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Research Article

C=C-Ene-Reductases Reduce the C=N Bond of Oximes

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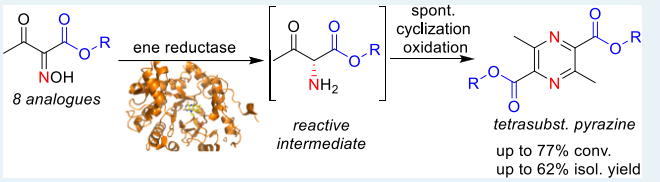
ABSTRACT: Although enzymes have been found for many reactions, there are still transformations for which no enzyme is known. For instance, not a single defined enzyme has been described for the reduction of the C=N bond of an oxime, only whole organisms. Such an enzymatic reduction of an oxime may give access to (chiral) amines. By serendipity, we found that the oxime moiety adjacent to a ketone as well as an ester group can be reduced by ene-reductases (ERs) to an intermediate amino group. ERs are well-known enzymes for the reduction of activated alkenes, as of α,β-unsaturated ketones. For the specific substrate used here, the amine intermediate spontaneously reacts further to tetrasubstituted pyrazines. This reduction reaction represents an unexpected promiscuous activity of ERs expanding the toolkit of transformations using enzymes.



KEYWORDS: *biocatalysis, ene-reductases, oxime, amine, pyrazine*

INTRODUCTION

The repertoire of chemical reactions gets constantly expanded,[1] including the spectrum of biocatalytic transformations by, for example, (i) exploiting light[2] or (ii) evolving enzymes for new reactions.[3] However, the biocatalytic transformation of an oxime to an amine has not been observed using a defined enzyme.

The oxime functionality can be chemically transformed *via* various established reactions[4] such as rearrangements[5] or chemical reductions, the latter leading to amines,[6] imines,[7] aziridines,[8] or hydroxylamines.[9] Biocatalytic transformations of oximes have been reported to give carbonyl compounds or alcohols only using baker's yeast, alcohol dehydrogenases, or ene-reductases (ERs).[10] In these cases, the oxime was most likely transformed enzymatically to the corresponding imine, which was subsequently hydrolyzed spontaneously to the carbonyl compound. Other reports indicated that whole-cell organisms such as Baker's yeast or anaerobic organisms may enable the reduction of oximes to afford the corresponding hydroxylamines and/or amines or even pyrazines,[11] but the involved enzymes have never been identified. Here, we report the first enzymatic reduction of an oxime to an amine using ERs.

RESULTS AND DISCUSSION

Searching for a defined enzyme to reduce the C=N double bond of an oxime, we suspected first that imine reductases[12] may be well-suited enzymes. Six randomly selected imine reductases (see the Supporting Information for details) were tested for the reduction of the model compound **1a**. However, no transformation was observed in any case, which supports a previous report on unsuccessful reduction of oximes with imine reductases.[13] Looking closer to the structure of the investigated activated oxime **1a**, the C=N double bond conjugated to a carbonyl reminded us of α,β-unsaturated carbonyl compounds, which are typical substrates for flavin-dependent ERs.[14] In general, ERs have been described to reduce C=C double bonds, which are activated *via* a broad set of electron-withdrawing functionalities such as carbonyls (aldehydes, ketones, carboxylic acids, and esters), nitriles, cyclic imides, and nitro groups. Although **1a** does not possess a C=C but a C=N double bond, several purified ERs[15−19] were screened for their activity toward the model compound **1a** (Scheme 1 and Table 1). Surprisingly, all ERs investigated transformed substrate **1a** in the presence of a NADPH recycling system [glucose dehydrogenase (GDH)/glucose]. Isolation of the obtained product revealed the formation of the tetrasubstituted pyrazine **2a** as the sole detectable product. Pyrazines have previously been obtained, for example, by chemoenzymatic reaction sequences involving transaminases.[20]

In the absence of GDH/glucose, product formation of **2a** corresponded to the amount of NADPH present (Table S2). In the absence of NADPH, no product formation was detectable at all. Furthermore, no product was detected (i) when omitting the ER, (ii) when the ER was substituted for a catalytic amount of FMN, and (iii) when the ER was substituted for a stoichiometric amount of NADPH. This indicated that this transformation is catalyzed by the ERs.

