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FEATURE ARTICLE

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Nonconventional regeneration of redox enzymes – a practical approach for organic synthesis?

Wuyuan Zhang and Frank Hollmann *

Oxidoreductases have become useful tools in the hands of chemists to perform selective and mild oxidation and reduction reactions. Instead of mimicking native catalytic cycles, generally involving costly and unstable nicotinamide cofactors, more direct, NAD(P)-independent methodologies are being developed. The promise of these approaches not only lies with simpler and cheaper reaction schemes but also with higher selectivity as compared to whole cell approaches and their mimics.

Introduction

Oxidoreductases are Nature's catalysts to perform redox reactions, ranging from functional group conversion to introduction of new functional groups. Therefore, oxidoreductases are increasingly recognised as valuable tools in chemical synthesis.

Mechanistically, most oxidoreductases fall into two major categories: (1) enzymes catalysing hydride reactions (*i.e.* oxidising or reducing a starting material by transfer of a hydride ion) and (2) oxyfunctionalisation reactions (*i.e.* introducing activated oxygen species into the starting material). In both reaction types, transfer of redox equivalents is vital to sustain the enzymes' catalytic cycles. Natural sources or sinks for redox equivalents

may imply complications for the synthetic application of oxidoreductases. For example, native nicotinamide cofactors (NAD(P)) are comparably expensive, necessitating efficient *in situ* regeneration systems to allow for their use in catalytic amounts. Alternatively, NAD(P) together with an *in situ* regeneration system may be omitted by substituting NAD(P) with an artificial source or sink of reducing equivalents. Hence, NAD(P)-independent reaction schemes bear the promise of simpler reactions, which has triggered intensive research efforts for more than 3 decades now.

The aim of this feature article is to present and critically evaluate the most common 'non-conventional' regeneration systems. We will focus on those systems that explicitly aim at synthetic applications. Therefore, bioanalytical and bioenergy applications will not be covered.¹

This contribution is divided into two sections dealing with oxidative and reductive regeneration systems.

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Oxidative regeneration of oxidases

Oxidases catalyse a dehydrogenative oxidation of alcohols² or amines³ (recently also thiols have been reported).^{4,5} The most common oxidases comprise a flavin or a Cu-prosthetic group performing the hydride abstraction from the substrate (Fig. 1). In the course of the reaction the prosthetic group itself gets reduced and regeneration of the oxidised form generally occurs, with molecular oxygen yielding hydrogen peroxide as a stoichiometric by-product.

The latter may be an issue mainly for two reasons. First, H₂O₂ may negatively interfere with biocatalysts *via* spontaneous oxidation of labile amino acid residues. This issue, however, can be circumvented by the co-application of catalase to dismutate H₂O₂ into O₂ and H₂O. Second, and maybe more importantly, oxygen availability usually becomes the overall rate-limiting factor of the reaction.^{6–9} Hence, the full catalytic potential of a given oxidase may not be accessible because O₂-diffusion limits the overall reaction rate.

O₂, however, can be replaced by other electron acceptors to regenerate the oxidised enzyme prosthetic group. For example, ferrocenes,^{10–12} quinoids,^{13–15} conducting polymers¹⁶ or redox active inorganic catalysts¹⁷ have been reported. Electrochemical regeneration of the oxidised mediators represents a promising approach to circumvent the above-mentioned rate limitation as here – in principle – the electrode surface area and mediator concentration, and consequently the reoxidation rate, can be controlled (Fig. 2).¹⁸ From an early stage on, glucose oxidase has been studied intensively, especially having biosensor applications in mind.^{17,19–23} But also cholesterol oxidase^{14,15} and other oxidases^{13,24,25} have been investigated. Steckhan and coworkers have pioneered the synthetic application of indirect electrochemical regeneration of oxidases (Fig. 2).^{10–12} To facilitate electrochemical communication between the oxidases' active sites and the anode, ferrocene-mediators proved to be very efficient. Excellent turnover numbers (TONs) for biocatalysts approaching 500 000 and respectable mediator turnover numbers of 500 were achieved.

