A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

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Accepted Article

Title: Asymmetric Ketone Reduction by Imine Reductases

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To be cited as: *ChemBioChem* 10.1002/cbic.201600647

Link to VoR: http://dx.doi.org/10.1002/cbic.201600647

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Asymmetric Ketone Reduction by Imine Reductases

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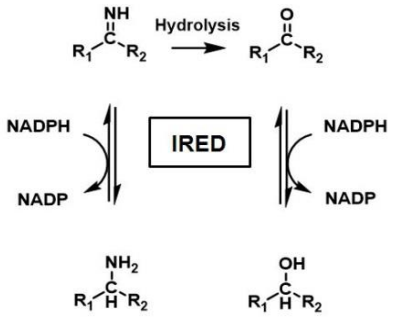
Abstract: The rapidly growing area of asymmetric imine reduction by imine reductases (IREDs) has provided alternative routes to chiral amines. Here we report the expansion of the reaction scope of IREDs by showing the stereoselective reduction of 2,2,2-trifluoroacetophenone (**1**). Assisted by an *in silico* analysis of energy barriers, we evaluated asymmetric hydrogenations of carbonyls and imines considering the influence of substrate reactivity on the chemoselectivity of this novel class of reductases. We report on the asymmetric reduction of C=N as well as C=O bonds catalyzed by members of the IRED enzyme family.

The biocatalytic, stereocontrolled addition of hydrogen from NAD(P)H to α,β-unsaturated carbonyl compounds, cyclic/acyclic imines and aldehydes/ketones during asymmetric catalytic transformation is a highly efficient and competitive alternative for the synthesis of chiral products[1]. In this respect, the enzymatic asymmetric reduction of imines by NADPH-dependent imine reductases (IREDs) has been intensively developed in recent years. To date more than 30 IREDs are characterized as catalyzing intramolecular asymmetric reductions of various cyclic imines[2,3]. In addition, IREDs were recently utilized as chiral catalysts for reductive aminations, demonstrating high chemoselectivities for reducing the *in situ* formed imine intermediate while leaving the C=O bond unaffected[4,5]. In the course of the establishment of the IRED database (Imine Reductase Engineering Database; http://ired.biocatnet.de/), we identified that most imine reductases functionally are annotated as dehydrogenases. However, the examination of the putative dehydrogenase activity for IREDs from *Streptosporangium roseum*, *Streptomyces turgidiscabies* and *Paenibacillus elgii* using glycerol-3-phosphate and 6-phosphogluconate as substrates showed no dehydrogenase activity[6]. We and others have recently demonstrated that IREDs appear to be incapable of reducing C=O bonds[7,8]. Conversely, to this day no asymmetric reduction of imines by dehydrogenases or carbonyl/keto reductases (KREDs) has been reported[2]. This led us to explore the relationship between IREDs and their highly related carbonyl-reducing counterparts like hydroxylisobutyrate and ß-hydroxyacid dehydrogenases[6,9]. The question of how proteins in nature evolve new functions is of fundamental significance. Herein, we describe the first example of the asymmetric reduction of activated ketones by two enantiocomplementary IREDs (Scheme 1).



Scheme 1. Imine reductase-catalyzed asymmetric reduction of imines and ketones.

The aim of this study was to investigate the promiscuous behavior of IREDs in respect of ketone reductions by elucidating the electronic effects of substituents on substrate molecules and their influence on catalysis. IRED-catalyzed asymmetric hydrogenation reactions using 2,2,2-trifluoroacetophenone (**1a**), benzoyl cyanide (**1b**), acetophenone (**1c**) as substrates were examined. The fluorinated aromatic carbonyl compound **1a** was transformed with 57.4% and 23.8% conversion into the corresponding α-(trifluoromethyl)benzyl alcohol (**2a**) by the purified *R*- and *S*-selective IREDs, respectively (Table 1). Both IREDs formed the *S*-enantiomer of **2a** with excellent enantioselectivities up to 96% (Table 1). No product formation was detected with benzoyl cyanide (**1b**) and acetophenone (**1c**) as substrates (Table 2, entry 10 and 11). We assume the size of the cyano group of benzoyl cyanide to cause a non-productive orientation of the substrate in the binding pocket due to steric hindrance. Despite the fact that acetophenone is one of the model substrates for alcohol dehydrogenases and KREDs[10–12], however, only a few dehydrogenases are described for the synthesis of enantiopure fluorinated alcohols from **1a**[12–15]. Activities between 21 and 99% were observed in the reduction of **1a** with alcohol dehydrogenases[13–15].

