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Extended Catalytic Scope of a Well-Known Enzyme: Asymmetric Reduction of Iminium Substrates by Glucose Dehydrogenase

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NADP(H)-dependent imine reductases (IREDs) are of interest in biocatalytic research due to their ability to generate chiral amines from imine/iminium substrates. In reaction protocols involving IREDs, glucose dehydrogenase (GDH) is generally used to regenerate the expensive cofactor NADPH by oxidation of D-glucose to gluconolactone. We have characterized different IREDs with regard to reduction of a set of bicyclic iminium compounds and have utilized ^{1}H NMR and GC analyses to determine degree of substrate conversion and product enantiomeric excess (*ee*). All IREDs reduced the tested iminium compounds to the corresponding chiral amines. Blank experiments without IREDs also showed substrate conversion, however, thus suggesting an iminium reductase activity of GDH. This unexpected observation was confirmed by additional experiments with GDHs of different origin. The reduction of C=N bonds with good levels of conversion (>50%) and excellent enantioselectivity (up to >99% *ee*) by GDH represents a promiscuous catalytic activity of this enzyme.

In general, a promiscuous enzyme can be defined as one "that does things it is not expected to do".[1] In this context, the term "pluripotent" describes members of the family of short-chain dehydrogenases/reductases (SDRs) that are capable of accepting more than their physiological substrate.[2] Spanning several EC classes, the SDR superfamily constitutes one of the largest enzyme families, with more than 46000 members.[3,4] Unrelated SDR members usually share very low protein sequence similarity, often as low as 15–30% in pairwise comparison. Even so, SDR proteins possess highly similar three-dimensional structures, an α/β-folding pattern (Rossmann-fold motif) for nucleotide binding, and chain lengths of 250–350 amino acids, with most such proteins existing as homodimers or homotetramers.[3–5]

Although pluripotency/promiscuity can be regarded as a typical feature of some SDRs involved in the metabolism of xenobiotics, many biological functions are controlled by SDRs that are assumed to be highly substrate-specific and monofunctional. Glucose dehydrogenase (GDH), a prototypical member of the SDR family, for example, was previously thought solely to catalyze NAD(P)-dependent oxidation of D-glucose to gluconolactone.[6] Until recently, GDH was not known to be active with non-sugar substrates.[7]

GDH is a component of a well-established method for nicotinamide cofactor regeneration.[8] Thus, this enzyme is often applied to provide NAD(P)H for the characterization of, for example, imine reductases (IREDs), a recently discovered group of enzymes identified as catalyzing the asymmetric reduction of cyclic or (generated in situ) open-chain imines.[9,10] IREDs have a broader substrate range than imine- or iminium-reducing enzymes involved in primary metabolism or alkaloid biosynthesis and have therefore been investigated as potential biocatalytic tools for an approach to chiral amines.[11]

Here we describe the serendipitous identification of GDHs from different organisms as catalysts for the asymmetric reduction of iminium salts. Prerequisite for this finding was the characterization of purified IREDs, thus excluding background reactions, and the selection of appropriate substrates.

Although IREDs have been extensively characterized for the reduction of cyclic imines, there are few reports on the reduction of iminium salts.[12,13] We aimed to characterize *R*- and *S*-selective IREDs for the reduction of cyclic iminium salts. Starting from the amino acid sequences of known IREDs as a template, two putative IREDs from *Streptomyces virginiae* were chosen, in addition to 17 already described IREDs from different microorganisms.[14,15] The genes were purchased as synthetic codon-optimized genes with an additional N-terminal His-tag and overexpressed in *Escherichia coli* BL21-Gold (DE3) cells (see the Supporting Information). Subsequently, all IREDs were purified by Ni-NTA affinity chromatography. IRED activity of the two newly identified enzymes from *S. virginiae* was confirmed by the reduction of 2-methyl-4,5-dihydro-1-pyrroline (data not shown). The substrate range and reaction stereoselectivity of the 19 bacterial IREDs towards a set of isoquinolinium substrates **1**–**3** were determined with use of D-glucose/GDH for NADPH regeneration (Scheme 1).

