Structural and Biochemical Studies Enlighten the Unspecific Peroxygenase from *Hypoxylon* sp. EC38 as an Efficient Oxidative Biocatalyst

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**ABSTRACT:** Unspecific peroxigenases (UPOs) are glycosylated fungal enzymes that can selectively oxidize C–H bonds. UPOs employ hydrogen peroxide as the oxygen donor and reductant. With such an easy-to-handle cosubstrate and without the need for a reducing agent, UPOs are emerging as convenient oxidative biocatalysts. Here, an unspecific peroxigenase from *Hypoxylon* sp. EC38 (HspUPO) was identified in an activity-based screen of six putative peroxigenase enzymes that were heterologously expressed in *Pichia pastoris*. The enzyme was found to tolerate selected organic solvents such as acetonitrile and acetone. HspUPO is a versatile catalyst performing various reactions, such as the oxidation of prim- and sec-alcohols, epoxidations, and hydroxylations. Semipreparative biotransformations were demonstrated for the nonenantioselective oxidation of racemic 1-phenylethanol rac-1b (TON = 13 000), giving the product with 88% isolated yield, and the oxidation of indole 6a to give indigo 6b (TON = 2800) with 98% isolated yield. HspUPO features a compact and rigid three-dimensional conformation that wraps around the heme and defines a funnel-shaped tunnel that leads to the heme iron from the protein surface. The tunnel extends along a distance of about 12 Å with a fairly constant diameter in its innermost segment. Its surface comprises both hydrophobic and hydrophilic groups for dealing with substrates of variable polarities. The structural investigation of several protein–ligand complexes revealed that the active site of HspUPO is accessible to molecules of varying bulkiness with minimal or no conformational changes, explaining the relatively broad substrate scope of the enzyme. With its convenient expression system, robust operational properties, relatively small size, well-defined structural features, and diverse reaction scope, HspUPO is an exploitable candidate for peroxigenase-based biocatalysis.

**KEYWORDS:** substrate recognition, protein tunnels, biocatalytic oxidation, monooxygenase, peroxigenase

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**INTRODUCTION**

The C–H bond is relatively inert, and its oxidation is one of the most challenging conversions in chemistry. The cytochrome P450s have been historically considered as a particularly attractive group of enzymes to perform oxidative reactions targeting C–H groups. Their reaction mechanism relies on the reductive activation of molecular oxygen through a stepwise catalytic cycle that involves the formation of the highly oxidized and reactive oxoferryl intermediate (Scheme 1). The direct interaction between this so-called compound I and the substrate affords substrate oxygenation. Cytochrome P450s require a reducing agent, such as NAD(P)H, and auxiliary flavoproteins that respectively donate and transport electrons. The mandatory presence of this complex protein machinery makes cytochrome P450s prone to the “oxygen dilemma”: the complexity of the electron-transport mediators inevitable promotes uncoupling pathways that waste reducing equivalents and significantly lower the catalytic turnover. These limitations, summed to the low operational stability as purified proteins, hampered the use of cytochrome P450s as general catalysts for large-scale biocatalytic applications. Several solutions have been proposed to overcome these issues. They include various strategies such as the usage of whole microbial cells, the regeneration of the reduced nicotinamide cofactor, and the coexpression with NADPH-regenerating systems and the exploitation of engineered cytochrome P450s endowed with peroxigenase activities.

In 2004, Ullrich et al. documented the discovery of a new secreted enzyme from the fungus *Agrocybe aegerita*. After its
initiation classification as a haloperoxidase, it was finally recognized as an unspecific peroxygenase (UPO) and considered as the first member of a new sub-subclass of oxidoreductases (E.C 1.11.2.1). This milestone discovery has been followed by an increasing interest in UPOs, which were defined as the “generational successors” to P450s. UPOs are highly glycosylated, naturally secreted, fungal heme-enzymes that combine a wide versatility in terms of substrate scope with a relatively simple reaction mechanism that does not need an external electron donor. In their reaction cycle, compound I is formed directly from the reaction between H2O2 and the protoporphyrin IX in the enzyme core (Scheme 1). The remarkable feature of this reaction is the dual function of the H2O2 cosubstrate, acting as both an oxygen donor and reductant.

To date, just the crystal structures of two peroxygenases are available, namely, one from A. aegerita (PDB: 2YOR for wild-type and SOXU for the PaDa-I secretion variant) and another from Marasmius rotula (PDB: SFUK; unpublished). Both crystal structures highlighted and elucidated the distinctive features of UPOs whose core is mainly composed of α-helices that organize the active site around the heme iron center. The active site is mainly cladded with aromatic residues, conferring a preference for mildly hydrophobic substrates. UPOs have been demonstrated to be active as oxygenases on many compounds, ranging from the aliphatic propane and fatty acids to the aromatic naphthalene, benzene, phenethylamine, and styrene. A number of strategies have been described to improve the challenging expression of these enzymes.

Strain Generation. Plasmids were obtained from bisy (bisy GmbH, Hofstaehen, Austria) and generated as described in ref 40. These vectors are optimized to drive the expression of the full-length protein fused with the Mating-Factor signal sequence [MKSLSFLALGFGSTLYYS], allowing the secretion of the target polypeptide under the control of a bidirectional promoter PPDF. The plasmids were transformed in Pichia pastoris, strain BSYBG11 (bisy GmbH, Hofstaehen, Austria), to afford the integration of the target cassette into the recipient genome by homologous recombination.

Small-Scale Protein Expression. Single colonies of the transformed P. pastoris cells, grown on YPD agar (1% yeast extract; 2% peptone; 2% dextrose; 2% agar) and selected with Zeocin (final 100 μg/mL), were picked, inoculated in 45 mL of buffered minimal dextrose medium (100 mM potassium phosphate pH 6; 1.34% YNB; 4 × 10⁻³% biotin; 1% dextrose), and incubated at 28°C with 200 rpm shaking for 60 h. The induction was then started by the addition of 5 mL of buffered minimal methanol medium (100 mM potassium phosphate pH 6; 1.34% YNB; 4 × 10⁻³% biotin; 0.5% methanol). After 12, 24, and 36 h, 0.5 mL of pure methanol was added to the culture medium. After a total of 180 h of cultivation, the cell cultures were centrifuged for 15 min at 8000 rpm.

Activity-Based Screenings. The cell-free supernatant containing the secreted enzymes was screened for activity using two assays.

2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic Acid) Di-ammonium Salt (ABTS) Assay. The assay mix solution consisted of 1 mL of 20× ABTS (440 mg of ABTS dissolved in 50 mL of 50 mM sodium acetate pH 4.5) and 19 mL of 200 mM sodium citrate pH 4.5. In the 96-well plate (Greiner), 15 μL of the cell-free supernatant was mixed with 140 μL of the assay solution and the reaction was started upon the addition of 1.75 μL of 30% H2O2 in each well. Product formation was monitored following the increase in absorbance at 405 nm (ε405 = 36 000 M⁻³ cm⁻¹) for 15 min.
**Naphthalene Assay.** The assay mix solution consisted of 2 mL of naphthalene (4 mM, stock solution prepared in pure acetone) and up to 10 mL with 200 mM citrate-phosphate pH 7. Each well contained 15 μL of the cell-free supernatant and 140 μL of the assay mix. The reaction was started upon the addition of 1.75 μL of 30% H₂O₂. Product formation was detected at 310 nm (ε₄₀₅ = 2030 M⁻¹ cm⁻¹).

