1. Introduction

Enzymes often rely on the presence of cofactors as molecular shuttles to transfer redox equivalents (e.g. electrons, hydrides), unstable species (carbanions, e.g. enolates, organometallics) or toxic molecules (e.g. ammonia) to catalyze a great variety of chemical reactions, as amino acid side chains alone cannot mediate all chemical transformations to sustain the essential processes of life. Among the redox-enzymes employed in biotransformations, the majority depends on nicotinamide acting as a hydride donor for C=O and C–C bond reductions.1 A smaller number uses a metal, most prominent Fe2+/3+, but also Mn2+/3+, V5+ and others, for single-electron transfer.2 Flavoenzymes, in particular, owe their redox activity to the ability of the flavin cofactor—either flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD)—to mediate one- or two-electron transfer processes, which are enabled by resonance-stabilized radical and/or anionic reactive intermediates.3 In contrast to nicotinamide, which can only participate in two-electron (hydride) transfer, the diversity of flavoenzyme chemistry is reflected by an astonishingly broad spectrum of reactions, ranging from oxidations, such as mono-, di-, and peroxydations, as well as halogenation and C–C formation, to an array of (one- or two-)electron reductions involving alkenes, arenes, nitro-groups and nitro-esters.3,4 The origin of this chemical versatility lies in the tricyclic isoalloxazine ring system of flavins. Although the redox potential for two-electron reduction of flavin lies at −200 mV, it can be modulated by the enzyme environment—often via (bi)covalent attachment of the flavin onto the protein5—to span a range from −400 mV to +60 mV, thus conferring diverse reactivity to the enzyme. Additionally, flavins react with molecular oxygen to affect C=C-epoxidation, C–H-hydroxylation and R-SO2H, R-NO2-, alcohol-, amine-, thioether- and Baeyer-Villiger-oxidations. Interestingly, flavoenzymes are also known to be involved in redox-neutral processes, such as C=C-isomerization, magnetoreception and light-responsiveness.6

Most of the flavin-dependent enzyme-catalyzed transformations have chemical counterparts. However, these often require conditions which do not allow sustainable and/or environmentally friendly processes.7 In particular, non-enzymatic catalyzed C=C-bond isomerization reactions often rely on the use of (transition)metal-based catalysts based on Ru, Rh, Pd, Ir,8 or AlCl3.9 Similarly, the catalytic asymmetric hydrogenation of alkenes traditionally involves transition-metal catalysts,10 as does the reduction of nitroaromatics,11 while the classical Nef-reaction requires the presence of a strong aqueous acid.12

2. Flexibility in hydride transfer

2.1 Reduction of activated alkenes

The most prominent application of flavoproteins is the two-electron reduction of activated C=C bonds by the so-called ene-reductases, which are homologues of the Old Yellow Enzyme (OYE) family of flavoproteins. Electronic activation is
provided by an electron withdrawing group such as carbonyl (ketone/aldehyde/acid/ester/lactone/cyclic imide), nitro or nitrite. The asymmetric reduction proceeds via transfer of [2H] in a Michael-type addition reaction operating in a trans-fashion. The hydride is provided by FMNH₂, while the proton (usually delivered from a conserved tyrosine residue) ultimately derives from the solvent. The oxidized flavin is reduced again at the expense of a nicotinamide cofactor to close the catalytic cycle (Fig. 1). Alternatively, flavin may be recycled via non-conventional electrochemical or light-mediated methods or by a non-natural hydride donor in a nicotinamide-free system.