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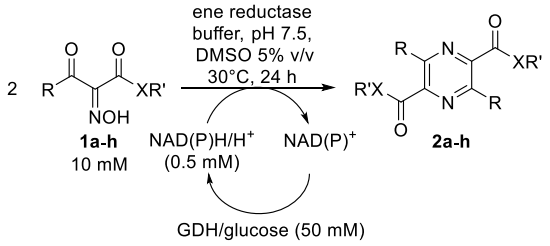
Scheme 1. Reductive Transformation of Activated Oximes by ERs



R	XR'
a: CH_3	OC_2H_5
b: CH_3	$OCH_2CH{=}CH_2$
c: CH_3	$OCH_2CH_2OCH_3$
d: CH_3	OC_4H_9
e: CH_3	OC_7H_{15}

R	XR'
f: CH_3	OCH_2Ph
g: CH_3	NHPh
h: C_2H_5	OC_2H_5
i: *n*-C_4H_9	OC_2H_5
j: Ph	OC_2H_5

Table 1. Transformation of the Oximes with Purified ERs

		enzyme[a]					
		product formation **2** [%][b,c,d]					
#	sub	OYE1	OYE2	OYE3	OPR3	XenA	FOYE
1	**1a**	64	68 (61)	66	40	15	61
2	**1b**	64	58	62	43	<1	64 (42)
3	**1c**	48	49	39	38	9	53 (53)
4	**1d**	50	51	53 (62)	41	17	51
5	**1e**	61	63	77 (57)	49	<1	77
6	**1f**	56	59	59 (50)	45	<1	53
7	**1g**	59	56	51	49	29	62 (35)
8	**1h**	18	17	28	16	38 (39)	14

[a]The enzymes originate from *Saccharomyces pastorianus* (OYE1),[15] *Saccharomyces cerevisiae* (OYE2 and OYE3),[16] *Arabidopsis thaliana* (OPR3),[17] *Pseudomonas putida* (XenA),[18] and *Ferrovum* sp. JA12 (FOYE).[19] [b]Reaction conditions: ER (200 μg/mL, 4 μM), 10 mM substrate, 0.5 mM NADPH, 50 mM glucose, 4 mg/mL GDH, 5% DMSO (v/v), 50 mM phosphate buffer, pH 7.5, 30 °C, 24 h, and 120 rpm. [c]Product formation is defined by the amount of substrate transformed to **2** as deduced from GC analysis using calibration curves. [d]Numbers in brackets represent isolated yield.

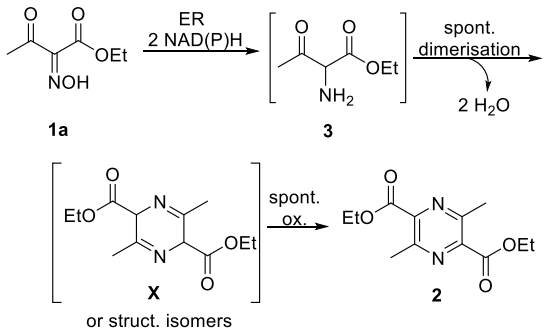
Subsequently, a library of β-keto α-oximo ester substrates and one β-keto α-oximo amide were investigated by varying the size of the alcohol part of the ester (R′) and the substituent R at the ketone side (substrates **1b**−**1h**, Table 1). Increasing the size of the ester (R′) moiety from ethyl (**1a**) to bigger groups such as benzyl (**1f**) led to a comparable amount of product formation for most enzymes, except XenA, which behaved differently and led to very low product formation for some esters. Substrate **1g**, a *N*-phenylamide, was converted in a comparable fashion like the related ester **1f**. Again XenA was different because for this enzyme, **1g** was the substrate, leading to the second best value for product formation. In general, it can be stated that the accepted scope of ester moieties is rather broad, as far as tested.

In contrast, extending the methyl group at the ketone moiety to an ethyl group (substrate **1h**) led to low product formation with almost all enzymes investigated (entry 8). The only exception was the ER XenA, which gave decent product formation of **2h** for this substrate (38%). However, when the size of the substituent at the ketone moiety (R) was extended further to an *n*-butyl or phenyl group (substrates **1i** and **1j**, see the Supporting Information for details), no pyrazine formation or any other product was detected with any enzyme. In general, the data in Table 1 show that all substrates were converted approximately equally well by OYE1, 2, and 3 and FOYE. Although XenA led to only moderate results for **1a**−**g**, it showed best performance for the ethyl ketone-derived substrate **1h** in comparison to all other enzymes tested.

