Biocatalysis

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Specific Photobiocatalytic Oxyfunctionalization Reactions**

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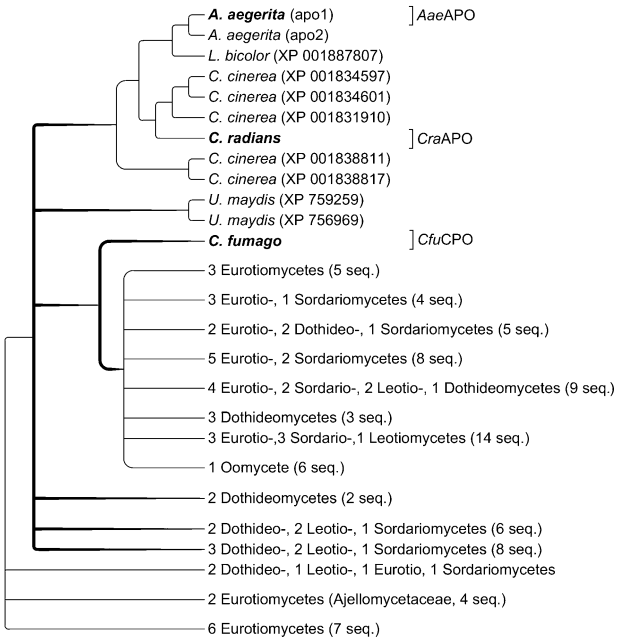
Specific oxyfunctionalization chemistry requires a delicate balance between reactivity and selectivity, and therefore still represents a major challenge in organic chemistry.[1] Cytochrome P450 monooxygenases (P450s) are widely considered as catalysts that solve this dilemma. Here, the reactive Compound I is embedded within a defined three-dimensional protein structure, thus enabling regio-, chemo-, and enantiospecific oxyfunctionalization reactions even on nonactivated hydrocarbons.[2] Their practical application, however, is limited to whole-cell biotransformations because of their cofactor dependency and their complex molecular architecture.[3] However, microbial transformations are not always straightforward to perform and suffer from intrinsic disadvantages such as reactant metabolization and toxicity, and often low productivities.

Great hope has been placed on the protein superfamily of heme/thiolate peroxidases to solve both challenges, the cofactor dependency as well as the complicated molecular architecture of P450s by using the hydrogen peroxide shunt.[4] Here, the catalytically active oxoferryl species is formed directly from H_2O_2 instead of from reductive activation of O_2. Seemingly, this allows simple circumvention of the abovementioned challenges and enables practical oxyfunctionalization procedures. Chloroperoxidase (CPO) from *Caldariomyces fumago* (*Leptoxyphium fumago*) represents the archetype of such heme/thiolate peroxidases.[4] In fact, thus far CPO is the only peroxidase used for oxyfunctionalization reactions.[4,5]

Unfortunately, CPO is active in sulfoxidation reactions only, whereas its performance in hydroxylation of C–H bonds and epoxidation reactions drops by several orders of magnitude.[4] Generally, CPO only performs a few hundred turnovers prior to loss of catalytic activity (see below), and nonactivated hydrocarbons such as cyclohexane are not converted at all by CPO.[4] Obviously, this disqualifies CPO as a broadly applicable catalyst for oxyfunctionalization chemistry. However, with more and more genome sequences becoming available, today, more than 100 putative heme/thiolate CPO analogues can be found in nucleotide databases (Scheme 1).[4]



Scheme 1. Simplified phylogenetic tree of the heme/thiolate superfamily. The names marked in bold refer to fungal species whose heme/thiolate enzymes have been purified and characterized. The other terms belong to taxonomic units of the fungal kingdom with putative CPO- or APO-like proteins found in databases.[4b]

Hence, there is a wealth of potential alternatives to CPO that are waiting to be discovered, and they potentially have more suitable catalytic properties for oxyfunctionalization.

Recently, a novel peroxidase from the basidomycetous fungus *Agrocybe aegerita* (*Aae*APO = *Agrocybe aegerita* aromatic peroxygenase) has been isolated and characterized.[6] *Aae*APO specifically converts aromatic hydrocarbons into the corresponding phenols and has recently been classified as an "unspecific peroxygenase" in the E.C. nomenclature (E.C.1.11.2.1).[7]

Herein we report that *Aae*APO is an active and versatile catalyst for enantiospecific hydroxylation and epoxidation reactions. Under nonoptimized reaction conditions *Aae*APO performance (in terms of turnover number) exceeds that reported for CPO by at least two orders of magnitude. Furthermore, we demonstrate that *Aae*APO, in contrast to CPO, can also hydroxylate nonactivated C–H bonds.

