A chemo-enzymatic oxidation cascade to activate C–H bonds with in situ generated H2O2

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Continuous low-level supply or in situ generation of hydrogen peroxide (H2O2) is essential for the stability of unspecific peroxygenases, which are deemed ideal biocatalysts for the selective activation of C–H bonds. To envisage potential large scale applications of combined catalytic systems the reactions need to be simple, efficient and produce minimal by-products. We show that gold-palladium nanoparticles supported on TiO2 or carbon have sufficient activity at ambient temperature and pressure to generate H2O2 from H2 and O2 and supply the oxidant to the engineered unspecific heme-thiolate peroxygenase PaDa-I. This tandem catalyst combination facilitates efficient oxidation of a range of C–H bonds to hydroxylated products in one reaction vessel with only water as a by-product under conditions that could be easily scaled.
Effective oxidative CH-activation of hydrocarbon substrates remains a challenge for both heterogeneous catalysis and biotechnology. Many potential heterogeneous catalysts suffer from low regioselectivity and are prone to overoxidation of the substrate. For example, the highly optimized commercial oxidation of cyclohexane typically produces cyclohexanol and cyclohexanone, which can be utilized as feedstocks for the production of adipic acid and caprolactam, together with considerable amounts of overoxidation products. In order to prevent diminishing yield due to overoxidation, conversion of cyclohexane has to be kept as low as 6%, leading to poor space-time yields and high recycling efforts. The radical reaction pathway of this oxidation cannot be manipulated to produce the alcohol selectively.

A number of enzymes have been characterized that do not suffer these shortcomings. Among these are unspecific peroxygenases (UPOs), extracellular fungal heme-thiolate enzymes that only use hydrogen peroxide \( (\text{H}_2\text{O}_2) \) as both oxygen donor and final electron acceptor to generate the activated oxygen species at the active site \( (\text{reactive Compound I}) \) \(^8\). Peroxygenases have been described as ideal catalysts for CH-activation because they are relatively stable with high efficiency toward \( \text{H}_2\text{O}_2 \) and in addition do not, like P450 monoxygenases, require ancillary flavoproteins and cofactors that need regeneration. However, a limitation to scale up of UPO based chemical transformations is that they can be easily deactivated by modest concentrations of \( \text{H}_2\text{O}_2 \), thus requiring a constant controlled supply of \( \text{H}_2\text{O}_2 \) at low concentrations. This makes the in situ production of low (mM) levels of \( \text{H}_2\text{O}_2 \) practically the most efficient way to carry out the transformation without the need to add large volumes of prepared commercial \( \text{H}_2\text{O}_2 \) solutions, which typically contain 50–70 wt\% \( \text{H}_2\text{O}_2 \).

Accordingly, several systems have been developed to supply \( \text{H}_2\text{O}_2 \) to UPOs and the closely related chloroperoxidase from *Caldariomyces fumago* (CPO) used for sulfoxidation and hydroxylation of benzylcyclohexenes. However, to date no system has been simple and robust enough to allow large scale applications. The generation of \( \text{H}_2\text{O}_2 \) with enzymes such as glucose oxidase (GOX) requires co-substrates that are transformed to side products (in the case of glucose oxidase, gluconic acid, gluconic acid) and as such produces a complex reaction mixture which is problematic. The GOX system is very (atom-)inefficient as only two of a potential 24 electrons are used to generate one \( \text{H}_2\text{O}_2 \) equivalent. Additionally, the production of gluconic acid in the same reaction vessel as the UPOs necessitates continuous pH control to maintain the activity of the UPOs. A recently described formate oxidase, AoFOx from *Aspergillus oryzae*, which uses sodium formate as source of reducing equivalents to produce \( \text{H}_2\text{O}_2 \), promises a significant improvement on GOX in terms of side product production, but still requires acid titration to maintain a constant pH.

