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Surfing the wave of oxyfunctionalization chemistry by engineering fungal unspecific peroxygenases

Alejandro Beltrán-Nogal¹, Israel Sánchez-Moreno¹, Daniel Méndez-Sánchez¹, Patricia Gómez de Santos², Frank Hollmann³ and Miguel Alcalde¹

Abstract

The selective insertion of oxygen into non-activated organic molecules has to date been considered of utmost importance to synthesize existing and next generation industrial chemicals or pharmaceuticals. In this respect, the minimal requirements and high activity of fungal unspecific peroxygenases (UPOs) situate them as the jewel in the crown of C–H oxyfunctionalization biocatalysts. Although their limited availability and development has hindered their incorporation into industry, the conjunction of directed evolution and computational design is approaching UPOs to practical applications. In this review, we will address the most recent advances in UPO engineering, both of the long and short UPO families, while discussing the future prospects in this fast-moving field of research.

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Introduction

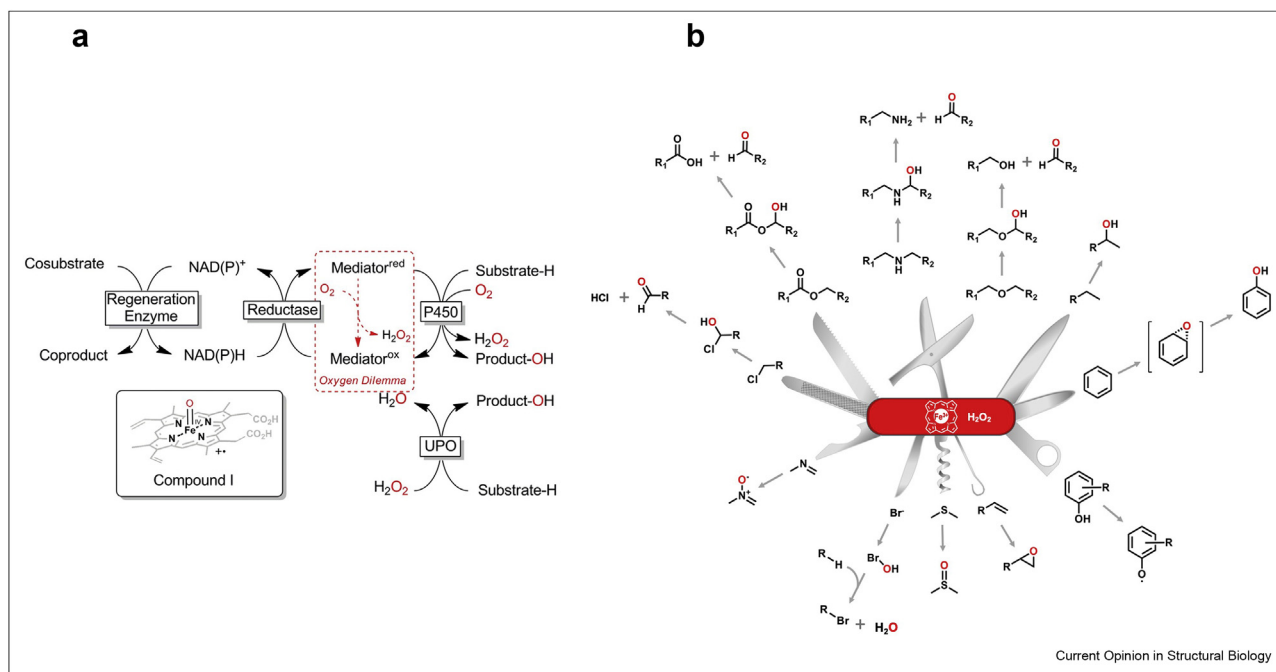
The majority of industrialized countries are seeking new ways to produce the chemicals on which our society has based its existence, ways that consume less resources, generate less waste and that are economically feasible. One particular chemical transformation that appears to be of paramount interest is the selective oxyfunctionalization of non-activated C–H bonds, which is still a mere pipedream in industrial biocatalysis. Indeed,

combining high reactivity with high selectivity remains the main stumbling block for chemical catalysis, although enzymes seem to be ‘naturally sculpted’ to manage this balancing act.

For several decades now, cytochrome P450 mono-oxygenases (in short P450s) have been the main focus of attempts to discover scalable reaction schemes, particularly given that oxyfunctionalization chemistry would benefit enormously from milder and more selective approaches [1,2]. Despite the Nobel prize winning developments of Frances H. Arnold and other pioneers in the field of protein engineering [3], P450s are still inefficient and not sufficiently robust to employ in large-scale production. The failure to take advantage of these enzymes is largely due to their catalytic mechanism involving reductive activation of molecular oxygen and the resulting need for complex and vulnerable electron transport chains in which a significant portion of reducing equivalents provided by NAD(P)H deviate in unproductive oxidative uncoupling and generation of reactive oxygen species [4], [Figure 1a](#). In recent years, a new superfamily of heme-thiolate mono(per)oxygenses has emerged, the fungal unspecific peroxygenase group (UPOs, EC 1.11.2.1), that is made up of stable, extracellular enzymes that do not suffer the drawbacks of P450s, while they do behave as self-sufficient biocatalysts in the most complex oxyfunctionalization reactions, [Figure 1b](#) [5–8].