In order to exclude the contribution of *E. coli*-based dehydrogenases, a cell-free protein expression system was applied by decoupling protein expression from the host and its metabolic pathways. An example for the application of the *in vitro* translation/transcription (IVTT) was lately shown by the group of Lutz, who prepared a cell-free mutant library of the ene reductase OYE1[16,17]. In biotransformations with the *in vitro* expressed *R*-selective IRED, 5% of **2a** was formed (Table 2). We used the PURExpress® In Vitro Protein Synthesis KIT from New England Biolabs. The reduced product formation of **2a** can be explained by a significantly reduced enzyme concentration in the reaction. Successful expression of the IRED was verified in biotransformations using the model substrate 3,4-dihydroisoquinoline (Figure S5, supporting information). Due to its high substrate specificity, imine reducing dihydrofolate reductase served as template in the negative control. As no reduction of **1a**

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10.1002/cbic.201600647

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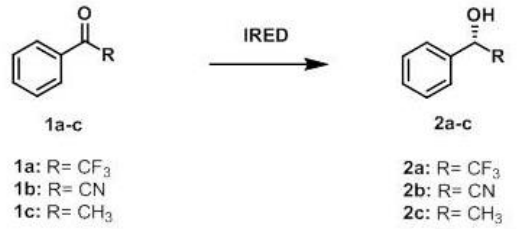
was observed in the subsequent biotransformations, reduction by components of the IVTT system can be excluded.

Table 1. Activities and selectivities of IREDs in the conversion of 2,2,2-trifluoroacetophenone **1a** into the corresponding alcohol α-(trifluoromethyl)benzylalcohol **2a** in comparison to selected controls.[a]



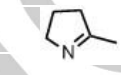
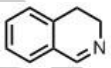
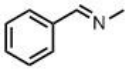
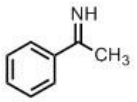
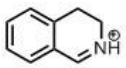
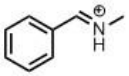
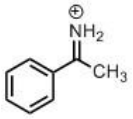
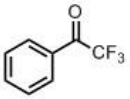
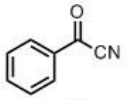
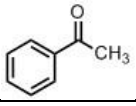
Catalyst	Cofactor	Formed product [%]	*ee* [%]
R-IRED-*Sr*	NADPH	57.4 ± 0.7	96 (*S*)
S-IRED-*Pe*	NADPH	23.8 ± 0.2	87 (*S*)
inactivated *R*-IRED-*Sr*	NADPH	< 1.0	-
BSA	NADPH	< 0.5	-
R-IRED-*Sr*	NADH	1.4 ± 0.3	-
R-IRED-*Sr* (expressed *in vitro*)	NADPH	4.5 ± 0.2 [a]	n.d.

[a] Product formation was calculated using GC areas from substrate and product normalized to an internal standard after 48 h by GC-MS as detailed in the supporting information. n.d. not determined

To ensure that the reduction of 2,2,2-trifluoroacetophenone (**1a**) to α-(trifluoromethyl)benzyl alcohol (**2a**) was performed by the IRED enzyme, further control experiments were performed. In reactions with inactive *R*-IRED-*Sr* or bovine serum albumin (BSA) with a nicotinamide cofactor regeneration system, only traces of **2a** were obtained (Table 1). Therefore, reduction by the NADPH cofactor can be excluded. In contrast to carbonyl reducing enzymes IREDs are described to be exclusively dependent on NADPH as cofactor[7]. It is thus not surprising that biotransformations applying NADH showed 40 times less product formation than those using NADPH (Table 1).