The volumetric activities of the enzymes were calculated as follows

\[ U = [(ΔAΔt⁻¹ × Vᵣ₋ₓ) × D)] / (Vₚₛₐ𝐦ｐ𝐥ₑ × εₑₘₓ × d) \]

where \( U \) is the unit per mL [μmol·mL⁻¹·min⁻¹], \( Vₐₓₒᵣₜ \) is the total assay volume [mL], \( ΔAΔt⁻¹ \) is the slope [ΔA min⁻¹], \( D \) is the dilution factor of the sample, \( d \) is the layer thickness [cm], \( Vₚₛₐ𝐦𝐩ˡₑ \) is the sample volume [mL], and \( εₑₘₓ \) is the extinction coefficient [mL·μmol⁻¹·cm⁻¹].

**Expression in Shaking Flasks.** Precultures were prepared by picking a single colony and inoculating it in 5 mL of YPD medium (1% yeast extract, 2% peptone, 2% dextrose) supplemented with Zeocin (100 μg/mL final) followed by an overnight incubation at 28 °C with 200 rpm shaking. After the initial growth, the preculture was split and 2.5 mL was added to 450 mL of buffered minimal dextrose medium and grown at 28 °C, 200 rpm shaking for 72 h. The induction was started with the addition of 50 mL of buffered minimal methanol medium, and every 12 h, for a total of 72 h, 5 mL of pure methanol was supplemented to the culture. The culture was then harvested by centrifugation at 8000 rpm for 15 min, and the resulting decanted supernatant was sterile-filtered prior to the test enzymatic activity as described in the previous paragraph.

**Fermentation and Purification.** HspUPO was expressed in *P. pastoris* using a 5 L glass vessel fermenter (Bioflo 3000) filled with basal salts medium (26.7 mL/L 85% phosphoric acid, 0.93 g/L CaSO₄·2H₂O, 14.9 g/L MgSO₄·7H₂O, 18.2 g/L K₂SO₄, 4.13 g/L KOH, 40 g/L glycerol, initial volume: 3 L) supplemented with 4.35 mL/L PTM1 trace salts and 4 x 10⁻⁵% biotin. The pH was set to 5.0 and kept constant with ammonium hydroxide solution (30%). The temperature was set to 28 °C. The fermentation batch phase was started by inoculating 0.2 L of *P. pastoris* preculture and grown for 16 h in a baffled flask on YPD medium at 200 rpm shaking and 28 °C. After 12 h, the glycerol in the basal salt medium was totally consumed, leading to a drop in the dissolved oxygen concentration, which was always kept above 30%. At this stage, the culture was supplemented with 50% (w/v) glycerol-feed containing 12 mL/L PTM1 trace salts and 4 x 10⁻⁵% biotin, until the cell density was 180 g/L. Methanol feed (0.5% v/v final concentration) was started at the end of the glycerol fed-batch phase, when the dissolved oxygen concentration spiked at 80%, and lasted for 72 h. At the end of the fermentation, the cell culture was clarified by centrifugation at 70 000 g for 20 min and the obtained supernatant was then decanted and filtrated with 0.22 μm filters (Millipore). Using a cross-flow device (Sartorius), as a final downstream process, the supernatant was then concentrated 6-fold, buffer-exchanged to 10 mM potassium phosphate pH 6, and stored at −20 °C. The polished supernatant was dialyzed overnight against 50 mM Tris–HCl pH 7.8 (buffer A) using a 10 kDa dialysis cassette (Thermo-Fisher). The day after, it was loaded by Akta System (Cytiva) equipped with a multilength detector (set at 280/350/420 nm) into a CaptoQ column (Cytiva) pre-equilibrated with buffer A. After washing (five column volumes) with buffer A, a linear ascending salt gradient (0~50% 1 M NaCl) was applied to elute bound proteins. Fractions containing the purified proteins were analyzed by SDS PAGE, and fractions containing the enzyme with an appropriate Reinheitszahl value (Abs₁₂₀/Abs₂₅₀; experimental value of 1.8 vs the theoretical value of 1.9) were pooled together. Deglycosylated HspUPO was prepared by incubating overnight with homemade 6xHis-EndoH deglycosylase (5 mg/mL) in the ratio of 3:1 (mgHspUPO:mgENDOH). While incubating, the protein mix was dialyzed against 50 mM Tris/HCl, pH 7.8, to remove the excess salt. The resulting sample was loaded on a Hi-Trap column (5 mL, Cytiva) to remove the His-tagged ENDOH, while the protein of interest was collected in the flow-through. Glycosylated and deglycosylated enzymes were concentrated with Amicon 3K (Millipore) until an appropriate volume, and then loaded on size-exclusion HiPrep Superdex 75 16/60 (Cytiva), previously equilibrated with storage buffer 50 mM Hepes pH 7.8 (Buffer B). Protein fractions were concentrated with Amicon 3K prior to crystallization screenings.

**Protein Crystallization and Structure Determination.** Extensive crystallization screenings were performed with several commercial kits and using an Oryx 8 robot (Douglas instruments) in sitting drop plates (Swissci, Molecular Dimension). Crystallization droplets were prepared with a 1:1 volume ratio by mixing 11 mg/mL protein in 50 mM Hepes pH 7.8 with the reservoir solution. Promising conditions were optimized by manually prepared sitting drop plates (Cryschem, Hampton), increasing the drop volume up to 1 μL. Using nylon loops (Hampton research), crystals were harvested and cryocooled in liquid nitrogen. Soaking experiments (0.5 h) with 1-phenylimidazole and styrene were performed in cryoprotectant solutions consisting of 20% PEG500 MME, 10% PEG20000, 0.1 M MES monohydrate pH 6.5, and the compound of interest (15 mM), followed by flash-freezing in liquid nitrogen. X-ray diffraction data were collected at Swiss Light Synchrotron in Villigen, Switzerland (SLS), at the European Synchrotron Radiation Facility in Grenoble, France (ESRF), and at the Diamond Light Source in Didcot, United Kingdom. Diffraction images were processed with XDS and Aimless of the CCP4i package. Structure solution was performed with Molrep using 5FUK as the searching model for molecular replacement. Atomic models were refined with Refmac5 and Coot. Figures were created using ChimeraX. Superpositions were performed with DALI.

**Activities and Steady-State Kinetic Analysis.** HspUPO activities were determined by absorbance-based methods using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), indole, 2,6-dimethoxyphenyl, 5-nitro-1,3-benzodioxole, 3,4-dimethoxybenzyl alcohol, and benzyl alcohol as substrates. The reactions were started by the addition of hydrogen peroxide (2 mM final concentration). Prior to steady-state kinetic experiments, optimal pH values were determined in McIlvaine (pH 3~8) and borate saline (pH 9) buffers. Specific activities were determined by measuring the initial rates of product formation with varying substrate concentrations. The assay mix consisted of 0.05~1.5 μM HspUPO and McIlvaine buffer pH 4 (ABTS and 3,4-dimethoxybenzyl alcohol), pH 5 (2,6-dimethoxyphenol), and pH 7 (indole, 5-nitro-1,3-benzo-dioxole, and benzyl alcohol). Measurements were carried out at 25 °C using a Cary100 spectrophotometer (Agilent), and product formation was followed monitoring the reactions at 405 nm for ABTS (ε₄₀₅ = 36 000 M⁻¹ cm⁻¹), 670 nm for indole (ε₆₇₀ = 4800 M⁻¹ cm⁻¹), 469 nm for 2,6-dimethoxyphenol (ε₄₆₉ = 4800 M⁻¹ cm⁻¹).
27 500 M⁻¹ cm⁻¹), 425 nm for 5-nitro-1,3-benzodioxole (ε₃25 = 9700 M⁻¹ cm⁻¹), 310 nm for 3,4-dimethoxybenzyl alcohol (ε₃1⁰ = 9300 M⁻¹ cm⁻¹), and 280 nm for benzyl alcohol (ε₂₈₀ = 1400 M⁻¹ cm⁻¹). Data were fit to the Michaelis–Menten equation and analyzed using GraphPad Prism software 6.0. Each point was assayed in duplicate.