Also due to the highly simplified regeneration scheme, UPO reactions, compared to P450 pendants, are much easier to control and optimize. As a result, while the catalytic performance of P450s still tends to range between several thousand and ten thousand catalytic turnovers, UPOs easily reach hundred thousand catalytic turnovers and probably more, suggesting they may soon offer better economic viability and even application for bulk chemical synthesis coming into reach [9–11]. Indeed, UPOs exhibit a remarkable industrial potential considering the steady increment of publications and patents relating UPOs and uses thereof over the last three years compared with the five previous years (from 10 to 24 publications and patents on average per year),

Figure 1



UPO catalytic advantages and portfolio of reactions. (a) Comparison of the molecular regeneration of P450 monooxygenases (upper) and UPOs (lower, right). Both enzyme classes utilize the same Compound I for oxygen-transfer reactions (lower, left), yet a much simpler mechanism of generation is used by peroxygenases. Furthermore, the reduced mediators in the mechanism used by P450 also directly react with O₂, thereby uncoupling the regeneration reaction from the oxyfunctionalization reaction and thus, limiting the efficiency of the overall reaction (Oxygen Dilemma). (b) The *swiss army knife*: As a result of the studies carried out since its discovery in 2004, and with nearly 400 substrates tested, UPO is now considered beyond doubt a *swiss-army knife* in oxyfunctionalization chemistry. Triggered simply by H₂O₂, which serves as the final electron acceptor and oxygen source, the promiscuity of UPOs is reflected by the huge portfolio of transformations they can perform, including aromatic, alkylic, (cyclo)aliphatic and heterocyclic hydroxylations, aromatic and aliphatic olefin epoxidations, ether cleavages (O-dealkylations), N-dealkylations, sulfoxidations, N-oxidations, deacylations (C-X bond cleavages) and halide oxidations/halogenations [5,9].

with a plethora of substrates and in reactions of commercial relevance. In this regard, enzyme engineering by directed evolution (*i.e.* iterative rounds of random mutation, DNA recombination and artificial selection) has proved to be a powerful strategy to obtain more efficient UPOs, adjusting exogenous genes to the subtleties of expression in a heterologous host, while opening up their full biocatalytic potential [8]. Most UPOs described to date may need to be engineered to meet the standards of a robust industrial biocatalyst, including high levels of heterologous functional expression, high stability under operational conditions -in terms of pH, temperature and the presence of non-aqueous media-, as well as the modification of chemo-, regio- and enantioselectivities. Besides, the unwanted peroxidative activity, the oxidative inactivation by H₂O₂ and the product overoxidation, are intrinsic UPOs' problems that deserve further research.

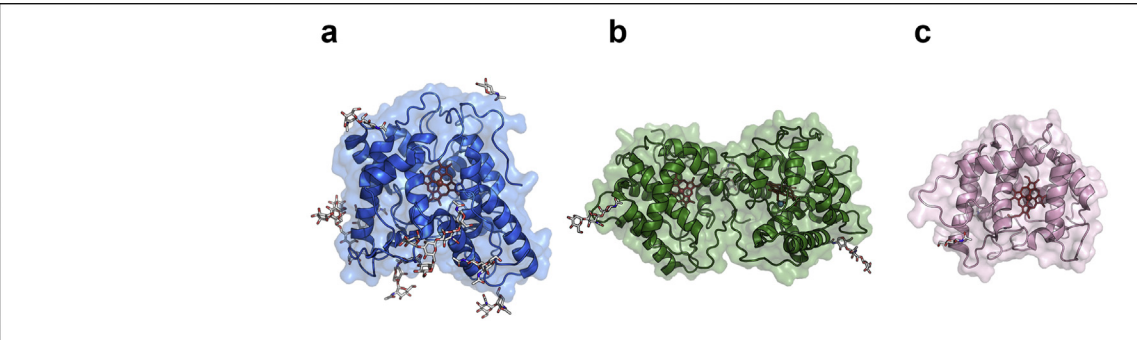
The natural function and distribution of UPOs, their mechanism of action, the palette of chemical reactions and biotechnological applications, have all been described extensively in recent years, and the reader is

referred to other excellent reviews addressing these topics [5–10,12–17]. We will focus here on the most recent findings in the field of UPO engineering, highlighting some relevant case studies involving the short and long UPO families (Figure 2), as well as offering a glimpse of the significant work carried out on artificial peroxygenases. In addition, we will share our perspectives for the present and future development of this promising area of research.

