Crossing the Border: From Keto- to Imine Reduction in Short-Chain Dehydrogenases/Reductases

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The family of NAD(P)H-dependent short-chain dehydrogenases/reductases (SDRs) comprises numerous biocatalysts capable of C=O or C=C reduction. The highly homologous noroxomaritidine reductase (NR) from Narcissus sp. aff. pseudonarcissus and Zt_SD from Zephyranthes treatiae, however, are SDRs with an extended imine substrate scope. Comparison with a similar SDR from Asparagus officinalis (Ao_SDR) exhibiting keto-reducing activity, yet negligible imine-reducing capability, and mining the Short-Chain Dehydrogenase/Reductase Engineering Database indicated that NR and Zt_SD possess a unique active-site composition among SDRs. Adapting the active site of Ao_SDR accordingly improved its imine-reducing capability. By applying the same strategy, an unrelated SDR from Methylobacterium sp. 77 (M77_SDR) with distinct keto-reducing activity was engineered into a promiscuous enzyme with imine-reducing activity, thereby confirming that the ability to reduce imines can be rationally introduced into members of the “classical” SDR enzyme family. Thus, members of the SDR family could be a promising starting point for protein approaches to generate new imine-reducing enzymes.

The NAD(P)H-dependent enzymatic reduction of C=N bonds represents an attractive approach to chiral 1°, 2°, and 3° amines.11 Whereas imine-reducing enzymes from metabolic pathways have been known for long,2–5 their strict substrate specificity make them less attractive for biocatalytic applications and has provoked the desire for biocatalysts with a relaxed substrate scope. Three different strategies have led to such imine-reducing enzymes: the identification of new enzyme families, enzyme engineering, and/or the exploitation of catalytic promiscuity.

By a screening approach, Mitsukura et al. discovered two Streptomyces strains capable of reducing the cyclic imine 2-methyl-1-pyrroline.11 The identification of the underlying NADPH-dependent oxidoreductases sparked the rise of the imine reductase (IRED) enzyme family.8,9 Bioinformatics approaches increased the availability of putative IRED-coding sequences, which are categorized in the Imine Reductase Engineering Database.8 IREDs are known to accept a broad range of cyclic imine compounds as substrates, with some catalyzing the reductive (alkyl)amination of carbonyls as well.9,10

Recently, the discovery of an enzyme family of native amine dehydrogenases (nat-AmDHs) expanded the portfolio of enzymes suitable for the reductive amination of carbonyls.12 These enzymes were identified by a sequence-driven approach. As nat-AmDHs are not related to IREDs, this exemplifies the natural diversity of imine-reducing enzyme families.

Protein engineering of enzymes facilitates the generation of variants with desired properties. Successful approaches in the context of NAD(P)H-dependent C=N reduction have been demonstrated recently. Bommarius and co-workers applied several rounds of protein engineering to change the substrate scope of an amino acid dehydrogenase. The result was a variant with four mutations that catalyzes the reductive amination of ketones instead of α-keto acids.13 Mutti and co-workers chose an ε-deaminating l-lysine dehydrogenase as a scaffold for the generation of amine dehydrogenases.14 Nestl and co-workers used β-hydroxy acid dehydrogenases as a starting point for mutagenesis experiments. Certain members of this enzyme family display a similarity to IREDs and possess promiscuous C=N reducing activity. This activity was enhanced by replacing an active site residue involved in the native catalytic reduction mechanism.15

Previously, we have reported on two homologous, promiscuous short-chain dehydrogenases/reductases (SDRs) from plants capable of reducing C=N and C=O bonds: noroxomaritidine reductase (NR) from Narcissus sp. aff. pseudonarcissus and Zt_SD from Zephyranthes treatiae.16 NR was originally identified as an enone reductase (C=C reduction) involved in alkaloid biosynthesis,16 highlighting the versatility of SDRs.

In general, members of the SDR family are well characterized with respect to carbonyl and enone reduction, and several candidates are used in biocatalytic applications. Protein engineering has mainly been used to modify cofactor preference,17 activity,18 stereoselectivity,19 or stability20 of SDRs. Notably,
Lygidakis et al. used protein engineering to address the catalytic scope of SDRs: the exchange of a single residue enabled the switch from C=C (enone) to C=O reduction in SDRs from *Mentha piperita*.  