Preparative biotransformations were performed at the 200 mL scale using the enzyme that gave the highest product formation on analytical scale. Products **2a** and **2d** gave the best isolated yields with 61 and 62%, respectively, whereas the isolated yield of pyrazines of **2g** and **2h** was 40%. Control experiments indicated that the formed products are stable in an aqueous solution under the conditions used and can be recovered quantitatively.

Next, we turned our attention to the possible reaction pathway. As described for pyrazine formation employing transaminases,[20] an α-amino keto motif can be expected to be an intermediate; thus, for the reaction of **1a**, α-amino-β-keto ester **3** might be the intermediate (Scheme 2).

Scheme 2. Proposed Reaction Pathway from 1a to Pyrazine 2a through ER-Catalyzed Oxime Reduction and Spontaneous Oxidation



The formation of compound **3** from the oxime (in equilibrium with the corresponding nitroso compound) formally requires two hydrides originating from NAD(P)H. Either an imine or a hydroxylamine might be formed after the first hydride transfer. Dimerization of **3** to **X**, followed by spontaneous oxidation, leads to the final product. As a first attempt to detect the intermediates, the biotransformation of **1a** was followed *via* NMR over a period of 30 min. When analyzing the obtained spectra, the peaks could be assigned to the oxime substrate, buffer components, glucose, and the pyrazine product (see the Supporting Information, Figure S48); however, no intermediate was detected with this method. In addition, following the reaction by HPLC−HRMS did not lead to any hint either.

In an attempt to get hold of the proposed intermediate **3**, a cascade reaction was envisioned to reduce the carbonyl group of the possible formed intermediate **3** by an alcohol dehydrogenase to obtain the threonine ester **4** (Scheme 3). Consequently, the cyclization of **3** would be circumvented and the formation of **4** would give an indirect proof for the formation of compound **3**. For this purpose, an ADH is required, which does not transform oxime **1a** but may transform any derivative. Fortunately, it turned out that ADH-A from *Rhodococcus ruber*[21] does not transform oxime **1a**. Therefore, any new product observed in the cascade must be due to ADH-catalyzed transformation of a compound formed by reduction with the ER.

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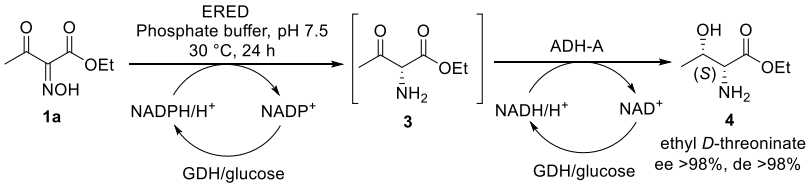
Scheme 3. Cascade Reaction to Give D-Threonine Ethyl Ester 4 for the Indirect Proof of Intermediate 3



Performing the cascade and analyzing the formed product by HR-MS (Figures S59 and S60) as well as after derivatization [as a *N*-benzoyl derivative (**5**)] by HPLC on a chiral stationary phase allowed to identify the newly formed product as a threonine derivative and to determine the absolute configuration of the stereocenters of the product **4** by comparison with independently derivatized stereoisomers of threonine (Figures S3 and S4). As expected, the ADH-A led to the formation of an (*S*)-configured alcohol moiety.[21] Interestingly, D-configuration was observed for the α-carbon for all ERs investigated with excellent optical purity (>98% ee and de, Table 2). Thus, the C=N bond was reduced in a stereoselective fashion, as only one stereoisomer of **4** was detected. The observed high stereospecificity again clearly supports that the reduction of the oxime is enzyme-catalyzed.

