Selective oxyfunctionalisation reactions catalysed by P450 monooxygenases and peroxygenases – A bright future for sustainable chemical synthesis

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Selective oxyfunctionalisation reactions catalysed by P450 monooxygenases and peroxygenases – A bright future for sustainable chemical synthesis

X. Xu, T. Hilberath and F. Hollmann

Abstract

Heme-dependent oxygenases (i.e. P450 monooxygenases and peroxygenases) are highly selective catalysts for the selective oxyfunctionalisation or organic compounds. Both enzyme classes exhibit mechanistic similarities (i.e. using so-called compound I (CpdI) as active oxidation species) and differences in how CpdI is formed. From the differences also practical differences arise which may influence the scalability, economic attractiveness and environmental impact of P450 monooxygenase- or peroxygenase-catalysed reactions.
In this contribution we propose a range of performance indicators to compare the potential of both enzyme classes.

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Keywords

Biocatalysis, Oxyfunctionalisation reactions, Peroxygenases, P450 monooxygenases.

Introduction

Oxyfunctionalisations comprise chemical transformations inserting oxygen atoms into (non-activated) C—H-, C—C-, C=C-bonds or onto heteroatoms. The general inertness of these bonds requires potent oxygen-transfer reagents such as high-valent metal-oxo complexes or organic peroxides. These reagents, however, are challenged by low selectivity resulting in complex product mixtures and isolation of the desired product can be tedious and time- and resource-intensive. Interestingly, many of the aforementioned oxyfunctionalising agents are 'biomimetic' i.e. they are inspired by natural catalysts such as iron- or flavin-dependent monooxygenases [1,2]. The fundamental difference between 'chemical' and enzymatic oxyfunctionalisation catalysts lies with the environment around the oxyfunctionalising catalyst. In the case of chemical catalysts this space is populated by simple ligands and solvent whereas enzymes provide a well-defined cavity (active site) that not only controls the orientation of the starting material towards the catalyst but also participates in the catalytic mechanism. Hence, it is not surprising that monooxygenases usually excel over their 'chemical' counterparts in terms of catalytic performance and selectivity.

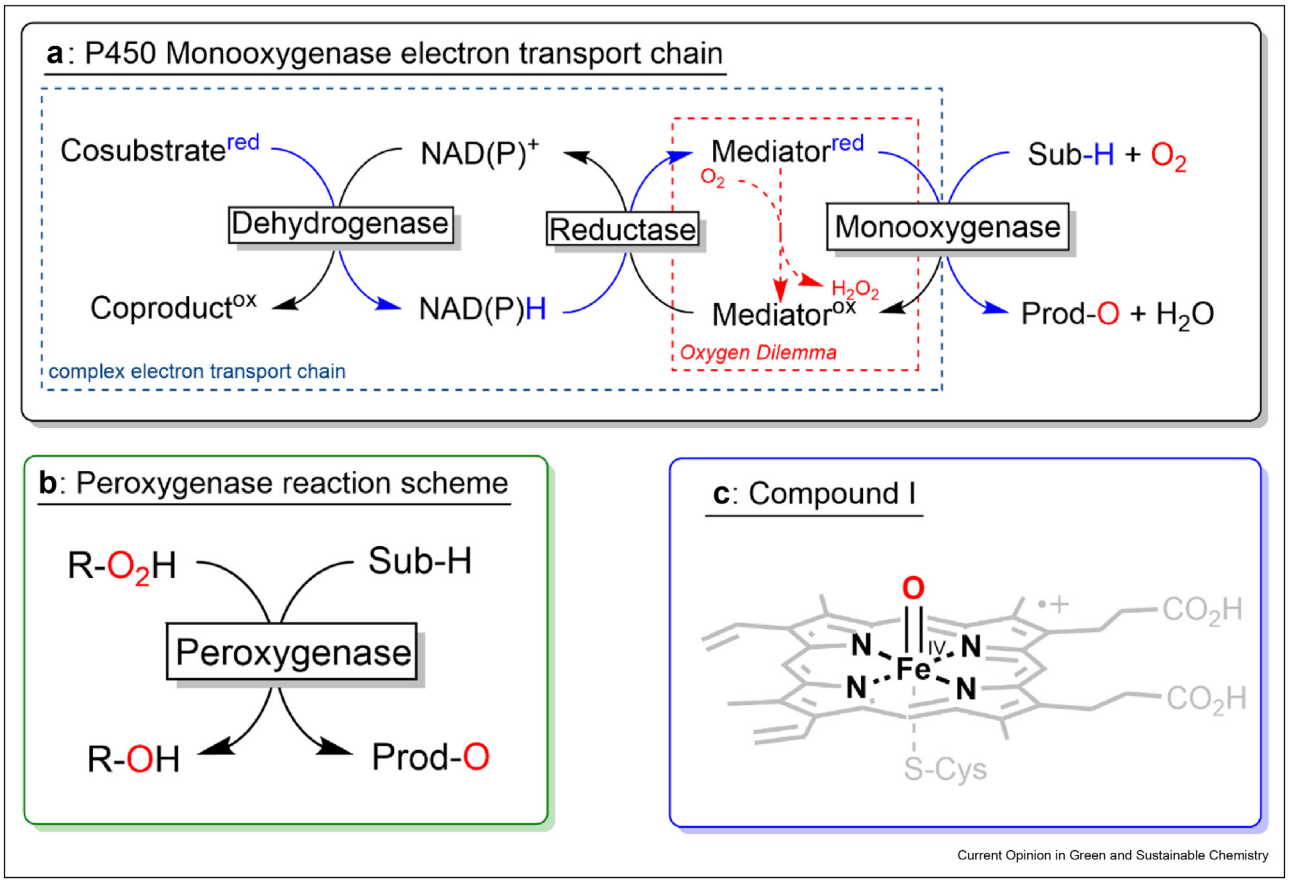
Especially cytochrome P450 monooxygenases have been the centre of attention for several decades now [3] but are increasingly challenged by so-called unspecific peroxygenases (UPOs) [4,5]. In this contribution we aim at providing a comparison between both enzyme classes with respect to their practical applicability based on recent scientific literature comparing their advantages and disadvantages.