**Temperature and Solvent Sensitivity.** HspUPO (final concentration 1 μM) was mixed with 200 mM sodium citrate pH 4.5 (final volume 400 μL). The mix was then split in 20 μL samples, and each of them was incubated, using a Thermocycler, at 35, 40, 50, and 60 °C. An aliquot from each sample was tested for enzyme activity at different time points (10, 30, 60, and 90 min). The blank measurement was performed by mixing HspUPO (final concentration 50 nm) and ABTS (final concentration 30 μM) in 200 mM sodium citrate pH 4.5, and the reaction was started upon the addition of H₂O₂ (2 mM final concentration). Tolerance to solvents was asayed with the ABTS and indole as substrates in the presence of different increasing concentrations of acetone, acetonitrile, and dimethyl sulfoxide (DMSO) (2, 5, 10, 20, and 30%). The mix used for the ABTS oxidation assay consisted of HspUPO (final concentration 50 nm), ABTS (final concentration 30 μM), and increasing concentrations of solvent in 200 mM sodium citrate pH 4.5. The mix used for the indigo production assay consisted of HspUPO (final concentration 1.5 μM), indole (final concentration 100 μM; stock solution solubilized in acetone or acetonitrile), and increasing concentrations of solvents in 50 mM potassium phosphate pH 7.4. The reaction was started by the addition of H₂O₂ (2 mM final concentration) and was carried out at 25 °C.

The equation used to calculate the residual activity (%) is

\[
(k_{\text{cat (obs)/}}/k_{\text{cat (standard)}}) \times 100
\]

where \(k_{\text{cat (obs)}}\) is the \(k_{\text{cat}}\) calculated for each temperature/solvent percentage and \(k_{\text{cat (standard)}}\) is the \(k_{\text{cat}}\) calculated with the standard assay condition.

**Biotransformations.** Reactions on a 1 mL scale were carried out in crimp top glass vials (1.5 mL volume) with a septum in horizontal adjustment in a benchtop incubator at 30 °C and 500 rpm. The reaction mixture contained HspUPO (7 μM) and the substrate (1a–6a, 10 mM, 50 μL of a 200 mM stock in acetonitrile, added last) in tricine buffer 100 mM, pH 7.5 with 5% (v/v) acetonitrile. The conversion was started by the continuous addition of in total 40 μL of a 0.5 M H₂O₂ solution in a reaction buffer (10 μL/h over 4 h for 1a–5a, 40 μL/h over 1 h for 6a, final concentration of H₂O₂: 2 mM, 2 equiv) with a kdScientific pump, equipped with 1 mL Omnisys-F syringes from BRAUN (Ø 4.7 mm) and 100 Sterican needles (Ø 0.80 mm × 120 mm). After completion of H₂O₂ addition, the reaction was extracted (3 × 20 mL ethyl acetate). The combined organic phases were dried over Na₂SO₄, the solvent was evaporated under reduced pressure, and the product was purified by flash chromatography (cyclodexhane/ethyl acetate, 9:1). The reaction vessel and adjustment were identical to the conditions described above for 1c. The reaction mixture contained HspUPO (10 μM) and indole 6a (46.8 mg, directly dissolved in reaction buffer, \(c_{\text{final}} = 20 \text{ mM}\) in tricine buffer 100 mM, pH 7.5. The conversion was started by the continuous addition of in total 0.8 mL of a 550 mM H₂O₂ solution in a reaction buffer (800 μL/h over 1 h, \(c_{\text{final}} = 22 \text{ mM}\), 1.1 equiv). After completion of H₂O₂ addition, the reaction was stopped by the addition of 40 μL of catalase from M. lysodeikticus (170 000 U/mL) and 15 min of incubation at 500 rpm at room temperature to quench the excess H₂O₂. The reaction solution was then transferred to two 15 mL plastic tubes and centrifuged to separate the insoluble indigo from the aqueous phase. The supernatant was removed and analyzed as stated for 1 mL scale biotransformations. The remaining indigo pellet was washed 3 times with deionized H₂O to remove any buffer salt residues, and the wet pellet was then dried by lyophilization. The structure of both preparative-scale biotransformation products 1c and 6b was verified by NMR.

## RESULTS AND DISCUSSION

**Peroxynasate Candidate Screening and HspUPO Isolation.** To identify an efficient and versatile UPO, six

| Table 1. Screening of UPO Candidates |
|-------------------------------------|
| source of origin | accession code | sequence identity (%) with ActUPO | relative activity ABTS/naphthalene |
| A. aegerita | B9W4V6c | 100 | 100/100 |
| Aspergillus brasiliensis | OJ73116.1c | 35 | 200/68 |
| Hypoxylon sp. | OTAS433.1c | 30 | 277/53 |
| (HspUPO) | | | |
| Podospora anserina | XP_00191126.1c | 28 | 111/19 |
| Daldinia sp. | OTB15755.1c | 33 | 22/17 |
| Aspergillus niger | QAS41520.2 | 34 | 270/30 |
| A. niger | XP_001390900.2d | 33 | 301/33 |

*UPO from A. aegerita (PDB ID: 2YOR). Activity of 15 μL of cell-free supernatant using ABTS and naphthalene, relative to the enzyme from A. aegerita, which is used as a reference. UniProt database.

**Database at the National Center for Biotechnology Information.**
enzyme candidates of fungal origin were tested. They were selected to represent a sufficiently diverse panel of putative peroxygenases as predicted from the similarity of their amino acid sequences to the sequence of UPO from A. aegerita. The enzymes were expressed as secreted recombinant proteins using P. pastoris with satisfactory secretion levels (0.2 g/L). We probed the peroxidase and peroxygenase activities of the candidate enzymes using two potential substrates: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; peroxidase assay, pH 4.5) and naphthalene (peroxynase assay, pH 7). For both substrates, convenient spectrophotometric assays were employed. Although all UPO candidates exhibited activities when expressed on a 0.45 L scale (Table 1), it became clear that when performing the protein expression on a 5 L scale, only the enzyme from Hypoxylon sp. EC38 (HspUPO) fully retained its heme cofactor when purified, while all other enzymes mostly became inactive. Thus, HspUPO emerged as the logical candidate for further biochemical and structural studies (see Figure S1 for details about the purification procedures and yields).

