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Characterization of three novel enzymes with imine reductase activity

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ABSTRACT

Imine reductases (IRED) are promising catalysts for the synthesis of optically pure secondary cyclic amines. Three novel IREDs from *Paenibacillus elgii* B69, *Streptomyces ipomoeae* 91-03 and *Pseudomonas putida* KT2440 were identified by amino acid or structural similarity search, cloned and recombinantly expressed in *E. coli* and their substrate scope was investigated. Besides the acceptance of cyclic amines, also acyclic amines could be identified as substrates for all IREDs. For the IRED from *P. putida*, a crystal structure (PDB-code 3L6D) is available in the database, but the function of the protein was not investigated so far. This enzyme showed the highest apparent *E*-value of approximately $E_{app} = 52$ for (*R*)-methylpyrrolidine of the IREDs investigated in this study. Thus, an excellent enantiomeric purity of >99% and 97% conversion was reached in a biocatalytic reaction using resting cells after 24 h. Interestingly, a histidine residue could be confirmed as a catalytic residue by mutagenesis, but the residue is placed one turn aside compared to the formally known position of the catalytic Asp187 of *Streptomyces kanamyceticus* IRED.

1. Introduction

A large number of secondary metabolites are composed of chiral secondary amines [1]. Their various physiological activities are explored and utilized by pharmaceutical companies [2]. Hence, many active ingredients of pharmaceuticals represent molecules having one or more stereogenic centres connected to a nitrogen atom. As a consequence, a variety of chemical methods for the enantioselective synthesis of optically pure secondary amines were developed during the last decades, e.g. organocatalytic or metal catalyzed hydrogenation of imines [3,4]. Biocatalysis is more and more accepted as an efficient synthesis strategy for regio- and stereospecific conversions, because it eliminates the use of environmentally toxic heavy metals, enables mild conditions and offers high selectivities [5]. Furthermore, protein engineering allows the fine-tuning of the employed enzymes to broaden their applicability [6]. In contrary to the preparation of primary amines, which can be accessed by various enzymes such as lipases, monoamine oxidases and especially by asymmetric synthesis with transaminases [7–10], for secondary amines there are fewer options: monoamine oxidases allow the deracemization of an amine by oxidizing one enantiomer to the imine, which in turn is reduced non-selectively in situ by chemical means to the amine racemate [11,12]. This leads to the enrichment of the non-reactive enantiomer up to 100%. In a second approach, cyclic *sec*-amines can be obtained in a two-step, one pot reaction if the substrate is carefully chosen: the presence of a halogen, ester or ketone moiety facilitates a spontaneous, intramolecular cyclization via nucleophilic substitution or imine formation [10,13–15]. Dependent on the strategy, a follow-up reductive step is necessary to yield the desired amine. A third option discovered recently are NADPH-dependent imine reductases (IRED) (Fig. 1). So far, only few IREDs were described as recombinant proteins and investigated for the asymmetric synthesis of secondary amines from the corresponding prochiral imines: the (*S*)-selective IRED originating from *Streptomyces* sp. GF3546 (SIR-Sgf3546) [16] and *Streptomyces aurantiacus* (SIR-Sau) [17], and the (*R*)-selective enzymes obtained from *Streptomyces* sp. GF3587 (RIR-Sgf3587) [18] and *Streptomyces kanamyceticus* (RIR-Ska). Crystal structures were solved for SIR-Sgf3546, SIR-Sau, and RIR-Ska [17,19]. During the revision of this manuscript, three additional enzymes were characterized originating from *Streptosporangium roseum* DSM 43021 (RIR-Sro), *Streptomyces turgidiscabies* (RIR-Stu) and *Paenibacillus elgii* B69 (SIR-Pel) [20]. The latter enzyme is one of the proteins that we characterized in detail in this study. Furthermore, the group of Thomas Ward designed artificial metalloenzymes that facilitate the reduction of imines by transfer hydrogenation [21,22].

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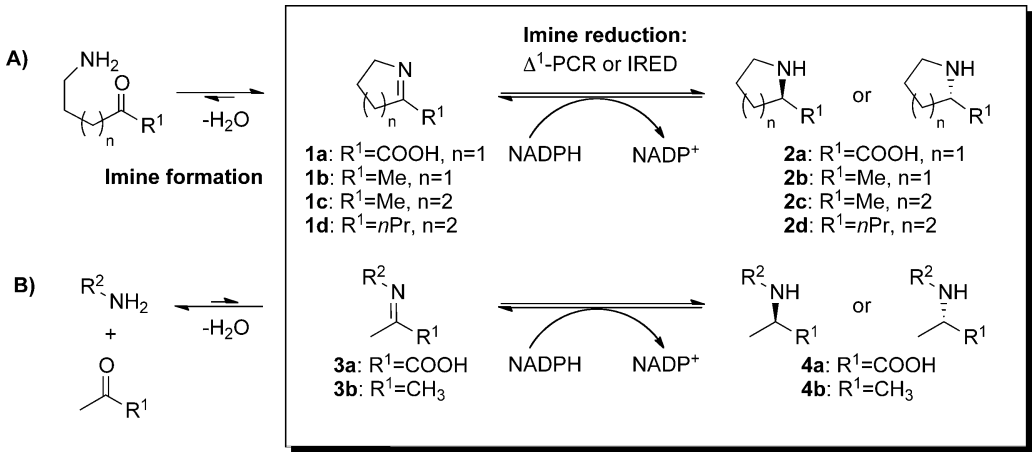

Fig. 1. Enzymatic imine reduction. Δ^1-Pyrrolin-carboxylate reductase (Δ^1-PCR) catalyzes the reduction of cyclic imines (reaction A) or acyclic imines (reaction B), R^1=COOH, n = 1–2. Isolated imine reductases (IRED) reduce the analogous cyclic substrates where R^1 is an alkyl substituent, e.g. a methyl group, and n = 1–3. The question, whether IREDs also act on acyclic substrates (reaction B) is addressed in this study. As the equilibrium favors the hydrolysis of acyclic imines in water (compared to the fairly stable cyclic imines), oxidative deamination of acyclic secondary amines is chosen in initial screening reactions (reverse reaction).