As an alternative, Hollmann and coworkers used the UPO from *Agrocybe aegerita* (AaeUPO) together with an oxidase/dehydrogenase cascade consisting of five enzymes to convert methanol to carbon dioxide, avoiding the accumulation of side products such as formic acid or formaldehyde from methanol oxidation. Although they obtained high total turnover numbers (TTNs) for the hydroxylation of ethylbenzene (294,700 mol mol\(^{-1}\)), the system still required the addition of nicotinamide adenine dinucleotide (NAD\(^+\)) as a cofactor, and a catalytic system requiring five different enzymes will only be economically feasible for production of high-value products due to the complexity of controlling the reaction and inhibition kinetics of five enzymatic processes operating in parallel. In the same direction, a biocatalytic cascade has recently been applied for the in situ generation of two equivalents of \( \text{H}_2\text{O}_2 \) from methanol, releasing formate as a byproduct, during the production of human drug metabolites, by an evolved AaeUPO mutant. The complex photobiocatalytic system Churakova et al. used with native AaeUPO for the hydroxylation of several substrates including ethylbenzene, cyclohexene and octane also still required flavin mononucleotide as a cofactor (FMN) and co-substrate (ethylenediaminetetraacetic acid) to supply reducing equivalents. Further recent papers by Hollmann and coworkers describe in situ \( \text{H}_2\text{O}_2 \) production by photocatalytic oxidation of methanol and water using various gold loaded TiO\(_2\) photocatalysts. When using recombinant AaeUPO (rAaeUPO) and methanol as electron donor they obtained TTNs in the order of 80,000 with ethylbenzene and 61,500 with cyclohexene. TTNs were about 50% lower when water was used as electron donor. However, the scale up of photocatalytic transformations is challenging and costly. Exposure of the enzyme to the photocatalyst was reported to cause significant loss in enzyme activity due to oxidative damage by \( \text{H}_2\text{O}_2 \) decomposition and reactive oxygen species being generated under light irradiation. This necessitated immobilization of the enzyme onto a solid carrier to minimize exposure to the photocatalytic TiO\(_2\) surface and increase lifetime. Horst et al. obtained a very high TTN of 400,000 for the hydroxylation of ethylbenzene with an electroenzymatic system using rAaeUPO and a gas-diffusion cathode that contained a \( \text{H}_2\text{O}_2 \) generating catalyst. This latter system demonstrated the potential of the UPOs for industrial application if \( \text{H}_2\text{O}_2 \) is generated in situ, although enzyme lifetime was linked to current density applied in the electrochemical cell, possibly due to the high \( \text{H}_2\text{O}_2 \) concentrations at the electrode surface resulting in a significant concentration gradient in the system and enzyme degradation due to localised high \( \text{H}_2\text{O}_2 \) concentrations.

A simple alternative to the enzymatic, photocatalytic and electrochemical methods described above would be to directly generate \( \text{H}_2\text{O}_2 \) from \( \text{H}_2 \) and \( \text{O}_2 \) with supported palladium (AuPd) heterogeneous catalysts, which have been shown to be able to achieve \( \text{H}_2 \) selectivity of >95% towards \( \text{H}_2\text{O}_2 \) making an atom efficient process possible and would represent an approach that does not require any significant re-design of current catalytic reactors to include light or electrical energy input. Continuous \( \text{H}_2\text{O}_2 \) generation from \( \text{H}_2 \) and \( \text{O}_2 \) by AuPd catalysts generally requires high pressures and subambient temperatures and often also the addition of acid or halide promoters, conditions that are not ideal for enzymes. Hence, a reaction conditions gap exists to couple the heterogeneous catalytic system with the current operational window of UPOs. Indeed, for \( \text{H}_2\text{O}_2 \) synthesis, temperatures are typically subambient (e.g. 2 °C), gas pressures are high (40 bar), and \( \text{pH} \) is low (<3), whereas for UPO activity, preferred conditions are ambient temperature and pressure and \( \text{pH} \) 6–7. Karmee et al. have nevertheless demonstrated the sulfoxidation of thioanisole by CPO in reactions using Pd/C for \( \text{H}_2\text{O}_2 \) generation at high pressure (130 bar) and 40 °C in supercritical carbon dioxide/water, which relies on setting up a biphasic scCO\(_2/\text{H}_2\text{O}\) phase separating the \( \text{H}_2\text{O}_2 \) synthesis, which occurs in the scCO\(_2\) phase and the biocatalysis, which occurs in the aqueous phase.