### Steady-State Kinetics on HspUPO

In the early stages of the project, we observed that addition of indole and H2O2 to the secreted media immediately led to a blue color, suggesting that HspUPO converts indole to indigo. Therefore, we initially chose indole to study the steady-state kinetics of a reaction that involves substrate oxygenation (peroxynase activity). We further used ABTS to probe a substrate-oxidation reaction (peroxidase activity). HspUPO oxidizes efficiently ABTS ($k_{cat} = 16.94 \text{ s}^{-1}$), whereas a slower, yet pronounced, catalytic turnover was observed for indole ($k_{cat} = 0.79 \text{ s}^{-1}$). Both ABTS and indole exhibited remarkably low $K_M$ values of $\sim 30–50 \mu\text{M}$ so that their activities reached the plateau at about 150 $\mu\text{M}$ concentrations (Figure 1 and Table 2). Based on these initial promising observations, we expanded our analysis to other potential substrates: benzyl alcohol, 2,6-dimethoxyphenol, 3,4-dimethoxybenzyl alcohol, and 5-nitro-1,3-benzodioxole. For all of these compounds, we observed good activities with $k_{cat}$ values in the range of $4.56–11.0 \text{ s}^{-1}$ for benzyl alcohol, $7.15–11.0 \text{ s}^{-1}$ for 3,4-dimethoxybenzyl alcohol, and $10.4–11.0 \text{ s}^{-1}$ for 5-nitro-1,3-benzodioxole. Table 2. Steady-State Kinetic Parameter

| substrate                  | $k_{cat}$ ($\text{s}^{-1}$) | $K_M$ ($\mu\text{M}$) | $k_{cat}/K_M$ ($\text{s}^{-1} \mu\text{M}^{-1}$) |
|----------------------------|-----------------------------|------------------------|-----------------------------------------------|
| ABTS                      | 16.94 ± 0.73                | 30 ± 3.65              | 0.57                                          |
| indole                     | 0.79 ± 0.055                | 54 ± 8.16              | 0.015                                         |
| benzyl alcohol             | 4.56 ± 0.39                 | 680 ± 0.14             | 0.006                                         |
| 2,6-dimethoxyphenol        | 11 ± 0.17                   | 57.5 ± 5               | 0.191                                         |
| 3,4-dimethoxybenzyl alcohol| 7.15 ± 0.58                 | 758 ± 100              | 0.01                                          |
| 5-nitro-1,3-benzodioxole   | 10.4 ± 0.55                 | 18 ± 3.42              | 0.57                                          |

**Figure 1.** Biochemical characterization of HspUPO. Michaelis–Menten curves for six substrates. ABTS and indole were evaluated also using the deglycosylated HspUPO (dashed lines).

**Table 2. Steady-State Kinetic Parameter**
range of 3−12 s⁻¹ (Table 2 and Figure 1). In summary, HspUPO proved to be active on several diverse substrates.

**Operational Robustness of HspUPO.** Besides the advantage of bypassing the need for expensive and demanding electron donors, the H₂O₂ cosubstrate may come at a price: the potential oxidative damage inflicted to the protein and reactants used in the biotransformations. Therefore, we probed the capacity of HspUPO to withstand relatively high hydrogen peroxide concentrations for prolonged incubation times. We conducted these experiments employing HspUPO with ABTS as the substrate (Figure 2A). Rewardingly, the enzyme retained 75 and 40% of the activity in the presence of 5 mM and 10 mM H₂O₂, whereas the optimal activity was observed at 2 mM H₂O₂ \( (k_{cat} \sim 8 \text{ s}^{-1}) \). A similar pattern was reported for UPO from A. aegerita.¹⁹ Our data on the Hypoxylon sp. enzyme thereby confirm that UPOs are not severely affected by elevated hydrogen peroxide concentrations as needed for efficient biotransformations.

Hydrophobic substrates require a range of solvents for improving substrate availability in the aqueous phase. Thus, the solvent tolerance of native HspUPO was assessed toward several commonly used solvents: DMSO, acetone, and acetonitrile. DMSO was hardly tolerated by HspUPO when assayed with ABTS as the substrate. Indeed, the residual activity dropped to near 0% already with 2% (v/v) DMSO (Figure 2B, green solid line). To provide additional validation to this finding, we...
investigated the impact of DMSO on indole conversion (Figure 2B, green solid line). With this substrate, the tolerance to DMSO was slightly better as compared to the activity loss registered for ABTS. As observed for other peroxygenases (i.e., *A. aegerita* UPO), DMSO appears to be an inhibitor of Hsp UPO, likely by binding to the iron center. In general, the activity was retained as long as the DMSO percentage was no higher than 5%.

On the other hand, acetone and acetonitrile boosted the activity of HspUPO toward ABTS by up to twofold (2% acetone) and sixfold (5% acetonitrile), respectively (Figure 2B). We speculate that the less polar acetone and acetonitrile cosolvents facilitate binding of the charged ABTS and thereby accelerate its conversion. Likewise, indole conversions were significantly affected by acetone and acetonitrile only at cosolvent concentrations above 10% (>50% decrease; Figure 2B). In summary, both acetone and acetonitrile may be applied at 10–20% concentrations without exceedingly large decreases in the enzymatic activities.

In addition to the sensitivity to the chemicals employed in bioconversions, critical factors are the pH and temperature. We found that HspUPO is optimally active at neutral pH with indole, 5-nitro-1,3-benzodioxole, and benzyl alcohol, whereas more acidic pH values are optimal for the conversions of ABTS (pH 4), 3,4-dimethoxybenzyl alcohol (pH 4), and 2,6-dimethoxyphenol (pH 5) (Figure 3A). Such pronounced variations in the pH dependency have been observed before for other peroxidases and peroxygenases.

Table 3. Biocatalytic Oxidation of Various Chemical Functionalities with Purified HspUPO

| Substrate | Reaction | Product | Conversion (%) | m/z |
|-----------|----------|---------|---------------|-----|
| 1         | Hydroxylation | 1a | 39 | 557 |
| 2         | Alcohol oxidation | 2a, 2b | >99 | 1428 |
| 3         | Epoxidation | 3a | 45 | 642 |
| 4         | Indole oxidation | 4a, 4b | >99 | 1428 |
| 5         | Epoxylation | 5a | 71 | 1014 |
| 6         | | 6a | 75-96 | 1071-1371 |

*Conversions are based on substrate recovery with the exception of 1a and 3a, where the conversions are based on product formation due to volatility of the substrate. Reaction conditions are as follows: substrate 10 mM, acetonitrile 5%, HspUPO 7 μM, H2O2 20 mM (2 equiv, continuous addition), tricine buffer 100 mM pH 7.5, Vfinal = 1 mL, 30 °C, 500 rpm, 4 h (1 h for 6a). All experiments were performed in triplicates. *Product ratio is determined by the peak area or calibration with the commercial reference material on GC or HPLC. *For analysis of trace oxidation products (missing 15% in the product ratio), see the Supporting Information. *Conversion is displayed as a range since replicates deviate more than 10%.
Recombinant HspUPO is a highly glycosylated protein with a sugar contribution of 28 kDa to the final molecular weight of 55 kDa (27 kDa for the fully deglycosylated isoform). In light of this feature, it was of interest to understand if and how the glycosylations influence the enzyme properties. The enzymatically generated deglycosylated HspUPO showed steady-state kinetic parameters practically identical to those measured with the native fully glycosylated enzyme (Figures 1 and S1). However, deglycosylation clearly proved to negatively impact the resistance of the enzyme to thermal inactivation (Figure 3B). It is interesting to observe that deglycosylation had virtually no effect on the temperature of unfolding ($T_m$) of 76 °C for both the

