Biocatalysis

International Edition: DOI: 10.1002/anie.201902380
German Edition: DOI: 10.1002/ange.201902380

Formate Oxidase (FOx) from *Aspergillus oryzae*: One Catalyst Enables Diverse H_2O_2-Dependent Biocatalytic Oxidation Reactions

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Abstract: *An increasing number of biocatalytic oxidation reactions rely on H_2O_2 as a clean oxidant. The poor robustness of most enzymes towards H_2O_2, however, necessitates more efficient systems for in situ H_2O_2 generation. In analogy to the well-known formate dehydrogenase to promote NADH-dependent reactions, we here propose employing formate oxidase (FOx) to promote H_2O_2-dependent enzymatic oxidation reactions. Even under non-optimised conditions, high turnover numbers for coupled FOx/peroxygenase catalysis were achieved.*

Enzymatic oxidation and oxyfunctionalisation reactions are currently receiving tremendous interest in the context of preparative organic chemistry.[1] Especially if selectivity is desired, enzymatic reactions often excel over the chemical counterparts. Amongst available biocatalysts, monooxygenases are of particular interest.[2] Monooxygenases, however, rely on molecular oxygen, which is reductively activated at the enzyme active site. The reducing equivalents required are mostly derived more or less directly from reduced nicotinamide cofactors (NAD(P)H). While issues regarding the in situ regeneration of NAD(P)H have largely been solved, so that it can be used catalytic amounts,[3] the so-called oxygen dilemma poses a more severe challenge:[4] many monooxygenases cannot utilise NAD(P)H directly but depend on single-electron mediators to transform the hydride transfer from NAD(P)H into two successive single-electron transport events. The reduced mediators, however, also directly interact with dissolved molecular oxygen and are re-oxidised (Scheme 1 a). As a consequence, reactive oxygen species are formed in a futile cycle that uncouples the regeneration reaction from the oxygenation reaction. In extreme cases, up to 95 % of the reducing equivalents provided by the co-substrate are wasted.[4]

A viable solution of the challenge outlined above is to make use of H_2O_2-dependent "Peroxizymes".[5] By using H_2O_2, Peroxizymes actually make use of the oxygen dilemma instead of being hampered by it (Scheme 1 b).

A range of enzymes are able to use H_2O_2. Peroxidases for example, represent model enzymes for H_2O_2-dependent oxidation,[6] polymerization,[7] or halogenation reactions.[8] More recently, peroxygenases have received a lot of attention for selective oxyfunctionalisation reactions.[9] In addition to these reactions, hydrolase-catalysed formation of peracids for H_2O_2-driven epoxidation[10] and Baeyer–Villiger oxidations[11] are becoming popular.

Enzymes, however, are also prone to oxidative inactivation by H_2O_2[12] which is why a broad range of in situ H_2O_2 generation methods have been investigated in recent years (Table S2 compares some established systems with respect to efficiency and waste generation). The goal is to provide the production enzymes with H_2O_2 at rates that allow high catalytic turnover while minimising the undesired oxidative inactivation by excess H_2O_2.[13] Today, glucose oxidase (GOx) is the catalyst of choice for in situ H_2O_2 generation.[14] It couples the oxidation of glucose to the reductive activation of O_2 to form H_2O_2 in a highly efficient and robust fashion. The GOx system, however, suffers from high levels of waste generation (196 g of gluconate waste per mol H_2O_2 equivalent are generated).[15] Additionally, practical issues such as the high viscosity of the reaction medium have to be dealt with at larger reaction scales. Formate would be a more suitable reductant for the reductive activation of O_2 (generating only