To support our experimental findings, we calculated energy barriers for hydride transfers from the nicotinamide subunit of NAD(P)H to a small panel of cyclic and acyclic imines and their corresponding iminium ions as well as aromatic carbonyl substrates (Table 2). We modelled the active center using 1-methylnicotinamide as hydride donor, substrate and one explicit water molecule embedded in a dielectric environment (detailed information in the supporting information). Due to the limitations of the model (only one water molecule present, no protein-substrate interactions, no protein environment), interpretation of the results is restricted to relative differences in activation energies. The calculated activation energies summarized in Table 2 show that two to three times lower energy barriers for iminium ions (Table 2, entry 5-8) than for imines (Table 2, entry 1-4) were obtained. These differences in reactivity are underlined by recent studies of Turner and Xu that demonstrated the reduction of iminium salts and the associated faster conversion of iminium ions in comparison with the corresponding imines[8,18]. According to our calculations, energy barriers for selected ketones are higher than for iminium ions of the chosen model substrates, however, lower than for imine compounds (Table 2). As expected the ketone reactivity is mostly influenced by the CF_3-group with the highest negative inducible effect.

Table 2. *In silico* calculated energy barriers in kJ/mol for the hydride transfer from the nicotinamide subunit of NAD(P)H to imine, iminium ion and ketone substrates.

Entry	Compound	Energy barrier [kJ/mol][a]
1		171.5
2		174.6
3		153.4
4		152.2
5		82.6
6		69.2
7		65.3
8		58.9
9		66.4
10		80.6
11		112.6

[a] Energy barriers were determined as detailed in the supporting information.

To compare our IRED activity for **1a** with the activity for characterized imine substrates, kinetic parameters were determined. The results showed that the catalytic efficiency of *R*-IRED-*Sr* for 2-methylpyrroline (2-MPN) being one of the model

substrates for IREDs was 600-fold higher than for **1a**. The k_{cat}/K_M values of 53.31 min^{-1} mM^{-1} for 2-MPN[6] and 0.085 min^{-1} mM^{-1} for **1a** reveal the difference in the reduction of imines and carbonyls. Moreover, the K_M value of 8.78 mM for **1a** is about eight times higher compared to the K_M of 2-MPN. Substrate inhibition has been reported for IREDs[19,20] and has also been observed for **1a**. *R*-IRED-*Sr* showed decreased activities at substrate concentrations above 7 mM as shown by the Michaelis-Menten plot (Figure S7, supporting information). Even though Grogan and Turner speculated in a recent publication that the reduction of imines might represent a promiscuous activity of IREDs[19], the determined kinetic constants indicate that IREDs have been evolved in nature for the reduction of C=N bonds, if in other substrates.

Reductases catalyzing asymmetric hydrogenations of ketones and imines are closely related. For both enzyme families NAD(P)H is described as cofactor providing the hydride ion for the reduction of the appropriate substrate. The binding of the nicotinamide cofactor not only influences protein dynamics but also constitutes a key structural component in organizing all catalytically important active site residues to form the binding pocket[21]. In comparison to carbonyl reducing enzymes, catalytic amino acid residues and the reduction mechanism of IREDs are still under debate. A generic proton-donating catalysis including hydride and proton transfer is expected for IREDs, closely related to the mechanism of dehydrogenases/KREDs[22]. NADPH is considered as the hydride donor, while the proton-donating residue remains elusive[3]. Hydride transfer prior to protonation seems unlikely due to the formation of a highly basic amide intermediate, which is difficult to stabilize[2]. Therefore two strategies for hydride- and proton-transfer can be envisaged: (i) the formation of an iminium ion through protonation of the imine, followed by addition of the hydride, or (ii) the concerted transfer of hydride and proton[2]. Commonly found zinc or iron ions functioning as Lewis acids in metal-containing alcohol dehydrogenases have so far not been observed in IREDs. IREDs are homodimeric proteins with each subunit binding one molecule of NADPH via a Rossmann-fold motif. First insights into the possible ligand-binding site and local residues of the IRED from *Amycolatopsis orientalis* influencing the catalytic activity were recently gained by the groups of Turner and Grogan. By co-crystallization with NADPH and the amine product 1-methyl-1,2,3,4-tetrahydrosioquinoline the ternary complex of the IRED was examined[23]. Latest investigations from Tawfik and co-workers on the evolution of the Rossmann-fold motif[24] and the structural similarity of IREDs to hydroxylisobutyrate and ß-hydroxyacid dehydrogenases[6,9] strongly suggest a common ancestry. Despite these common elements, IREDs and KREDs differ in their ability to reduce imines.