We recently demonstrated AuPd bimetallic catalysts can produce \( \text{H}_2\text{O}_2 \) in water at ambient temperature and 40 bar working pressure. The question arose whether this class of catalysts can be used to produce \( \text{H}_2\text{O}_2 \) to cross the conditions gap for a UPO catalyzed reaction at both ambient temperature and low pressure in phosphate buffered solution. Compared to the other solutions of in situ \( \text{H}_2\text{O}_2 \) formation this approach would provide an atom efficient reaction, using \( \text{H}_2 \) as the reductant rather than glucose in the case of the GOX approach resulting in much simpler reaction mixtures - as water is the only byproduct - requiring no extra \( \text{pH} \) control due to the presence of byproducts of other enzymatic cascade processes. In contrast to photocatalytic methods using...
Results

Compatibility of the heterogeneous and enzyme catalyst. Initial control reactions using GOX to generate H₂O₂ demonstrated that cyclohexene hydroxylation reactions to produce cyclohexanol could be carried out either using shaking or stirring to facilitate mixing. In addition, these reactions showed that the 0.5 wt% Au–0.5 wt% Pd/TiO₂ (1 mg ml⁻¹) catalyst had no negative effect on the PaDa-I reaction when GOX was used to produce H₂O₂ (Fig. 2a); demonstrating that the PaDa-I could still operate in the presence of the metal catalyst. Considering H₂O₂ production requires H₂ and O₂ mixtures, which are combustible between H₂ concentrations of 5 and 75%, H₂O₂ synthesis experiments were carried out in an open system by flowing 50 ml min⁻¹ of gas (2%, 80%, 90% H₂ in air) through an aqueous solution of metal catalyst (0.1 mg ml⁻¹). With 80% H₂ in air, a peak concentration of 25 ppm H₂O₂ (0.74 mM) was produced (Supplementary Fig. 1). The effect of the catalyst concentration was also investigated between 0.1 and 0.6 mg ml⁻¹ (Fig. 2b). At higher catalyst loadings, the maximum amount of H₂O₂ generated decreased while at lower loadings, the peak concentration was higher and the system was capable of producing a sustained concentration of H₂O₂. These results were promising, given that Molina-Espeja et al. had reported the Michaelis constant (Kₘ) of PaDa-I for H₂O₂ at pH 7 in 100 mM potassium phosphate buffer as 0.49 mM (17 ppm) when it was produced by Saccharomyces cerevisiae and 1.53 mM (53 ppm) when produced by Pichia pastoris. Thus, concentrations of 1 or 2 mM H₂O₂ (34 or 68 ppm) were used successfully in enzyme activity experiments. These concentrations compared with our results suggested that the AuPd catalyst would be active enough at ambient conditions to supply H₂O₂ in order for the PaDa-I to carry out the oxidation of cyclohexene at significantly lower precious metal catalyst loadings than previous studies.

System optimisation and substrate scope. Due to the volatile nature of substrates such as cyclohexane, a flowing system with gas bubbling is not optimal and relies on constant substrate addition to prevent complete depletion of the substrate. Following the feasibility experiments showing that combining the two catalytic systems was possible, reactions were carried out in sealed pressurized glassware with a range of substrates. Into the reactor was placed the substrate (10 mM), and the tandem catalyst system formed by the heterogeneous catalyst and the biocatalyst in buffered reaction media. All reactions were carried out with the same initial substrate and enzyme concentration limiting the TTN to ~30,000 in these studies. The system was pressurized to 2 bar under a pre-mixed gas mixture of 80% H₂ and 20% air and stirred at 250 rpm. Under these conditions H₂O₂ was produced over time (Fig. 3a) in a similar range 15–25 ppm to the flowing system over extended reaction times despite the lack of gas bubbling. The results in Fig. 3a demonstrate that under these conditions cyclohexene hydroxylation can be carried out to produce cyclohexanol with high yields (87%) and TTN of 25,300 with negligible overoxidation to cyclohexanone indicating that the metal H₂O₂ producing catalyst is not facilitating further overoxidation reactions as observed in our previous experiments using the gas flow reactor.