The engineering of long UPOs

In general terms, long UPOs are poorly expressed in heterologous hosts [10,18]. The lack of specific chaperones, the need for complex post-translational modifications (N- and/or C-terminal processing, the formation of S-S bridges, glycosylation) and the singularity of being heme-thiolate enzymes (*i.e.* carrying a cysteine residue as the axial [fifth] ligand of the iron-heme prosthetic group), along with harbouring a structural Mg²⁺ cation, are all important hurdles to be overcome for the successful functional expression of these enzymes. However, these drawbacks are currently being

Figure 2



Fungal source	<i>Agrocybe aegerita</i>	<i>Marasmius rotula</i>	<i>Hypoxylon</i> sp. EC38
Family	Long UPOs	Short UPOs	Short UPOs
Molecular weight	46 kDa	32 kDa (per monomer)	~28-30 kDa
Structure	Monomeric	Dimeric	Monomeric
Disulfide bridges	Intramolecular	Intermolecular	None
Amino acids lining heme channel	Mostly aromatic	Mostly aliphatic	Mostly aliphatic and hydrophilic
Acid-base catalytic pair	Arg189/Glu196	His86/Glu157	His110/Glu180
PDB	2YP1, 5OXU	5FUJ	7O2D

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Short and long UPOs. Up to date, all UPO genes have been exclusively found in the fungal kingdom, yet their natural functions are unclear. For instance, in the ligninolytic enzyme consortium secreted by white-rot fungi, which is formed by high-redox potential oxidoreductases (including laccases, peroxidases and H₂O₂ supplying enzymes [17]), UPOs may be involved in the latest stage of lignin degradation (e.g. O-demethylation), showing also potential roles as an 'external detoxifying agent' or in the synthesis of different metabolites, including antibiotics. As several UPO genes have been reported in selective white-rot fungi, their potential action during natural wood decay may expand their biotechnological applications beyond organic synthesis, including uses in the bioremediation of different pollutants in the near future. From a phylogenetic point of view, UPOs are sorted in two broad families, short and long UPOs that show different molecular sizes and structural organization. Despite both UPO families have highly conserved sequences at the active site (EGD-S-R-E for long UPOs and EHD-S-E for short UPOs), they show distinct active site shapes and reactivities that are responsible for different substrate profiles and product selectivity [7,12]. In general terms, short UPOs can transform bulky compounds (including steroids and long aliphatic substrates) while long UPOs have stronger preferences towards small aromatic compounds. Crystal structures of long UPO (AaeUPO (a)) and short UPOs (MroUPO (b) and HspUPO (c)) are shown together with their general characteristics.

tackled by protein and/or strain engineering, Table 1, Figure 3. The first true heme-thiolate peroxygenase reported came from the edible mushroom *Agrocybe aegerita* (AaeUPO), which was also the first UPO to be engineered in the laboratory, becoming a platform for peroxygenase chemistry. This enzyme was subjected to directed evolution to improve its functional expression in yeast (both *Saccharomyces cerevisiae* and *Pichia pastoris/Komagataella phaffii*), giving rise to the PaDa-I mutant that is expressed at ~ 8 mg/L in *S. cerevisiae* and over 200 mg/L in *P. pastoris* in a bioreactor [19,20]. Since then, PaDa-I has become a recurrent example in UPO engineering, not only working as a launching pad for multiple applications and the generation of new

mutants but also, providing important lessons for the functional expression of other novel UPOs, from the pivotal role of bearing a proper signal peptide to drive secretion and adopting expression mutations, to its use for UPO chimeragenesis (*vide infra*). The first pilot-scale production of PaDa-I was reported recently in a 2500 L fermentation of *P. pastoris*, paving the way for its transfer to industry [21]. More significantly, recent evolutionary campaigns of PaDa-I in conjunction with computational studies, such as ligand diffusion and QM/MM simulations, identified the JaWa and SoLo mutants to be highly active and regioselective for the production of aromatic compounds, in particular the agrochemical 1-naphthol and several human drug metabolites (HDMs)

Table 1

Recombinant and genetically engineered UPOs.