Unfortunately, this very promising approach has not found much attention so far!

Reductive regeneration of flavoenzymes

The flavin prosthetic group is very versatile with respect to the reactions it catalyses.²⁶ In the so-called old yellow enzymes

Fig. 1 Highly simplified representation of Cu- (upper) and flavin-dependent (lower) oxidases. The oxidised (left) enzymes perform an overall hydride abstraction from the starting materials (e.g. alcohols: X = O, amines: X = NH or thiols: X = S), yielding the corresponding carbonyl products and the reduced prosthetic groups (right). Regeneration of the oxidised enzymes naturally occurs aerobically, yielding H₂O₂ as a stoichiometric by-product.

Fig. 2 Electroenzymatic oxidation of *p*-alkyl phenols using *p*-cresol methylhydroxylase (PCMH) and ferrocenes as redox mediators between PCMH and the anode.^{11,12}

Fig. 3 The chemical versatility of flavoproteins.

reduced flavins mediate the (enantiospecific) reduction of prochiral, conjugated C=C-double bonds. In flavin-dependent monooxygenases, the reduced flavin reacts with molecular oxygen to form a hydroperoxoflavin species capable of a variety of (selective) oxyfunctionalisation reactions (Fig. 3). Naturally, the reduction of the flavin group is achieved through the natural NAD(P)H. The latter, however, can be substituted by other reductants, making flavoenzymes a fascinating playground for 'unconventional' regeneration systems!

Reductive regeneration of old yellow enzymes

Though principally known since decades²⁷ the so-called Old Yellow Enzyme (OYE)-like, flavin-dependent ene reductases have only been recently rediscovered thanks to the studies of Faber and Hauer.^{28,29} Today, OYEs are well-established tools for selective *trans*-hydrogenation of conjugated C=C-double bonds in preparative redox biocatalysis.^{30–32} Naturally, OYEs receive their reducing equivalents needed from reduced nicotinamide cofactors (NAD(P)H) (Fig. 4).

OYEs are rather promiscuous with respect to the source of the reducing equivalents (hydride) and also accept other

Fig. 4 Highly simplified mechanism of flavin-dependent ene reductases. An oxidised flavin represents the resting state of the enzymes. Upon reduction by the natural nicotinamide cofactor (NAD(P)H), a reduced, enzyme-bound flavin is formed, which transfers the hydride obtained from NAD(P)H to the conjugated C=C-double bond in a Michael-type reaction. Protonation of the resulting enolate generally occurs in a *trans*-fashion.

Table 1 Selection of NAD(P)H-independent regeneration methods for old yellow enzymes and related flavoreductases

Catalyst	Mediator	Cosubstrate	Ref.
FMN/ <i>hν</i>	FMN	EDTA	34 and 35
[Cp*Rh(bpy)(H ₂ O)] ²⁺	FMN	HCO ₂ H	36
CdSe/ <i>hν</i>	MV ²⁺	TEOA	37
TiO ₂ / <i>hν</i>	FMN	H ₂ O	38
ITO-cathode	— ^a	H ₂ O	39
[Cp*Rh(bpy)(H ₂ O)] ²⁺	FMN	Cathode	40
[Ru(bpz) ₂ (dClbpy)] ²⁺ / <i>hν</i>	MV ²⁺	TEOA	41
Rose bengal/ <i>hν</i>	Rose bengal	TEOA	42
—	—	mNADH	43–46
ATH	mNADH	HCO ₂ H	47
p-g-C ₃ N ₄ /CNT/ <i>hν</i>	—	H ₂ O	48

p-g-C₃N₄: protonated graphitic carbon nitride; CNT: carbon nanotubes; ATH: artificial transfer hydrogenase; FMN: flavin mononucleotide; MV²⁺: methyl viologen (1,1'-dimethyl-4,4'-bipyridinium); EDTA: ethylenediaminetetraacetate; TEOA: triethanolamine.^a Direct electron transfer to a surface-exposed heme group of the flavoenzyme.

reductants than NAD(P)H.³³ In recent years, a variety of alternative, NAD(P)H-independent regeneration systems for OYEs have been developed (Table 1). Photochemical approaches are particularly popular as they enable using unusual sacrificial electron donors such as amino alcohols/acids. Even water as a sacrificial electron donor is feasible. Also electrochemical regeneration appears to be attractive from commercial and environmental points of view (provided the power source is renewable-based).