The ability of biocatalysts to carry out chiral transformations is a major advantage over traditional heterogeneous systems. The hydroxylation of ethylbenzene was carried out over extended...
times with near full conversion being reached at 16 h (Fig. 3b). While in this case some overoxidation was observed when the reaction had consumed the majority of ethylbenzene, again a high TTN was observed (25,900) at 12 h with near complete selectivity to 1-phenylethanol. Chiral analysis of the 1-phenylethanol produced showed an e.e. of ~98% towards R-1-phenylethanol at 16 h, which is comparable to reported values concerning the peroxygenase alone indicating that the presence of the metal catalyst does not affect the stereoselectivity of the PaDa-I hydroxylation reactions (Supplementary Fig. 3)19. To demonstrate the possibility of achieving higher TTN a reaction was carried out with 20 mM starting concentration for 16 h resulting in 13.1 mM of 1-phenyl ethanol and 2.9 mM of acetophenone at a TTN of 51,400. To test the system at extended reaction times an additional reaction was carried out with an initial concentration of 30 mM ethylbenzene at the same metal and enzyme catalyst loadings. After an initial 16 h of reaction, a further 30 mM of substrate was added and gas re-charged and this was repeated after another 24 h - resulting in 90 mM of substrate addition over 64 h. The system was able to produce 46 mM of 1-phenyl ethanol and 14 mM of acetophenone - achieving a TTN of 201,000 showing that this is among the most efficient tandem heterogeneous H2O2 delivery—UPO systems reported to date.

The selective functionalization of isophorone provides a route to synthetic precursors of both fragrance molecules and the building blocks of carotenoids and tocopherols (Vitamin E)32,33. α-Isophorone is produced industrially on a large scale and selective hydroxylation to 4-hydroxyisophorone is a target transformation to produce ketoisophorone without the industrially executed detour of first isomerizing to β-isophorone34–36. The direct transformations have been reported using both homogeneous metal chlorides as catalysts at 80 °C in the presence of 80% H2 in air. Reaction Conditions: Solvent (H2O) 50 ml, ambient temperature and pressure, gas flow 30 ml min⁻¹, 500 rpm stirring. Errors associated in the measurement of H2O2 by titration were ± 3 ppm. Cyclohexane conversion to cyclohexanol using PaDa-I (15 U ml⁻¹) in potassium phosphate buffer (100 mM, pH 6) and H2O2 generated in situ by metal catalysts. Efficiency of different AuPd catalysts (0.5 mg ml⁻¹) in the presence of 77% H2 in air.

**Fig. 2 Feasibility of Coupling Catalytic Systems.** a Cyclohexanol production in control reactions to determine the effect of the metal catalyst on unspecific peroxygenase activity when H2O2 is generated using GOX. b H2O2 synthesis with various catalyst concentrations under flowing (30 ml min⁻¹) 80% H2 in air. Reaction Conditions: Solvent (H2O) 50 ml, ambient temperature and pressure, gas flow 30 ml min⁻¹, 500 rpm stirring. Errors associated in the measurement of H2O2 by titration were ± 3 ppm. c Cyclohexane conversion to cyclohexanol using PaDa-I (15 U ml⁻¹) in potassium phosphate buffer (100 mM, pH 6) and H2O2 generated in situ by metal catalysts. Efficiency of different AuPd catalysts (0.5 mg ml⁻¹) in the presence of 77% H2 in air. d Time course results for cyclohexanol formation by PaDa-I (15 U ml⁻¹) was coupled with in situ H2O2 generation by 2.5% Au-2.5% Pd/TiO2 (0.1 mg ml⁻¹) using a gas mixture of 77% H2 in air (solid diamonds) or by GOX (0.2 U ml⁻¹) using 200 mM glucose and air (circles). The GOX reactions were carried out using the same experimental setup as the metal catalyst reactions. Typically analysis of products was ± 0.2 mM as determined by GC (on duplicate experiments).
of radical initiators and biocatalytic transformations based on P450s requiring cofactors\textsuperscript{37,38}. As a proof of principle using our tandem catalyst system we were able to drive the hydroxylation of isophorone (30 mM) to produce a mixture of 4- and 7-hydroxyisophorone in roughly equal amounts over a 6 h period at a combined yield of 75\% (Fig. 3c). The unselective nature of the hydroxylation is inherent to the selectivity of the enzyme but acts as a demonstration that this transformation could be possible with a more selective peroxigenase, which could be engineered by new rounds of laboratory evolution. In all cases cyclohexane, ethylbenzene and isophorone reactions containing the metal catalyst producing H\textsubscript{2}O\textsubscript{2} without the presence of the PdA-I did not produce any hydroxylated products resulting from C–H activation as a result of the heterogeneous catalyst or the presence of H\textsubscript{2}O\textsubscript{2} alone (Supplementary Fig. 4). In addition, the activity of the metal catalyst producing H\textsubscript{2}O\textsubscript{2} alone towards the oxidation of cyclohexanol and 1-phenylethanol to the respective ketones was minimal (Supplementary Fig. 5).