Although only a few studies have addressed the technical aspects of ene-reductase-catalyzed biotransformations, some reactions have been investigated with a focus on productivity and upsaling. TTN values are generally low (usually <10⁴, as estimated from conversion data reported for non-optimized reactions), a phenomenon which has been associated with enzyme deactivation by the substrate, which significantly limits substrate loading. More sophisticated reaction engineering techniques, however, led to significant improvements in productivity, as shown in the preparation of ethyl (neering techniques, however, led to significant improvements in productivity, as shown in the preparation of ethyl (neering techniques, however, led to significant improvements in productivity, as shown in the preparation of ethyl (neering techniques, however, led to significant improvements in productivity, as shown in the preparation of ethyl (neering techniques, however, led to significant improvements in productivity, as shown in the preparation of ethyl (neering techniques, however, led to significant improvements in productivity, as shown in the preparation of ethyl (neering techniques, however, led to significant improvements in productivity, as shown in the preparation of ethyl (neering techniques, however, led to significant improvements in productivity, as shown in the preparation of ethyl

### 2.2 Reduction of activated alkenes

Ene-reductases from the OYE family have been extensively studied for their catalytic activity in the C=C bond reduction of activated alkenes and were implemented in various asymmetric synthetic strategies for the generation of chiral molecules in enantiopure form. In addition, OYE homologues were shown to catalyze the C=C bond reduction of electronically related yrones at the expense of NAD(P)H. This stereoselective reductive reaction furnished the corresponding trans-configurated enone, which was subsequently reduced to the fully saturated ketone.

Recombinant *E. coli* strains over-expressing OYE1 (*Saccharomyces pastorianus*), OYE2 (*Saccharomyces cerevisiae*), OYE3 (*Saccharomyces cerevisiae*) or NCR (*Zymomonas mobilis*) were used in the reduction of 4-phenyl-3-butyn-2-one (1) in the presence of NAD(P)H in excess. Depending on the relative rate of both steps, the reaction yielded (E)-4-phenyl-3-buten-2-one (2) and 4-phenyl-2-butanone (3) in various ratios: while NCR predominately furnished the fully reduced ketone without a trace of intermediate enone, OYE3 showed a strong substrate preference for the ynone over the enone.

### 2.3 Reduction of activated aromatics

The ability of flavoproteins to deliver a hydride onto electron-deficient carbon atoms is not restricted to C=C and C=C-bonds, but may likewise take place on electron-deficient aromatics. Elucidation of biodegradation pathways of polynitroaromatics, such as the toxic explosive 2,4,6-trinitrotoluene (TNT) (4), showed catalytic promiscuity of ene-reductases on nitro-aromatics. Apart from nitro-reduction of TNT (leading to the nitroso- and hydroxylamino-products, see Section 2.4), PETN reductase (pentaerythritol tetrinitrate reductase from *Enterobacter cloacae* PB2) could also direct a nucleophilic hydride attack directly onto the electron-deficient aromatic ring system. The latter process is remarkable, because it goes...
in hand with (energy-requiring) destruction of aromaticity. The first intermediate thus obtained is a mono-hydride-Meisenheimer complex (5), which was further reduced to an unstable dihydridom-Meisenheimer complex (6) (Fig. 3). Disappearance of the latter is dependent on the enzyme concentration and is accompanied by elimination of nitrite along with unidentified compounds. Alternatively, dimerization occurred with products derived from the concurrent nitro-reduction reaction (see Section 2.4) to yield secondary diarylamine accompanied by loss of nitrite, for which several pathways have been suggested. This activity, favored by the enhanced electrophility of the aromatic system by three nitro-substituents, was also found in XenB (Pseudomonas fluorescens), NemA (N-ethylmaleimide reductase from E. coli) and OPRI (Arabidopsis thaliana).

