H, 6 g/L substrate, crude enzyme preparations, 30 °C, 24 h.
[b] Control: sample without enzymes.
[c] Measured by GC–MS.

2.3. Transformation of oxime to alcohol

Therefore we turned our attention to the ADH-catalysed transformation of the oxime to the alcohol. Alcohol **4** can only be obtained here by reduction of the corresponding aldehyde **3** (Scheme 2), which is also indicated by the detected aldehyde **3** in selected experiments (Table 1, entries 6 and 7). In separate experiments it was proven that the suspected ADH preparations are indeed able to reduce aldehyde **3** to the alcohol **4** in the presence of an NAD(P)H recycling system. As a consequence aldehyde **3** is the obvious precursor for the formation of the alcohol **4** as depicted in Scheme 2. The next question concerns the formation of aldehyde **3**. Aldehyde **3** might be obtained by hydrolysis from aldoxime **1** as generally assumed in literature (Scheme 2, pathway 1).[3b,e,13]



Scheme 2. Pathways for transformation of aldoxime **1** to alcohol **4**.

Hydrolysis might be an unspecific spontaneous reaction, however since the blank experiments (Table 1, entry 1) as well as all other (hydrolytic) enzyme preparations (Table 2) did not lead to hydrolysis of aldoxime **1** to afford the corresponding aldehyde, formation of aldehyde **3** can only be attributed to the activity of the ADHs. In case the ADHs are indeed catalysing this transformation of the oxime via hydrolysis, it can be expected that no reducing equivalents are required: in a test experiment aldoxime **1** was incubated in the presence of various ADHs (ADH-A, PpADH and RasADH) in the absence of NAD(P)H recycling. To our surprise no hydrolysis occurred, thus no aldehyde was formed (neither alcohol, only isomerisation was observed) even if $NAD(P)^+$ was present. As a consequence it was concluded that the ADH in combination with the reduced cofactor might be responsible for the observed transformation. Additional tests showed that NAD(P)H itself is not able to reduce aldoxime **1** spontaneously in the absence of ADH, therefore both ADH and NAD(P)H were required to transform the aldoxime **1** to the aldehyde **3** by *reduction*. The only feasible reduced intermediate one can think of is an imine intermediate, which then spontaneously hydrolyses to yield the aldehyde (Scheme 2, pathway 2).

Attempts to trap the imine intermediate by reduction to the amine employing $NaBH_3CN$, Raney nickel or H_2/Pd/C were not successful; however this was not surprising since additional chemical reduction experiments employing phenylacetaldehyde in the presence of huge excess of ammonia showed that the possible imine intermediate is much too unstable to be trapped at all in water; thus not a trace of amine could be detected when testing the chemical reduction of a possible imine in a mixture of phenylacetaldehyde and ammonia in aqueous solution. This is supported by literature, since no preparative metal-catalysed reductive amination of comparable aldehydes/ketones with ammonia in water at reasonable conditions can be found. Anyway, since aldehyde **3** is an intermediate and since the oxime is reduced (and not hydrolysed), the imine is the only possible unstable intermediate to be expected. Related reductive transformations of oximes to imines have previously been achieved by chemical methods[14] and have been observed by a reconstituted liver microsomal system of a pig liver CYP2D enzyme (NADH-benzamidoxime reductase).[15] Therefore, we propose a reduction pathway via the imine for the ADH-catalysed transformation of oxime **1**. Related observations were reported recently for flavo-proteins for the reduction of aliphatic nitro-compounds leading finally to aldehydes and not to amines or hydroxylamines.[16]

The sequential reduction of the aldehyde to the alcohol was much faster than the aldoxime reduction, since only traces of the aldehyde could be detected in selected cases (LK-ADH and

Table 3
Testing the promiscuous oxime reducing activity with purified strep-RasADH

Entry	Catalyst	Reagents	*Z*-**1**[c] (%)	*E*-**1**[c] (%)	Aldehyde **3**[c] (%)	Alcohol **4**[c] (%)
1	—	NADPH	95	5	<0.1	<0.1
2	—	Glucose	95	5	<0.1	<0.1
3	—	GDH	93	7	<0.1	<0.1
4	strep-RasADH[a,b]	NADPH, glucose, GDH	58	33	1	6

[a] Reaction conditions: Tris/HCl buffer (pH 7.5, 50 mM), 1 mM NADPH, 6 g/L substrate, GDH, glucose, 30 °C, 24 h.
[b] ADH from *Ralstonia* sp. DSM 6428 with strep-tag, purified by affinity chromatography.
[c] Measured by GC–MS.