Since *Aae*APO, like all heme-dependent enzymes, is easily inactivated by H_2O_2, strict control over the in situ

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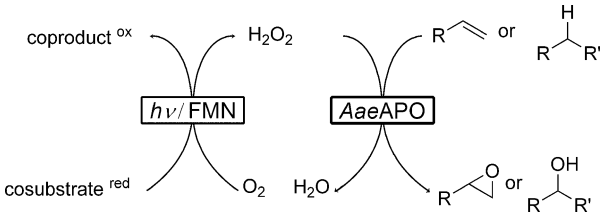
H_2O_2 concentration is essential. Therefore, we chose to produce H_2O_2 using our recently developed photochemical in situ H_2O_2 generation system.[8] Overall, a photobiocatalytic oxyfunctionalization system was designed (Scheme 2).



Scheme 2. Photobiocatalytic oxyfunctionalization.

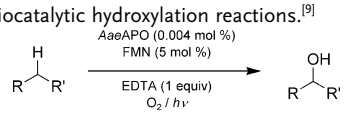
In a first set of experiments we investigated the *Aae*APO-catalyzed hydroxylation of ethylbenzene. We chose flavin adenine mononucleotide (FMN, 50 μM) as a photocatalyst. Unless stated otherwise, ethylenediaminetetraacetic acid (EDTA; 1 mM) served as a source for stoichiometric amounts of reducing equivalents (co-substrate). Upon irradiation with visible light the photoexcited flavin oxidizes EDTA, and the reduced flavin is then reoxidized by molecular oxygen (from ambient atmosphere), thus forming H_2O_2 which is utilized by *Aae*APO (40 nM) for oxyfunctionalization (Scheme 2).

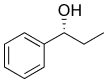
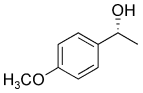
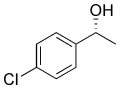
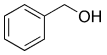
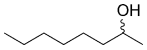
Rewardingly, a high degree of regio-, enantio-, and chemospecificity was observed: (*R*)-1-phenylethanol was obtained in greater than 97% optical purity (Table 1, entry 1) as the sole product. Additional oxidation to acetophenone was observed only after complete depletion of ethylbenzene. Notably, the second oxidation step proceeded at a significantly decreased rate (less than 5% of the initial hydroxylation rate; Table 1).

Compared to the use of stoichiometric amounts of H_2O_2, the photochemical generation of H_2O_2 in situ yielded somewhat reduced rates (ca. one third; Table 1, entry 2). This rate reduction was compensated for by the increased robustness of the enzymatic hydroxylation reaction: Whereas *Aae*APO lost more than 75% of its initial activity in the first 10 minutes when using stoichiometric amounts of H_2O_2, stable hydroxylation activity was observed for at least 5 hours with the in situ generated H_2O_2. Preliminary results using immobilized *Aae*APO in a two-liquid phase system indicated that the production period might easily be prolonged to several days and could attain more than 200000 turnovers for *Aae*APO (data not shown).

We additionally characterized the influence of each of the reaction components upon the rate of the photoenzymatic hydroxylation reaction. As expected, increasing the light intensity or the FMN concentration increased the overall reaction rate, whereas [EDTA] had only a negligible effect. This evidence supports the assumption that the in situ concentration of the photoexcited FMN is rate limiting overall. It should be noted that at a FMN concentration above approximately 0.05 mM no significant increase in the hydroxylation rate was observed.[9] This observation can be attributed to fast depletion of dissolved molecular oxygen. The phase transfer of O_2 into the reaction phase then becomes rate limiting overall. Reaction engineering measures overcoming this technical limitation of the present reaction setup are currently being implemented in our laboratory.

Table 1: Photobiocatalytic hydroxylation reactions.[9]



Entry	Product	TF [min^{-1}][a]	TN[b]	*ee* [%][c]
1		399 (11)	11470	>97
2[d]		1252 (138)	6000	97
3		270 (6)	6430	93
4		385 (18)	17260	90
5		303 (12)	11500	>99
6		366 (6)	17520	85
7		180 (15)	39200	–
8		182 (17)	17900	–
9		5.3 (6)	1040	n.d.

[a] Values are for *Aae*APO. Values in parentheses denote overoxidation rates observed upon complete conversion of the starting materials. [b] Values are for *Aae*APO. [c] Determined by chiral GC. Details are in the Supporting Information (Ref. [9]). The value is for that of the *R* enantiomer. [d] Results from the use of stoichiometric amounts of H_2O_2. n.d. = not determined, TF = turnover frequency, TN = turnover number.