Mechanistic similarities and differences between P450 MOs and UPOs

Both enzyme classes make use of compound I (CpdI, Scheme 1C) as active reagent to insert an oxygen atom into their substrates. The mechanism, via which CpdI is formed, however, differs significantly between both enzyme classes. P450 monooxygenases utilise molecular oxygen as source of O and activate it reductively (Scheme 1A). The reducing equivalents required for this are obtained from NAD(P)H via more or less complex electron transport chains [6,7]. The nicotinamide cofactor itself (for economic reasons) is applied in catalytic amounts and regenerated *in situ* by the enzymatic oxidation of a stoichiometric co-substrate. It should also be mentioned that the reduced species of the electron transport chain spontaneously react with O_2 (needed for the catalytic reaction) and thereby partially uncouple the electron supply from the monooxygenase reaction (*Oxygen Dilemma*) [7]. As a consequence, mostly super-stoichiometric amounts of the sacrificial electron donor ($Cosubstrate^{red}$) are necessary.

Peroxygenases are much simpler as they directly utilise reduced oxygen in the form of hydrogen peroxide or organic hydroperoxides (Scheme 1B).

Scheme 1



Comparison of substrate oxidation via Compound I (c) formation in cytochrome P450 monooxygenases (a) and peroxygenases (b).