The main motivation mentioned in most of the contributions shown in Table 1 is to circumvent the costly nicotinamide cofactor together with the corresponding (enzymatic) regeneration system. Yet, another advantage of such NAD(P)H-independent regeneration systems lies in their higher chemoselectivity: unless highly purified, OYEs are often 'contaminated' with alcohol dehydrogenases, which in a NAD(P)H-dependent manner reduce carbonyl groups (Fig. 5). Most direct regeneration systems for

Fig. 5 Chemoselectivity issues observed using classical NAD(P)H-driven C=C-bond reductions with non-highly purified OYEs.

Fig. 6 Turning an amino acid oxidase (AAOx) into a 'reductive aminase' by indirect electrochemical reduction of the AAOx.⁴⁹

OYEs do not regenerate NAD(P)H and/or ADHs and therefore promote the desired C=C-bond reduction only.

Another example of direct reductive regeneration of a flavoenzyme was reported by Kawabata and coworkers (Fig. 6).⁴⁹ By indirect electrochemical regeneration, these authors reversed the reactivity of an amino acid oxidase, turning it into a catalyst for enantiospecific reductive amination of α -ketoacids.

Though impressive turnover numbers for the viologen mediator (36 000), highly catalytic activities of the immobilised AAOx (> 50% of the 'natural activity') for the reverse reaction and excellent optical purities are reported, this system – unfortunately – has not found great interest yet.

Reductive regeneration of flavin-dependent monooxygenases

Flavin-dependent monooxygenases rely on reductive regeneration of the flavin cofactor for reductive activation of molecular oxygen and specific incorporation into the respective substrate (Fig. 7).^{50,51}

Hence, in principle, they are attractive targets for non-NAD(P)H-dependent regeneration. Schmid and coworkers, for example, reported use of synthetic NADH mimics to regenerate the FAD-dependent 2-hydroxy biphenyl-3-monooxygenase (Fig. 8).⁵² Though catalytic turnover was demonstrated, the system proved to be rather inefficient in use of the reducing equivalents provided by mNADH as more than 85% of the reducing equivalents were non-productively channelled into H₂O₂ generation (with NADH, this number was less than 10%).

Apparently, in the case of HbpA, the natural nicotinamide cofactor not only serves as a reductant for the catalytic FAD

Fig. 7 Simplified mechanism of flavin-dependent monooxygenases. Upon reductive activation (at the expense of NAD(P)H) the flavin cofactor/prosthetic group reacts with molecular oxygen to form a 4a-(hydro)peroxoflavin, which performs the oxyfunctionalisation.

Fig. 8 *o*-Hydroxylation of phenols using 2-hydroxy biphenyl-3-monooxygenase (HbpA) using either the natural nicotinamide cofactor (NADH) or synthetic *N*-benzyl nicotinamide (mNADH) as a reductant.

prosthetic group but is also involved in the stabilisation of the intermediate 4a-hydroperoxoflavin.^{53–55} The very simple mNADHs are not capable of interacting with the 4a-hydroperoxoflavin, leading to an increased elimination of H₂O₂ instead of O-transfer to the substrate. Possibly, a new, structurally adjusted generation of mNADHs will overcome this limitation. In the case of phenylacetone monooxygenase (PAMO), catalytic turnover using NADPH-independent regeneration (*via* photogenerated, reduced flavins) was observed only in the presence of small amounts of the oxidised cofactor (NADP⁺).^{56,57} Possibly, here as well, the enzyme-bound cofactor exhibited a stabilising effect on the intermediate 4a-hydroperoxoflavin and thus favoured productive turnover (Baeyer–Villiger oxidation) over unproductive decomposition into the enzyme resting state and H₂O₂.⁵⁸