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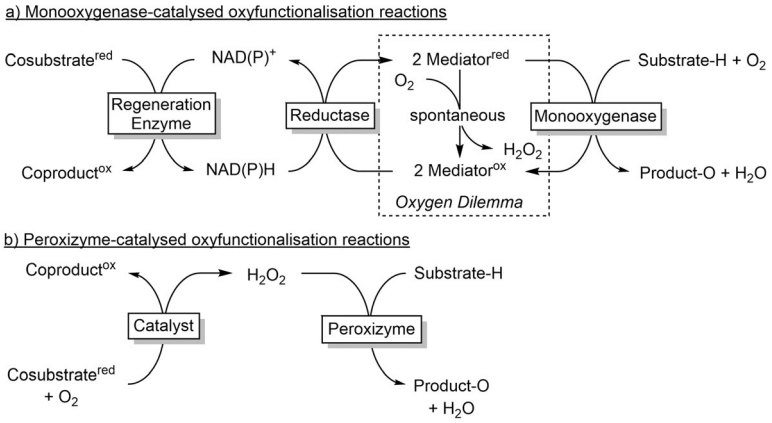
Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.201902380.



Scheme 1. Biocatalytic oxyfunctionalisations using monooxygenases (a) or peroxizymes (b). Monooxygenases often are prone to the oxygen dilemma while peroxizymes productively make use of the oxygen dilemma.

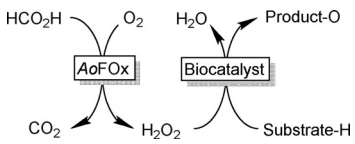
44 g of volatile and therefore not accumulating CO_2 waste per mol H_2O_2 equivalent). The systems available today, however, either rely on bioincompatible transition-metal catalysts,[16] or are too complex[15,17] or too elaborate[18] to be practical.

Recently, a formic acid oxidase from *Aspergillus oryzae* (*Ao*FOx) has been reported as the first member of the glucose-methanol-choline (GMC) oxidoreductase superfamily that oxidizes formic acid instead of simple alcohols.[19] This enzyme features an optimum pH range from 2.8–6.8 and a k_{cat} value of 82 s^{-1} over that range. It contains an unusual 8-formyl flavin adenine dinucleotide (FAD) cofactor, which is formed in situ from FAD through self-oxidation. Its unique catalytic properties render *Ao*FOx a promising candidate for H_2O_2-dependent enzymatic reactions. We therefore set out to evaluate the potential of *Ao*FOx as a catalyst to promote H_2O_2-dependent biocatalytic oxidation reactions (Scheme 2).

*Ao*FOx was prepared according to a previously published procedure.[19] In short, *Ao*FOx was expressed in recombinant *Escherichia coli* and partially purified to remove catalase. Overall, from 1 L culture broth, 38 mg of purified enzyme were obtained within 1 day (Figure S1 in the Supporting Information).

Having *Ao*FOx in hand, we decided to first apply this enzyme for some selective oxyfunctionalisation reactions catalysed by the recombinant evolved unspecific peroxygenase from *Agrocybe aegerita* (r*Aae*UPO) heterologously expressed in *Pichia pastoris*.[20] As model reaction, we first focused on the selective hydroxylation of ethyl benzene into (*R*)-1-phenylethanol. A preliminary optimisation of the reaction conditions (Figure 1) revealed that the bienzymatic cascade operates optimally in slightly acidic reaction media (pH 6, Figure 1a, Figure S2), which is in line with the reported preferences of the enzymes.[21] An apparent optimal temperature of 25 °C was determined (Figure 1b, Figure S3). Between 20 and 35 °C, the initial rates of the overall system were largely temperature-independent (Figure S2) but the reaction ceased sooner at elevated temperatures. At 40 °C for example, no further product formation was observed after 2 h. In contrast, steady product accumulation occurred at 30 °C or lower. This behaviour can be attributed to the comparably poor thermal robustness of wt-*Ao*FOx.

We determined an apparent optimal formate concentration of 200 mM (Figure 1d, Figure S4), which represents a compromise between the relatively high K_M value of wt-*Ao*FOx at this pH[19d–f] and the decreasing peroxygenative activity of UPOs at higher formate concentrations.[22]



Scheme 2. The formate oxidase from *Aspergillus oryzae* (*Ao*FOx) enables in situ H_2O_2 generation from formate and ambient oxygen to promote a broad range of biocatalytic oxidation/oxyfunctionalisation reactions.