Parental type ^a	Family	Variant	Expression host ^b	Engineering method	Relevant achievements	Ref
AaeUPO	Long	PaDa-I mutant ^c	<i>S. cerevisiae/P. pastoris</i>	Random mutagenesis, <i>in vivo</i> shuffling and signal peptide engineering by MORPHING ^e	Functional expression, improved activity and stability; general platform for UPO engineering	[19,20]
AaeUPO	Long	Recombinant ^d	<i>M. oryzae</i>	Fusion to <i>M. oryzae</i> signal peptide	Functional expression	[59]
PaDa-I mutant	Long	JaWa mutant	<i>S. cerevisiae/P. pastoris</i>	Random mutagenesis, <i>in vivo</i> shuffling, StEP ^f	Improved synthesis of 1-naphthol with high regioselectivity and reduced peroxidative activity	[22]
PaDa-I mutant	Long	WamPa mutant	<i>S. cerevisiae/P. pastoris</i>	Random mutagenesis, <i>in vivo</i> shuffling, neutral genetic drift, site directed recombination <i>in vivo</i>	Improved activity in organic co-solvents	[28,29]
PaDa-I mutant	Long	JEd-I mutant	<i>S. cerevisiae/P. pastoris</i>	Site saturation mutagenesis	Improved kinetic parameters for peroxidative and peroxygenative substrates; high resolution crystal structures unveiled the pattern of substrate binding.	[26]
PaDa-I mutant	Long	Clones 8 and 23	<i>S. cerevisiae/P. pastoris</i>	Combinatorial site saturation mutagenesis	Determination of the origin for peroxidative activity in long UPOs	[33]
PaDa-I, GmaUPO, CciUPO	Long	Chimeras I-VI	<i>S. cerevisiae</i>	Combinatorial Golden Gate cloning	High-throughput GC-MS method to detect activity against tetralin; first reported UPO chimeras	[27]
JaWa mutant	Long	SoLo mutant	<i>S. cerevisiae/P. pastoris</i>	MORPHING ^e and combinatorial mutagenesis	Improved synthesis of HDMs ^g with high regioselectivity	[23,24]
SoLo mutant	Long	SoLo-AAO fusion	<i>S. cerevisiae/P. pastoris</i>	UPO-AAO Enzyme fusion libraries with peptide linkers and leaders	Self-sufficient UPO system for the synthesis of HDMs ^g	[25]
PabUPO-I	Long	Grogu mutant	<i>S. cerevisiae/P. pastoris</i>	Adopting secretion mutations by site directed recombination <i>in vivo</i> and signal peptide switching	Functional expression and unusually broad pH stability and activity profiles	[30]
PabUPO-II	Long	Recombinant ^d	<i>S. cerevisiae/P. pastoris</i>	Signal peptide switching	Functional expression and unusually broad pH stability and activity profiles	[30]
PabUPO-I, CglUPO	Long and short	Recombinant ^d	<i>E. coli</i>	Fusion proteins with SUMO (Small Ubiquitin-related Modifier) and TRX (thioredoxin)	Functional expression	[40]
PaDa-I, short and long evolved UPO variants	Long and Short	12 Evolved UPO variants (EVOKit)	<i>P. pastoris</i>	EvoShuffler	Evolved UPOs by EvoEnzyme, S.L.; uses in lead diversification, HDMs synthesis, production of fine chemicals	https://evoenzyme.com
CciUPO	Long	Recombinant ^d	<i>Aspergillus oryzae</i>	n.d.	Recombinant peroxygenase by Novozymes A/S	[13]
HinUPO	Short	Recombinant ^d	<i>Aspergillus oryzae</i>	n.d.	Recombinant peroxygenase by Novozymes A/S	[13]
HspUPO	Short	Recombinant ^d	<i>P. pastoris</i>	Attachment to a mating-factor secretion sequence, use of a hyper secretory <i>P. pastoris</i> strain	Functional expression, biochemical characterization, crystallization with different ligands	[34]

<i>Mro</i> UPO	Short	Recombinant ^d	<i>S. cerevisiae/P. pastoris</i>	Signal-peptide shuffling	Functional expression; Modular Golden Gate-based UPO secretion system	[36]
<i>Mro</i> UPO	Short	I153T and I153F–S156F mutants	<i>E. coli</i>	Site-directed mutagenesis	Modulated epoxidation vs. hydroxylation selectivity in fatty acids	[39]
<i>Cg</i> UPO	Short	Recombinant ^d	<i>S. cerevisiae/P. pastoris</i>	Signal-peptide shuffling	Functional expression. Modular Golden Gate-based UPO secretion system	[36]
<i>Mth</i> UPO	Short	Recombinant ^d	<i>S. cerevisiae/P. pastoris</i>	Signal-peptide shuffling	Functional expression. Modular Golden Gate-based UPO secretion system	[36,37]
<i>Mth</i> UPO	Short	L60F, F59Q-L60F–S159G and L60F–S159G-A161F mutants	<i>S. cerevisiae</i>	Site-saturation mutagenesis	Chemo- and regioselective oxidations of benzylic and aromatic compounds	[43]
<i>Mth</i> UPO	Short	L60A, F154V and A161L mutants	<i>S. cerevisiae</i>	Site-saturation mutagenesis	Selective alkene epoxidation and shifted regioselectivities towards the synthesis of 2- and 1-octanol	[44]
<i>Tte</i> UPO	Short	Recombinant ^d	<i>S. cerevisiae/P. pastoris</i>	signal peptide shuffling	Functional expression; modular Golden Gate-based UPO secretion system	[36,37]
<i>Mhi</i> UPO	Short	Recombinant ^d	<i>P. pastoris</i>	Promoter/signal peptide shuffling	Functional expression; episomal expression system for directed UPO evolution in <i>P. pastoris</i>	[37]
<i>Mfe</i> UPO	Short	Recombinant ^d	<i>P. pastoris</i>	Promoter/signal peptide shuffling	Functional expression; episomal expression system for directed UPO evolution in <i>P. pastoris</i>	[37]
<i>Dca</i> UPO	Short	Recombinant ^d	<i>P. pastoris</i>	Promoter-signal peptide shuffling	Functional expression, episomal expression system for directed UPO evolution in <i>P. pastoris</i>	[37]
<i>Dca</i> UPO	Short	Recombinant ^d	<i>E. coli</i>	None	Functional expression	[38]
<i>Cvi</i> UPO	Short	Recombinant ^d	<i>E. coli</i>	None	Functional expression	[38]
<i>Cvi</i> UPO	Short	F88L and T158F mutants	<i>E. coli</i>	Site-directed mutagenesis	Selective oxidation of unsaturated fatty acids of different chain lengths	[42]