RasADH). Since carbonyl reduction is the 'natural' activity of ADHs, it is understandable that it proceeds considerably faster than the promiscuous aldoxime reduction. This is in agreement with previous enzyme promiscuity studies comparing natural and promiscuous activity showing that the latter is much slower.[17]

2.4. Tests with purified ADH

To ensure that the observed activity is not catalysed by other interfering enzymes possibly present in the crude (commercial) ADH preparations, a strep-tagged ADH from *Ralstonia* sp. DSM 6428 (strep-RasADH) was prepared and purified by affinity chromatography. This pure strep-RasADH was used for the transformation of aldoxime **1** employing NADPH as cofactor and glucose dehydrogenase (GDH) with glucose for cofactor recycling (Table 3). Only in the presence of strep-RasADH and NADPH recycling the formation of alcohol **4** and a trace of aldehyde **3** could be detected (entry 4), proving that indeed only the promiscuous activity of the alcohol dehydrogenase is responsible for reducing aldoxime **1** to finally yield the primary alcohol **4**.

Since the reduction step of the aldoxime did not afford the stable hydroxylamine **2**, but the unstable imine (which hydrolysed spontaneously), it can be concluded that the hydride of NAD(P)H is transferred to the nitrogen atom of the oxime moiety and not to the carbon atom, probably due to the poorer electrophilicity of the carbon atom of the oxime moiety and the changed polarisation of the nitrogen atom by the proximity of the negatively polarised hydroxyl group. As observed for the reduction of carbonyls, ADHs transfer the hydride to the atom next to the negative polarised oxygen, which is in the case of carbonyls a carbon but for oximes it is the nitrogen atom; another reason for the hydride transfer to the nitrogen might result from the spatial arrangement between substrate, NAD(P)H and enzyme.

3. Conclusion

In summary, we have described the first example of a catalytic chemo-promiscuous activity of alcohol dehydrogenases, namely the reduction of aldoxime **1** to the unstable imine intermediate, which hydrolyses to give the aldehyde intermediate. ADH-catalysed reduction of the aldehyde yielded finally the corresponding primary alcohol resembling overall reductive bio-deoximation.[18] As a consequence the results suggest that the hydride of NAD(P)H is transferred to the nitrogen atom of the oxime moiety and not to the carbon atom; only transfer of the hydride to the nitrogen can lead to the proposed imine, which can then hydrolyse to the aldehyde; transfer of the hydride to the carbon would result in the formation of stable hydroxylamine, which was not observed. The results explain why various attempts of microbial oxime reductions aiming at the preparation of amines were unsuccessful,[3] since the promiscuous ADH activity interfered. Furthermore, it could be deduced that the transformation of oximes to aldehydes/ketones by fermenting yeast possessing a lot of ADHs did not proceed via hydrolysis but through reduction catalysed by the ADHs.