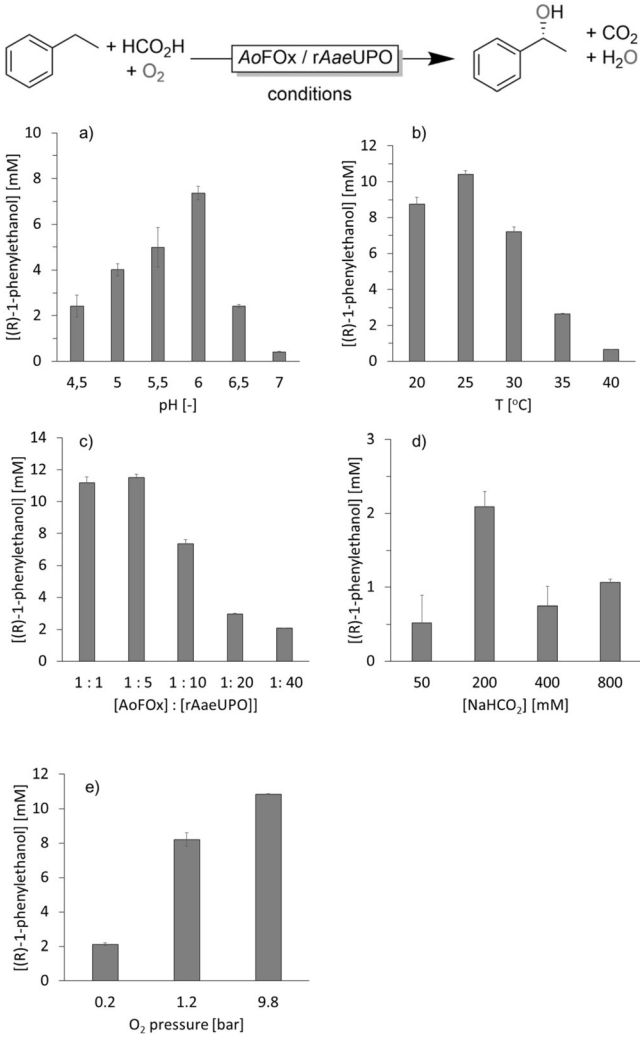


Figure 1. Characterisation of the reaction parameters that influence the efficiency of the bienzymatic hydroxylation of ethyl benzene. Individual reaction conditions are given in the captions of Figures S2–8.

The relative ratio of (H_2O_2-generating) *Ao*FOx and (H_2O_2-consuming) r*Aae*UPO had a very pronounced effect on the efficiency of the overall reaction system (Figure 1c, Figure S5). The highest initial rate was observed at an equimolar ratio of the two enzymes, albeit at the expense of poor long-term stability of the overall system (after 5 h, no further product formation was observed; Figure S5). Lower ratios of *Ao*FOx to r*Aae*UPO gave lower productivity but significantly greater robustness. At a ratio of 1:5, stable product formation for at least 24 h was observed.

The availability of molecular oxygen had a significant influence on the overall reaction (Figure 1e). Under ambient atmosphere without stirring, an O_2 transfer rate of 0.84 ± 0.03 mM h^{-1} was estimated (Figure S7), which limits the productivity of the overall system. Increasing the O_2 availability by increasing the O_2 partial pressure in the headspace of the reaction dramatically increased the productivity of the overall reaction more than ten-fold (Figure 1e, Figure S8).

It is worth mentioning that appropriate negative controls (i.e. reactions leaving out either one of the enzymes or reactions in the absence of formate) were performed for all of the reactions reported. With the sole exception of CytC-catalysed sulfoxidation, where traces of sulfoxide were also observed in the absence of CytC, the control reactions gave no product formation.

