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A NADH-accepting imine reductase variant: Immobilization and cofactor regeneration by oxidative deamination

Martin Gand[a], Christian Thöle[a], Hubertus Müller[a], Henrike Brundiek[b], Ghader Bashiri[c], Matthias Höhne[a,*]

[a] Institute of Biochemistry, Greifswald University, Felix-Hausdorff-Str. 4, 17487 Greifswald, Germany
[b] Enzymicals AG, Walther-Rathenau-Straße 49a, 17489 Greifswald, Germany
[c] Structural Biology Laboratory and Maurice Wilkins Centre for Molecular Biodiscovery, School of Biological Sciences, The University of Auckland, Auckland 1010, New Zealand

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ABSTRACT

Engineering cofactor specificity of enzymes is a promising approach that can expand the application of enzymes for biocatalytic production of industrially relevant chemicals. Until now, only NADPH-dependent imine reductases (IREDs) are known. This limits their applications to reactions employing whole cells as a cost-efficient cofactor regeneration system. For applications of IREDs as cell-free catalysts, (i) we created an IRED variant showing an improved activity for NADH. With rational design we were able to identify four residues in the (*R*)-selective IRED from *Streptomyces* GF3587 (IR-Sgf3587), which coordinate the 2′-phosphate moiety of the NADPH cofactor. From a set of 15 variants, the highest NADH activity was caused by the single amino acid exchange K40A resulting in a 3-fold increased acceptance of NADH. (ii) We showed its applicability using an immobilisate obtained either from purified enzyme or from lysate using the EziG™ carriers. Applying the variant and NADH, we reached 88% conversion in a preparative scale biotransformation when employing 4% (*w*/*v*) 2-methylpyrroline. (iii) We demonstrated a one-enzyme cofactor regeneration approach using the achiral amine *N*-methyl-3-aminopentanone as a hydrogen donor co-substrate.

1. Introduction

Active pharmaceutical ingredients (APIs), agrochemicals and other fine chemicals often bear one or more stereogenic centers connected to a nitrogen atom (Breuer et al., 2004; Welsch et al., 2010). Amines represent a structurally diverse class of compounds, with especially secondary and tertiary amines existing in various cyclic or acyclic scaffolds. Therefore, a variety of chemical methods for the asymmetric synthesis of enantiopure amines were developed, albeit their eco-efficient preparation remains challenging (Ghislieri and Turner, 2014). Options for secondary amine synthesis include organocatalytic or transition-metal catalyzed hydrogenation of imines (Gamenara and Domínguez de Maria, 2014; Hsieh et al., 2012; Li and Xiao, 2008). Even though much progress was made in the last decades, numerous (de)protection steps and the application of expensive and toxic metals (Nugent and El-Shazly, 2010) are necessary. Enzymatic approaches facilitate regio- and stereospecific conversions and thus bear a great potential to be developed into efficient synthesis strategies, avoiding eco-toxic reagents (Buchholz et al., 2005). Primary amines can be accessed by various enzymes (Kohls et al., 2014). For secondary amines there are fewer options: monoamine oxidases (MAO) allow the deracemization of an amine by oxidizing one enantiomer to the imine, which is then non-selectively reduced *in situ* by a chemical or a second enzymatic step to the amine racemate (Heath et al., 2015). A second approach uses the spontaneous, intramolecular cyclization *via* nucleophilic substitution or imine formation of cyclic *sec*-amines in a two-step, one-pot reaction, if the substrate is carefully chosen (Girardin et al., 2013; Kroutil et al., 2013). A third option is the use of imine reductases (IREDs) (Leipold et al., 2015; Turner, 2014). These enzymes allow two “types” of reactions: (i) the reduction of a cyclic imine to a cyclic *sec*-amine (Leipold et al., 2013) or (ii) the reductive amination of a ketone with a primary amine yielding a *sec*-amine, where a proof of concept (Huber et al., 2014; Scheller et al., 2015) and preparative applications were demonstrated very

* Corresponding author at: Institute of Biochemistry, Greifswald University, Felix-Hausdorff-Str. 4, 17487 Greifswald, Germany.
E-mail addresses: henrike.brundiek@enzymicals.com (H. Brundiek), g.bashiri@auckland.ac.nz (G. Bashiri), Matthias.Hoehne@uni-greifswald.de (M. Höhne).

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Table 1
Enzyme activity of the wild type IR-Sgf3587 and the cofactor-binding mutants.

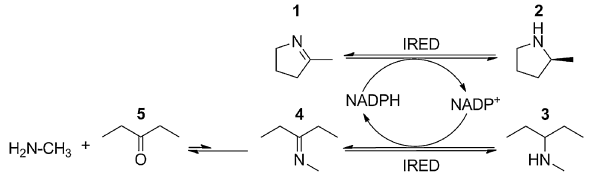
Mutant	N35D	R36L	S37V	K40A	Ratio NADH/NADPH	Specific activity NADPH in% [100% = 490 mU/mg]	Specific activity NADH in% [100% = 15 mU/mg]
WT	–	–	–	–	0.03	100 ± 22	100 ± 66
1	X	–	–	–	–	n.d.	n.d.
2	–	X	–	–	–	38 ± 14	n.d.
3	–	–	X	–	–	67 ± 4	n.d.
4	–	–	–	X	0.08	126 ± 12	337 ± 90
5	X	X	–	–	–	n.d.	n.d.
6	–	X	X	–	–	n.d.	n.d.
7	–	–	X	X	5.5	0.4 ± 0	83 ± 45
8	X	–	X	–	0	2 ± 0	n.d.
9	X	–	–	X	–	n.d.	n.d.
10	–	X	–	X	1.7	0.4 ± 0	26 ± 4
11	–	X	X	X	–	n.d.	n.d.
12	X	X	X	–	–	n.d.	n.d.
13	X	X	–	X	–	n.d.	n.d.
14	X	–	X	X	–	n.d.	n.d.
15	X	X	X	X	–	n.d.	n.d.