The substrate scope was increased to include a wide range of C–H functionalization and is shown in Table 1 for a series of unoptimized reactions. Again, in the case of tetralin and propylbenzene negligible C–H activation was observed in the presence of the metal catalyst generating H\textsubscript{2}O\textsubscript{2} without the PdA-I (Supplementary Fig. 6). Oxygenated products were produced by the tandem catalyst system for this wide range of substrates and with high enantiomeric excess (>99\%), (Supplementary Fig. 7) typical of the reaction previously carried out with the PdA-I alone, demonstrating that the system does not change the specificity of the biocatalyst but simply provides a continuous feed of low-level H\textsubscript{2}O\textsubscript{2} to facilitate clean hydroxylation. Reactions of styrene with the tandem catalysis system act as a demonstration of more complicated cascades that can be built by combing the two catalyst systems (Supplementary Fig. 8a). The AuPd catalyst used has no activity towards styrene epoxidation but is able to reduce styrene to ethylbenzene at these conditions (Supplementary Fig. 8b) leading to R-1-phenylethanol production with high enantiomeric excess (Supplementary Fig. 8c) through enzymatic hydroxylation along with styrene oxide. This reaction network of coupled C=C bond reduction and subsequent hydroxylation could be exploited to produce chiral alcohols from alkenes or mixtures of epoxides and chiral alcohols with proportions controlled by the design of the heterogeneous catalyst or reaction conditions. As with many process involving H\textsubscript{2}O\textsubscript{2} in situ reactions H\textsubscript{2} selectivity is challenging. The reported cyclohexane hydroxylation H\textsubscript{2} to cyclohexanol selectivity is of the order of 10\% (Supplementary Discussion 1); however, we have previously shown that high selectivity can be engineered through effective catalyst design\textsuperscript{30,39} and detailed understanding of the reaction kinetics and will be a key feature of any process intensification.

The results reported here demonstrate the compatibility of AuPd chemo catalysts with the recently discovered UPOs to be
used for hydroxylation of unactivated sp\(^3\) carbons through the in situ generation of H\(_2\)O\(_2\). In this case we have demonstrated the potential for reactions that are operated commercially at a range of scales from commodity to fine chemicals. We consider that this chemo-enzymatic approach has potential for a wide range of applications in tandem reactions due to the ease of adaptation into current reactor technology and infrastructure, lower energy input as there is no requirement for illumination compared to recent photocatalytic approaches and inherent efficiency of the process in only producing water as a byproduct without the requirement for organic molecules such as MeOH as a supply of electrons. The nature of the catalyst materials as both effective hydrogenation and oxidation catalysts significantly extends the scope of the reaction cascades that can be considered and can open the pathways to efficient tandem heterogeneous/biocatalytic processes.

**Methods**

**Catalyst preparation.** A typical preparation of 1% AuPd/TiO\(_2\) was carried out according to the following procedure\(^{29}\). The requisite amounts of HAuCl\(_4\)·3H\(_2\)O solution (Sigma–Aldrich, 12.25 mg ml\(^{-1}\)) and PdCl\(_2\)·HCl solution (Sigma–Aldrich, 6 mg ml\(^{-1}\); HCl concentration: 0.58 M) were mixed in a 50 ml round-bottom flask, the volume of the solution was adjusted using deionized water to a total volume of 16 ml and immersed into an oil bath on a magnetic stirrer hot plate. The solution was stirred at 1000 rpm and the temperature of the oil bath was raised from room temperature to 60 °C over a period of 10 min. At 60 °C, metal oxide support material [1.98 g TiO\(_2\) (Degussa Evonik P25)] was added slowly over a period of 8–10 min with constant stirring. The subsequent slurry was stirred at 60 °C for an additional 15 min. Following this, the temperature of the oil bath was raised to 95 °C for 16 h leaving a dry solid. The solid powder was ground thoroughly. 400 mg of the sample was reduced at 10 °C min\(^{-1}\) under a steady flow of gas (5% H\(_2\)/Ar). 2.5% Au-2.5% Pd/TiO\(_2\), 2.5% Au-2.5% Pd/C and 5% Au/C were prepared by wet impregnation methods followed by calcination at 400 °C for 3 h.