The so-called two-component flavomonooxygenases exhibit an unusual molecular architecture comprising two distinct enzymes responsible for reductive activation of the flavin cofactor and the actual oxygenation reaction with a diffusible, reduced flavin cofactor (Table 2). Furthermore, representatives of this enzyme class catalyse a range of preparatively interesting oxidative transformations such as hydroxylation of phenols,⁵⁹ epoxidation

Table 2 Styrene monooxygenase as an example for a two-component flavomonooxygenase. The reduced FADH₂ cofactor binds to the monooxygenase subunit (StyA), where it activates O₂ and reacts with the styrene starting material to form enantiomerically pure epoxides. A: the ‘traditional’ regeneration pathway including regeneration of FADH₂ through NAD(P)H (catalysed by the reductase, StyB) and *in situ* regeneration of NAD(P)H by, e.g., formate dehydrogenase (FDH)-catalysed oxidation of formic acid. B: the direct regeneration of FADH₂

Cosubstrate	Catalyst(s)	TON ^a	Ref.
HCO ₂ H	FDH/NAD ⁺ /StyB/FAD	2867	67
HCO ₂ H	[Cp ⁺ Rh(bpy)(H ₂ O)] ²⁺ /FAD	Up to 750	68
Cathode	FAD	Up to 1600	69 and 70
mNADH	FAD	Up to 950	71

^a TON = [mol_{product} mol_{StyA}^{−1}]; mNADH: *N*-benzyl nicotinamide.

of C=C-double bonds^{60–64} and, most interestingly, selective halogenation of activated aromatics.^{65,66}

This class of enzymes appears to be predestined for non-conventional regeneration methods as these significantly simplify the rather complicated electron transfer chain. In particular, Schmid and coworkers have extensively explored the possibilities of simplified regeneration schemes using styrene monooxygenase as a model system (Table 2).

Table 2 shows that simplified regeneration of StyA (as a representative of the two-component flavin monooxygenases) is possible using various flavin reduction methods. Direct regeneration has also been demonstrated to be feasible with other representatives of two component flavin monooxygenases such as the tryptophane-7-halogenase.⁷² The productivities of the unconventional regeneration approaches come close to the productivity of the ‘natural system’ but all still fall short by one or two orders of magnitude in terms of turnover numbers envisioning economical preparative-scale transformations. Further research on more robust and efficient reaction schemes is necessary. In particular, the *Oxygen Dilemma* remains an unsolved issue in the case of two component flavin monooxygenases.⁷³ The diffusible, reduced flavin rapidly reacts with molecular oxygen (needed for the monooxygenation reaction), thereby uncoupling the regeneration reaction from the production reaction. Though this issue can to some extent be controlled by adjusting the rates of regeneration and oxygenation reactions, still significant proportions of the reducing equivalents are wasted in a futile (and potentially harmful) side reaction yielding H₂O₂. Some hope has been put

on O₂-stable reduced deazaflavins.⁷⁴ However, further inspection showed that such deazaflavins are O₂-stable only in their fully reduced form, while semiquinone intermediates (as occurring, e.g., during electrochemical or photochemical regeneration) are O₂-labile.⁷⁵ Clearly, more intense research efforts will be necessary to solve this issue.

The *Oxygen Dilemma* also represents a major challenge for P450 monooxygenase-catalysed oxyfunctionalisation reactions (*vide infra*).

Reductive regeneration of heme-enzymes

From a synthetic point-of-view, metal containing enzymes comprise an even more interesting class of enzymes as compared to the above-discussed flavoenzymes. While the latter are promising catalysts for the oxyfunctionalisation of activated starting materials, metal-dependent monooxygenases are capable of selective oxyfunctionalisation of non-activated C–H bonds. A variety of metal-containing mono- and dioxygenases are known,⁷⁶ but today by far the most widely used class of enzymes are the so-called P450 monooxygenases. Therefore, we will largely focus on these enzymes.