^a *Aae*UPO, UPO from *Agroclybe aegerita*; *Gma*UPO, UPO from *Galerina marginata*; *Ccl*UPO, UPO from *Coprinopsis cinerea*; *Pab*UPO-I, UPO paralog from *Psathyrella aberdarensis*; *Pab*UPO-II, UPO paralog from *Psathyrella aberdarensis*; *Cg*UPO, UPO from *Chaetomium globosum*; *Hin*UPO, UPO from *Humicola insolens*; *Hsp*UPO, UPO from *Hypoxylon* sp. EC38; *Mro*UPO, UPO from *Marasmius rotula*; *Mth*UPO, UPO from *Myceliophthora thermophila*; *Tte*UPO, UPO from *Thielavia terrestris*; *Mhi*UPO, UPO from *Myceliophthora hinnulea*; *Mfe*UPO, UPO from *Myceliophthora fergusii*; *Dca*UPO, UPO from *Daldinia caldarium*; *Cvi*UPO, UPO from *Collariella virescens*.

^b *S. cerevisiae*, *Saccharomyces cerevisiae*; *P. pastoris*, *Pichia pastoris*; *M. oryzae*, *Magnaporthe oryzae*; *A. oryzae*, *Aspergillus oryzae*; *E. coli*, *Escherichia coli*.

^c PaDa-I mutant is also referred in many studies as to rAaeUPO.

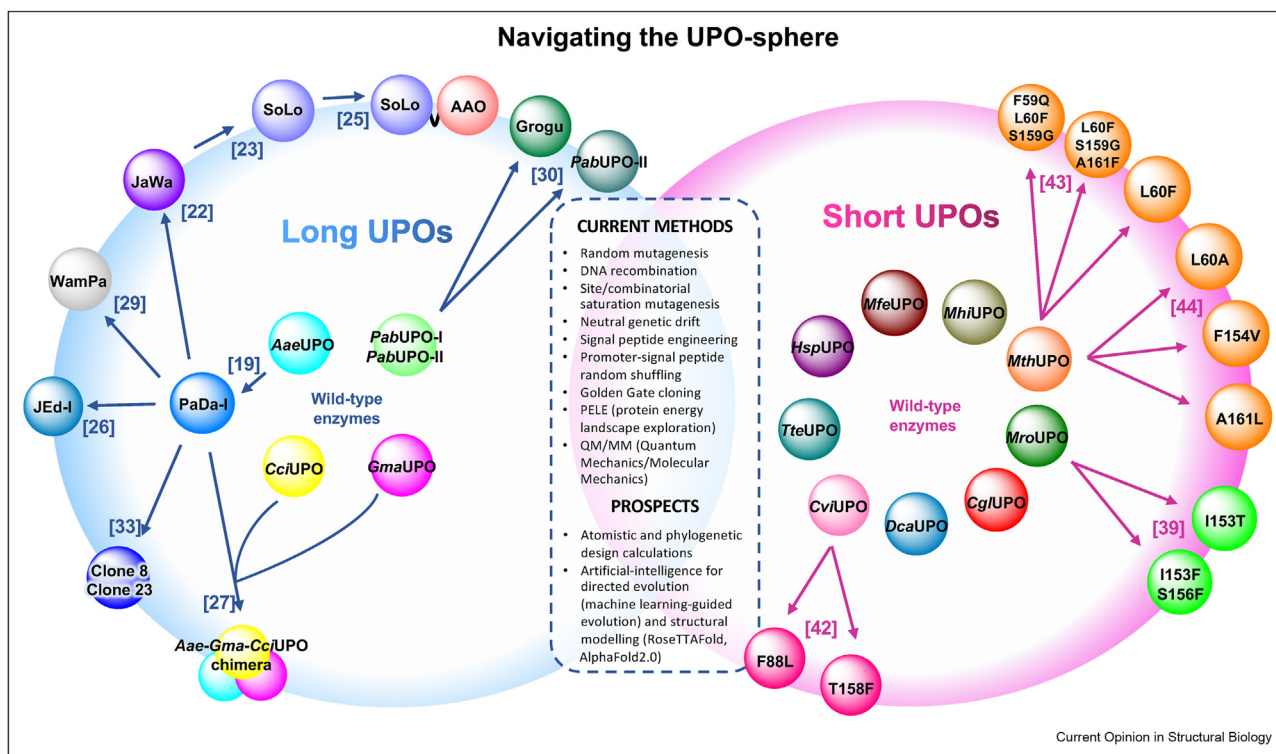
^d Recombinant: native (non-mutated) enzyme expressed in a heterologous host.

