FOx News

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FOx News: Towards Methanol-driven Biocatalytic Oxyfunctionalisation Reactions

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The novel formate oxidase from *Aspergillus oryzae* (*Ao*FOx) is a useful catalyst to promote H_2O_2-dependent oxyfunctionalisation reactions. In this contribution we exploit the substrate promiscuity of *Ao*FOx to fully oxidise methanol and formaldehyde to CO_2 and drive peroxygenase-catalysed stereoselective oxyfunctionalisation reactions. The highly atom efficient H_2O_2 generation system also enabled high catalytic turnover of the peroxygenase production enzyme.

Selective oxyfunctionalisation of C–H-bonds is one of the most challenging reactions in organic chemistry. In recent years, peroxygenases have emerged as promising catalysts for this reaction enabling a broad range of regio- and stereoselective oxyfunctionalisation reactions.[1] Like the well-known cytochrome P450 monooxygenases[2] peroxygenases utilise a highly oxidised heme Fe-oxo complex (Compound I) to electrophilically insert an oxygen atom into the starting material. While P450 monooxygenases depend on molecular oxygen and rather complicated electron transport chains to attain the reductive activation of O_2 (yielding Compound I), peroxygenases use already reduced H_2O_2 for the same goal. Hence, peroxygenases appear as simpler, more easily applicable alternatives to P450 monooxygenases. However, such as all heme enzymes, peroxygenases are rapidly inactivated by H_2O_2, necessitating controlled provision with H_2O_2 to balance the H_2O_2-dependent catalytic activity and the (likewise H_2O_2-dependent) inactivation reaction.[3] *In situ* generation of H_2O_2 through catalytic reduction of O_2 is one of the most promising approaches, which, however, also necessitates a sacrificial co-substrate to provide the reducing equivalents needed for the reductive activation of O_2. Envisioning large-scale preparative applications, H_2O_2 generation systems producing no or innocuous wastes are preferred. Electrochemical generation of H_2O_2, for example, is an attractive means to drive peroxygenase reactions.[4] Also, simple reductants such as H_2[5] or H_2O[6] appear promising. Methanol would be a very suitable sacrificial electron donor as it is readily available, easy to handle and, in principle, can be fully oxidised to CO_2 to provide reducing equivalents for 3 equiv. H_2O_2 per equiv. MeOH.[7] Similarly, complete oxidation of methanol to regenerate reduced nicotinamide cofactors has been reported.[8]

Recently, we reported that the formate oxidase from *Aspergillus oryzae* (*Ao*FOx) is an efficient catalyst for the *in situ* generation of H_2O_2 to drive peroxygenase-catalysed oxyfunctionalisation reactions.[9] Encouraged by the very promising results, we originally aimed at a further characterisation of *Ao*FOx. To our surprise, while evaluating methanol as potential co-solvent, we found that *Ao*FOx also exhibited a methanol oxidase activity. We therefore further investigated *Ao*FOx to drive peroxygenase catalysed oxyfunctionalisation reactions using either methanol, formaldehyde or formic acid as sacrificial electron donor (Figure 1). As production enzyme we chose the recombinant, evolved peroxygenase from *Agrocybe aegerita* (r*Aae*UPO).[10]

Using 250 mM of either MeOH, HCHO or HCO_2H as sacrificial reductant, hydroxylation of ethyl benzene or cyclohexane as well as epoxidation of *cis*-ß-methyl styrene to (*R*)-1-phenyl ethanol, cyclohexanol and (1R,2S)-*cis*-ß-methyl styrene oxide, respectively, was observed (Figure 1). *Ao*FOx exhibited a low, but detectable methanol oxidase activity.

Interestingly, *Ao*FOx appeared to be promiscuous with respect to the oxidation state of the C1-substrate but accepted no other starting material such as ethanol or propanol (Supporting Information, Figure S8).