The higher versatility mentioned above, however, also comes at the expense of rather complicated electron supply chains. The catalytic mechanism of P450 monooxygenases involves two sequential single electron transfer steps (Fig. 9).

This is not compatible with NAD(P)H, being an obligate hydride donor. As a consequence, P450 monooxygenases naturally depend on relay systems transforming the NAD(P)H-dependent hydride transfer reaction into two single electron transfer steps mediated by small redox proteins containing FeS-clusters or flavins (Fig. 10). A reductase catalyses the transformation from hydride transfer into two succinct single electron transfer steps.

Fig. 9 Simplified P450 mechanism. Fe^{III} represents the resting state, which in the first step is reduced to Fe^{II}. After O₂-binding, a second single electron reduction and elimination of water, so-called compound I (the oxygenating species) is formed. After O-atom transfer to the enzyme-bound substrate, the enzyme returns into the resting state and the product is released.

Fig. 10 General scheme of the electron transport chain supplying P450 monooxygenases (P450 MO) with the reducing equivalents. NADH serves as a general reductant (and is regenerated itself e.g. *via* formate dehydrogenase (FDH)-catalysed oxidation of formic acid). As the heme-Fe^{III} cannot accept a hydride equivalent from NAD(P)H directly, a relay system (reductase, mediator) is needed to transform the hydride donation.

Generally, these components are individual proteins; however, in some cases such as the P450 monooxygenase from *Bacillus megaterium* (P450BM3) they can be included in a single polypeptide chain.

Electrochemical processes are single electron transfer reactions by nature as well. Hence, electrochemical methods are predestined to take up the electron transport chain at the level of the mediator (Fig. 10) and thus result in drastically simplified reaction schemes. Various research groups have demonstrated catalytic turnover of P450 monooxygenases either *via* direct electrochemical communication between the heme-group and an cathode^{77,78} or using mediated electron transfer.^{79–83}

Vilker and coworkers have pioneered the cathodic regeneration of P450 monooxygenases (Fig. 11).^{82,83} The authors, however, also demonstrated one of the major challenges of this approach: the high reactivity of molecular oxygen. O₂ is an inevitable part of the P450 catalytic cycle. At the same time it also reacts quickly with the reduced components of the electron transport chain and is easily reduced cathodically.⁷³ This *Oxygen Dilemma* not only lowers the current efficiencies of electroenzymatic processes but also yields reactive oxygen species that have to be dealt with in order to ensure enzyme stability.

One possible solution to this challenge is to control the O₂ level in the reaction mixture e.g. *via in situ* generation *via* anodic water oxidation as demonstrated by Vilker and coworkers (Fig. 11).^{82,83}

Fig. 11 Indirect electrochemical regeneration of P450cam with *in situ* generation of molecular oxygen.^{82,83}

Fig. 12 A Ru-photocatalyst covalently attached to a P450 monooxygenase for direct electron transfer from the photoreduced Ru^{II} -centre to the heme-active site of the P450 monooxygenase.

A similar approach (with a flavin-dependent monooxygenase) by Schmid and coworkers also proved to be a doable solution.⁷⁰

Also increasing the electron transfer rate between the mediator molecule and the P450 heme-site represents a very promising approach to address the *Oxygen Dilemma*.^{84–92}

Mechanistically, photochemical reduction is very similar to electrochemical regeneration of P450 monooxygenases. Hence it is not very astonishing that also (in)direct photochemical regeneration of P450 monooxygenases has been investigated. Natural and synthetic flavins, for example, enable catalytic turnover of P450BM3 in ω -1 hydroxylation of fatty acids.^{74,93}

Cheruzel and coworkers intensively investigated the photochemical regeneration of P450BM3 using covalently attached $\text{Ru}(\text{bpy})_3$ photosensitisers (Fig. 12).^{94–100} Protein engineering proved to be efficient in optimising the electron transfer from the attached Ru^{II} centres to the catalytic heme and thus yielded respectable turnover numbers of 1000 for the monooxygenase.