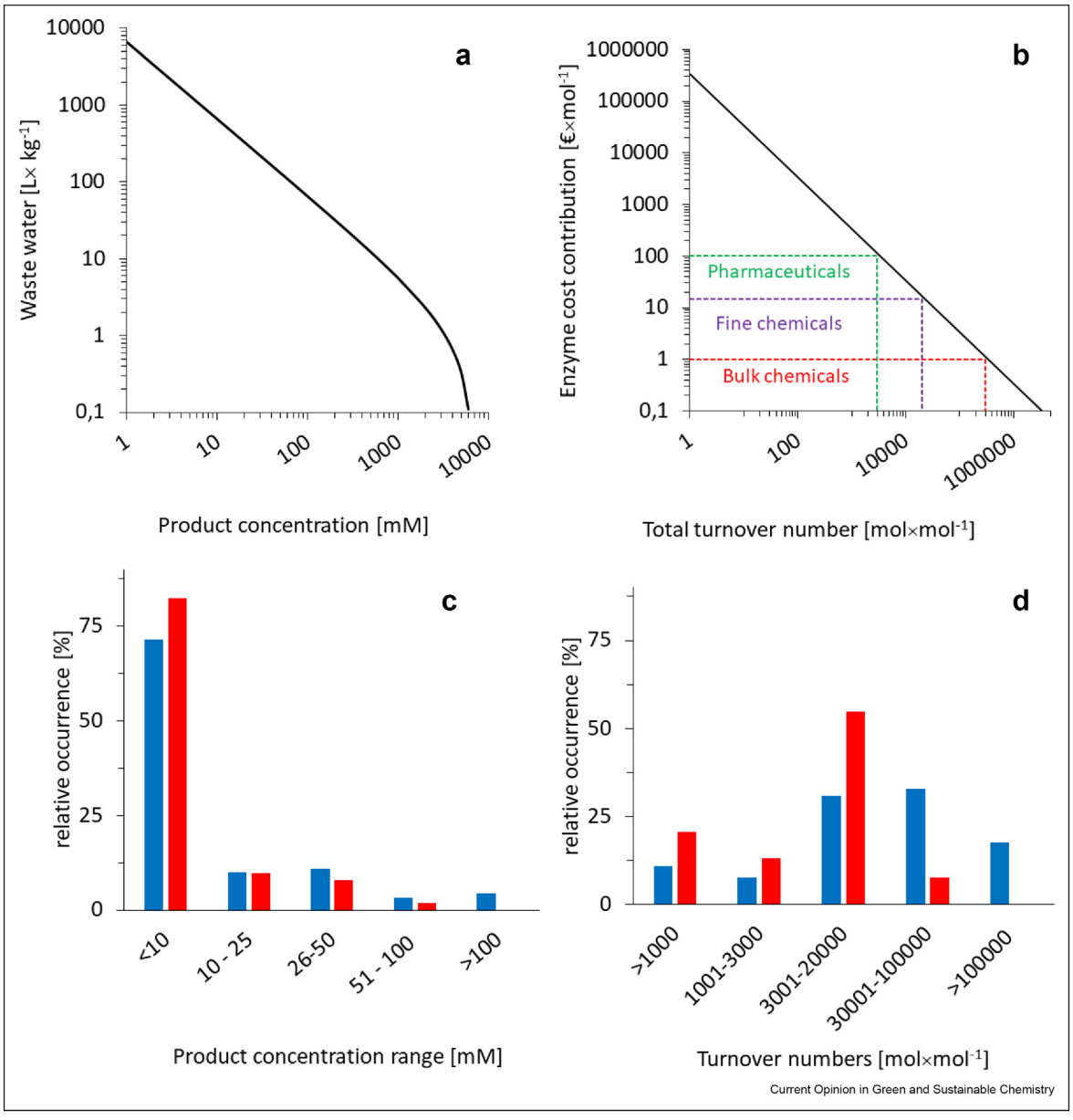
A comparison based on performance indicators

Evaluating the environmental impact of a given (catalytic) reaction is a complex task for which many factors have to be taken into account. Nevertheless, we believe that already some simple indicators such as catalyst total turnover number (TTN) and achievable product concentration already give a reasonable indication about the economic and environmental attractiveness of a given catalytic reaction.

The importance of product concentration is rather obvious: highly diluted reaction mixtures not only use the production infrastructure insufficiently but also result in high amounts of solvent wastes and necessitate additional (time-, resource- and energy-intensive) downstream processing operations to obtain the product of interest (Figure 1a). Also, it should be taken into account that energy required for heating/cooling, mixing etc. is mostly spent on the solvent. Consequently, maximisation of the product concentration is mandatory from both an economic and environmental point-of-view. As shown in Figure 1C, the majority of recent P450- and peroxygenase-publications deal with highly diluted reaction mixtures with product concentrations beneath 10 mM corresponding to the solvent (i.e. aqueous buffer) representing more than 99% of the reaction mixture. However, in the case of P450 monooxygenases, the highest product concentrations reported so far lie around 50–60 mM [8,9]. The situation is more promising in the case of peroxygenases with more and more examples of non-aqueous applications reported, culminating in 360 mM as the, so far, highest product concentration. Particularly attractive about peroxygenases is that they can be applied under non-aqueous reaction conditions thereby circumventing the low solubility issue of many reagents of interest in aqueous media [10–14]. Nevertheless, in both cases significant improvements in final product titres are mandatory. Fortunately, the tools (such as multi-phase reactions and other non-conventional reaction media) principally exist [15,16] and are waiting to be implemented more in biocatalytic oxyfunctionalisation chemistry.