### Table 4. Crystallographic Statistics

| HspUPO complex      | imidazole (7O1R) | 1-phenylimidazole (7O1X) | S-1,2-propanediol (7O1Z) | MES (7O2D) | styrene (7O2G) |
|---------------------|-----------------|--------------------------|--------------------------|------------|----------------|
| resolution range    | 48.2–1.3        | 48.0–1.6                 | 48.0–1.8                 | 29.8–2.6   | 52.6–2.1       |
| space group         | $P4_12_2$       | $P4_12_2$                | $P4_12_2$                | $P4_12_2$  | $P4_12_2$     |
| unit cell axes (Å)  | 71.7 153.7      | 71.5 153.2               | 71.4 152.6               | 73.1 154.1 | 71.9 154.5    |
| total reflections   | 2,556,180 (120,423) | 687,067 (35,537)        | 478,559 (28,188)        | 325,802 (42,673) | 302,461 (26,523) |
| unique reflections  | 99,264 (4837)   | 53,428 (2629)            | 37,610 (2196)            | 12,383 (1603) | 23,855 (1968) |
| resolution (Å)      | 48.0–1.3        | 48.0–1.6                 | 48.0–1.8                 | 29.8–2.6   | 52.6–2.1       |
| space group         | $P4_12_2$       | $P4_12_2$                | $P4_12_2$                | $P4_12_2$  | $P4_12_2$     |
| unit cell axes (Å)  | 71.7 153.7      | 71.5 153.2               | 71.4 152.6               | 73.1 154.1 | 71.9 154.5    |
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| unique reflections  | 99,264 (4837)   | 53,428 (2629)            | 37,610 (2196)            | 12,383 (1603) | 23,855 (1968) |

Values in parentheses are for reflections in the highest resolution shell. $R_{merge} = \sum |I_i - \langle I \rangle| / \sum I_i$, where $I_i$ is the intensity of the $i$th observation and $\langle I \rangle$ is the mean intensity of the reflection. The resolution cutoff was set to $CC_{1/2} > 0.3$, where $CC_{1/2}$ is the Pearson correlation coefficient of two "half" data sets, each derived by averaging half of the observations for a given reflection.

![Figure 4. Crystal structure of HspUPO in complex with imidazole.](A) Ribbon diagram of the overall conformation of HspUPO. Protein, heme carbon atoms, magnesium ion, and imidazole are colored, respectively, in cyan, maroon, lawn green, and orange. "N" and "C" outline the N- and C-termini, respectively. The C-terminal residues 251–261 are disordered. (B) The $\alpha$-helices $\alpha_C$, $\alpha_G$, and $\alpha_J$ shape the active site.](B)
The reactivity of ketones has been explored extensively, with values up to 99% and TON = 10,600 efficiency. This reactivity is with only traces of conversion to ethylbenzene to the secondary benzyl alcohols (ee values up to 99%) and TON = 10,600.

Enantiomers; alternatively, using complementary enzymes (mostly alcohol dehydrogenases) can be very attractive because biocatalysts oxidizing both functional groups were investigated under the optimal conditions as suggested by the above-described experiments (Table 3 and Supporting Information). Ethylbenzene, a common test substrate for hydroxylation, was regioselectively oxidized at the benzylic position to give the corresponding ketone, acetophenone. Additional reactions indicate that first C–H oxidation in the allylic position must have occurred prior to further oxidation. The oxidation of styrene was also performed on a 20 mL scale with increased substrate concentrations ranging from 75 to 96% (entry 7). The synthesis of HspUPO also has the potential to compare the productivity of these biocatalysts toward indigo production. For instance, the enzymatic preparation of indigo has been described in two patents, whereby an unspecific peroxidogen from Humicola insolens was able to give 72.4% indigo formation based on limiting cosubstrate H2O2 (2 mM) in a 100 mL reaction. HspUPO surpasses H. insolens UPO in terms of absolute indigo production while also displaying a lower byproduct formation. Nevertheless, it should be stated that the varying reaction setups and conditions make it difficult to reliably compare the productivity of these biocatalysts toward indigo production.

In summary, this first tapping of the potential of the catalyst clearly indicates that HspUPO can catalyze various reactions such as hydroxylation in the benzylic position, alcohol oxidation, and epoxidation with high efficiency. Thus, the protective role of glycosylation becomes manifested only upon long incubations and by probing the enzymatic activities rather than protein denaturation. Activities may sense multiple factors, such as the loss of the heme, and not only the global protein unfolding as interrogated by the thermal denaturation methods. In essence, protein glycosylation contributes to the operational robustness of the enzyme.

**Biotransformations.** To characterize the reaction potential of HspUPO, various substrates displaying a broad scope of functional groups were investigated under the optimal conditions as suggested by the above-described experiments (Table 3 and Supporting Information). Ethylbenzene, a common test substrate for hydroxylation, was regioselectively oxidized at the benzylic position to give the corresponding ketone, acetophenone. Indeed, when tested racemic phenylethanol rac-1b, the alcohol was oxidized with high efficiency, giving exclusively the ketone product 1c with >99% conversion (entry 2).

The oxidation of 1a was also performed on a semipreparative scale (20 mL) with increased substrate concentration (100 mM), yielding 210 mg of 1c (88% isolated yield, 92% GC-yield, TON = 13 143). Thus, HspUPO transforms both phenylethanol enantiomers efficiently. This is worth noting since the well-characterized A. aegerita UPO converts ethylbenzene to the secondary benzyl alcohols (ee values up to 99% and TON = 10 600) with only traces of α-ketones. The reactivity of HspUPO toward phenylethanol can be very attractive because biocatalysts oxidizing both enantiomers of a sec-alcohol are highly desired, e.g., in the amination of sec-alcohols. In various studies, two stereo-complementary enzymes (mostly alcohol dehydrogenases) had to be employed to ensure the oxidation of both enantiomers; thus, here, HspUPO may offer an alternative.

The benzylic primary alcohol 2a (veratryl alcohol) was oxidized to the corresponding carboxylic acid 2c as the main product, whereby at the conditions employed, significant amounts of aldehyde were also present (entry 3). The structures of minor side products formed were not elucidated. However, the masses indicate most likely demethylation of the substrate and dimerization. The ability of HspUPO to convert veratryl alcohol to the corresponding acid marks another substantial difference with A. aegerita UPO that mostly produces the aldehyde.

Turning to alkenes, cyclohexene 3a underwent epoxidation as the main reaction, giving cyclohexene oxide 3b as the dominant product in the product mixture (69%, entry 4). Additional products like the epoxy ketone 3d indicate that first C–H oxidation in the allylic position must have occurred prior to further oxidation. The oxidation of styrene 4a went to completion, leading to the corresponding (S)-epoxide (S)-4b (10% ee) as the main product (91%, entry 5). These numbers favorably compare with A. aegerita UPO, which was reported to convert up to 71% styrene, giving the epoxide with an ee of 7% and TON of 7900. Introducing a chloro-substituent in the para-position of styrene (substrate 5a) resulted in a switch of the enantiomer formed in excess by HspUPO, thus giving the (R)-epoxide with moderate ee (30%, entry 6).

Indole 6a can be considered as a substrate with high potential for application since it is a precursor to indigo. HspUPO oxidized 6a to indigo 6b within only 1 h of incubation and conversions ranging from 75 to 96% (entry 7). The synthesis of 6b was also performed on a 20 mL scale with increased substrate concentration (20 mM), yielding 51 mg of the isolated product (98%, TON = 2813). A comparison with other reports using unspecific peroxigenases shows that HspUPO is a very promising biocatalyst for indigo production. For instance, the enzymatic preparation of indigo has been described in two patents, whereby an unspecific peroxidogen from Humicola insolens was able to give 72.4% indigo formation based on limiting cosubstrate H2O2 (2 mM) in a 100 mL reaction. HspUPO surpasses H. insolens UPO in terms of absolute indigo product concentration while also displaying a lower byproduct formation. Nevertheless, it should be stated that the varying reaction setups and conditions make it difficult to reliably compare the productivity of these biocatalysts toward indigo production.

In summary, this first tapping of the potential of the catalyst clearly indicates that HspUPO can catalyze various reactions such as hydroxylation in the benzylic position, alcohol oxidation, and epoxidation with high efficiency. Side reactions indicate also the potential for hydroxylation in the allylic position as well as demethylation.