2.4 Nitro-group reduction: nitroaromatics

The biodegradation of nitroaromatics usually proceeds via nitro-group reduction, catalyzed by flavin-dependent nitroreductases. Two types of these enzymes have been identified, mostly in bacterial strains. Type II nitroreductases catalyze nitro-group reduction via single-electron transfer yielding an unstable nitro-anion free radical. The latter undergoes spontaneous re-oxidation by \( O_2 \) producing superoxide, which accounts for the oxygen-sensitivity of these enzymes. In contrast, oxygen-insensitive (type I) nitroreductases act via a two-electron transfer and include NfsA and NfsB from E. coli, SnrA and CnR from Salmonella enterica serovar Typhimurium TA1535, PnA and PnB from Pseudomonas putida, and YcnD from Bacillus subtilis (NfrA2). Nitroreductases are regarded as typical detoxification enzymes because they act on a remarkably broad range of nitroaromatics, such as nitrofurazone, nitrofurantoin or 5-(1-aziridinyl)-2,4-dinitrobenzamide (an anticancer prodrug known as CB1954). 2,4,6-Trinitrotoluene (7) is widely accepted by most nitroreductases and OYE homologues, which sequentially reduce the nitro-group to the corresponding nitroso- and hydroxylamino-compounds. Condensation of the latter products with intermediates originating from the competing pathway via reduction of the aromatic ring (see Section 2.3) usually leads to the formation of various dimers. Overall, the outcome of the reductive biodegradation of nitro-aromatics is not only determined by the relative rates of enzymatic and spontaneous steps, but also by the stability of intermediates towards conditions in a given environment (such as pH, temperature, and concentrations).

It is noteworthy that nitroreductases and ene-reductases, though structurally different, display reciprocal substrate promiscuity. While OYE homologues are strong in the nitro-group reduction of TNT, Salmonella typhimurium...
2.5 Nitro-group reduction: nitroaliphatics (bio-Nef-reaction)

Like aromatic nitro-groups, also aliphatic nitro analogs (12) were readily reduced by OYEs. Several ene-reductases could transfer a hydride directly onto the substrate nitrogen atom, yielding a highly unstable aliphatic sec-nitroso compound (13) which spontaneously tautomerized to form the corresponding more stable oxime (14). The latter was further attacked at the nitrogen atom by FMNH₂ to form a water-labile imine (15), which rapidly hydrolyzed to yield the corresponding carbonyl compound (16) and ammonia as the final stable end-products of this cascade (Fig. 5). Overall, this resembles a biocatalytic equivalent to the Nef-reaction. The instability of the intermediates prevented isolation and/or detection of the nitroso and imine products. ¹²

In the case of nitroalkenes, the bio-Nef pathway and the traditional C–C bond reduction occurred sequentially, as exemplified with 1-nitro-2-phenylpropene (17). All ene-reductases tested delivered a mixture of 1-nitro-2-phenylpropane (obtained as the initial product from alkene reduction), and oxime and/or aldehyde (both resulting from subsequent reduction of the nitro-alkane via the Nef-pathway). Interestingly, the most active enzymes were those previously identified as xenobiotic reductases, naturally acting on nitro-compounds: XenA (Pseudomonas putida II-B 86), NerA (glycerol trinitrate reductase from Agrobacterium radiobacter) and PETN reductase. ¹² Oxophytodienoic acid reductase (OPR3, Solanum lycopersicum) and morphine reductase (Pseudomonas putida M10) also showed significant activities.

