The kinetic mechanism of 5-(hydroxymethyl)furfural oxidase

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Abstract

The FAD-containing 5-(hydroxymethyl)furfural oxidase (HMFO) is of great interest for biocatalytic applications because of its wide range of substrates, including hydrated aldehydes, primary alcohol and thiols. An example is the ability of HMFO to perform three consecutive oxidation reactions. Converting 5-(hydroxymethyl)furfural (1) into 2,5-furandicarboxylic acid (4), makes the enzyme attractive for the production of biobased polymers.

In this chapter, the kinetic mechanism of HMFO will be described. Pre-steady state and steady state analyses reveal that the reductive half-reaction is rate limiting in the oxidation of the model substrate benzyl alcohol. Reoxidation rates of the product-enzyme complex were higher than those for the free enzyme, supporting a ternary complex mechanism for HMFO.
Oxidation reactions can be performed by a wide variety of enzymes. Most, but not all, oxidative enzymes rely on a cofactor for their activity. One of the major classes of these enzymes use a flavin cofactor for catalysis, in most cases in the form of flavin adenine dinucleotide (FAD). FAD-containing oxidases and dehydrogenases can perform the same oxidation reaction, but differ in electron acceptor. The common feature of all FAD-containing oxidases is the use of molecular oxygen as electron acceptor, while dehydrogenases rely on alternative electron acceptors instead. Oxidases and dehydrogenases can be structurally and functionally related, and only subtle differences in the active site distinguish between both enzyme types.

The glucose-methanol-choline oxidoreductase (GMC) family is one of many families containing FAD-containing oxidases. Well known members of the GMC family are glucose oxidase (EC 1.1.3.4) and cholesterol oxidase (EC 1.1.3.6). The natural substrates for GMC-members are alcohols, which are oxidized in a two electron oxidation to the corresponding carbonyl group. Some of the formed aldehydes undergo a spontaneous hydration reaction, yielding a gem-diol. Aryl alcohol oxidase (EC 1.1.3.7), choline oxidase (EC 1.1.3.17), and 5-(hydroxymethyl)furfural oxidase (HMFO, EC 1.1.3.47) have been shown to act on these gem-diol, resulting in the formation of a carboxylic acid.

The kinetic scheme of flavoprotein oxidases can be broken down into two half reactions, both consisting of multiple steps. For many members of the GMC-family, the kinetic mechanism has been studied in detail. These studies are facilitated by the absorbance changes of the FAD cofactor depending on its redox state or charge transfer complexes.

The first half-reaction is the reductive half-reaction, in which the enzyme gets reduced. The dissociation constant ($K_d$) is the ratio of $k_1/k_2$. The upper, red, part of the cycle is the oxidative half-reaction when a ternary complex mechanism applies. The lower red part of the cycle shows the steps in an oxidative half-reaction for a ping-pong mechanism.

Scheme 1. The kinetic scheme of flavoprotein oxidases. The steps shown in black depict the reductive half-reaction, in which the enzyme gets reduced. The dissociation constant ($K_d$) is the ratio of $k_1/k_2$. The upper, red, part of the cycle is the oxidative half-reaction when a ternary complex mechanism applies. The lower red part of the cycle shows the steps in an oxidative half-reaction for a ping-pong mechanism.
substrate Cα to the flavin. The most supported mechanism is however the hydride transfer mechanism. In this mechanism, the reaction is initiated by an active site base which abstracts a proton from the alcohol of the substrate. This is followed by hydride transfer from the Cα to the flavin. In GMC-type oxidases, the active site base is a conserved histidine [8,15,16] H467 in HMFO (chapter 2). For some enzymes the proton abstraction takes place simultaneously with hydride transfer and a concerted mechanism applies [17]. In other enzymes the reaction is stepwise, meaning hydride transfer takes places after proton abstraction [18].