**Three-Dimensional Structure of HspUPO.** Knowledge of the three-dimensional structure became of interest to gain further insights into the substrate and reactivity profiles featured by HspUPO. Glycosylated proteins may be problematic for crystallization, and HspUPO was no exception as no crystals were obtained with the native enzyme. The hurdle was overcome by crystallizing the deglycosylated protein, which we found to be enzymatically active and sufficiently stable for biochemical analysis. Indeed, deglycosylated HspUPO (Figure S1) readily crystallized, enabling the structure determination of several ligand complexes of the heme-bound holoenzyme. All of the crystal structures were solved at high resolutions ranging from 1.3 Å to 2.5 Å (Table 4).
Overall, HspUPO can be described as a highly ordered and compact protein, whose many helices completely wrap the heme prosthetic group. In agreement with size-exclusion chromatography data (Figure S1), the analysis of the crystal packing indicates that the protein is monomeric. The HspUPO three-dimensional structure comprises 10 α-helices and two short β-strands, which we labeled following the current literature (αA: 40–48; αB: 60–71; αC: 75–88; αD: 99–103; αE: 129–138; αF: 146–163; αG: 171–188; αH: 198–207; αI: 212–214; β1: 143–145; β2: 195–197). Two molecules of N-acetylglucosamine were found to be linked to Asn133 and Asn161 (Figure 4A,B). The N-terminal residues 1–24 form the signal peptide and were predicted to be cleaved during the protein maturation process. Indeed, the first ordered amino acid is Ser25. A noticeable feature is the high degree of order displayed by the protein structure as a whole. All protein residues display clearly defined electron density. Only the C-terminal residues, spanning from 251 to 261, do not reveal any electron density owing to their flexible conformation.

The active site is a tube-shaped closed tunnel and is finely circumscribed by three α-helices (αC, αG, αJ) (Figures 4B and 5A). The iron atom of the heme sits at the dead-end of the tunnel. The prosthetic group is coordinated by the thiol side chain of Cys39, the axial ligand, and is firmly anchored to the protein through extensive interactions. A heme propionate group is bound to magnesium, which is further coordinated by two side chains (Glu109 and Ser113), a backbone nitrogen (His110), and two water molecules, defining an octahedral coordination sphere (Figure 5B).

Further active site analysis portrays three core side chains, His110, Glu180, and Phe176 above the Fe center (Figure 5C). Phe176 provides a steric block that coordinates and channels the substrate toward the iron atom of the heme, whereas the His110-Glu180 pair has been proposed to play a leading role in the catalytic cycle by promoting compound I formation (Scheme 1).

Based on sequence similarities, HspUPO belongs to the class I—so-called “short”—peroxygenases. Comparison of HspUPO with the structure of UPO from A. aegerita, a class II (long)
Figure 7. HspUPO active site in complex with (A) imidazole, (B) S-1,2-propanediol, (C) MES, (D) 1-phenylimidazole, and (E) styrene. Left panels: weighted 2Fo – Fc electron density contoured at 1.4σ. Right panels: two-dimensional schematic diagram of the interactions between ligands and the protein residues. Green lines indicate van der Waals contacts, whereas gray lines indicate metal coordination.

peroxynasenase, shows that the two enzymes share a similar overall structure, with a root-mean-square deviation of 2.1 Å for 201 Cα atoms (Figure 6A).

However, the comparison also reveals a few evident differences. The long (class II) A. aegerita UPO features a 70-residue α-helical C-terminal extension and a less compact structure compared to that of the short (class I) HspUPO. Moreover, the active-site shaping helices αC, αG, and αJ of HspUPO are shifted by about 3–4 Å and reoriented by ∼10° (Figure 6B). This rearrangement is coupled to several amino acid replacements, particularly on αC and αG. Although the hydrophobic nature of these amino acids is mostly conserved, the bulkiness of their side chains is not, as detailed in Figure 6C,D. This results in a generally narrower substrate-binding site in HspUPO. Another cluster of critical variations is found in the residues that directly surround the iron center above the heme. The catalytic acid—base pair (Arg189-Glu196) of A. aegerita UPO is replaced by Gly173 and Glu180 in HspUPO, whereas Gly123 of A. aegerita UPO is replaced by His110 in HspUPO (Figure 6E). As a result of these amino acid replacements, the His110 side chain of HspUPO spatially overlaps on the Arg189 side chain of the A. aegerita structure. This observation validates the prediction that in the short (class I) UPOs, the catalytic role of “charge stabilizer” is played by a class I-specific conserved histidine (His110 in HspUPO) rather than by an arginine, as found for the class II enzymes (Arg189 in A. aegerita UPO). In summary, besides an identical fold and similar overall conformations, the two peroxynasenes differ in the fine details of the active site, and these differences can have far-reaching implications in the modulation of their substrate scopes. The narrow shape of the HspUPO site may well explain its preferences for aromatic substrates with small substituents, as evidenced by our bioconversion experiments (Table 3).

Different Ligands and Same Protein Conformation.

The main aim of the structural analysis was to rationalize the substrate profile exhibited by HspUPO. Toward this aim, it was very helpful that crystals of deglycosylated HspUPO were found in many conditions and could be successfully used for data collection experiments. Inspection of the resulting electron densities revealed that various ligands were bound in proximity to the iron. Remarkably, the bulkiness and polarities of these ligands differ substantially, ranging from a small alcohol [(S)-1,2-propanediol] to a small heterocycle (imidazole) to a larger molecule [MES; 2-(N-morpholino)ethanesulfonic acid]. In the crystal structure of the imidazole complex (Figure 7A), a ligand nitrogen directly coordinates the heme iron. Similarly, (S)-1,2-propanediol and MES bind to the heme through their hydroxyl and ether oxygens, respectively (Figure 7B,C). In light of the preference for aromatic substrates exhibited by HspUPO, we then decided to further investigate the ligand acceptance by considering aromatic ligands. We first took advantage of the propensity of heme to bind imidazole by conducting a soaking experiment with 1-phenylimidazole (Figure 7D), a double-ring imidazole-substituted molecule. As with imidazole, 1-phenylimidazole was found to coordinate the iron center with its nitrogen. Moreover, its aromatic substituent interacts with Phe176 through an edge-to-face interaction whereby the two aromatic rings are oriented orthogonal to each other. We next solved the structure of the enzyme bound to styrene, possibly the most efficiently converted substrate (Table 3 and Figure 6E). Also in this case, the ligand sits on top of the heme with its ethyl side chain oriented toward the iron and the edge of its aromatic ring facing the edge of the Phe176 side chain. This binding mode is similar to that previously found in A. aegerita UPO and is fully compatible with catalysis leading to epoxidation styrene.