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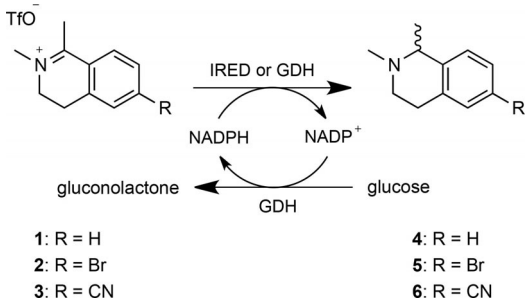


1: R = H
2: R = Br
3: R = CN

4: R = H
5: R = Br
6: R = CN

Scheme 1. Reduction of the iminium compounds **1**–**3** by IRED or GDH. Reaction conditions: 0.6 mg mL^{-1} purified enzyme, 10 mM substrate (**1**, **2**, or **3**), 20 mM D-glucose, 0.5 mM $NADP^+$, 2 mM $MgCl_2$, 0.26 mg mL^{-1} GDH in 100 mM HEPES buffer, pH 7.5, 30 °C, 20 h.

A ^{1}H NMR-based assay was used for determination of the enzymatic activity and product formation immediately after removal of the proteins by filtration (10 kDa cutoff). This method facilitated the simultaneous identification of the hydrophilic substrates and lipophilic products in the crude reaction mixture. The aromatic signals of the substrates and products, and the signals of the C-1 methyl group (in the case of products **4**–**6** a doublet in the aliphatic region), were clearly separated from each other and from those of other contaminants (see the Supporting Information). Furthermore, this procedure enabled concurrent qualitative and semiquantitative determination of the degree of conversion.

Compounds **1**–**3** were accepted by all tested IREDs (Table S7 in the Supporting Information). Several IREDs, such as IR_4 from *Kribbella flavida* (WP_012921542.1) and IR_20 from *Streptomyces tsukubaensis* (WP_006347397.1), showed acceptable levels of conversion (27–74%) for each substrate. The absolute configuration of product **4** was determined by vibrational circular dichroism (VCD). For this purpose, the transformation of **1** in the presence of IR_20 was performed on a preparative scale (50 μmol), yielding oily **4**. A VCD spectrum of the neat liquid was recorded and compared with the calculated spectrum of (*R*)-**4**, thus establishing the absolute *R* configuration (Figure 1). With the assumption of an identical reduction mechanism and, additionally, from the uniform retention time order in the chiral-phase gas chromatograms (later eluting enantiomer; see the Supporting Information), the *R* configuration is postulated for the products of IR_20. Accordingly, IR_4 gave access to the *S* enantiomers of products **4**–**6** (earlier eluting enantiomers; Tables S8 and S9).

The enantiomeric excesses (*ee* values) of the enzymatic products **4**–**6** were determined by chiral-phase GC analysis (Figure S11 and Table S9). Many of the transformations with high levels of conversion also revealed high *ee* values for the products. Surprisingly, significant levels of conversion (9 and 20%) were also obtained for substrates **2** and **3,** as well as high *ee* values for the products **5** and **6** (83 and 84% *ee*), in the blank experiments without IRED enzymes. Although nonspecific reduction by the (chiral) cofactor NADPH could, per se, explain product formation in the blank experiments, this should result in products with only slight *ee*. Instead, the high

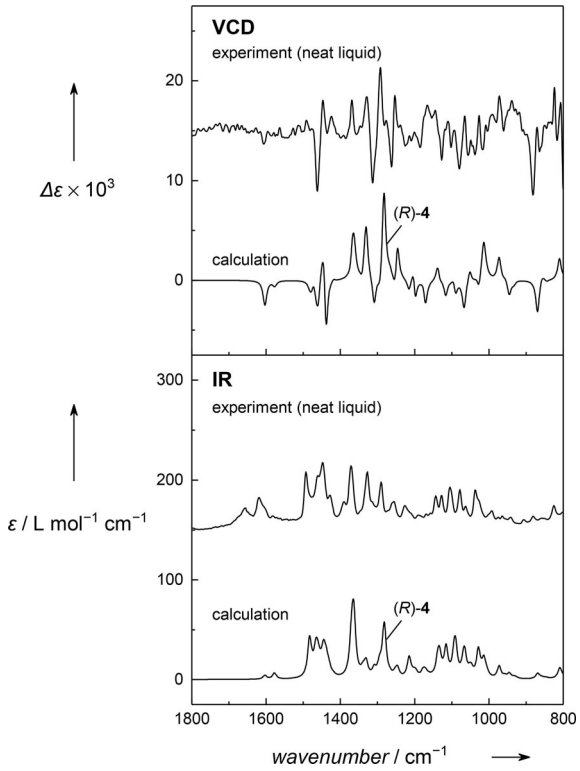


Figure 1. Experimentally measured IR and VCD spectra in comparison with spectra calculated for (*R*)-**4** at the B3LYP/6–31 + G(d,p) level in Gaussian 09.[16] The excellent agreement in positions and signs of the experimentally measured and calculated VCD bands confirms the *R* configuration.