^e MORPHING (Mutagenic Organized Recombination Process by Homologous IN vivo Grouping).

^f StEP (Staggered Extension Process).

^g HDMs (Human Drug Metabolites); n.d. not described.

Figure 3



Navigating the UPO sphere. With more than 4000 UPO sequences deposited in genomic databases, the number of recombinant UPOs produced and engineered in the laboratory is steadily increasing. Indeed, directed evolution is shifting the paradigm of adapting processes to the UPO available, to adapting the UPO to a given process. Such an approach is expanding the application of UPOs to a larger number of new reactions and positioning them, little by little, in a privileged position to compete with standard chemical catalysts [8,9]. On the left are the recombinant long UPOs expressed and the variants engineered in recent years, whereas an analogous diagram of their short family counterparts is shown on the right. Current engineering methods and future trends are highlighted in the central box. In brackets are shown the associated references (see also Table 1).

[22–24]. The latter are of considerable interest to the pharmaceutical industry, as the synthesis of HDMs on a preparative scale is a fundamental part of the pharmaceutical manufacturing pipeline and a new offspring of UPO mutants has just been commercially available, Table 1. The potential use of the SoLo mutant has been further explored by creating a chimeric fusion with an evolved aryl alcohol oxidase (AAO) that acts as a H_2O_2 supplier [25]. One of the benefits of this UPO_AAO fusion is that the AAO partner feeds the UPO partner *in situ*, providing controlled and stoichiometric amounts of H_2O_2 that limit UPO oxidative inactivation. This inactivation is a mechanistic problem known as suicide inactivation, whereby H_2O_2 is the enzyme's co-substrate but paradoxically, it is also a strong inhibitor of its activity. Indeed, the rate of H_2O_2 feeding in peroxygenase driven reactions has been explored in depth over recent years through photo-, chemo- electro- and biocatalysis (*i.e.* enzyme cascade reactions) [12 and references therein]. However, bringing the two active sites close together in a single polypeptide seems to generate a substrate tunnelling effect that boosts the efficiency of oxyfunctionalization reactions [25].

The possibility of obtaining functional chimeric UPOs by assembling UPO blocks from different species has also generated interest. The sequences of PaDa-I, *Galerina marginata* UPO (*GmaUPO*) and *Coprinopsis cinerea* UPO (*CciUPO*), with more than 60% identity between them, were rationally split on the basis of PaDa-I crystal structure [26], giving rise to five subunits of secondary structural elements that could be randomly shuffled. The library of chimeric UPOs generated in this way was screened for the hydroxylation of tetralin using a multiple injection GC–MS high-throughput screening (HTS) assay [27]. Only four of the 243 chimeric possibilities were active with tetralin, with an over-representation of the PaDa-I sequence blocks in the structure of the selected functional chimeras. Future endeavours in this direction should perhaps focus on creating more complex sets of UPO chimeras, with less bias towards the PaDa-I mutant, such that new hybrid properties may arise among the variants.

In addition, the PaDa-I mutant has recently been used as a template to generate a palette of different mutants using diverse library creation methods, in particular by

neutral genetic drift (an engineering strategy based on accumulating neutral mutations that produce similar phenotypes with different genotypes) and classic *in vivo* DNA shuffling. In this way, a collection of neutrally evolved mutants with improved stability against temperature and the presence of co-solvents has been generated [28]. These variants were then further evolved toward a final mutant that is active and stable in the presence of high concentrations of organic solvents of different chemical nature and polarities, which can therefore be used in transformations of many hydrophobic compounds [29]. The activity and stability of this mutant, referred to as WamPa, was enhanced in the presence of acetonitrile, acetone, dimethyl sulfoxide and methanol. Significantly, the mutations were filtered by site directed recombination to achieve optimum epistasis, and they were mostly situated at the protein's surface and in the neighbourhood of the heme access channel.