This set of enzyme–ligand structures corroborates the biotransformation experiments. All ligands are located in the same region at the bottom of the funnel-shaped tunnel leading to the heme in direct contact with the iron. The ligand–protein contacts comprise extensive van der Waals interactions involving the side chains that decorate the innermost segment of the tunnel. As shown by the 1,2-propanediol complex, HspUPO is able to bind alcohols as the hydroxyl group can orient itself...
toward the iron (Figure 7B). This is in line with the capacity of oxidizing several prim- and sec-alcohols (entries 2 and 3 of Table 3). The structure with MES reveals how a cyclic aliphatic compound can bind in the active site, providing a clue about the binding of a substrate such as cyclohexene, which is efficiently oxidized by HspUPO (entry 4 of Table 3). The binding of 1-phenylimidazole rationalizes the efficiency in indole conversion exhibited by HspUPO, as the two compounds have a similar steric hindrance and comprise a heterocyclic ring. Together with the 1-phenylimidazole complex, the structure with styrene shows how an aromatic ring can snugly fit at the bottom of the tunnel to establish several van der Waals interactions with the surrounding residues. A noticeable feature arising from these structural studies is that the binding of this rather diverse panel of ligands does not cause any conformational change in the active site. The only detectable variation is a slight, 0.1 Å, shift of the Phe176 side chain upon binding of 1-phenylimidazole. Moreover, none of the ligands forms hydrogen-bond interactions with the surrounding protein groups, as inspected with MAESTRO (Figure 7). In the absence of highly specific interactions, the bound substrates are unlikely to be anchored in a tightly restrained single orientation. This feature may explain the low stereo- and enantioselectivities of the reactions, as outlined by the conversions of alcohol and styrene compounds, respectively (entries 2, 5, and 6 of Table 3). Along these lines, it is interesting to notice that the surface of the tunnel has a double-face nature, which can be appreciated by looking at Figure 8. Along the entire 12 Å path, one side of the tunnel surface is mostly hydrophobic and populated by aromatic and aliphatic side chains, whereas the opposite side is more hydrophilic as it comprises hydrophilic side chains or main chain atoms (Figure 8C). Such a fine partition is evident also at the tunnel opening, where the side chains are arranged to form two semi-circles. One is mostly hydrophobic, whereas the other is more hydrophilic, with a prevalence of positively charged groups (Figure 8A,B). With its bipartite nature and funnel shape, the active site of HspUPO seems perfectly tailored for dealing with substrates that are not exceedingly hydrophobic and bulky or require a precise constellation of hydrogen-bonding groups to enable their recognition.

CONCLUSIONS

The increasing interest in UPOs stems from their ability to operate using hydrogen peroxide only as the cosubstrate without the need for electron donors or auxiliary electron-transporting subunits. After an initial testing of six candidates, the UPO from Hypoxylon sp. EC38 was identified as a suitable enzyme. HspUPO can be expressed in high yields as a secreted recombinant protein using P. pastoris. In addition to the ease of expression and purification, HspUPO can withstand widely used organic solvents such as acetone and acetonitrile. Moreover, the enzyme can easily operate at a temperature of 35 °C although operational stability at high temperatures will probably need to be further improved. We further notice that HspUPO can sustain catalysis at relatively high mM concentrations of hydrogen peroxide. This is the critical feature that makes peroxynases so attractive and distinguishes them from cytochrome P450s.

HspUPO is applicable for the transformation of various functional groups. Remarkably, HspUPO exhibits relatively low $K_M$ values, in the double-digit μM range, and yet, we could not detect substrate inhibition in steady-state assays. These reactivities can be framed in the context of the three-dimensional structure. HspUPO displays a characteristic funnel-shaped tunnel that leads from the surface to the heme. The enzyme has a well-ordered overall structure, and this type of rigid and compact conformation finds a counterpart in the absence of detectable conformational changes upon binding of diverse ligands. No specific hydrogen-bonding interactions are found in any of the five enzyme–ligand complexes that have been crystallographically characterized. With a bipartite nature that provides hydrophobic and hydrophilic groups for interactions,
the tunnel thereby functions as a molecular sieve, promoting the diffusion of small-to-medium-sized substrates that can reach to and react with the oxoferryl (compound 1) intermediate (Scheme 1). Oxygenation is therefore afforded by promoting the encounter between the activated oxygen of the oxoferryl and the substrate in the highly confined environment at the end of the catalytic tunnel, which is in line with the low \( K_M \) values. Such a combination of a rigid and structurally well-defined protein scaffold with an initial portion of known substrates highlights HspUPO as a biocatalyst with high potential for the development of designer peroxygenases targeting specific substrates.

**ASSOCIATED CONTENT**

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.1c03065.

Protein purification protocols, analytical methods, GC and HPLC traces (PDF)

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**Author Contributions**

L.R. performed all activity measurements, purifications, kinetics, and crystallographic studies. C.R. performed the cloning and initial expression and screening experiments. A.S. performed the biotransformations. K.E. carried out protein preparations. W.K., A.G., and A.M. designed and supervised the research. The manuscript was written with contributions from all authors.

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**Notes**

The authors declare no competing financial interest.

**ABBREVIATIONS**

UPO, unspecific peroxygenase; HspUPO, Hypoxylon sp. UPO; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

**REFERENCES**

(1) Newhouse, T.; Baran, P. S. H C-H Bonds Could Talk: Selective C-H Bond Oxidation. Angew. Chem., Int. Ed. 2011, 50, 3362–3374.

(2) Sheldon, R. A.; Norton, M. Green chemistry and the plastic pollution challenge: towards a circular economy. Green Chem. 2020, 6310–6322.

(3) Sheldon, R. A.; Woodley, J. M. Role of Biocatalysis in Sustainable Chemistry. Chem. Rev. 2018, 118, 801–838.

(4) Clomburg, J. M.; Crumbley, A. M.; Gonzalez, R. Industrial biomanufacturing: The future of chemical production. Science 2017, 355, No. aag0804.

(5) Li, Z.; van Beilen, J. B.; Duetz, W. A.; Schmid, A.; de Raad, A.; Griengl, H.; Witholt, B. Oxidative biotransformations using oxygenases. Curr. Opin. Chem. Biol. 2002, 6, 136–144.

(6) de Montellano, P. R. Oxygen Hydroxylation by Cytochrome P450 Enzymes. Chem. Rev. 2009, 110, 932–948.

(7) Krest, C. M.; Onderko, E. L.; Yosca, T. H.; Calixto, J. C.; Karp, R. F.; Livada, J.; Rittle, J.; Green, M. T. Reactive Intermediates in Cytochrome P450 Catalysis. J. Biol. Chem. 2013, 288, 17074–17081.

(8) Smith, G. C. M.; Tew, D. G.; Wolf, R. Dissection of NADPH-cytochrome P450 oxidoreductase into distinct functional domains. Proc. Natl. Acad. Sci. U.S.A. 1994, 91, 8710–8714.

(9) Holtmann, D.; Hoffmann, F. The Oxygen Dilemma: A Severe Challenge for the Application of Monooxygenases? ChemBioChem 2016, 17, 1391–1398.

(10) Holtmann, D.; Fraaije, M. W.; Arends, I. W.; Opperman, D. J.; Hoffmann, F. The taming of oxygen: biocatalytic oxyfunctionalisations. Chem. Commun. 2014, 50, 13180–13200.

(11) Park, H.; Park, G.; Jeon, W.; Ahn, J. O.; Yang, Y. H.; Choi, K. Y. Whole-cell biocatalysis using cytochrome P450 monooxygenases for biotransformation of sustainable bioresources (fatty acids, fatty alkanes, and aromatic amino acids). Biotechnol. Adv. 2020, 40, 107504.

(12) Hollmann, F.; Arends, I. W. C. E.; Buehler, K. Biocatalytic Redox Reactions for Organic Synthesis: Nonconventional Regeneration Methods. ChemCatChem 2010, 2, 762–782.

(13) Mifsud, M.; Gargiulo, S.; Iborra, S.; Arends, I. W. C. E.; Hollmann, F.; Corma, A. Photobiocatalytic chemistry of oxidoreductases using water as the electron donor. Nat. Commun. 2014, 5, 3145.