n.d.: no detectable activity (<0.4 mU/mg).

recently (Wetzl et al., 2016). In the previous years, several research groups discovered a large number of IREDs (Gand et al., 2014; Huber et al., 2014; Li et al., 2015; Man et al., 2015; Mitsukura et al., 2013, 2011, 2010; Rodríguez-Mata et al., 2013; Scheller et al., 2015; Wetzl et al., 2015). In addition, an imine reductase engineering database with more than 1000 putative IREDs is established (Scheller et al., 2014). Until now the crystal structures of six different IREDs have been solved (Gand et al., 2014; Huber et al., 2014; Man et al., 2015; Rodríguez-Mata et al., 2013). All hitherto discovered IREDs of this superfamily show a strong preference for NADPH as the cofactor.

Although the above-mentioned IREDs were applied for the biocatalytic synthesis of several amines (Grogan and Turner, 2016; Schrittwieser et al., 2015), the function of these enzymes in the metabolism of the host organisms has yet not been elucidated. In nature, the synthesis of various secondary amines by reduction of a C=N bond occurs by several enzymes. One example is the biosynthesis of antibiotics, such as tomaymycin (Li et al., 2009) and chlortetracycline (Nakano et al., 2004) by different *Streptomyces* strains, where an enzyme with imine reductase activity is involved. In this case, F_{420}-H_2 is used as cofactor for the reduction of the C=N bond. F_{420} has some structural similarities with FAD, but performs hydride transfers like NAD(P)$^+$, and is also found in other metabolic reactions such as antibiotic biosynthesis or methanogenesis. Whether the NADPH-dependent IREDs can additionally accept F_{420} was not reported until now.

For biocatalytic applications there is often the motivation to (i) work with cell-free extract or purified enzymes, sometimes as immobilisates, if an avoidance or the suppression of side reactions is necessary, and (ii) use a cheap cofactor/cofactor regeneration system to reduce the costs of the application. If available, the usage of NADH is preferred over NADPH, because the non-phosphorylated cofactor is cheaper than the phosphorylated one. In some cases, a NADH-dependent enzyme is not available, but protein engineering can be used to create such a variant. In the past, this has been demonstrated for several cases, such as γ-diketone reductase, where the acceptance from NADPH to NADH could be altered (Katzberg et al., 2010). This strategy in the case of IREDs would allow to utilize them together with other NADH-dependent enzymes in cascades, *e.g.* in a similar manner Turner and colleagues have recently coupled a keto reductase and an amine dehydrogenase (Mutti et al., 2015). Alternatively, 1-benzyl-1,4-dihydronicotinamide (BNAH) was developed as an alternative synthetic cheap cofactor, in which the sugar phosphate moiety is replaced by a benzyl group (Paul et al., 2013). This cofactor can be successfully used by enoate reductases and monooxygenases (Paul et al., 2015). A last option to avoid additional cofactor-regenerating



Scheme 1. Cofactor regeneration using a one-enzyme approach: imine reduction is coupled with oxidative deamination of an acyclic secondary amine.

enzymes is to add a hydrogen donor co-substrate to recycle the bound cofactor in a one-enzyme approach. This strategy is successfully applied with alcohol dehydrogenases, where excess of isopropanol is used to regenerate the reduced cofactor.

For immobilization purposes, which facilitate the reusability of the biocatalyst, many different classes of carriers and methods have been developed (Bornscheuer, 2003; Buchholz and Klein, 1987; Homaei et al., 2013; Sheldon and van Pelt, 2013). A relatively new carrier option is the EziG™ carriers (Enzyme immobilized on Glass) that chelate metal ions on their porous surface, facilitating the selective binding of His_6-tagged proteins from crude cell lysate. The applicability of these carriers has been demonstrated for transaminases, lipases and one Baeyer-Villiger-monooxygenase (Cassimjee et al., 2014).

To develop cell-free IRED applications, we used the enzyme from (*R*)-selective *Streptomyces* GF3587 (IR-Sgf3587) as model enzyme in current study and investigated the acceptance of alternative cofactors, generated a NADH-accepting variant, performed immobilization and investigated the one-enzyme approach for cofactor regeneration by oxidative deamination of an achiral secondary amine.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Fluka (Buchs, Switzerland), Sigma (Steinheim, Germany), Carl-Roth (Karlsruhe, Germany) and Merck (Darmstadt, Germany). The gene encoding the imine reductase IR-Sgf3587 was available from previous studies (Gand et al., 2014). The primers were supplied by Life Technologies (Carlsbad, CA, USA). Competent *E. coli* TOP10 and BL21 (DE3) cells were purchased from Life Technologies.