Next, we explored the enzyme and product scope of the *Ao*FOx-catalysed H_2O_2 generation system to promote various H_2O_2-dependent biocatalytic oxidation reactions (Figure 2).

First, we investigated some peroxygenase-catalysed hydroxylation and epoxidation reactions. The proposed H_2O_2-generation system enabled excellent catalytic performance of the peroxygenase used. Both product concentrations and r*Aae*UPO-turnover numbers were at least as high as for previous methods using more complicated H_2O_2 generation systems.[15,18,20d,23]

The stereospecific hydroxylation of ethyl benzene was performed on a semi-preparative scale, yielding 434 mg of (*R*)-1-phenylethanol (> 99% *ee*, see the Supporting Information for further details). A very satisfactory turnover number for the *Ao*FOx of more than 300000 was achieved, which suggests that this in situ H_2O_2 generation system is economically feasible. It is also worth mentioning that up to 31 ± 3 mM (*R*)-1-phenylethanol was produced (Figure S6), which is one of the highest numbers observed so far using *rAae*UPO.[15] It should be mentioned here that in case of volatile reagents, imperfect mass balances were observed upon prolonged reaction times. We believe that this is a technical issue that will be overcome in future scale-up experiments.

Cytochrome C (CytC), another heme-containing protein capable of catalysing H_2O_2-driven oxygen transfer reactions, especially sulfoxidation,[16] was evaluated next. Compared to the turnover numbers observed with r*Aae*UPO, the numbers achieved with CytC appear rather low. However, these numbers are still significantly higher than those achieved previously using other H_2O_2-generation systems.[16] The lack of enantioselectivity in the sulfoxidation of thioanisol is in accordance with previous reports.[16] It should be kept in mind here that the natural role of CytC is not that of an enzyme but rather that of an electron-transport protein.

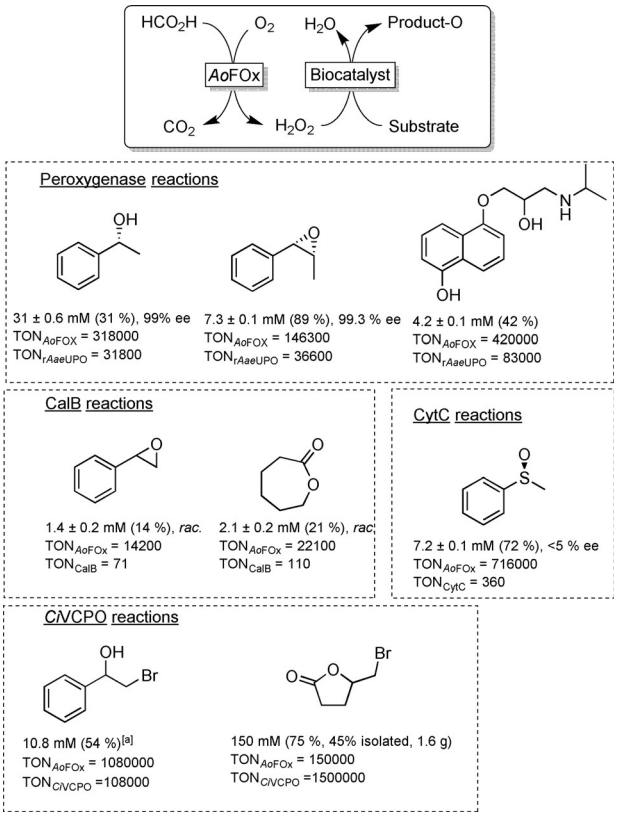


Figure 2. The scope of *Ao*FOx-driven peroxizyme reactions: The peroxygenase from *Agrocybe aegerita* (r*Aae*UPO) enabled selective hydroxylations and epoxidations; lipase B from *Candida antarctica* (CalB) mediated chemoenzymatic epoxidation and Baeyer–Villiger oxidations; cytochrome C (CytC, a heme-containing electron-transport protein) was applied to the sulfoxidation of thioanisole; and V-dependent chloroperoxidase from *Curcuvaria inaequalis* (*Ci*VCPO)-initiated hydroxyhalogenation and halolactonisation reactions. For details about the reaction schemes and experimental results, please refer to the respective section in the Supporting Information. Yields shown are calculated from the product concentration divided by the initial starting material concentration.