We suspected a poor affinity of wild-type *Ao*FOx for MeOH to account for the low product formation observed in these preliminary experiments (Figure 1). We therefore determined

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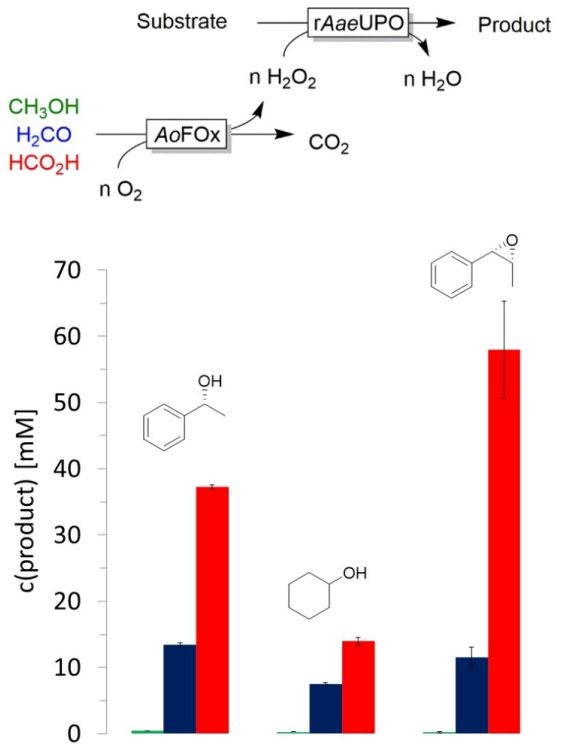

Figure 1. Comparison of r*Aae*UPO product formation when either 250 mM methanol, formaldehyde or formate is applied. Conditions: c(substrate) = 100 mM, c(r*Aae*UPO) = 1 μM, c(*Ao*FOx) = 1 μM, 25 °C, 100 mM KPi buffer (pH 6.0), 600 rpm, 24 h.

the kinetic parameters of *Ao*FOx for the oxidation of methanol and formaldehyde (Table 1).

Indeed, the catalytic performance of *Ao*FOx on formaldehyde and especially methanol falls back behind its natural formate oxidation activity. Instead of interpreting this as a disadvantage, we rather see the advantage of this relative activity as it, in principle, results in low *in situ* concentrations of harmful (HCHO) or acidifying (HCO_2H) intermediate oxidation states of the sacrificial cosubstrate. However, even the comparably low methanol oxidase activity under saturation conditions corresponded to a specific activity of 0.43 U mg^{-1}.

Robust and efficient reaction schemes involving peroxygenases need a balancing of the H_2O_2 generation reaction rate and the (H_2O_2-consuming) peroxygenase reaction rate. On the one hand, high H_2O_2-generation rates are desirable to attain high space-time-yield. On the other hand, accumulation of H_2O_2 must be avoided in order to minimise the oxidative inactivation of the prosthetic heme group. We therefore investigated the influence of the methanol concentration on the overall reaction system (Figure 2). As expected from the kinetic measurements (Table 1), increasing the methanol concentration increased the overall product formation (Figure 2A). A methanol concentration around 10 % (vol/vol, approx. 2.5 M) appeared optimal. We attribute the decreasing product formation at higher methanol concentrations to a decreasing long-term stability of either *Ao*FOx or r*Aae*UPO (or both) in aqueous methanol solutions. We therefore used a methanol concentration of 2.5 M for further experiments. Under these conditions, *Ao*FOx-catalysed H_2O_2 generation appeared to be overall rate-limiting, as up to at least 1000 nM *Ao*FOx (equimolar to r*Aae*UPO) the productivity of the system increased linearly with the increasing AoFOx concentration (Figure 2B, Figure S12). Higher productivities may be accessible upon further increase of the *Ao*FOx concentration. However, to avoid possible H_2O_2 accumulation, we continued with the more conservative equimolar ratio of *Ao*FOx and r*Aae*UPO.

As shown in Figure 3, robust oxyfunctionalisation of all three model starting materials over at least 5 days could be achieved using methanol as sacrificial electron donor. Very promising turnover numbers for r*Aae*UPO and *Ao*FOx of up to 49000 were observed (equimolar concentrations of biocatalysts used). The differences in the overall rates of cyclohexane hydroxylation, ethyl benzene hydroxylation and *cis*-β-methyl styrene epoxidation (0.05 mMh^{-1}, 0.23 mMh^{-1} and 0.32 mMh^{-1},

Table 1. Kinetic parameters for the *Ao*FOx-catalysed oxidation of methanol, formaldehyde and formic acid.