Table 2. Product Formation (4) and Stereochemical Outcome of the Cascade Reactions

enzyme	**4** (%)[a,b]	ee [%][c]	de [%][c]	abs. config. **4**
OYE1	54	>98	>98	(2*R*,3*S*)
OYE2	46	>98	>98	(2*R*,3*S*)
OYE3	40	>98	>98	(2*R*,3*S*)
OPR3	38	>98	>98	(2*R*,3*S*)
XenA	20	>98	>98	(2*R*,3*S*)
FOYE	29	>98	>98	(2*R*,3*S*)

[a]Reaction conditions: 10 mM substrate, ER (200 μg/mL, 4 μM), ADH-A (2 mg/mL heat purified enzyme), 0.5 mM NADPH, 0.5 mM NADH, 50 mM glucose, 4 mg/mL GDH, DMSO (5% v/v), 50 mM phosphate buffer, pH 7.5, 30 °C, 24 h, and 120 rpm. [b]Product formation of 4 is defined as the amount of substrate transformed to **4** analyzed as a *N*-benzoyl derivative by HPLC. [c]Ee and de were determined by HPLC analysis on a chiral phase.

The pyrazine core is present in a multitude of naturally occurring flavor and fragrance compounds, which usually contribute to a roasted, nutty, or chocolate-like aroma.[22] Pyrazines have been synthesized *via* various chemical methods[23] including recent biocatalytic protocols using transaminases.[20a,b] In addition to the olfactory properties, various pyrazines have potential applications in the medical sector.[24] For instance, liguzinediol (**5**) [(3,6-dimethylpyrazine-2,5-diyl)dimethanol] has recently been discovered as a potential agent for the treatment of heart failure with fewer side effects than traditional agents[24a,25] and is hence a suitable candidate for clinical studies. Using the approach described here, liguzinediol (**6**) was synthesized in a two-step chemoenzymatic reaction sequence (Scheme 4).

In the first step, the ER produced the pyrazine ester **2d**, which was isolated and subjected to chemical reduction using sodium borohydride giving 80 mg of liguzinediol (**6**), which corresponds to an overall yield of 45% over two steps. This example demonstrates the applicability of the promiscuous activity[26] of ERs for the transformation of oximes.

CONCLUSIONS

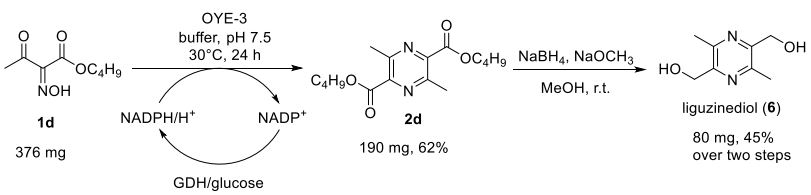
Herein, we report the reduction of an oxime to an amine by a specific enzyme, namely, ERs. Selected biocatalytic transformations of oximes resulted in general in the formation of alcohols or aldehydes when using defined enzymes; few cases report the formation of amines using wild-type cells, however, without knowing the involved enzyme(s). This study presents the first biocatalytic reduction of oximes to an amine using a defined enzyme. The reaction was found to work using a variety of structurally different ene-reductases and eight β-keto α-oximo ester or amide substrates. The oxime moiety of the substrates tested was reduced to amine and then reacted further spontaneously to pyrazines because of the nature of the substrate. The intermediate amine was trapped in situ by reducing the neighboring ketone group to an alcohol after amine formation, thereby avoiding the spontaneous cyclization. Therefore, the reaction sequence is suggested to involve a 2-fold reduction of the oxime moiety, yielding an α-amino-β-keto ester intermediate. Both enzymatic steps (carried out by ADH and ER) showed very high stereoselectivity, yielding the trapped intermediate in >98% ee and de and showing the ERs to be (*R*)-selective toward these substrates. The reaction was also shown to be feasible on a preparative scale (2 mmol), providing the pyrazine products in decent yields. The application of this reaction was demonstrated in a chemoenzymatic synthesis of liguzinediol. The reduction of oximes to amines exploiting the promiscuous activity of ER demonstrates the potential of biocatalysis for non-natural reactions extending the biocatalytic toolbox for synthetic applications.[27]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.0c03755.

Scheme 4. Chemoenzymatic Synthesis of Liguzinediol (6)



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Synthesis of substrates and references, details on biotransformations, protein expression, analytics, and NMR and MS spectra (PDF)

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S.V. and W.B.B. contributed equally.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ADH, alcohol dehydrogenase; ER, ene-reductase; GDH, glucose dehydrogenase; OYE, old yellow enzyme

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