Our research related to SDR-catalyzed C=N reduction was fortified by the observation that glucose dehydrogenase (GDH) is capable of reducing iminium compounds (Scheme 1). This ability was unexpected: GDH was originally identified as solely acting on sugar substrates. However, our finding along with the above-noted examples of promiscuous C=N reduction suggest that other enzymes may fulfill the prerequisites to behave as imine-reducing enzymes. We therefore hypothesized that a rational design of imine-reducing activity in SDRs should be possible, which is backed by the previous examples of SDR engineering.

Here, we report the generation of a new imine-reducing enzyme resulting from a rational mutagenesis approach. Inspired by the unique active site compositions of NR and Zt_SD, four mutations transformed an unrelated SDR with distinct keto-reducing activity into a promiscuous enzyme with additional imine-reducing activity. In addition to our previous results and literature examples, this pattern was extended by polar residues that are common in the substrate binding site of NR and Zt_SD (N102, C149, T/S199; standard positions 98, 145, 197), but do not occur at the equivalent positions in Ao_SD (T124, I171, V221). These positions were considered to be promising targets for engineering of imine-reducing activity (Figure 1, Table 1).

Hence, six mutations (F122Y, T124N, I171C, A172C, L180H, V221S) were expected to improve the imine-reducing activity of Ao_SD. The mutations were gradually introduced by site-directed mutagenesis, and the variants were tested as purified enzyme resulting from a rational mutagenesis approach. Inspired by the unique active site compositions of NR and Zt_SD, four mutations transformed an unrelated SDR with distinct keto-reducing activity into a promiscuous enzyme with additional imine-reducing activity. In addition to our previous results and literature examples, this pattern was extended by polar residues that are common in the substrate binding site of NR and Zt_SD (N102, C149, T/S199; standard positions 98, 145, 197), but do not occur at the equivalent positions in Ao_SD (T124, I171, V221). These positions were considered to be promising targets for engineering of imine-reducing activity (Figure 1, Table 1).

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Figure 1. Catalytic triad (blue), previously proposed flanking residues (green), and NR/Zt_SD consensus positions (dark red) of A) Zt_SD and B) Ao_SD.

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**Scheme 1.** Stages of SDR-catalyzed imine reduction. The serendipitous identification of iminium-reducing activity of glucose dehydrogenase (GDH); the exploitation of the catalytic promiscuity of noroxomaritidine reductase (NR); generation of imine-reducing activity in an unrelated SDR by mutagenesis (this study).
conversion was determined by 1H NMR spectroscopy, while the low solubility of the substrate impeded the determination of the specific activity. To monitor changes of the ketoreductase activity, the specific activity of the variants was determined with (R)-3-methylcyclohexanone (5) as a substrate (Table 2). The twofold variant (F122Y/L180H) with exchanges of flanking residues at standard positions 96 and 156 displayed a slightly improved conversion of 1 (6%) and a sixfold decreased ketoreductase activity compared to the wild-type enzyme. Next, a cysteine residue at standard position 146 was introduced, resulting in a further increase in conversion of 1 to 9%. Interestingly, this variant displayed an elevated ketoreductase activity compared to the 2-fold variant. The exchange of valine at standard position 197 by serine did not influence the imine-reducing capability, but the additional substitution of threonine at standard position 98 by asparagine resulted in a 5-fold variant with the highest conversion of 1 (17%). Its ketoreductase activity was reduced to 13% of the wild-type activity. Introducing a cysteine at standard position 145 to complete the sixfold variant resulted in a decrease in imine-reducing activity and a further reduction in ketoreductase activity to 1% of the wild-type activity. Thus, the five mutations F122Y/T124N/A172C/L180H/V221S significantly promoted the imine-reducing capability of Ao_SDR, although it was still lower than the capability of NR and Zt_SDR.

To confirm the functional relevance of the flanking and consensus positions (Figure 1, Table 1), an in silico screening for SDRs with imine-reducing activity was performed assuming a similar structure of “classical” SDRs even at low sequence identity.[28] Therefore, the Short-Chain Dehydrogenase/Reductase Engineering Database[29] was scanned separately for sequences with tyrosine, asparagine, cysteine, cysteine, histidine, or threonine/serine at standard positions 96, 98, 145, 146, 156, or 197, respectively. Interestingly, none of the 130000 SDR sequences had more than three matching positions.