	CH_3OH oxidation	H_2CO oxidation	HCO_2H oxidation[11]
k_{cat} [s^{-1}]	0.46 ± 0.02	8.28 ± 0.12	82
K_M [mM]	3300 ± 400	380 ± 16	160

Experimental conditions: c(*Ao*FOx) = 42 nM, 25 °C, 50 mM KPi buffer (pH 6.0), c(O_2) = 0.25 mM (1 atm air), 10 U horseradish peroxidase, 1 mM ABTS (see SI for further details).

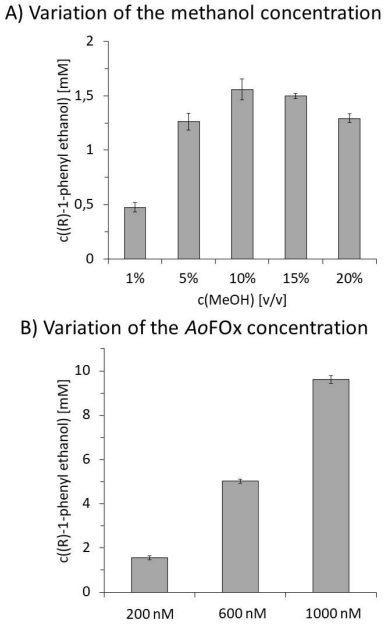

Figure 2. Influence of (A) methanol concentration on product formation over 24 h and (B) *Ao*FOx: r*Aae*UPO ratio on product formation rate. Conditions: 10 %(v/v) methanol, c(ethylbenzene) = 100 mM, c(r*Aae*UPO) = 1 μM, c(*Ao*FOx) = see x-axis, 25 °C, 100 mM Kpi buffer (pH 6.0), 600 rpm.

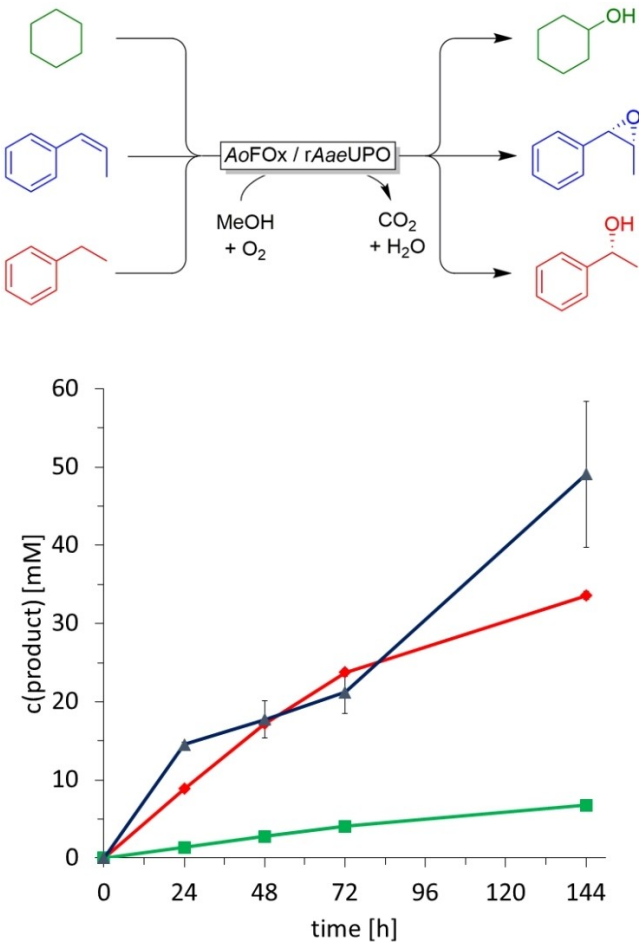

Figure 3. Time-courses of the selective oxyfunctionalisation of (●) ethyl benzene, (▲) *cis*-β-methylstyrene and (■) cyclohexane using the MeOH/*Ao*FOx/r*Aae*UPO cascade. Conditions: 10%(v/v) methanol, c(substrate) = 100 mM, c(r*Aae*UPO) = 1 μM, c(*Ao*FOx) = 1 μM, 25 °C, 100 mM KPi (pH 6.0), 600 rpm.