In the present study, we surveyed the hydride transfer to C=N and C=O containing substrates. Theoretically calculated activation energies encourage the speculation that imines are protonated prior to the hydride transfer from NADPH. We demonstrated that our selected iminium ions are more reactive than the corresponding imines (Table 1). This is in accordance with the work of Mayr describing the electrophilicity of benzaldehyde-derived iminium ions and their higher reactivities compared to substituted imines[25]. In pursuit of this trend, IREDs showed higher initial rates in transformations with iminium ions than with the corresponding imines[8,18]. Regarding speculations of the role of amino acid residues for C=N protonation in IREDs[9,23], there is some supporting evidence based on mutations of putative catalytically important aspartic acid residues displaying reduced catalytic efficiencies over wild type enzyme[6]. Residual activities can be attributed to the partly protonated imines in solution at pH 7. However, reductive amination reactions were conducted at pH 9, where protonated imines are not expected in solution. Product formation up to 60% under these conditions[5] strongly suggests imine protonation in the active site as part of the catalytic mechanism. Efforts to determine crucial amino acid residues for catalysis in IREDs were not yet successful. We hypothesize a common orientation of substrate and cofactor for favourable hydride and proton transfer in IREDs and carbonyl-reducing enzymes. In this respect, fitting of imines into the binding pocket of carbonyl reducing enzymes might result in steric clashes with amino acid residues that normally interact with the free electron pairs of oxygen. Furthermore, the substrate binding site of dehydrogenases/KREDs might prevent productive hydride transfer from the nicotinamide ring of NAD(P)H to alternate imine substrates, because of unfavorable distances. Our theoretical and experimental data have provided considerable information on the size and electronic requirements for the asymmetric reduction of imines and ketones with IRED catalysts. The substitution of the keto functionality by a trifluoromethyl group increases the substrate reactivity and thus, facilitates the asymmetric reduction of ketones. The asymmetric hydrogenation of substituted ketones highlights the difficulties in understanding the balance between steric and electronic factors that govern the outcome of organic transformations. We consequently conclude that by employing chemical tools the chemoselectivity of IREDs can be driven.

In summary, we describe the first example of the promiscuous imine reductase-catalyzed asymmetric reduction of a highly reactive carbonyl compound. Assisted by *in silico* calculations of energy barriers for the hydride transfer from the nicotinamide subunit of NAD(P)H to several imines and their corresponding iminium ions, the present results contribute to a deeper understanding of the reaction mechanism of imine reductases and their evolution.

Acknowledgements

We express our cordial thanks to Prof. Dr. Bernhard Hauer and Dr. Stephan Hammer for the fruitful discussions. We also acknowledge financial support from the European Union and the EFPIA companies' in kind contribution for the Innovative Medicine Initiative under Grant Agreement No. 115360 (Chemical manufacturing methods for the 21st century pharmaceuticals industries, CHEM21).

Keywords: biocatalysis • asymmetric reduction • imine reductase • promiscuity • carbonyl compounds

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10.1002/cbic.201600647

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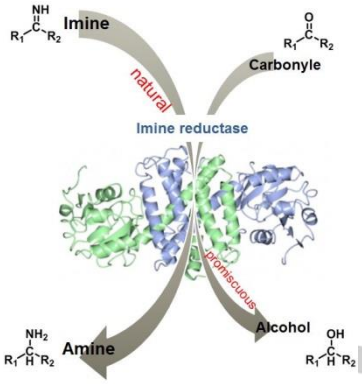
IRED *vs.* KRED: Asymmetric reduction of a highly reactive carbonyl compound has been accomplished using an imine reductase. Kinetics and *in silico* studies have offered mechanistic insights, allowing an understanding of this promiscuous behaviour.



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Chemoselectivity of Imine Reductases Driven by the Inherent Reactivity of Functional Groups

Layout 2:

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