Nevertheless, six protein sequences from Amaryllidaceae transcriptomes from the 1000 Plants (1KP) project (Table S8) matched at least four of the six flanking/consensus positions (Table 1). One of these six proteins, the SDR from *Phycella cyranthoides* (Pe_SDR, 88% sequence identity to NR, M98 instead of N), was tested and showed imine-reducing activity (Table S9). As the proposed pattern occurs only in Amaryllidaceae SDRs, thus representing a tiny subgroup of a large enzyme family, it might be regarded as a specific solution for imine reduction. Nevertheless, we hypothesized that the pattern derived from NR and Zt_SDR is exploitable and transferable to other SDRs.

To challenge this hypothesis, we elected to test SDRs as wild types and engineered for imine-reducing activity according to the established pattern. From the database hits, we chose an uncharacterized SDR from *Methyllobacterium* sp. 77 (*M77_SDR*) containing Y96 and T197 (35% sequence identity to NR). As a second candidate, a GDH from *Bacillus subtilis* subsp. *subtilis* str. 168 (*Bs_GDH*) was selected, which is known to be active towards a highly reactive iminium compound.[30]

Based on the protein sequence of *M77_SDR* and the engineered variants *M77_SDR_opt* (F97N, A142C, I143C, S151H) and *Bs_GDH_opt* (E96Y, P98N, V146C, H147C, F155H, N196S), synthetic genes were ordered codon-optimized for expression in *Escherichia coli*. The genes were cloned into pET28a by In-Fusion cloning, overexpressed in *E. coli* BL21-Gold(DE3) using auto-induction medium,[34] and purified by Ni–NTA affinity chromatography. Despite several first-shell residues being exchanged, the “optimized” variants remained soluble.

The catalytic scope of the candidates was explored by testing three imine compounds (1–3), one iminium compound (4), and two keto substrates (5, 6), alongside the glucose/GDH NADPH regeneration system (Table 3). Both *Bs_GDH* and *Bs_GDH_opt* were active with ketone 5, but neither catalyzed C= \( \text{N} \) reduction of the tested substrates 1–3. Moreover, *Bs_GDH_opt* lost the GDH activity and the ability to reduce the activated derivative of iminium compound 4 (data not shown).[16]

*M77_SDR* proved to be an active enzyme and converted ketones 5 and 6 quantitatively with moderate stereoselectivity, displaying high activity with 5 (28.1 \( \text{U mg}^{-1} \)). These results indicate that *M77_SDR* is a ketoreductase, corroborated by the finding that no C= \( \text{N} \) reduction was observed with substrates 1–

### Table 1. Comparison of the amino acids on flanking (green) and consensus positions (red) in NR, Zt_SDR, and Ao_SDR. The enzymes share the SDR-typical catalytic triad (blue).

| Flanking/consensus positions | Catalytic triad |
|-------------------------------|-----------------|
| standard position             |                 |
| NR                            |                 |
| Y100                          | 51              |
| N102                          | 39              |
| C149                          | 51              |
| C150                          | 50              |
| Zt_SDR                        |                 |
| Y100                          | 45              |
| N102                          | 51              |
| C149                          | 47              |
| C150                          | 45              |
| Ao_SDR                        |                 |
| F122                          | 39              |
| T124                          | 47              |
| I171                          | 41              |
| A172                          | 39              |

| Mutations | Specific activity with ketone 5 \( \text{[mU mg}^{-1}] \) | Conversion of imine 1 \( \text{[H NMR]} \) \( \% \) |
|-----------|----------------------------------------------------------|----------------------------------|
| wild type | 331.9 ± 94                                               | < 5 %                            |
| twofold   | F122Y/L180H                                             | 577 ± 23                         | 6                               |
|           | F122Y/A172C/L180H                                        | 1012 ± 29                        | 9                               |
| fourfold  | F122Y/A172C/L180H/V221S                                  | 307 ± 11                         | 9                               |
| fivefold  | F122Y/T124N/A172C/L180H/V221S                             | 410 ± 54                         | 17                              |
| sixfold   | F122Y/T124N/A172C/L180H/V221S                             | 31 ± 2                           | 9                               |