respectively) can be attributed to the relative activity of the peroxygenases for these substrates.[12]

Overall, with the current contribution we have demonstrated that methanol-driven peroxygenase reactions are principally possible using just one biocatalyst (*Ao*FOx), in addition to r*Aae*UPO. At present, the major limitation of this approach is the comparably high K_M value of the wild-type *Ao*FOx for MeOH, which may be addressable through protein engineering. Envisioning preparative scale applications, further issues are likely to occur such as the poor solubility of O_2 in aqueous media, which we will address by either using non-aqueous reaction media[13] and/or innovative reactor concepts such as the bubble column[14] or flow-chemistry[15] to attain higher k_L values for O_2.

Nevertheless, already at present stage, the catalytic performance of the biocatalysts with turnover numbers as high as 49000 for r*Aae*UPO and *Ao*FOx, respectively represent a good starting point for further characterisation and optimisation of the reaction system.

Experimental Section

Enzyme preparation. Recombinant expression and purification of the evolved unspecific peroxygenase mutant from *A. aegerita* in *P. pastoris* was performed following a previously described procedure.[10a] Formate oxidase from *Aspergillus oryzae* RIB40 (*Ao*FOx) was produced recombinantly in *E. coli* BL21(DE3) as reported before with slight modifications.[9] During desalting step with HiTrap, an additional buffer exchange was applied by using a phosphate potassium buffer (25 mM, pH 6.0) for elution of the target enzyme of the column. A final protein concentration of 2.16 ± 0.06 mg mL^{-1} was measured by BCA assay. *Ao*FOx purity of approximately 60% was determined by SDS-PAGE. With consideration of the extinction absorption at 472 nm,[16] a molar protein concentration of 21 μM was calculated.

Enzyme activity assay. *Ao*FOx kinetics were determined indirectly by (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ABTS assay. ABTS assay was performed with 50 mM of acetate buffer at pH 4.5 or phosphate buffer at pH 6.0, respectively. A final concentration of 1 mM of ABTS, 10 U mL^{-1} of HRP (horseradish peroxidase) and different concentration of methanol or formaldehyde were used. ABTS was either dissolved in 50 mM acetate buffer (pH 4.5) or 50 mM phosphate buffer (pH 6.0). HRP was stored in 25 mM phosphate buffer (pH 7.5). The reactions were measured with an UV-Vis spectrometer at 420 nm for 1 min at a controlled temperature of 25 °C. For calculation, an extinction coefficient of 36 000 M^{-1} cm^{-1} was used.

Enzymatic reaction. Reactions of 200 μL volume were performed in GC glass vials of 1.5 mL. Thermoshakers were used for temperature and stirring control. All components were mixed and the reactions started by the addition of either methanol, formaldehyde or formate. For each time point, a full reaction is extracted with ethyl acetate (equivolume) containing 5 mM n-octanol as internal standard. The extraction is then dried over $MgSO_4$ and analysed by gas chromatography (temperature profiles in supporting information). Further information for the negative control can be found in supporting information.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Biocatalysis · Formate oxidase · Methanol · Oxyfunctionalisation · Peroxygenases

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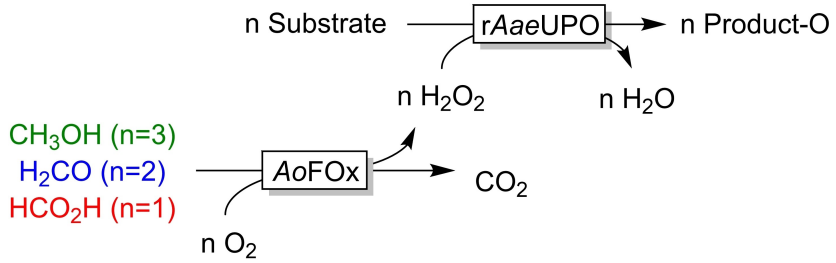

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