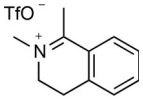
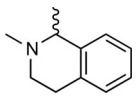
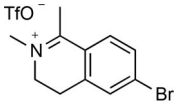
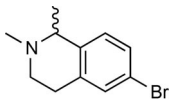
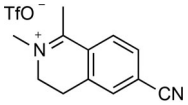
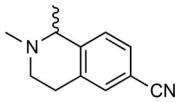
ee values provide evidence of asymmetric reduction of the prochiral substrates.

To elucidate the enantioselective background reaction, additional control experiments with substrate **3**, which showed the highest "background reaction" in the NMR assay, were performed. The presence of equimolar or 2 equivalents of NADPH, without any enzyme, gave 4 and 7% conversion, respectively. As expected, the product **6** was obtained in almost racemic form in both transformations (*ee* < 20%). Hence, the noted asymmetric induction must arise from enzyme control.

Accordingly, GDH-catalyzed transformation of substrates **1**–**3** was tested with increased amounts of GDH (0.023 mg mL^{-1}, 0.23 mg mL^{-1}, 1.15 mg mL^{-1}), in the absence of any IRED. The results (Table 1) clearly showed a correlation between enzyme concentration and conversion along with product *ee*. The decreased *ee* values of the products at lower enzyme concentration can be explained in terms of a higher contribution of nonenzymatic reduction by NADPH.

With the highest enzyme concentration tested, the reduction of **3** led to virtually enantiopure product (*R*)-**6** (> 99% *ee*), with 57% conversion. Moreover, this result explains the moderate to low *ee* values (< 50%) obtained for products **5** and **6** in assays with *S*-selective enzymes, as the accumulation of the *R*-configured products from the more or less pronounced GDH background reaction would partially cancel the gain from IRED catalysis. Correspondingly, replacement of the GDH/glucose regeneration system should result in higher *ee* values of the

Table 1. Conversion of **1**, **2**, and **3** by glucose dehydrogenase (GDH) and resulting enantiomeric excesses (*ee* values) of the products.[a]

Substrate	Product	GDH[b] conc. [mg mL^{-1}]	Conv. [%]	*ee* [%] (absolute config.)
 **1**	 **4**	0.023	0	–
		0.23	0	–
		1.15	9	n.d.[c]
 **2**	 **5**	0.023	4	28 (*R*)
		0.23	20	68 (*R*)
		1.15	28	86 (*R*)
 **3**	 **6**	0.023	9	86 (*R*)
		0.23	16	97 (*R*)
		1.15	57	>99 (*R*)

[a] Reaction conditions: 10 mM substrate (**1**, **2**, or **3**), 20 mM D-glucose, 0.5 mM $NADP^+$, 2 mM $MgCl_2$ in 100 mM HEPES buffer, pH 7.5, 30 °C, 20 h. [b] GDH-105 (Codexis). [c] Not detected.

S products under such IRED catalysis conditions. IR_10, which showed the most prominent *S* selectivity of the tested IREDs, was chosen for further biotransformations with a stoichiometric supply of NADPH, instead of the regeneration system. The substantially improved *ee* values of the formed products (*S*)-**5** and (*S*)-**6** confirmed our hypothesis (Table S13).

To verify the unexpected results, purified GDH from *Bacillus subtilis* (evocatal, Monheim am Rhein) was tested (see the Supporting Information). These experiments confirmed the asymmetric induction and enzymatic transformation of the iminium salts by GDH, resulting in high *ee* values of product (*R*)-**6** (86–99% *ee*) with up to 57% conversion. In experiments with high concentrations of commercial GDH preparations [2.6 mg mL^{-1} GDH-105 (Codexis, Redwood City, CA) or GDH-2 (Roche)] the reduction of imines (non-*N*-methylated) was observed as well (see the Supporting Information).

In summary, we have shown that all tested IREDs, including the two newly identified enzymes from *S. virginiae*, are capable of reducing the bicyclic iminium compounds **1**–**3**. The high reactivity of the iminium salts towards reduction was probably helpful for elucidation of the entirely unexpected asymmetric reduction of a C=N bond by GDH, usually regarded as a "classical" NADPH-dependent SDR.[17] Hence, we have demonstrated that the substrate scope of GDH is broader than initially suspected, underscoring our previous finding of GDH-catalyzed reduction of naphthoquinone derivatives.[7,18] The enantioselectivity of the iminium reduction is strikingly high, even being higher than for many IRED-catalyzed transformations.