The second half-of the catalytic cycle is the oxidative half-reaction, in which the reduced FAD cofactor is reoxidized and the product is released. In oxidases the reduced flavin is reoxidized by molecular oxygen, yielding hydrogen peroxide as by product. The reaction of reduced flavin with molecular oxygen is spin forbidden. To overcome this, the reduced flavin donates first one electron to molecular oxygen, thus forming a radical pair. Spin inversion within the radical pair occurs and afterwards the second electron is transferred, completing the oxidative half-reaction [19].

Because both reoxidation and product release happen within the oxidative half-reaction, two possible routes can apply (Scheme 1). A so called ping-pong mechanism applies when the product is released before the enzyme reacts with oxygen. If, on the other hand, the oxygen reacts with the enzyme-product complex, a ternary complex mechanism applies. Both mechanisms have been described for members of the GMC family, and the mechanism can dependent on the substrate studied [20–23]. When the reoxidation rate of the enzyme-product complex is comparable to the rate of product release from this complex, a combination of both ping-pong and ternary complex mechanism can apply [24].

In this chapter, mechanistic studies on HMFO are presented. HMFO is active on a wide range of primary, preferably aromatic, alcohols and thiols [14,25]. Here, the oxidation of benzyl alcohol to benzaldehyde is chosen as a model reaction. Insights from steady state kinetics and pre-steady state kinetics are combined with observed kinetic isotope effects to gain a detailed view on both the reductive and oxidative half-reaction of HMFO. This resulted in the elucidation of the kinetic mechanism of HMFO.

**Results**

To gain insight into the kinetic mechanism of HMFO, the oxidation of benzyl alcohol to benzaldehyde was studied (Scheme 2). In addition, the oxidation of D₂-benzyl alcohol was studied. In this substrate, the protons on the Cα of the alcohol are substituted for deuterium.

From the steady state kinetic parameters (Table 1), it can be seen that benzyl alcohol is oxidized more rapid than its deuterated analogue, with a primary kinetic isotope effect (KIE) of 4.8 on the $k_{cat}$ value. This is in line with the mechanism of substrate oxidation when the reduction rate is, partially, rate limiting. During catalysis one proton from the alcohol group

$$\text{Scheme 2. The oxidation of benzyl alcohol (left) and D₂-benzyl alcohol (right) by HMFO.}$$
Table 1. Kinetic parameters of HMFO.

<table>
<thead>
<tr>
<th>parameter(^{(a)})</th>
<th>benzyl alcohol</th>
<th>(\text{D}_2\text{-benzyl alcohol})</th>
<th>isotope effect(^{(b)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k_{\text{cat}}) (s(^{-1}))</td>
<td>32 ± 0.07</td>
<td>6.6 ± 0.13</td>
<td>4.8</td>
</tr>
<tr>
<td>(K_M) (mM)</td>
<td>2.2 ± 0.15</td>
<td>2.2 ± 0.13</td>
<td>1.0</td>
</tr>
<tr>
<td>(k_{\text{cat}}/K_M) (s(^{-1}) mM(^{-1}))</td>
<td>15</td>
<td>3.0</td>
<td>5.0</td>
</tr>
<tr>
<td>(K_{\text{in}}) (mM)</td>
<td>0.13 ± 0.026</td>
<td>0.033 ± 0.027</td>
<td>3.9</td>
</tr>
<tr>
<td>(k_{\text{red}}) (s(^{-1}))</td>
<td>60 ± 2.2</td>
<td>13 ± 0.54</td>
<td>4.6</td>
</tr>
<tr>
<td>(K_O) (mM)</td>
<td>1.7 ± 0.26</td>
<td>1.4 ± 0.25</td>
<td>1.2</td>
</tr>
<tr>
<td>(k_{\text{red2}}) (s(^{-1}))</td>
<td>6.1 ± 0.42</td>
<td>4.0 ± 0.15</td>
<td>1.5</td>
</tr>
<tr>
<td>(K_{D2}) (mM)</td>
<td>2.5 ± 0.67</td>
<td>1.9 ± 0.28</td>
<td>1.3</td>
</tr>
</tbody>
</table>