High catalyst turnover numbers (i.e. number of catalytic cycles) are desirable from an economic point-of-view to

Figure 1



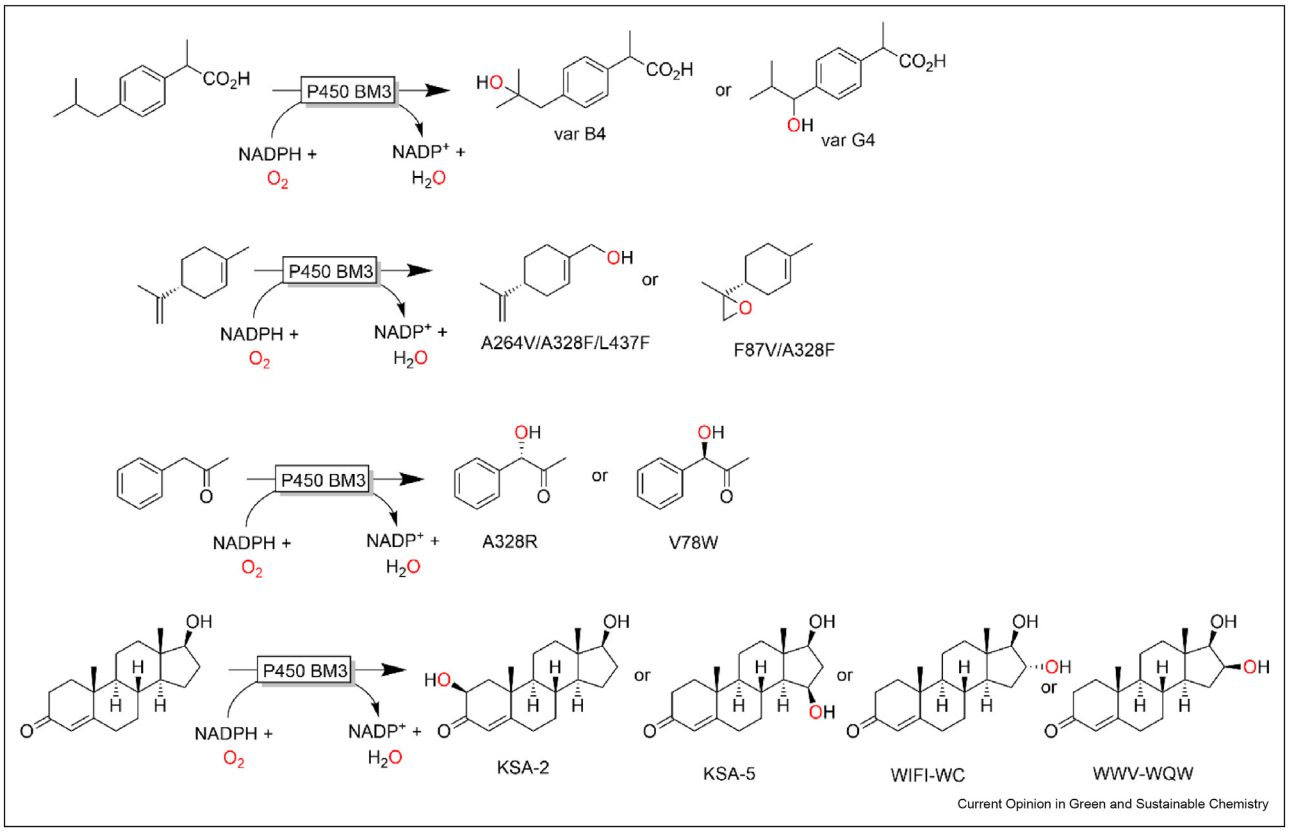
Performance indicators for biocatalytic oxyfunctionalisation reactions. a: Solvent (water) wastes generated with varying product concentrations; b: Influence of biocatalyst TN on its contribution to the final product (assumptions made: M_W(Enzyme) = 50 kDa, Enzyme price: 1000 € kg^{-1} [17], M_W(Product) = 150 g mol^{-1}); c&d: results of a literature analysis covering 91 peroxygenase reactions and 53 P450 monooxygenase reactions.