Park and coworkers demonstrated the direct photochemical regeneration of a P450 monooxygenase.¹⁰¹ Eosin Y as a photocatalyst was included in host cells together with the sacrificial electron donor (TEOA). Though product titers are comparably low, this study represents nice proof-of-concept. Further contributions of this group, particularly towards the photocatalytic regeneration of nicotinamide cofactors, are discussed elsewhere.¹⁰²

Overall, mimicking the natural P450 monooxygenase pathway, *i.e.* reductively regenerating the oxygenase subunit for reductive activation of molecular oxygen, has so far been rather limited by comparably low turnover numbers of the enzymes. Generally, TONs reported for fully enzymatic systems are in the range of several ten thousands. TONs of artificial regeneration systems range approximately one to two orders of magnitude lower. We believe that the *Oxygen Dilemma* represents the major obstacle here by deviating reducing equivalents provided by single electron mediators primarily to direct O_2 reduction.

Oxidative, O_2 -independent regeneration of monooxygenases may represent a solution to this challenge. Indeed, oxidative regeneration of heme monooxygenases has been reported (Fig. 13).^{88,103,104} In principle, oxidative regeneration of P450 monooxygenases would not only avoid uncoupling challenges observed in the presence of O_2 . Even more, it would also circumvent O_2 -availability issues originating from the poor aqueous solubility of O_2 . Unfortunately, so far, this very promising approach, however, has not been further developed. Possibly, stability issues of the intermediate $\text{Fe}^{\text{IV}} = \text{OH}$ species limit the feasibility.

Fig. 13 Highly simplified reaction scheme for oxidative regeneration of P450 monooxygenases. The scheme was adopted from Udit *et al.*⁸⁸

Another, even simpler approach for O_2 -independent P450 reactions is to make use of the H_2O_2 shunt pathway.^{76,105} The $\text{Fe}^{\text{III}}\text{-OO}(\text{H})$ intermediate species of the P450 mechanism (Fig. 9) can also be obtained from reaction of the enzymes' resting state with (already reduced) H_2O_2 . Unfortunately, however, the majority of P450 monooxygenases exhibit extremely low robustness towards trace amounts of H_2O_2 and therefore is not applicable to this seemingly very simple regeneration method. Arnold and coworkers addressed this issue *via* protein engineering with moderate success.¹⁰⁵ In addition, some naturally occurring P450 monooxygenases also exhibit some peroxxygenase activity.^{106–113} Peroxygenases are heme-enzymes naturally utilising the H_2O_2 shunt pathway to form the catalytically active compound I (*vide infra*). Cong and coworkers hypothesised that P450 monooxygenases lack a catalytic base in the enzyme active site to mediate the deprotonation of the initial H_2O_2 adduct to the Fe^{III} resting state (Fig. 14).¹¹⁴ They suggested using decoy molecules containing a terminal imidazole moiety to (partially) compensate for this lacking base.

Very impressive increases in the TNs of P450BM3 were achieved in the presence of such artificial catalytic bases.¹¹⁴ Further developments of this very interesting approach may eventually lead to truly efficient P450 peroxxygenase reactions.

Peroxygenases themselves are increasingly receiving interest as catalysts for preparative oxyfunctionalisation reactions.^{115–117} While principally being capable of the same reactions as the above-discussed P450 monooxygenases, they do not rely on complicated electron transport chains and hence are much easier to apply preparatively. This ease of application is also

Fig. 14 Simplified scheme of the peroxxygenase mechanism.