\(^{(a)}\) All experiments were performed at 25 °C in a 50 mM potassium phosphate buffer. The steady state experiments were performed at atmospheric oxygen concentrations.\(^{(b)}\) The isotope effect was calculated by dividing the value obtained for benzyl alcohol by the value for \(\text{D}_2\text{-benzyl alcohol}\).

and a hydride from the C\(\alpha\) are abstracted from the substrate. In \(\text{D}_2\text{-benzyl alcohol}\), there is a deuterium on the C\(\alpha\) position (Scheme 1). Since a deuterium-carbon bond is stronger than a hydrogen-carbon bond, the oxidation of \(\text{D}_2\text{-benzyl alcohol}\) will be slower. In contrast to an effect seen on the \(k_{\text{cat}}\), there is no KIE on the \(K_M\) value for the oxidation of benzyl alcohol or \(\text{D}_2\text{-benzyl alcohol}\).

To assess the redox state of the FAD cofactor during steady state, the absorbance of the flavin was monitored in a stopped flow apparatus at atmospheric oxygen concentration. From these enzyme monitored turnover experiments, it becomes clear that the enzyme is mainly in the oxidized state when benzyl alcohol is converted (Figure 1). When enzyme monitored turnover experiments were performed with \(\text{D}_2\text{-benzyl alcohol}\) as substrate, the oxidized state was even more dominant. Taken together, the steady state kinetic data suggest that the rate limiting step is, at least partially, limiting the rate of catalysis.

Figure 1. Redox state of the FAD cofactor during the oxidation of benzyl alcohol and \(\text{D}_2\text{-benzyl alcohol}\). The absorbance change was measured at 456 nm. HMFO (10 μM) was mixed with benzyl alcohol (solid black line), \(\text{D}_2\text{-benzyl alcohol}\) (dotted line). The percentages are based on fully oxidized enzyme, as obtained when mixing HMFO without substrate (grey line).
Pre-steady state analysis of the reductive half-reaction was carried out using both benzyl alcohol and D<sub>2</sub>-benzyl alcohol as substrates in anaerobic conditions. These experiments reveal a similar KIE on the rate of reduction, the \( k_{\text{red}} \) (Table 1). Similar to the \( K_M \) value obtained from the steady state analysis, there is no isotope effect on the dissociation constant (\( K_d \)). The decrease of absorbance of FAD, reflecting the reduction of the cofactor by benzyl alcohol, could not be fitted satisfactorily using a single exponential equation. The main contributing term, around 80 percent, was used to calculate the \( k_{\text{red}} \) as displayed in Table 1. The second exponential term was also clearly substrate concentration dependent, with a maximal value of 6.0 s<sup>-1</sup>. This rate is, looking at the steady state parameters, too slow to be of catalytic relevance. For D<sub>2</sub>-benzyl alcohol, the same double exponential behavior was observed. In this case the contribution of the second exponential term was higher, contributing for around 40 percent. Again the rate was substrate concentration dependent with a maximal value of 4.0 s<sup>-1</sup>. In contrast to the \( k_{\text{red}} \), there is hardly an KIE on this kinetic parameter.

In addition to the steady state and the reductive half-reaction, also the oxidative half-reaction was investigated into more detail. The reoxidation of substrate reduced HMFO was measured at different concentrations of molecular oxygen. The slope of the linear relationship between reoxidation rate and the concentration of molecular oxygen yield the oxidation rate (\( k_{\text{ox}} \)) as displayed in Scheme 1. This scheme shows that reoxidation can take place after product release (\( k_{\text{ox2}} \)) or with the product still bound to the protein (\( k_{\text{ox1}} \)). To differentiate between those two mechanisms, the reoxidation reaction was performed in the absence or presence of saturating conditions of the product benzaldehyde. The reoxidation of HMFO in the presence of product (\( k_{\text{ox1}} \)) is 189 s<sup>-1</sup> mm<sup>-1</sup>. This over 3.5 times faster than reoxidation of the enzyme without product bound, as the \( k_{\text{ox2}} \) is only 53 s<sup>-1</sup> mm<sup>-1</sup> (Figure 2). Reoxidation of the enzyme-product complex is therefore favored, suggesting a ternary complex mechanism for HMFO.