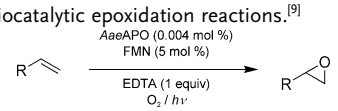
Like ethylbenzene, several derivates were also converted at similarly high rates and selectivities (Table 1).[9] In any case alkylbenzenes were converted smoothly at the benzylic position with good to excellent enantiospecificities (Table 1, entries 3–6). In the case of toluene (Table 1, entry 7), slow accumulation of the aldehyde product was observed upon full consumption of the starting material. Even more interestingly, nonactivated alkanes such as cyclohexane and *n*-octane were converted by *Aae*APO (Table 1, entries 8 and 9). Cyclohexane was hydroxylated at approximately 50% the rate of alkylbenzenes with a slightly increased relative overoxidation rate (ca. 9% of the initial hydroxylation rate), which again only occurred at full consumption of the initial starting material. In contrast, *n*-octane was converted rather sluggishly.

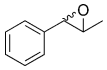
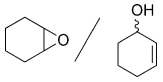
Under the nonoptimized reaction conditions we determined a TN of at least 30000 for *Aae*APO. In comparison, the TNs determined for CPO (under optimized reaction conditions) are generally 2–3 orders of magnitude lower. Substituting *Aae*APO with CPO in our hands did not yield any product formation. It should be mentioned here that we used CPO in the same concentration as *Aae*APO (40 nM) whereas the CPO-based epoxidation methods generally utilized more

than 100-fold higher CPO concentrations (1–10 μM). Furthermore, to the best of our knowledge, no CPO-catalyzed oxidation of nonactivated alkanes such as cyclohexane or *n*-octane has been reported yet.

Next, we evaluated the performance of the photoenzymatic system in epoxidation reactions. As model substances we chose styrene and its derivates. As shown in Table 2, there

Table 2: Photobiocatalytic epoxidation reactions.[9]



Entry	Product	TF [min^{-1}]	TN	*ee* [%][a]
1		92	10390	4.6
2		28	1830	40
3		< 2	< 500	4
4		228	4730	> 99 (2*S*,3*R*)
5		181	3970/1980	–/n.d.

[a] Determined by chiral GC. Details are in the Supporting Information (Ref. [9]).

was a distinct dependency of activity and selectivity upon the substitution pattern of the C=C bond. Terminal alkenes were converted at reasonable rates albeit with poor enantiospecificity (Table 2, entries 1 and 2). The *cis*-β-methyl styrene was converted efficiently while its *trans* isomer was converted at less than 1% of the rate of the *cis* isomer (Table 2, entries 3 and 4). It is worth mentioning here that styrene oxides were the only products detectable.

Aliphatic alkenes were converted less specifically. For example, cyclohexene was converted into the corresponding epoxide and allylic alcohol at a ratio of approximately 2 to 1 (Table 2, entry 5). Similarly, conversion of (*R*)-limonene and 1-octene yielded complex product mixtures (not shown). Currently, we can only speculate about the nature of these products, but allylic hydroxylation and/or epoxidation combined with hydrolysis products is likely to give rise to these (undesired) side products. Identification of these by-products is currently underway.

In contrast to CPO, *Aae*APO was not a very efficient sulfoxidation catalyst. When using thioanisol as the substrate, a TF(AaeAPO) of only 35 min^{-1} with concomitantly decreasing enantiospecificity (*ee* < 70%) was observed.

Finally, we evaluated some alternatives for EDTA as the sacrificial electron donor.[9] In contrast to CPO, the enantiospecificity of *Aae*APO was not impaired by substituting EDTA by, for example, formate.[9]

Overall, we have demonstrated that the peroxidase from *Agrocybe aegerita* (*Aae*APO) is a versatile oxyfunctionalization catalyst. Its performance in hydroxylation is unparalleled by chemical catalysts.[1] In epoxidation activity and selectivity strongly depend upon the substitution pattern of the C=C bond. Nevertheless, *Aae*APO appears to be an interesting complementary tool to, for example, the Jacobsen-type asymmetric epoxidation catalysts.[1f]

The in situ generation of H_2O_2 by photochemical reduction of O_2 proved to be beneficial for sustaining robust oxyfunctionalization activity over several hours. Under non-optimized reaction conditions the enzyme turnover numbers exceed those of comparable systems (P450s and CPO) by several orders of magnitude. Furthermore, preliminary results indicate that hundreds of thousands of turnovers can be achieved after reaction engineering. Therefore, we are convinced that *Aae*APO is a preparatively very valuable catalyst that might give a fresh impetus to biocatalytic oxyfunctionalization chemistry. Additional studies to substantiate *Aae*APO's full potential for preparative organic chemistry are currently underway. Also, it should be very interesting to additionally explore new peroxidases for organic chemistry (Scheme 1) and rationalize the structural basis for the striking difference in activity between CPO and *Aae*APO.

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