Another important H_2O_2-driven reaction is the so-called perhydrolase reaction of lipases.[1b,24] In short, a lipase catalyses the perhydrolysis of carboxylic (esters) to yield a reactive peracid, which in turn can undergo Baeyer–Villiger oxidations of ketones or Prilezhaev oxidations of C=C-double bonds. Our proposed *Ao*FOx H_2O_2-generation system proved to be applicable in principle to drive these reactions (Figure 2). Using the lipase B from *Candida antarctica*, CalB) together with octanoic acid as cocatalyst gave catalytic turnover in the chemoenzymatic Baeyer–Villiger oxidation of cyclohexanone as well as the chemoenzymatic epoxidation of styrene. However, compared to the other systems investigated here, rather low turnover numbers for the biocatalyst were observed. This can be attributed to the low affinity of CalB towards H_2O_2 in aqueous systems[25] resulting in low CalB activity under the conditions chosen. Further investigations aiming at higher in situ H_2O_2 concentrations are currently ongoing.

Finally, we evaluated *Ao*FOx to promote halogenation reactions catalysed by the V-dependent haloperoxidase from

Curvularia inaequalis.[8b,c,26] The hydroxyhalogenation of styrene gave acceptable results in terms of product yield and catalyst performance. Again, the volatility of the reagents impaired the final product concentration and thereby the catalytic numbers. A completely different picture evolved, however, when using 4-pentenoic acid as starting material. Here, a perfect mass balance was observed and full conversion of the starting material into the desired bromolactone was observed. We also scaled up this reaction to the gram scale: Starting from 200 mM 4-pentenoic acid, 150 mM of the desired bromolactone was obtained, which could be separated from the reaction mixture by simple extraction (Figure S14). Thus, 1.6 g of the pure product was obtained.[27]

In conclusion, we present herein the proof-of-concept for a simple H_2O_2 generation system based on formate oxidases such as the FOx from *Aspergillus oryzae* (*Ao*FOx). This system stands out in terms of practical simplicity and excellent performance, even at this early stage of development. Furthermore, the turnover numbers achieved with AoFOx exceed those of established systems by orders of magnitude (Table S2).

Three decades ago, the introduction of formate dehydrogenases as NADH regeneration catalysts ushered in a new era in bioreduction catalysis.[28] We are convinced that formate oxidases will have a similar impact for biooxidation/functionalisation catalysis. Further developments in our laboratories will focus on further engineering *Ao*FOx (in particular, a lower K_M value towards formate is highly desirable) and further characterisation and optimisation of the synthetic schemes to fully explore its synthetic potential.

Acknowledgements

The authors gratefully acknowledge funding by the European Research Commission (ERC consolidator grant, No. 648026), the European Union (H2020-BBI-PPP-2015-2-1-720297), the Netherlands Organisation for Scientific Research (VICI grant No. 724.014.003), the National Science Foundation (NSF) of the United States (grant IIP-1540017) and the Comunidad de Madrid Synergy CAM Project Y2018/BIO-4738-EVOCHIMERA-CM.

Conflict of interest

The authors declare no conflict of interest.

Keywords: biocatalysis · formate oxidase · hydrogen peroxide · oxidation · oxyfunctionalisation

How to cite: *Angew. Chem. Int. Ed.* **2019**, *58*, 7873 – 7877
Angew. Chem. **2019**, *131*, 7955 – 7959

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Manuscript received: February 22, 2019
Accepted manuscript online: April 4, 2019
Version of record online: April 30, 2019