minimise the cost contribution and environmental impact of the catalyst to the final product (Figure 1B). According to Woodley et al. [17] a minimal turnover number for an enzyme (produced on large scale) of roughly 3.000, 20.000 and 1.000.000 can be estimated to achieve acceptable cost contributions for pharmaceuticals, fine chemicals and bulk chemicals, respectively (Figure 1b). Furthermore, it should be kept in mind that the catalyst preparation causes environmental impact by consuming resources and energy [18,19]. As shown in Figure 1d the majority of both, P450 monooxygenase- and peroxygenase TNs fall more into the range of pharma- and fine-chemicals. Again, however, only with peroxygenases higher TNs approaching those required for the synthesis of bulk chemicals have been reported yet.

Substrate scope/engineering and recombinant expression

Today, a variety of different P450s and variants thereof are known and available for organic chemists [20,21]. Currently, online databases include more than 300,000 P450 sequences from all parts of the tree of life

Scheme 2



Selected examples of tailored P450 (BM3) selectivity achieved via enzyme engineering [24]. hydroxylation of a) ibuprofene [25], b) limonene [26,27], c) phenylacetone [28], d) testosterone [29,30].

underlining the enormous diversity of P450 biocatalysts [22]. Furthermore, enzyme engineering has proven an efficient tool to tailor P450 monooxygenases to the substrate scope-, selectivity- or stability needs of organic chemists [20,23]. Scheme 2 gives some examples of engineered P450 BM3 to selectively hydroxylate complex starting materials at user-defined positions thereby overcoming its natural selectivity [24].

With peroxygenases we currently cannot access such a wealth of diversity. Though principally thousands of putative peroxygenase genes have been identified [31] only a handful of them have so far been functionally expressed and initially characterised [4]. Future characterisation studies will reveal whether peroxygenases are competitive in covering the broad substrate spectrum and diverse product selectivities of P450 enzymes.

Currently, the chemical space of selective oxyfunctionalisation accessible via P450 monooxygenases is enormous and approaches truly rational design. Compared to this, peroxygenase-catalysis still largely relies on wild-type enzymes, limiting their synthetic applicability.

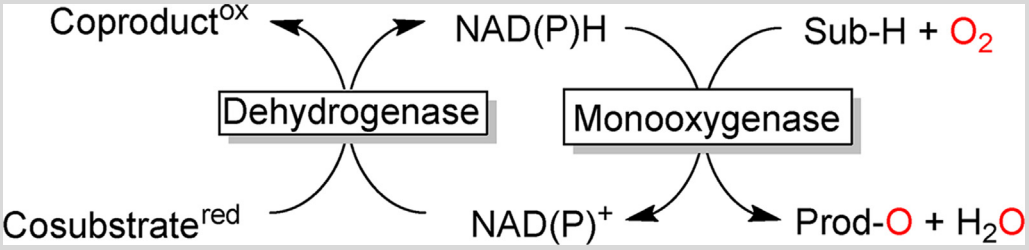
Recombinant expression systems are crucial for both, cost-efficient production of biocatalysts and their engineering. Especially prokaryotic P450 monooxygenases can nowadays be expressed in simple bacterial expression systems such as *Escherichia coli* putting the basis for mutant libraries and large-scale production [3,32]. Comparable, robust and simple expression systems for peroxygenases are still lacking today which complicates functional expression of larger libraries [4,33,34]. The enzyme titres achievable especially for eukaryotic peroxygenases are in the range of 0.5–7.5 μmol L^{-1} (corresponding to approx. 25–375 mg L^{-1}) [33–35], which, compared to typical enzyme yield achievable with recombinant *E. coli* systems (often significantly above 5–10 g L^{-1}) [36] leaves room for improvement. Nevertheless, *E. coli*-based expression systems appear more favourable for enzyme production than *Pichia* simply for the dramatically reduced fermentation times (2–3 day compared to ca. 2 weeks).