To further differentiate whether the catalytic cycle of HMFO proceeds via a ternary complex or a ping-pong mechanism, the steady state parameters of HMFO on benzyl alcohol were determined at three different concentrations of molecular oxygen (Figure 3). Double reciprocal plots of the obtained kinetic data clearly show diverging lines, indicative for a ternary complex mechanism. Similar experiments were performed for D<sub>2</sub>-benzyl alcohol, but using only two concentrations of molecular oxygen. The obtained results support a ternary complex mechanism.

![Figure 2](image-url). Reoxidation of reduced HMFO in the absence and presence of the product benzaldehyde. In the presence of benzaldehyde the \( k_{\text{ox1}} = 189 \text{ s}^{-1} \text{ mm}^{-1} \). Reoxidation of the reduced enzyme in the absence of product is lower, with a \( k_{\text{ox2}} \) of 53 s<sup>-1</sup> mm<sup>-1</sup>.
Figure 3. Bi-substrate steady state kinetics for HMFO. Double reciprocal plot of the oxidation of benzyl alcohol by HMFO at three different concentrations of O₂. The oxidation rate of different concentrations of benzyl alcohol by 50 nM HMFO was measured at different concentrations of oxygen: 258 μM (circles), 536 μM (squares) and 1280 μM (diamonds). All experiments were performed at 25 °C.

In addition to the wild type enzyme, a mutant enzyme containing a mutation close to the FAD cofactor was analyzed. In this mutant, W466 is substituted for phenylalanine. The mutation causes a decreased $k_{cat}$ for primary alcohols (see chapter 4). From the enzyme monitored turnover experiments it becomes clear that the oxidative half-reaction is affected by this mutation. In the mutant only 45 percent of the enzyme is in the oxidized state, 35 percent lower than the wild type enzyme. The reoxidation of the mutant is therefore relatively slow, showing the importance of W466 in modulating reactivity with molecular oxygen in the active site of the enzyme.

Conclusions and discussion

HMFO is a very versatile oxidase because it is active on a wide range of substrates. To date its physiological substrate is however unknown. Of special interest is the ability of HMFO to perform up to four consecutive oxidations starting on one diol substrate (chapter 3). The ability to use the product of one oxidation reaction as the substrate for the another oxidation reaction is depending on the hydration of the formed product. When the subsequent aldehyde product is hydrated, the formed gem-diol serves as a substrate for a new oxidation reaction in which the carboxylic acid is formed.

The oxidation of the gem-diol product is however not desired for mechanistic studies, as it might complicate the analysis of the experiments. For this reason, the oxidation of benzyl alcohol to benzaldehyde was studied. Only one percent of the product benzaldehyde is present in its hydrated gem-diol form when dissolved in water. Benzaldehyde is therefore not a good substrate for HMFO for a subsequent oxidation reaction. Here, pre-steady state and steady state was analyzed to gain insight into the catalytic cycle of HMFO, focusing on both the reductive and the oxidative half-reactions.

A significant primary kinetic isotope effect is observed on the rate of reduction. This translates into an isotope effect in the overall reaction as seen in the $k_{cat}$ values for benzyl alcohol and D₂-benzyl alcohol. The reductive half-reaction is therefore rate limiting in the
catalytic cycle, which is confirmed by enzyme monitored turnover reactions. The enzyme is predominantly (80 percent) in the oxidized state, during steady state which shows that reoxidation is faster than reduction. Experiments with the deuterated substrate underline this. Because the rate limiting step (the \( k_{\text{red}} \)) for this reaction is even slower than for the normal substrate, the enzyme is around 95 percent oxidized during catalysis.