The main challenges for applying IREDs in biocatalysis are (i) only few enzymes are available, (ii) they display a low activity and (iii) some enzymes are reported to show a narrow substrate specificity, especially the (*R*)-selective imine reductase from *Streptomyces* GF3587 (RIR-Sgf3587) [18]. It was suggested earlier that the 'true' natural substrate has not yet been identified [19], or that imine reduction might be a promiscuous activity.

To address the above-mentioned problems, we characterized three proteins similar in sequence or structure to RIR-Ska or SIR-Sgf3546. For their characterization, we screened a broad panel of possible substrates. Besides the substrates of the hydroxyisobutyrate dehydrogenase superfamily (3-hydroxyisobutyrate, 6-phosphogluconate) and cyclic amines, we also examined various alcohols, ketones, aldehydes, amino acids, *prim*- and *sec*-amines.

An additional question is whether IREDs are restricted towards cyclic imine substrates. In principle, imine formation from a ketone and a primary amine of choice would allow the synthesis of acyclic secondary amines. This would be a desirable reaction. During the preparation of this manuscript, Huber et al. published a proof of principle for the reductive amination of three methyl alkyl ketones with methylamine using SIR-Sgf3546 and SIR-Sau: product formation could be detected with conversions ranging from 0.1% to 9%. However, the enzymes showed a very low catalytic turnover of 1.7 molecules/week for the best substrate. Thus, these enzymes are not yet applicable for preparative reactions at larger scale [17]. Another example is described where whole cells were applied to reduce acyclic imines [23], but a huge cell mass (18 g for 0.18 mmol substrate) was needed and the amino acid sequence of the acting catalyst is unknown. In nature, this synthetic strategy is applied for the synthesis of various secondary amines, such as opines. In this case an α-keto acid reacts with an amino acid and the formed imine is reduced stereoselectively to yield an opine [24]. These products usually contain two or more carboxylate functionalities, but these polar groups are often not desired in pharmaceutically active *sec*-amines. Another reaction yielding secondary amino acids is catalyzed by Δ^1-pyrroline-carboxylate-reductase (Δ^1-PCR), which is involved in proline/lysine metabolism: the cyclic substrate Δ^1-pyrroline-carboxylate **1a** is reduced to proline **2a** (Fig. 1A). Interestingly, Δ^1-PCR also reduces acyclic imines like **3a** formed by in situ condensation of α-keto acids like pyruvate with methyl- and ethylamine to the *N*-alkylated α-amino acid **4a** as a side reaction [25] (Fig. 1B). As the model substrate of IREDs, 2-methylpyrroline **1b**, differs from **1a** only by the replacement of the carboxylic acid group by an alkyl substituent, we hypothesized that IREDs could also be active towards the analogous acyclic substrates **3b** and **4b** (Fig. 1B). Therefore, a second aim of this study was to investigate, whether IREDs are able to perform this reaction either as kinetic resolution, or preferentially, in an asymmetric synthesis by generating a stereocenter via reductive amination.

2. Methods and materials

2.1. General

All chemicals were purchased from Fluka (Buchs, Switzerland), Sigma (Steinheim, Germany), Carl-Roth (Karlsruhe, Germany) and Merck (Darmstadt, Germany). The pSGX3 plasmid bearing an insert of a putative reductase with unknown function, (NCBI Gene identification number GI: 283807276, PDB-accession code 3L6D) was kindly provided by the New York Structural Genomics Research Consortium, Department of Biochemistry of Albert Einstein College of Medicine, New York, USA). The genes of the previously described imine reductases SIR-Sgf3546 (GI: 460838082) and RIR-Sgf3587 (GI: 505423772) and two putative reductases identified by BLAST search (GIs: 498183793 and 496688866) were ordered as synthetic genes (GenScript, Piscataway, USA) as subcloned constructs in the pET28b(+) vector, which creates a *N*-terminal His_6-tag for purification purposes. 2-Methylpiperideine was synthesized according to Leipold et al. [26] and for the synthesis of 2-propylpiperideine, the same synthesis procedure was adapted (compare Section 1.1, supplementary information).

2.2. Protein biosynthesis and purification

Kanamycin was used in all cultivations as antibiotic. In a typical procedure, *E. coli* BL21 (DE3) cells transformed with pET28b_IRED were inoculated in LB medium from an overnight culture (1:100, v/v) and were grown at 30 °C until an OD_{600} of 0.2–0.5 was reached. Protein expression was initiated by the addition of IPTG to a final concentration of 0.2 mM. Eight hours after induction, the cells were harvested (4000 g, 4 °C, 10 min) and resuspended in sodium phosphate buffer (50 mM, pH 7.5). The cells were lysed with a French press (Thermo Fisher Scientific, Waltheim, MA, USA) and centrifuged to separate the cell debris from the soluble protein. The His_6-tagged IREDs were purified by metal affinity chromatography using an ÄKTA purifier (GE Healthcare, Chalfont St Giles, UK) with a HisTrap™ FF 5 mL column (GE Healthcare). In a