(14) Fujishiro, T.; Shoji, O.; Nagano, S.; Sugimoto, H.; Shiro, Y.; Watanabe, Y. Crystal Structure of H2O2-dependent Cytochrome P450SPOr with Its Bound Fatty Acid Substrate: insight into the regioselective hydroxylation of fatty acids at the α position. J. Biol. Chem. 2011, 286, 29941–29950.

(15) Girvan, H. M.; Poddar, H.; McLean, K. J.; Nelson, D. R.; Hollywood, K. A.; Levy, C. W.; Leys, D.; Munro, A. W. Structural and catalytic properties of the peroxygenase P450 enzyme CYP152K6 from Bacillus methanolicus. J. Inorg. Biochem. 2018, 188, 18–28.

(16) Pickl, M.; Kurakin, S.; Cantú Reinhard, F. G.; Schmid, P.; Pöcheim, A.; Winkler, C. K.; Krottul, W.; de Visser, S. P.; Faber, K. Mechanistic Studies of Fatty Acid Activation by CYP152 Peroxygenases Reveal Unexpected Desaturase Activity. ACS Catal. 2019, 9, 565–577.
(17) Yu, D.; Wang, J.-B.; Reet, M. T. Exploiting Designed Oxidase—Peroxxygenase Mutual Benefit System for Asymmetric Cascade Reactions. J. Am. Chem. Soc. 2019, 141, 5655–5658.

(18) Dunham, N. P.; Arnold, F. H. Nature's Machinery, Repurposed: Expanding the Repertoire of Iron-Dependent Oxigenases. ACS Catal 2020, 10, 12239–12255.

(19) Ullrich, R.; Nünke, J.; Scheibner, K.; Spantzel, J.; Hofrichter, M. Novel Haloperoxidase from the Agaric Basinidium Agrobacterium aegerita Oxidizes Aryl Alcohols and Aldehydes. Appl. Environ. Microbiol. 2004, 70, 4575–4581.

(20) Hofrichter, M.; Kellner, H.; Pecyna, M. J.; Ullrich, R. Fungal unspecific peroxgenases: heme-thiolate proteins that combine peroxidase and cytochrome p450 properties. Adv. Exp. Med. Biol. 2015, 851, 341–368.

(21) Püllmann, P.; Knorrtscheidt, A.; Munch, J.; Palme, P. R.; Hoehenwarter, W.; Marillonnet, S.; Alcalde, M.; Westermann, B.; Weissenborn, M. J. A modular two yeast species secretion system for the production and preparative application of unspecific peroxgenases. Commun. Biol. 2021, 4, No. 562.

(22) Gomez de Santos, P.; Cervantes, F. V.; Tieves, F.; Plou, F. J.; Hollmann, F.; Alcalde, M. Benchmarking of laboratory evolved unspecific peroxgenases for the synthesis of human drug metabolites. Tetrahedron 2019, 75, 1827–1831.

(23) Wang, Y.; Lan, D.; Durraní, R.; Hollmann, F. Peroxgenases en route to becoming dream catalysts. What are the opportunities and challenges? Curr. Opin. Chem. Biol. 2017, 37, 1–9.

(24) Shoji, O.; Watanabe, Y. Peroxygenase reactions catalyzed by cytochromes P450. J. Biol. Inorg. Chem. 2019, 14, 529–539.

(25) Piontek, K.; Strittmatter, E.; Ullrich, R.; Hofrichter, M.; Plattner, D. A. Structural Basis of Substrate Conversion in a New Aromatic Peroxygenase: Cytochrome P450 functionality with benefits. J. Biol. Chem. 2013, 288, 34767–34776.

(26) Ramirez-Escudero, M.; Molina-Espeja, P.; Gomez de Santos, P.; Hofrichter, M.; Sanz-Aparicio, J.; Alcalde, M. Structural Insights into the Substrate Promiscuity of a Laboratory-Evolved Peroxygenase. ACS Chem. Biol. 2018, 13, 3259–3268.

(27) Bordeaux, M.; Galameau, A.; Drone, J. Catalytic, Mild, and Selective Oxo-functionalization of Linear Alkanes: Current Challenges. Angew. Chem., Int. Ed 2012, 51, 10712–10723.

(28) Gutiérrez, A.; Babot, E. D.; Ullrich, R.; Hofrichter, M.; Martínez, A. T.; Del Rio, J. Regioselective oxidation of fatty acids, fatty alcohols and other aliphatic compounds by a basidiomycete heme-thiolate peroxidase. Arch. Biochem. Biophys. 2011, 514, 33–43.

(29) Kluge, M.; Ullrich, R.; Dolge, C.; Scheibner, K.; Hofrichter, M. Hydroxylation of naphthalene by aromatic peroxgenases from Agrobacterium aegerita proceeds via oxygen transfer from H2O2 and intermediary epoxidation. Appl. Microbiol. Biotechnol. 2009, 81, 1071–1076.

(30) Zhang, W.; Li, H.; Younes, H. H. S.; Gomez de Santos, P.; Tieves, F.; Grogan, G.; Palbst, M.; Alcalde, M.; Whitwood, A. C.; Hollmann, F. Biocatalytic Aromaticity-Breaking Epoxidation of Naphthalene and Nucleophilic Ring-Opening Reactions. ACS Catal. 2021, 11, 2644–2649.

(31) Molina-Espeja, P.; Canellas, M.; Plou, F. J.; Hofrichter, M.; Lucas, F.; Guallar, V.; Alcalde, M. Synthesis of 1-Naphthol by a Natural Hydroxylation of naphthalene by aromatic peroxygenase from Agrobacterium aegerita. ACS Catal. 2020, 10, 12239–12255.

(32) Kluge, M.; Ullrich, R.; Hofrichter, M. Benzene oxidation and oxygenation by the peroxygenase of Agrobacterium aegerita. AMB Express 2013, 3, No. S.

(33) Tonin, F.; Tieves, F.; Willot, S.; van Troost, A.; van Oosten, R.; Bresestraat, J. van, Pelt-S; Alcalde, M.; Hollmann, F. Pilot-Scale Production of Peroxygenase from Agrobacterium aegerita. Org Process Res Dev. 2021, 25, 1414–1418.

(34) Kluge, M.; Ullrich, R.; Scheibner, K.; Hofrichter, M. Stereoselective benzylic hydroxylation of alkybenzenes and epoxidation of styrene derivatives catalyzed by the peroxygenase of Agrobacterium aegerita. AMB Express 2013, 3, No. S.
(53) Fabara, A. N.; Fraaije, M. W. An overview of microbial indigo-forming enzymes. *Appl. Microbial. Biotechnol.* 2020, 104, 925–933.

(54) Kalum, L.; Lund, H.; Hofrichter, M.; Ullrich, R. Enzymatic Preparation of Indigo Dyes and Intermediates. WO2014122109A12014.

(55) Tovborg, M.; Hofrichter, M.; Poraj-Kobielska, M.; Lund, H. Enzymatic Preparation of Indigo Dyes and In Situ Dyeing Process. WO2018002379A22017.

(56) Kellner, H.; Luis, P.; Pecyna, M. J.; Barbi, F.; Kapturska, D.; Kruger, D.; Zak, D. R.; Marone, R.; Vadenbol, M.; Hofrichter, M. Widespread occurrence of expressed fungal secretory peroxidases in forest soils. *PLoS One* 2014, No. e95557.

(57) Bell, J. A.; Cao, Y.; Gunn, J. R.; Day, T.; Gallicchio, E.; Zhou, Z.; Levy, R.; Farid, R. PrimeX and the Schrödinger computational chemistry suite of programs. *Int. Tables Crystallogr.* 2012, 534–538.