As the use of GDH, even in small amounts for cofactor regeneration, resulted in the unambiguous conversion of iminium compounds **2** and **3** into the amine products with high *ee* values, the D-glucose/GDH cofactor regeneration system has to be considered non-innocent. Thus, it could have a significant influence on the apparent results achieved with a primarily tested enzyme, such as an IRED. Therefore, experiments relating to the reduction of reactive iminium compounds in which IREDs are applied alongside GDH should be validated with respect to the cofactor regeneration system, particularly in cases in which crude cell IRED extracts or whole-cell cofactor regeneration are used.

Our finding for GDH is in line with a recent example of promiscuous SDR activity: Kutchan and co-workers discovered a C=C-bond-reducing SDR with an imine reductase side activity.[19] Moreover, in *Catharanthus roseus* a medium-chain alcohol dehydrogenase homologue that possesses iminium-reducing activity has been found.[20] Conversely, Nestl and co-workers most recently proposed a carbonyl reductase (side) activity by two IREDs exposed to a highly reactive ketone substrate.[21]

Nevertheless, the observation that both GDH and IREDs reduce iminium compounds cannot easily be explained. Members of both enzyme types are oxidoreductases and share the Rossmann fold for cofactor binding as a common structural feature. Comparison of GDH with known and structurally analyzed IREDs (data not shown), however, does not reveal any major similarity. Sequence analysis indicated that 17 of the 19 tested IREDs exhibit the active-site motif suggested by Pleiss and co-workers.[22] Nonetheless, the exact role of these residues or even their participation in the reduction of imine/iminium substrates has not been fully elucidated. In contrast, GDH has the SDR-typical catalytic triad Ser-Tyr-Lys,[23] whereas IREDs lack a lysine residue in their active site, highlighting the differences between these enzyme types. Moreover, the postulated mechanism of reduction by dihydrofolate reductase[24] includes activation of the imine through protonation by a water molecule, which might be another clue that, in general, the main function of imine-reducing enzymes is to provide close proximity for hydride transfer.[22] Thus, reduction of the tested iminium compounds by IREDs, as well as by GDH, cannot be explained in terms of simple structural characterization and/or sequence alignment without implementation of other factors, such as dynamic contributions or substrate–enzyme interactions. Rather, our results and the literature examples noted indicate that the focus on IREDs should not lead to the neglect of SDRs and other NAD(P)H-dependent oxidoreductases as templates for the identification of new imine-reducing enzymes.

Experimental Section

GDHs of different origin were tested on an analytical (0.5 mL) scale. GDH-105 (Codexis) was applied from a freshly prepared stock solution [2.33 mg mL^{-1} in HEPES buffer (100 mM, pH 7.5)], and GDH from *Bacillus subtilis* (evocatal) as a crude cell lysate or as the purified enzyme. The substrate was added from a stock solution (1 M in MeOH) to different amounts of enzyme, resulting in a 10 mM substrate concentration. Each reaction was started by addition of a mixture (250 μL) containing the other reaction components dissolved in HEPES buffer (pH 7.5, 100 mM). The reaction mixture consisted of GDH, substrate **1**, **2**, or **3** (10 mM), D-glucose (20 mM), $NADP^+$ (0.5 mM), and $MgCl_2$ (2 mM). After incubation for 20 h at 30 °C and 850 rpm, the reaction was stopped by removing the enzyme (filtration with 10 kDa cutoff or centrifugation after heat-

ing). Positive and negative controls were undertaken. Degrees of conversion were determined by ^{1}H NMR spectroscopy after addition of D_2O (10%). For chiral-phase GC analysis, the reaction mixtures were basified with NaOH (8 M) and extracted twice with EtOAc.

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Keywords: biotransformations · enantioselectivity · enzyme promiscuity · oxidoreductases · tertiary amines

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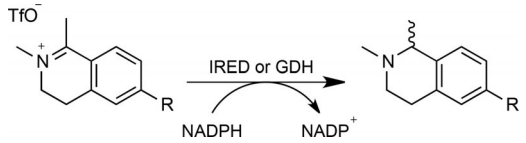

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Doing it all: Glucose dehydrogenase (GDH) was applied as a cofactor regeneration system in the characterization of imine reductases (IREDs) in the reduction of bicyclic iminium compounds. Both enzyme types were found to be capable of reducing such substrates.

Extended Catalytic Scope of a Well-Known Enzyme: Asymmetric Reduction of Iminium Substrates by Glucose Dehydrogenase