2.6 Nitro-group reduction: nitroalkenes (oxazete formation)

While the reduction of activated alkenes by ene-reductases is usually highly chemo-selective with hydride transfer onto Cβ, flexibility in the substrate binding may afford alternate binding modes favoring hydride attack on the nitrogen atom in the case of β,β-disubstituted nitroalkenes (e.g. 17), where hydride delivery on Cβ is sterically impeded (compare with Section 2.5). In turn, this leads to two competing reactions, i.e. C=C bond vs. nitro-group reduction, which potentially occur simultaneously on the same substrate, as opposed to the bio-Nef reaction on nitroalkenes, where C=C bond and nitro-group reductions are sequential. Simultaneous competing C=C-bond and nitro-group reductions were observed in the reduction of 1-nitro-2-phenylpropene (17): reduction of the nitro group to the corresponding nitroso-alkene (18) was followed by fast s-cis/s-trans-isomerization, affording a highly unstable s-cis-nitroso-alkene (19). The latter spontaneously underwent electrocyclization to rac-4-methyl-4-phenyl-1,2-oxazete (20) as the major product (Fig. 6). At elevated temperature, the modestly stable 1,2-oxazete underwent retro-[2 + 2]-cycloaddition to furnish HCN and acetophenone (21). ¹⁰ From a range of ene-reductases tested, oxazete formation was predominant with XenA, while PETN reductase favored the bio-Nef pathway after reduction of the C=C bond. Overall, only β,β-di-substituted nitroalkenes were found to undergo reduction of the nitro-group to form the corresponding racemic oxazete. NerA, XenA, PETN reductase and OPR3 were identified as a subset of OYE homologues showing structural similarities most likely responsible for their dual activity on nitroalkenes with regard to C=C and nitro-group reductions. In particular, the presence of ‘hydrophobic hot-spots’ in the region around loop β5 and loop β6 was identified as determinant for specific nitro group reduction, allowing two concurrent binding modes required for dual catalytic activity. ⁴¹ Along with the favored substrate binding mode, the enzyme kinetics eventually determine the biodegradation pathways of nitroalkenes, as the bio-Nef reaction only occurs after C=C bond reduction, while oxazete formation results from preferential nitro-reduction over C=C bond reduction.

2.7 Reductive nitrate ester cleavage

Like nitro-aromatics, nitrate esters are primarily man-made xenobiotics which have been extensively used as explosives during the two World Wars. Glycerol trinitrate (GTN) (22), also erroneously termed nitroglycerine, and pentaerythritol tetranitrate (PETN) (23) are two common organic nitrate esters, for which biodegradation pathways involving OYE homologues have been identified. They both proceed via reductive denitration in a net two-electron transfer, liberating nitrite and the corresponding alcohols (Fig. 7). PETN reductase, NerA, OYE1
as well as XenA and XenB, for instance, catalyze the reductive liberation of 1–2 mol of nitrite from GTN at the expense of NAD(P)H, while 2 mol of nitrite are usually obtained from PETN. Mechanistically, the reduction occurs via hydride attack onto the N atom of nitrate ester (24), followed by spontaneous tautomerization to yield the corresponding nitrite ester (25). The latter is hydrolytically very labile and is non-enzymatically converted to the alcohol with concomitant release of nitrite. The possibility that the mechanism proceeds via a radical mechanism has been postulated. 

Additionally, PETN reductase was also found to be active on structurally related N-nitramines, such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX, ‘Royal Demolition Explosive’) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), however to a much lesser extent compared to nitrate esters.

2.8 Reductive dehydrohalogenation of activated haloalkenes

In an attempt to render insufficiently activated α,β-unsaturated carboxylic esters more reactive towards ene-reductases, the effects of additional electron-withdrawing halogen substituents on the C=C bond were investigated. The latter were expected to enhance C=C bond activation and thus increase ene-reductase activity on such substrates. While this was found to be extremely efficient on α-halo-α,β-unsaturated carboxylic esters of type 30, this strategy failed for β-halo-analogs 26 and 28 due to the high instability of the corresponding saturated β-halo-esters 27 and 29 obtained from the bioreduction. The enhanced acidity of the α-proton combined with the presence of a halogen on Cβ led to fast dehydrohalogenation via spontaneous elimination of hydrohalic acid. On α,β,γ-tri-substituted halo-acrylic esters (26) the sequence proceeded until complete removal of halogen on Cβ took place (Fig. 8).

Enzyme-based stereocontrol in the last C=C bond reduction step (30) allowed the formation of both methyl 2-halopropio-nate enantiomers (31) in high enantiopurity (up to >99% ee). Overall, this pathway resembles a reductive dehalogenation of β-halogenated acrylic esters.