**Unspecific peroxygenase preparation.** Evolved AueUPO (PaDa-I variant) designed in *Saichromyces cerevisiae* was over produced in *Pichia pastoris* in a bioreactor and purified to homogeneity (Reinheitszahl value [Rz] \(\frac{A_{418}}{A_{280}}\) \sim 2.4)\(^{27,28}\). Enzyme activities was determined using ABTS as substrate. Reactions were done in triplicate. 20 \(\mu\)l PaDa-I was added to 180 \(\mu\)l ABTS reaction mixture (100 mM sodium citrate–phosphate pH 4.4 with 0.3 mM ABTS and 2 mM H\(_2\)O\(_2\)) and substrate conversion was followed by measuring the absorption at 418 nm (\(\epsilon = 418 \times 36000 \text{ M}^{-1} \text{ cm}^{-1}\)). The PaDa-I concentration was appropriately diluted to give rise to linear enzyme kinetics. One unit is defined as the amount of enzyme that convert 1 pmol of substrate in 1 min.

**H\(_2\)O\(_2\) synthesis testing in flowing system.** H\(_2\)O\(_2\) synthesis activity was evaluated at ambient temperature and pressure using a 200 ml dreschel bottle. 50 ml 0.1 M potassium phosphate buffer pH 6 was added containing catalyst concentrations ranging from 0.1 to 1 mg ml\(^{-1}\). During stirring using a magnetic stirrer, H\(_2\) in air mixtures were bubbled (30 ml min\(^{-1}\)) through the bottle at compositions ranging from 2 to 90% by vol H\(_2\) controlled by a Brooks Mass Flow Controllers (Bamm–ability region of H\(_2\) in air 5–75 vol%). At periodic intervals aliquots were taken and the concentration of H\(_2\)O\(_2\) was determined by titration against an acidified dilute Ce(SO\(_4\)) solution using ferroin as an indicator.

| Substrate | Reaction Time/h | Product Concentration/mM | TTN x10\(^3\)(a) (Yield\%) (b) |
|-----------|----------------|-------------------------|---------------------------------|
|            |                |                         |                                 |
|            |                |                         |                                 |

Table 1 Substrate scope of tandem heterogeneous and biocatalytic system

PaDa-I (15 U ml\(^{-1}\)) was coupled with in situ H\(_2\)O\(_2\) generation by 0.5% Au-0.5% Pd/TiO\(_2\) (0.1 mg ml\(^{-1}\)) using a gas mixture of 80% H\(_2\) in air in a sealed system under 2 bar total pressure. Substrate concentration was 10 mM unless otherwise stated.

(a) TTN of C-H activation reaction accounting for secondary oxidation products.

(b) 

| Substrate | Reaction Time/h | Product Concentration/mM | TTN x10\(^3\)(a) (Yield\%) (b) |
|-----------|----------------|-------------------------|---------------------------------|
|            |                |                         |                                 |
|            |                |                         |                                 |

**Table 1 Substrate scope of tandem heterogeneous and biocatalytic system**
Cyclohexane oxidation testing. mmol cyclohexanol (M) refers to mmol cyclohexanol produced per unit volume of the aqueous phase. Under some conditions, premature cyclohexane exceed the solubility limits resulting in two phases being present.

Reactions using glucose oxidase for in situ H₂O₂ production. Biotransformations using an enzyme driven system for the in situ production of H₂O₂ were performed on a 1 ml scale in 10 ml amber vials. Glucose oxidase (GOD) (Aspergillus niger, Sigma–Aldrich) generates H₂O₂ by the conversion of glucose to gluconic acid. The reaction mixtures contained: 15 U ml⁻¹ GOD, 0.1 U ml⁻¹ glucose oxidase (a 10 U ml⁻¹ premix in buffer was prepared and 10 µl ml⁻¹ GOD were used to setup the biotransformation) and 200 mM glucose in 100 mM potassium phosphate buffer (pH 6). Cyclohexane was added as substrate at a concentration of 100 µl ml⁻¹ to start the reaction. The reactions were shaken or stirred with a magnetic stirrer bar at 200 rpm, 20 °C for 2 h. Reaction mixtures were extracted with 2 × 500 µl ethyl acetate containing 2 mM 2-decanol as internal standard and subjecting samples to GC analysis (Supplementary Method 1). The effect of the metal catalyst on the PaDa-l/GOX reactions were investigated by adding 1 mg of metal catalysts to stirred reactions.