It is worth noting that PaDa-I has also helped us to understand the determinants underlying the functional expression and activity of other UPOs. Very recently, two unusual acidic UPO paralogs from the East African ink-cap *Candolleomyces (Psathyrella) aberdarensis (PabUPO)* were engineered, adopting the evolved signal peptide from PaDa-I alongside many of the mature PaDa-I mutations that foster secretion by yeast [30]. Both *PabUPO* genes were satisfactorily expressed in yeast, producing 300 mg/L in a bioreactor together with the highest volumetric activity ever reported for a native or recombinant peroxygenase (60,000 U/L, using veratryl alcohol as a substrate). Of particular interest is the use of proper signal peptides that can guide the nascent UPO polypeptide through the secretory route of the eukaryotic host for a successful exocytosis. Most of the cases describing the heterologous expression of ligninolytic enzymes in yeast are based on the use of the α -factor prepro-leader from *S. cerevisiae* or chimeric evolved versions that help improve secretion yields. By contrast, all UPOs expressed to date show preferences for native signal peptides which can be switched among different species (*vide infra*) or that can be adapted to the particularities of the secretion route of the host by laboratory evolution, like in the case of the PaDa-I and the *PabUPO* paralogs.

While functional heterologous expression and oxidative stability are areas that have received much attention, the coexistence of peroxidative and peroxygenative activities in UPOs is also an important trait worthy of further study [31]. Conceptually, UPOs are considered hybrid enzymes of generic peroxidases and P450s, as they can perform both one electron oxidation of phenolics (peroxidative activity) and two electron oxidation reactions (*i.e.* oxygen insertions into organic compounds through their peroxygenative activity). Unfortunately, the convergence of both these activities within the same

protein scaffold makes UPOs unsuited to several industrial applications, since the product of this peroxygenative activity may be a substrate for its peroxidative activity. Thus, this dual activity can severely affect final production yields and complicate downstream processes. Modifications to the heme access channel can affect the relative peroxygenative/peroxidative activities [22,24,31,32]. The origin of UPO's peroxidative activity has been isolated by combining computational simulations with saturation mutagenesis, highlighting a flexible loop at the heme funnel as the main region responsible for this activity. In turn, the influence of potential long-range electron transfer (LRET) pathways was ruled out, being these typical of ligninolytic peroxidases in which catalytic aromatic Trp/Tyr residues at the protein surface are connected to the heme by LRET [33]. Future engineering undertakings to improve specific reactions involving aromatic compounds should focus on dampening the peroxidative activity of UPO via the heme access channel while retaining and even improving its peroxygenative activity. Rather than engineering a universal UPO with quenched peroxidative activity, we visualize an ensemble of mutants created *à la carte* for each specific reaction, particularly given that the relationship between both activities is dependent of the starting substrate.

The engineering of short UPOs

Although long UPOs have been those predominantly subjected to protein engineering over the past few years, they are gradually sharing this stage with their short UPO counterparts, Table 1, Figure 3. Unlike long UPOs, the heterologous expression of short UPOs is considerably easier and through different engineering approaches numerous recombinant enzymes of this family have been functionally expressed in heterologous hosts, mainly yeast. Very recently, the short UPO from *Hypoxylon* sp. EC38 (*HspUPO*) was cloned and functionally expressed in *P. pastoris*. This *HspUPO* was preceded by a mating-factor secretion sequence that drives protein secretion at titres of 200 mg/L in a bioreactor, allowing it to be characterized thoroughly at the biochemical and structural level. *HspUPO* was the fourth UPO successfully crystallized, together with wild-type *AaeUPO*, its evolved mutant PaDa-I, and the wild type short UPO from *Marasmius rotula (MroUPO)* [26,34,35]. In addition, four new recombinant short UPOs (*MroUPO*, UPO from *Chaetomium globosum (CgUPO)*, UPO from *Myceliophthora thermophila (MthUPO)*, and UPO from *Thielavia terrestris (TteUPO)*), have been expressed in *S. cerevisiae* and *P. pastoris*. Their heterologous expression was achieved by developing a modular shuffling system based on the Golden Gate cloning tool, whereby 17 different signal peptides were fused in a combinatorial way to eight different UPO sequences to identify the constructs most efficiently expressed by split-GFP (green fluorescent protein) screening [36].

Interestingly, using the same combinatorial fusion approach, 17 signal peptides and 11 different promoters were evaluated to find the optimal combination for the functional expression of other short UPOs by *P. pastoris* (*Myceliophthora hinnulea* (MhiUPO), *Myceliophthora fergusii* (MfeUPO) and *Daldinia caldarium* (DcaUPO)), while introducing an episomal expression system for the directed UPO evolution in *P. pastoris* [37]. Expression of short UPOs in *Escherichia coli* as a host has been also reported for *MroUPO*, *DcaUPO* and *Collariella* (*Chaetomium*) *virescens* (*CviUPO*) although at considerably lower levels [38,39]. The lack of a well-developed secretory apparatus in *E. coli* seems to affect recombinant UPO production in this host. New approaches to resolve this problem include the use of SUMO (Small Ubiquitin-related Modifier) and TRX (thioredoxin) fusion proteins to prevent their accumulation in inclusion bodies, and to enhance their solubility and stability, as recently reported with *CglUPO* [40].