2.9 Reduction of O₂ forming H₂O₂: Weitz–Scheffer oxidation (activated alkenes and thioethers)

Although the physiological role of ene-reductases has not been clearly identified yet, it is believed that OYE homologues are generally involved in oxidative stress response as detoxification enzymes. The nature of the physiological reductant has been determined, and most enzymes accept NADH and NADPH, often equally well, although some differences in kinetic parameters have been occasionally observed. However, oxidants capable of reacting with the enzyme-bound reduced flavin are very diverse, highlighting the catalytic promiscuity of this class of enzymes. Molecular oxygen has also been found to act as the substrate, although its reactivity is usually lower compared to activated alkenes. In some cases, however, this so-called ‘NAD(P)H oxidase activity’ predominates, thus producing hydrogen peroxide, which spontaneously reacts with compounds such as enones and thioethers, thereby producing the corresponding epoxides and sulfoxides (Fig. 9). Since this Weitz–Scheffer reaction does not require the enzyme’s active site, the products are formed in a non-stereoselective fashion and racemic mixtures are obtained.

![Fig. 7](Image) Reductive nitrate ester cleavage catalyzed by ene-reductases; GTN: glycerol trinitrate, PETN: pentaerythritol tetranitrate.

![Fig. 8](Image) Sequential reductive dehydrohalogenation of β-haloacrylic ester derivatives mediated by ene-reductases.

![Fig. 9](Image) Weitz–Scheffer epoxidation of enones and oxidation of thioethers mediated by NAD(P)H-FMN-dependent oxidoreductases in the presence of O₂.
Though molecular oxygen is known to be accepted as oxidant by several ene-reductases,\textsuperscript{37,49} this side reaction was particularly predominant with YcnD (nitro-reductase from \textit{Bacillus subtilis}, Lot6p [NAD(P)H:FMN-dependent reductase from \textit{Saccharomyces cerevisiae} active on azo-compounds and quinones] and YhdA (NADPH:FMN-dependent oxido-reductase from \textit{Bacillus subtilis} with azoreductase activity), all displaying the typical ping-pong bi-bi mechanism of OYE homologues. Ketoisophorulus subtilis active on azo-compounds and quinones and YhdA (NADPH:FMN-dependent oxidoreductase from \textit{Saccharomyces cerevisiae} active on azo-compounds and quinones) were epoxidized to various extent (up to 77% conversion with ketoisophorone using YcnD) while thioanisole (39) was selectively oxidized to the racemic sulfoxide (35) in up to 80% conversion with YcnD, but no sulfone was detected. Interestingly, non-activated C=C bonds were not reactive (e.g. in styrene, α-methylstyrene, or the isolated C=C bond in citral). Performing the reaction in the presence of H$_2$O$_2$-degrading catalase or under an inert (argon) atmosphere suppressed product formation, thus confirming the chemical nature of this pathway.\textsuperscript{50}

3. Redox-neutral hydrogen transfer

3.1 Disproportionation of activated alkenes

While investigating the formation of long wavelength charge-transfer bands upon mixing of OYE1 with phenolic compounds, Massey \textit{et al.} discovered that OYE1 catalyzed the oxidative aromatization of several compounds, including 2-cyclohexene (40), 3-oxodecalin-4-ene (ODE) (42) and 3-oxodecalin-4-ene-10-carboxaldehyde (ODEC), via a dehydrogenation reaction. With 2-cyclohexene or ODE, a redox-neutral disproportion (‘dismutation’) reaction was observed (Fig. 10A/B): while one molecule of substrate was reduced to the corresponding saturated ketone—cyclohexanone (41) or 3-oxodecalin (43), respectively—as expected from the well-known ene-reductase activity, one equivalent of 40 or 42 was dehydrogenated yielding an unstable dienone, which spontaneously tautomerized to the aromatic species phenol or 3-hydroxy-6,7,8,9-tetrahydrophenanthrene (HTN) (44), respectively. Overall, this reaction seems to be a shared characteristic of OYE homologues\textsuperscript{51} and does not proceed with net consumption of NAD (P)H, as FMN shuttles electrons directly between the two substrate molecules serving as hydrogen-acceptor and -donor.\textsuperscript{52} The synthetic potential of this system was investigated further by testing appropriate hydrogen-donor molecules in order to by-pass the requirement for nicotinamide cofactor and a second enzyme for its recycling in the reduction of various activated alkenes (Fig. 10C).\textsuperscript{53} N-Phenyl-2-methylmaleimide (45) could be asymmetrically reduced by YqjM to (R)-N-phenyl-2-methylsuccinimide (46) in a decent conversion (51%) and >99% ee, using equimolar amounts of cyclohexane-1,4-dione (47) as a sacrificial hydrogen-donor. Dimethyl citraconate (48) was reduced to a lower extent (20% conversion) yielding dimethyl (R)-2-methylsuccinate (49) with excellent stereoselectivity (>99% ee). Overall, cyclohexane-1,4-dione (47) appeared to be a superior hydrogen donor than 3-methylcyclohexenone and offers an excellent coupled-substrate FMN-recycling approach for nicotinamide-free C=C bond reduction protocols, where the driving force lies in the spontaneous aromatization of the oxidized co-product.\textsuperscript{53} In this process, the flavin cofactor plays a dual redox-role, being able to oxidize and reduce two different substrates in an ordered and sequential fashion.