Reactions using metal catalysts for in situ H₂O₂ production. To evaluate PaDa-l reactions using H₂O₂ generated in situ by metal catalysts, sets of three simultaneous reactions were performed on a 10 ml scale using 100 ml dreschel bottles. H₂ and air from two gas reservoirs were mixed continuously. Distinct concentrations of H₂ in air (4, 77 and 98%) were obtained by connecting the rubber tubes from the two reservoirs with a polypropylene tubing y-connector and then adjusting the gas flow from each reservoir by means of needle valves and air was being continuously gas chromatography and adjusted if necessary. The gas inlets terminated below the liquid surfaces so that the gas mixture was bubbled through the stirred reaction mixtures at a rate of 30 ml min⁻¹. The reaction mixtures contained 15 U ml⁻¹ PaDa-l, different concentrations (0.5, 0.25 and 0.1 mg ml⁻¹) of the different metal catalyst (2.5 wt% Au – 2.5 wt% Pd/ TiO₂, 0.5 wt% Au – 0.5 wt% Pd/TiO₂, 2.5 wt% Au – 2.5 wt% Pd on carbon and 5 wt% Au on carbon) in 100 mM potassium phosphate buffer (pH 6) and 100 µl ml⁻¹ cyclohexane. The catalysts were weighed out directly into the glass vessels and then the buffer was added. Immediately before starting the reactions, enzyme and cyclohexane were added. The reactions were started by switching on the gas flow. The reactions were stirred with a magnetic stirrer bar at 200 rpm and ambient temperature (19.5–20.5 °C). To compensate for the evaporation of the cyclohexane, additional cyclohexane (500 µl) was added every 15 min. Product formation was monitored after 2 h (or longer times) by extracting with 2 × 2.5 ml ethyl acetate containing 2 mM 2-decanol as internal standard and subjecting samples to GC analysis (Supplementary Method 1). The reactions were shaken or stirred with a magnetic stirrer bar at 200 rpm and ambient temperature (19.5–20.5 °C). The reactions were started by the addition of 1 ml cyclohexane and ran for 2 h and 8 h, substrate (300 µl) was added every 15 min. Product formation was monitored by extracting with 2 × 2.5 ml ethyl acetate containing 2 mM 2-decanol as internal standard and subjecting samples to GC analysis.

Assay for residual peroxygenase activity. Residual activity was evaluated using the peroxygenase assay with 5-nitro-1,3-benzoxazole (NBD) as substrate and determining endpoint values. The assay mixture contained potassium phosphate buffer (100 mM, pH 7.0) (500 µl), water (380 µl), NBD (5 mM in acetonitrile) (100 µl), enzyme solution (10 µl) and hydrogen peroxide (100 mM) (10 µl). Assays were incubated at room temperature for 5 min and then stopped with 10 M NaOH (100 µl). Absorbance was read at 514 nm and activities calculated using ε₄₅₂ = 11400 M⁻¹ cm⁻¹. Percentage residual activity was calculated relative to activity measured after 2 min.

Chiral GC analysis. Data were acquired on an Agilent 7890 A fitted with a Restek Rt-beta-DExsm fused silica capillary column (30 m × 0.25 μm i.d.) and a flame ionization detector (FID). Samples (5 µl) were injected at 200 °C in a 10:1 split at 26.7 ml /min total flow. The oven was equilibrated for 5 min before and after each analysis. For phenylethanol samples the oven was held at 50 °C for 1 min before heating to 200 °C at 5 °C/min and holding at 200 °C for 5 min. For other compounds, the oven was held at 100 °C for 1 min before heating to 200 °C at 3 °C /min and holding at 200 °C for 5 min.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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