Cosubstrates/coproducts

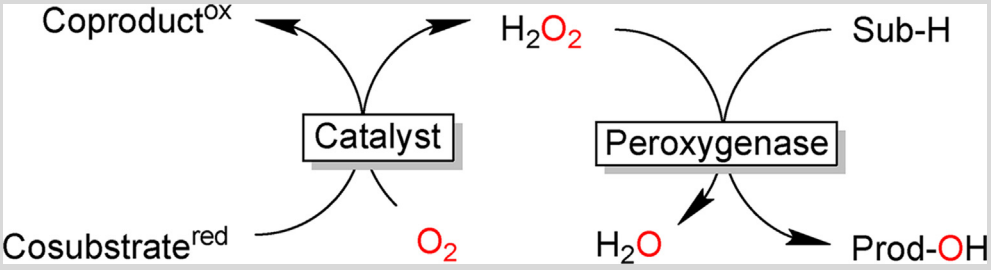
As redox reactions, oxyfunctionalisation reactions require stoichiometric cosubstrates. P450 monooxygenases require molecular oxygen and reducing equivalents for their catalytic mechanism whereas

Table 1

Examples for P450 monooxygenase- and peroxygenase-in situ regeneration systems.



Cosubstrate/coproduct	Dehydrogenase	Waste [g mol$^{-1}_{NAD(P)H}$]	Refs
Glucose/Gluconolactone	GDH[a]	176	[39–42]
Formic acid/CO_2	FDH[a]	44	[43,44]
Isopropanol/Acetone	ADH[a]	58	[45]
H_2/–	Hase[a]	–	[46,47]



Cosubstrate/coproduct	Catalyst/energy source	Waste [g mol$^{-1}_{H2O2}$]	Ref
MeOH/CO_2	AlcOx, FDM, FDH, NAD, FMN/hν[a] FOx[a]	14.7	[48,49]
H_2O/O_2	TiO_2/hν		[50]
	–/plasma		[51]
	–/^{60}Co or ^{238}U		[52]
	Bi_2Te_3/heat		[53]
	BiOCl/ultrasound		[54]
e^-/'none'	Cathode	'none'[b]	[55–58]
H_2/–	Hase[a]	–	[59]

[a] GDH: glucose dehydrogenase, FDH: formate dehydrogenase, ADH: alcohol dehydrogenase, Hase: hydrogenase, AlcOx: alcohol oxidase, FDM: formaldehyde dismutase, NAD: nicotinamide adenine dinucleotide; FMN: flavin mononucleotide.

[b] depending on the CO_2 footprint of the electricity used.

peroxygenases rely on hydrogen peroxide or organic hydroperoxides (Scheme 1). Envisioning larger scale applications of either enzyme class, the cosubstrate/coproduct selection has a significant impact on the economics and environmental footprint of the process.

In the case of P450 monooxygenases, glucose is a popular sacrificial electron donor because the corresponding regeneration enzyme is very active, easy to express and recycles both NAD and NADP-cofactors [37]. One issue however is the low atom efficiency of glucose as sacrificial electron donor: only one out of the 6 available carbon atoms is actually oxidised, resulting in large amounts of waste (Table 1). Moreover, the medium acidification caused by the hydrolysed by-product (gluconic acid) makes external pH control necessary. Furthermore, the viscosity of concentrated aqueous glucose solutions may pose practical difficulties for stirring and pumping such solutions. Therefore, the large-scale applicability of glucose dehydrogenase is questionable. A few other relevant cosubstrates are listed in Table 1. Overall, a broad range of practical *in situ* NAD(P)H regeneration systems are available today. They enable lab-scale applications of P450 monooxygenases and industrial applications for the synthesis of fine chemicals and active pharmaceutical intermediates but show little potential for larger scale implementation for cost- and waste reasons.

To promote peroxygenase reactions, stoichiometric supply with H_2O_2 or organic hydroperoxides is needed. As H_2O_2 also inactivates the enzymes either controlled dosage or *in situ* generation of H_2O_2 via O_2 reduction is generally applied (Table 1) [38].

As mentioned above, large-scale applications of monooxygenases or peroxygenases have to rely on readily available, easy to handle, cost-effective and low waste-

Table 2

Qualitative comparison of P450 monooxygenases and peroxygenases from a synthetic application perspective.