4. Experimental section

4.1. General

Phenylacetaldehyde, and hydroxylamine hydrochloride and all other reagents and solvents were purchased with the highest purity and used as received either from Aldrich–Sigma–Fluka (Vienna, Austria) or Lancaster (Frankfurt am Main, Germany). Alcohol dehydrogenase from *R. erythropolis* (RE-ADH, Nr. 04.11, 33.8 U/g), glucose dehydrogenase (GDH) from *Bacillus megaterium*, and esterase BS3 from *Bacillus stearothermophilus* (Nr. 16 U/g) were purchased from Jülich Chiral Solutions GmbH, a Codexis company (Germany). Alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH, Nr. 06543, 0.4 U/mg), lipase from *Aspergillus niger* (194 U/g), alcohol dehydrogenase from equine liver (HLADH, Nr. A8435, 1.33 U/mg), and bovine albumin (BSA, A-2153) were obtained from Aldrich–Sigma–Fluka (Vienna, Austria). Lipase from *Candida antarctica* B (CAL-B) was purchased from Novozymes. Alcohol dehydrogenase from *Rhodococcus ruber* DSM 44541 (ADH-'A') was commercially available from Codexis Inc or can be prepared as *Escherichia coli*/ADH-A preparation as previously reported.[9] Overexpressed *P. pantotrophus* ADH (PpADH)[8] and *Ralstonia* sp. ADH (RasADH)[10] were obtained as previously described and used as lyophilised powder.

TLC plates were run on silica gel Merk 60 F_{254} and visualised by UV or by spraying with $KMnO_4$ solution. NMR spectra were measured on a Bruker AMX spectrometer at 360 MHz.

4.2. GC–MS analysis

GC–MS analyses were performed on an HP 6890 Series GC system equipped with a 5973 mass selective detector and a 7683 Series injector. The following column was used: Agilent 19091S-433 (30 m, 0.25 mm, 0.25 μm film) and the method used was: 1.3 mL/min, 100 °C, then 10 °C/min until 300 °C (held 0.5 min).

4.3. Synthesis of *E*- and *Z*-phenylacetaldoxime 1

Phenylacetaldehyde (200 μL, 1.79 mmol) was dissolved in ethanol (5 mL). Hydroxylamine hydrochloride (249 mg, 3.58 mmol) was dissolved in pyridine (435 μL, 5.37 mmol) and added to the phenylacetaldehyde solution. The reaction mixture was stirred for 24 h at room temperature and monitored by thin-layer chromatography (ethyl acetate/petroleum ether 1:3, v/v). The reaction mixture was evaporated, water (20 mL) was added and the mixture was extracted twice with ethyl acetate. The combined organic layers were washed with water and brine and dried (Na_2SO_4). After filtering, the organic solvent was concentrated by evaporation under reduced pressure and the product was purified via *flash* chromatography (ethyl acetate/petroleum ether 1:5, v/v) leading to a mixture of *E*- and *Z*-**1** isomers. Upon recrystallisation with chloroform and hexane, the *Z*-isomer crystallised in about 95% purity. The yield was 47% [R_f=0.24 (ethyl acetate/petroleum ether 1:3, v/v). GC–MS (EI): 135 ($M^{+\cdot}$), 117 ($M^{+}-OH$)]. ^1H NMR ($CDCl_3$) showed a mixture of *E*- and *Z*-isomers. Compound *Z*-**1** (88%): δ 3.78

(d, J=5.3 Hz, 2H), 6.94 (t, J=5.3 Hz, 1H), 7.33 (m, 4H), 8.88 (br s, 1H) and Compound *E*-**1** (12%): δ 3.58 (d, J=6.3 Hz, 2H), 7.57 (t, J=6.4 Hz, 1H), 7.33 (m, 4H), 8.35 (br s, 1H).

4.4. Example procedure for the enzymatic screening

Lyophilised cells of *E. coli*/ADH-'A' (20 mg), 2-propanol (32 μL) as hydrogen donor and the substrate (6 g/L) were suspended in Tris·HCl buffer [500 μL, pH 7.5, 50 mM, 1 mM NADH] in Eppendorf tubes (1.5 mL) and shaken at 30 °C and 600 rpm for 24 h. The reaction was stopped by extraction with ethyl acetate (2×0.5 mL). The organic layer was separated from the cells by centrifugation (2 min, 13,000 rpm) and dried (Na_2SO_4). Composition of the product mixture was determined by GC–MS.