As a rising number of new short UPOs are heterologously expressed, they are being engineered with more strict control of their regio-, enantio- and chemoselectivity. The *MroUPO* expressed in *E. coli* has been rationally designed for the selective oxyfunctionalization of unsaturated fatty acids (oleic, linoleic and α -linoleic acids): Relevant catalytic residues at the entrance of the substrate channel were mapped by computational simulation and single/double mutants were identified that differed in their epoxidation/hydroxylation ratios, while also displaying improved regio- and chemoselectivities [39,41]. A similar approach was followed to design a double mutant of *CviUPO* expressed in *E. coli* in order to mimic the active site of the homologous *CglUPO*, acquiring analogous selectivity [42].

The *MthUPO* expressed in yeast was studied by molecular dynamics simulations, assigning 9 positions at the entrance of the active site that were relevant for the chemoselective oxidation of naphthalene-derivative compounds [43]. After saturation mutagenesis and screening, single and double mutants with increased regio- and chemoselectivities were identified. Those mutant variants were benchmarked with octane, cyclohexane and cyclohexene as substrates, observing distinct patterns of oxidation and/or epoxidation depending on the variant assayed. This finding is not unexpected if we take into account that the variants were screened with a very different substrate, which ultimately highlights the first law of directed evolution, *i.e.* 'you get what you screen for' [44].

Artificial peroxygenases and *de novo* peroxygenase design

An interesting alternative to the design of natural UPOs, is the use of artificial enzymes that are capable of catalysing peroxygenase or peroxygenase-like activities [14].

Two lines of research have been followed in this regard: (i) opening up the catalytic repertoire of promiscuous, non-peroxygenase enzymes through engineering so that they can make use of peroxide to catalyse C–H oxyfunctionalizations; and (ii) the development of *de novo* peroxygenases from different computationally designed scaffolds (protein and non-protein based). In terms of the former, some P450 mutants can harness a peroxide shunt pathway to work like peroxygenases, although much less efficiently than natural UPOs [45,46]. It was recently demonstrated that P450 BM-3 from *Bacillus megaterium* can be converted into a peroxygenase by simply adding a decoy molecule (N-(ω -imidazolyl)-hexanoyl-L-phenylalanine) that mimics the active site of *AaeUPO* for the hydroxylation of small organic compounds [47]. Another enzyme that has shown mild promiscuous peroxygenase activity is the cofactor-independent 4-oxalocrotonate tautomerase (4-OT) from *Pseudomonas putida*. Engineered 4-OT variants were screened for enantioselective epoxidation of α,β -unsaturated aldehydes using H_2O_2 or *t*-BuOOH as oxidants. This approach led to the discovery of a triple mutant with a 60-fold enhancement in activity [48].

The *de novo* design of peroxygenases has focused on the use of metalloporphyrin-containing proteins (mimochromes) as protein scaffolds. Mimochromes are based on the miniaturization of the active site of human haemoglobin, mimicking the two parallel α -helices involved in metalloporphyrin binding. These artificial metalloenzymes can act as peroxygenases, catalysing the sulfoxidation of phenyl thioethers [49], as well as the selective oxidation of indoles [50]. Following a similar approach, the TM1459 cupin superfamily protein from *Thermotoga maritima*, a promiscuous metal-binding enzyme, was used as the departure point to design an osmium peroxygenase capable of dihydroxylating different alkenes [51]. Non-protein scaffolds, like G-quartet based systems, have been explored to construct DNazymes capable of binding heme groups that catalyse peroxidase-like reactions, such as thioether oxidation [52].

Conclusions and future directions

We envisage a bright future for the engineering of UPOs in the quest for new functionalities, activities and selectivities. Led by the appearance of artificial intelligence (AI) algorithms to more accurately predict protein structure (DeepMind's AlphaFold2.0, RoseTTAFold), exciting possibilities for UPO design will be unlocked in the forthcoming years at the time that experimental effort will be reduced by performing a more efficient structure-guided evolution [53,54]. With the number and accuracy of the prediction algorithms increasing rapidly, the nascent association between Rosetta design and phylogenetic inference methods will unfold the true potential of UPOs as synthetic tools [55]. We also foresee the use of paleoenzymology *-i.e.*

ancestral sequence reconstruction and resurrection-aimed at opening unexplored avenues for the laboratory evolution of more promiscuous and stable ancestral UPOs [56]. This engineering tool-box will be completed in the near future with the arise of machine-learning-guided directed evolution [57,58]. Taken together, the courtship of cutting-edge directed evolution and computational design should forge a breakthrough in organic chemistry, producing *ad-hoc* peroxygenases that will catalyse unprecedented chemical reactions in a manner that has not been seen to date.

Conflict of interest statement

Nothing to declare.

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