	P450 monooxygenases	Peroxygenases
Ease of application	+ a broad range of (whole cell) reaction systems are available – cell-free reactions are complex	+ self-sufficient catalysts
Substrate scope/selectivity	+ very broad substrate and reaction scope	– largely limited to the selectivity of wt-enzymes
Cosubstrate/coproduct	– stoichiometric cosubstrates are needed generating additional wastes – the *Oxygen Dilemma* still needs to be solved	+ *in situ* H_2O_2 generation systems are at hand to minimise oxidative inactivation
Availability	+ huge variety of engineered and wild type enzymes	– currently rather limited
Engineering	+ well-established enzyme engineering to tailor substrate scope	+ new enzymes (from natural or man-made diversity) are constantly being added

generating energy sources to promote the biocatalytic oxyfunctionalisation reaction. Electrochemical regeneration using emission-free electrical power would represent an environmentally attractive solution to the 'regeneration issue'. First trials were reported in the late 1990s by Vilkner and coworkers [60]. Unfortunately, ever since then there has not been fundamental improvements of the productivity and robustness of direct or indirect electrochemical regeneration of P450 monooxygenases. Possibly the direct cathodic reduction of O_2 poses a too large technical hurdle to be solved at more than a proof-of-concept level. In contrast, this direct cathodic O_2 reduction to H_2O_2 offers, in the case of peroxygenases, a promising approach for scalable and robust reaction schemes [55,58].

Hydrogen represents another promising, waste-free cosubstrate. With the advent of O_2-tolerant hydrogenases [61] now also H_2-driven P450 monooxygenase- [46,47] and peroxygenase-driven [59] oxyfunctionalisation reactions are coming into reach. This approach, however, is still in its infancy and necessitates further development to be able to judge its practicability.

Finally, water-derived reducing equivalents would be an elegant method to drive P450 monooxygenases and peroxygenases. The thermodynamic and kinetic inertness of water oxidation, however, necessitates external energy sources and catalysts. In the case of monooxygenases, natural photosynthesis appears to be the most promising route *en route* to a water-driven oxyfunctionalisation chemistry [62–65]. The energy repertoire for *in situ* H_2O_2 generation from water and O_2 to drive peroxygenases is somewhat broader ranging from visible light [50,66,67] via γ-radiation (e.g. from nuclear waste) [52], cold plasma [51] to waste-heat [53]. Also wastes such as microplastics or lignin can serve as alternative electron donors [68]. Again, the early stage of development of the aforementioned approaches makes it difficult to predict if they will ultimately be applicable on a large-scale.

Conclusions and outlook

P450 monooxygenases and peroxygenases bear an enormous potential for selective chemical oxyfunctionalisation chemistry. Both enzyme classes exhibit specific advantages and disadvantages over the other (Table 2).

Practical application of peroxygenases is much simpler than of P450 monooxygenases as complex and vulnerable regeneration systems can be avoided simply by adding H_2O_2 (or organic hydroperoxides) or utilising one of the various *in situ* H_2O_2 generation systems. As a consequence, turnover numbers reachable with peroxygenase-catalysts appear to exceed those of P450 monooxygenases, which gives the former an advantage in terms of cost-contribution of the enzyme catalyst to the final product. Also the application of peroxygenases in non-aqueous reaction systems appears more straight-forward (to date), which is important to reduce solvent wastes and increase the productivity of the reactions [10,12,13]. This approach, however, is still in its infancy. Particularly the low activity of the immobilised peroxygenases calls for further improvements [69].

P450 monooxygenases, on the other hand, excel in terms of recombinant expression and ease of setting-up larger mutant libraries. As a consequence, many P450 monooxygenase mutants with tailored selectivity are available whereas this number is much smaller in case of peroxygenases.

Overall, right now, it is not possible to define a 'clearly more promising' enzyme class. Future will tell if either challenge (applicability issues of P450 monooxygenases or the 'engineering gap' of peroxygenases) will be solved faster increasing the attractiveness of either enzyme class.

Disclosure statement

Given their role as Guest Editor, Frank Hollmann had no involvement in the peer review of the article and has no access to information regarding its peer-review. Full

responsibility for the editorial process of this article was delegated to John Woodley.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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