4.5. Cloning, expression and purification of tagged *Ralstonia* sp. ADH

4.5.1. Construction and cloning of a StrepII tagged Ralstonia sp. ADH gene. Standard molecular-biology procedures were performed according to literature.[19] The StrepII tag was introduced at the 3′ end of the RasADH gene by PCR using pEam_RasADH[10] as template. The primers were ADH-f 5′-ATGTATCGACTATTAAACAAAACAGC-3′ and ADH-StrepII-r5′-TTA**TTTTTCGAACTGCGGGTGGCTCCAAGCGCT**-GACCTGGGTCAATCCACCGTCC-3′ (StrepII Tag sequence in bold). The PCR product was purified and cloned into pEamTA.[20] The resulting plasmid pEam_tagged-RasADH was used to transform *E. coli* DH5α, the strain was arbitrarily designated as *E. coli*/tagged-RasADH.

4.5.2. Preparation of lyophilised cells containing recombinant catalyst E. coli/RasADH. Cultivation medium LB-amp: Luria broth (25 g/L, Sigma L-3522), ampicillin sodium salt (100 mg/L, Sigma A9518-5 G), KH_2PO_4 (1.4 g/L, Fluka 60220) and K_2HPO_4 (4.4 g/L, Fluka 60355).

E. coli/RasADH was stored at −86 °C in a glycerol/LB-amp 15:85 solution. Prior to use it was plated on LB-amp, a single colony was plated again on LB-amp (16 h, 37 °C), before a loop of cells were used to inoculate 250 mL of LB-amp medium in 1 L baffled shake flasks. After incubation for 24 h at 30 °C at 130 rpm an OD of ∼5 was reached and the expression of ADHs was induced by the addition of IPTG (450 mg/L, 2 mM final concentration, preqlab Biotechnologie GmbH 37-2020) and again, ampicillin sodium salt (100 mg/L) was added. The incubation was performed at 20 °C to avoid the formation of inclusion bodies for 24 h at 130 rpm. The cells were harvested by centrifugation (8000 rpm, 3000 g, 20 min, 4 °C), the medium was decanted and the cells were resuspended in water, shock-frozen (liquid nitrogen) and lyophilised.

The same procedure was used for overexpression of tagged-RasADH.

4.5.3. Purification of tagged-RasADH. The purification was performed following the instruction of the supplier of the column (IBA, BioTAGnology, purification of strep-tag fusion proteins with Strep-Tactin matrices, version PR03-0002).

E. coli/tagged-RasADH cells were grown in LB media (2 L) with ampicillin (100 mg/L) at 30 °C for 24 h. Afterwards, additional ampicillin (100 mg/L) and IPTG (2 mM) were added and cells were grown at 20 °C for 24 h. Then, the media was centrifuged, the cells were washed with a buffer W as described by the supplier (buffer W, Tris 100 mM, NaCl 150 mM, EDTA 1 mM, pH 8), and the cells were suspended in 50 mL of the same buffer.

Cells disruption was performed by ultrasonication: 16 min, 1 s on, 2 s off, 50% amplitude, then centrifuged and the supernatant was taken for the strep-tag purification.

A Strep-Tactin Superflow catridge (5 mL bed volume, Cat. No. 2-2112-001, IBA) was equilibrated by passing 10 mL of buffer W (IBA, BioTAGnology). Then, the supernatant was microfiltered and passed through the column. The column was washed with buffer W (25 mL) to remove the remaining *E. coli* proteins.

The ADH was eluted with another buffer (buffer E, Tris 100 mM, NaCl 150 mM, EDTA 1 mM, 2.5 mM desthiobiotin, pH 8) and eight fractions of 2.5 mL were collected separately. Afterwards, activity (propiophenone, NADPH) was tested with every fraction to identify the ADH. SDS-page analysis showed a single band.

4.6. Procedure of enzymatic conversion using purified tagged-RasADH

Purified tagged-RasADH (475 μL), substrate **1** (6 g/L), DMSO (25 μL), NADPH (1 mM), GDH (1 mg), and glucose (13 mg) were placed in an Eppendorf tube (1.5 mL). The reaction mixture was shaken at 30 °C and 600 rpm for 24 h and stopped by extraction with ethyl acetate (2×0.5 mL). The organic layer was separated and dried (Na_2SO_4). Composition of the product mixture was determined by GC–MS.

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