3.2 C=C bond isomerization

Examples of reduced FMN involved in non-redox rearrangement or isomerization reactions include isopentenyl-diphosphate isomerases type 2 (IDIs-2),\textsuperscript{54} which catalyze the reversible isomerization of isopentenyl diphosphate (IPP) (50) to dimethylallyl diphosphate (DMAPP) (51), both compounds being central activated intermediates in the mevalonate pathway for the biosynthesis of terpenoids (Fig. 11A). Likewise, cis-trans isomerases catalyzing (E/Z)-isomerization of alkenes are involved in the biosynthesis of D-carotenoids,\textsuperscript{55} such as lycopene-β-cyclase.\textsuperscript{56} IDIs-2 act through acid–base catalysis via a protonation/deprotonation sequence mediated by the flavin.\textsuperscript{57} In a related fashion, FMN$_2$H$_2$ was shown to act as an acid/base catalyst hydrogen-shuttle in an unexpected C=C-isomerization reaction catalyzed by ene-reductases (Fig. 11B). OYE homologues EBP1 (\textit{Candida albicans}), OYE1, OYE2, and KYE (\textit{Klyveromycetes lactis}) were found to mediate the isomerization of a terminal alkene (α-methylene-γ-butyrrolactone) (52) to the more stable internal olefin isomer 3-methylfurane-2(5H)-one (53). The competing C=C-bond reduction of both isomers leading to 2-methyl-γ-butyrrolactone (54) was detected as a minor pathway (66–78% of isomerization vs. 20–27% of reduction product). With OYE2, the reduction of the thermodynamically more stable endocyclic isomer 53, which cannot be isomerized, was found to be about three times slower than the

![Image](14x290 to 26x354)

*p"C-Resonance of cyclohexene: B: disproportionation of 3-oxodecalin-4-ene (ODE) catalyzed by ene-reductases; C: nicotinamide-independent reduction of enones with cyclohexene-1,4-dione as a hydrogen donor.*

*Fig. 10 A: Disproportionation of cyclohexene; B: disproportionation of 3-oxodecalin-4-ene (ODE) catalyzed by ene-reductases; C: nicotinamide-independent reduction of enones with cyclohexene-1,4-dione as a hydrogen donor.*
role in the reduction of toxic nitro-aromatics. Based on these similar reductive detoxification properties, it is not surprising that some xenobiotic reductases share catalytic characteristics of both ene- and nitro-reductases. In addition to redox catalysis, these flavin-dependent enzymes are also involved in chemo-enzymatic cascade-reactions, where the reactivity of intermediates often opens unexpected deviations to new pathways. Finally, examples of unusual reactions involving the flavin as a dual (non-)redox catalyst highlight the importance of these reductases beyond their well-studied traditional reductive activity.

Acknowledgements

This project was performed within the DK ‘Molecular Enzymology’ and financial support from the Austrian Science Fund FWF (Vienna, project W9) is gratefully acknowledged.

References