[a] Reaction conditions: 0.0085–0.055 mg mL\(^{-1}\) Ao_SDR variant, 1 mM ketone 5, 250 \( \mu \text{M} \) NADPH, 1% (v/v) DMDSO, HEPS buffer (100 mM, pH 7.5), 30 °C; mean of triplicate. (b) Reaction conditions: 1 mg mL\(^{-1}\) Ao_SDR variant, 10 mM imine 1, 20 mM D-glucose, 0.5 mM NADPH, 0.25 mg mL\(^{-1}\) Bs_GDH, HEPS buffer (100 mM, pH 7.5), 30 °C, 20 h.
4. As proposed, M77_SDR_opt catalyzed both C=O and C=N reduction. While displaying reduced ketoreductase activity (1.36 U/mg with S), M77_SDR_opt accepted imine 1 (6% conversion), imine 2 (28% conversion, ee > 99% (R)-2a), and iminium 4 (20% conversion, ee 86% (R)-4a) as substrates. Interestingly, relative to the wild-type, M77_SDR_opt showed an improved stereoselectivity (ee > 93%) with regard to the alcohol products 5a and 6a.

In summary, we have shown that imine-reducing activity can be introduced into members of the “classical” SDR enzyme family. We implemented an amino acid pattern, obtained from the imine-reducing SDRs NR and Zt_SDR, in the unrelated ketoreductase M77_SDR, the latter with no obvious link to imine reduction. The resulting 4-fold variant M77_SDR_opt catalyzed as a new activity C=N reduction at the expense of C=O reduction activity. This proof-of-concept extends the scope of SDR engineering towards imine reduction.

The identified pattern enables imine reduction; however, it is not necessarily sufficient as seen for Bs_GDH_opt. The protein scaffold of the engineering target also impacts activity as it determines a) the arrangement of the introduced amino acids and b) elements that can influence or are required for activity, such as structural flexibility or long-range electrostatic interactions. The results obtained for Pc_SDR indicate that standard position 98 tolerates slight variations, which is in line with the results of the alanine scan of Zt_SDR. This has been shown for standard position 146 and might also apply for other positions of the pattern. Moreover, this illustrates the complexity of the rationale behind imine reduction catalyzed by SDRs, as well as the need for a more comprehensive knowledge of the underlying pattern and its influence on the structure–function relationship.

In a more general context, this work underscores that one SDR scaffold (e.g., NR, M77_SDR_opt) can meet the requirements for different reductive activities (here C=O, C=N reduction). With the latter activity being engineered, our work has revealed that the catalytic scope of SDR enzymes can be manipulated, which is in line with the results by Lygidakis et al. concerning C=O and enone reduction. Moreover, our results show that the starting activity is not a prerequisite for obtaining a desired novel functionality, here imine reduction. This encourages to consider SDRs as scaffolds for generating enzymes which catalyze challenging transformations that are not easily amenable by known biocatalysts.

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Conflict of Interest

The authors declare no conflict of interest.

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Table 3. Conversion of substrates 1–6 with Bs_GDH/M77_SDR and variants Bs_GDH_opt/M77_SDR_opt as well as stereochemistry of the resulting products 1a–6a (in parentheses).[a]

| Enzyme            | 1a   | 2a   | 3a   | 4a   | 5a   | 6a   |
|-------------------|------|------|------|------|------|------|
| Bs_GDH            | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Bs_GDH_opt        | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| M77_SDR           | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| M77_SDR_opt       | 6a   | 28   | (ee > 99%, R) | n.d. | (99% (59% cis) | > 99 (93% cis) | > 99 (99% cis) |
| Reaction conditions: 1 mg mL⁻¹ SDR, 10 mM substrate, 20 mM [NADP⁺], 0.25 mM mg⁻¹ mL⁻¹ Bs_GDH, 30 °C, 20 h, HEPES buffer (100 mM, pH 7.5), 30 °C, 20 h. In assays with Bs_GDH a concentration of 1.25 mg mL⁻¹ Bs_GDH in total was used; reactions with M77_SDR and M77_SDR_opt and S contained a malate dehydrogenase/l-malate as cofactor regeneration system. [b] Conversions were determined by 1H NMR spectroscopy as were the de values of 5a. [c] Conversion and ee values were determined by chiral-phase HPLC analysis. [d] ee values were determined by chiral-phase GC analysis. n.d. = no product detected. – = not determined due to low conversion.

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Willing to learn: In the context of enzymatic asymmetric reduction SDRs are mainly known for their ability to reduce ketones, rather than imines. However, both activities are based on the same catalytic triad. Using a rational approach inspired by two imine-reducing SDRs, the keto-reducing M77_SDR was equipped with imine-reducing capability.