Table 3 Selection of some peroxxygenase reactions driven by *in situ* H₂O₂ generation

Product	Cat	Cosubstrate	Peroxygenase	TON	Ref.
1	FMN/ <i>hν</i>	EDTA	<i>AaeUPO</i>	11 500	118
	AOx/FDM/FDH/3HB6H/NAD	MeOH	<i>AaeUPO</i>	295 000	119
	Au-TiO ₂	MeOH	<i>AaeUPO</i>	80 000	120
	Au-TiO ₂	H ₂ O	<i>AaeUPO</i>	30 000	121
	Cathode	—	<i>AaeUPO</i>	124 000	122
2	FMN	mNADH	P450 _{BSβ}	200	106
	FMN/ <i>hν</i>	EDTA	P450 _{BSβ}	200	107
3	FMN/ <i>hν</i>	EDTA	OleT	10	113
4	FMN/ <i>hν</i>	EDTA	<i>CfCPO</i>	22 000	123 and 124
	Cathode	—	<i>CfCPO</i>	250 000	125 and 126
	Pd(0)	H ₂	<i>CfCPO</i>	n.d.	127
5	Cathode	—	<i>CfCPO</i>	11 00 000	128–130

reflected by the generally much higher turnover numbers reported for peroxxygenases as compared to P450 monooxygenases. Table 3 summarises some recently reported preparative peroxxygenase examples. Efficient *in situ* generation of H₂O₂ is critical to achieve high TNs with peroxxygenases. On the one hand, H₂O₂ has to be supplied in sufficient amounts to maintain the catalytic cycle. On the other hand, excess H₂O₂ needs to be avoided to prevent oxidative inactivation of the catalytic heme group.

Table 3 impressively demonstrates that peroxxygenases (when combined with an efficient *in situ* H₂O₂ generation system) are capable of highly efficient oxyfunctionalisation reactions.

Future developments revealing new peroxxygenases (either from natural or from man-made diversity) with new reactivities and products together with even more improved H₂O₂ generation systems will certainly start a new era in biocatalytic oxyfunctionalisation chemistry!

Finally, it is also worth mentioning that also flavomonooxygenases should be accessible for the peroxide shunt pathway. The natural flavin, however, is not sufficiently electrophilic to react with H₂O₂ to form the catalytically active 4a-hydroperoxoflavin. Therefore, Fraaije and coworkers utilised an N⁵-alkylated flavin derivative bound to a riboflavin binding protein to constitute a chiral 'active site' for H₂O₂-driven sulfoxidation reactions (Fig. 15).¹³¹

Even though the turnover numbers and enantioselectivity obtained were rather modest (in the non-optimised system), this represents a very interesting proof-of-concept study potentially

paving the way to H₂O₂-driven, flavomonooxygenase-catalysed oxyfunctionalisation reactions!

Conclusions

Oxidoreductases are promising tools for organic chemists to perform selective oxidation, oxyfunctionalisation and reduction reactions.

Naturally, these enzymes need a constant supply with redox equivalents, which is mostly accomplished more or less directly *via* the natural nicotinamide cofactor. In many oxidoreductases (*e.g.* alcohol dehydrogenases) the nicotinamide cofactor is directly involved in the catalytic mechanism and therefore can hardly be replaced by simpler (cheaper) synthetic electron-donors or -acceptors. Other enzymes, for example, many flavin-dependent reductases and -monooxygenases, however, utilise NAD(P)H merely as a reductant. In these cases, very efficient, artificial electron transport chains could be established, enabling higher reaction rates and higher selectivity as compared to the 'natural system'.

Also P450 monooxygenases in principle can be regenerated in an NAD(P)H-independent fashion. Here, however, the *Oxygen Dilemma* poses a significant challenge *en route* to efficient systems. A satisfactory solution is urgently needed (also for natural systems).

Finally, the hydrogen peroxide shunt pathway is enjoying renewed popularity, especially thanks to new and efficient peroxxygenases.

Conflicts of interest

There are no conflicts to declare.

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Fig. 15 Towards a hydrogen peroxide shunt pathway for flavoenzymes.

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