Steady state experiments were performed on benzyl alcohol and D\(_2\)-benzyl alcohol, at different concentrations of molecular oxygen. Plotting the observed rates against the substrate concentration gives rise to divergent lines in a double reciprocal plot, which indicative for a ternary complex mechanism. In addition, a ternary complex mechanism is also supported by the reoxidation experiments. Reoxidation of the enzyme is faster in the presence of product. The rate of reoxidation in the absence of product is slower than the reduction rate. This would not yield the enzyme predominantly in the oxidized state during catalysis. Based on this, the \( k_{\text{ox2}} \) cannot be part of the catalytic cycle, and therefore a ping-pong mechanism cannot apply (Scheme 1). The determined \( k_{\text{ox1}} \), the rate of reoxidation in the presence of product, is around 50 s\(^{-1}\) at ambient oxygen concentration. This is still too low to explain why HMFO is predominantly in the oxidized state during catalysis. Probably, the \( k_{\text{ox1}} \) is underestimated by the experiments because the concentration of product used was too low to saturate the active site of the reduced enzyme. To measure the real \( k_{\text{ox1}} \), higher concentrations of the product benzaldehyde should be used, although this might be difficult due to the limited solubility of benzaldehyde in water. Alternatively, sequential mixing stopped-flow could be used to determine the rate of reoxidation of the enzyme-product complex.\(^{[24]}\) Even when \( k_{\text{red}} \) and \( k_{\text{ox1}} \) have similar rates, FAD can still be mainly in the oxidized state during catalysis. In this case, the product release (\( k_2 \) in Scheme 1) is relatively slow, trapping the enzyme in the oxidized state. This is in our case not in agreement with the similar KIE seen on both the \( k_{\text{cat}} \) and the \( k_{\text{red}} \), which suggests that the rate of reduction is rate limiting.

As mentioned before, the wild type enzyme is predominantly in the oxidized state during catalysis. A large effect on the redox state was found in a mutant of HMFO, containing the substitution of a tryptophan to a phenylalanine (W466F). This residue is close the FAD cofactor (Figure 2 in chapter 4), and in the wild type enzyme this tryptophan residue prohibits the oxidation of secondary alcohols due to steric hindrance. Introduction of the smaller residues phenylalanine or alanine resulted in activity towards secondary alcohols, but at the same time reduces the activity on primary alcohols (Table 2 and 3 of chapter 4). The reduced activity towards primary alcohols can be attributed to improper positioning of the primary alcohol in the W466F mutant. The mechanistical studies performed here also show that the activity might be impaired because the oxidative half-reaction is affected by this mutation. Similar results were obtained for aryl alcohol oxidase (EC 1.1.3.7). Here, the wild type enzyme has a phenylalanine in this position. The mutant enzyme F501A in which more space is created, similar to W466F in HMFO, shows reduced reactivity with oxygen. The F501W mutation on the other hand increases the reactivity.\(^{[26]}\) W466 in HMFO and F501 in aryl alcohol oxidase therefore have a similar role in modulating oxygen reactivity, and positioning the substrate in the active site.

The steady state parameters for benzyl alcohol displayed in Table 1 differ from those presented in Table 1 of chapter 2, especially for the \( k_{\text{cat}} \) value. In this chapter the oxidation rate is measured by using the absorbance increase upon product formation. This results in a \( k_{\text{cat}} \) value which is 2.5 fold higher than presented in chapter 2. In chapter 2, the kinetic parameters were determined using the depletion of molecular oxygen. Both analysis methods rely on the
direct measurement of one of the oxidation reaction, and are therefore less prone to errors than an indirect assay. The discrepancy found for the oxidation rate of benzyl alcohol by HMFO is not limited to this enzyme-substrate combination. Different enzymes and substrates were analyzed and in all cases the rates measured using oxygen depletion underestimated the rate with a factor of 2 to 2.5. The data obtained using oxygen depletion should therefore be used with care.

This study shows that the oxidation of benzyl alcohol by HMFO follows a ternary complex mechanism and the reductive half-reaction is rate limiting. Even though the experiments performed here focus on benzyl alcohol, a similar mechanism likely applies to the oxidation of 5-(hydroxymethyl)furfural (1) and its derivatives. Because similar \( k_{\text{cat}} \) values are found for benzyl alcohol and for instance 5-(hydroxymethyl)furfural (1), also here the reductive half-reaction is probably limiting the rate of catalysis.

**Material and Methods**

**Protein expression and purification**

For HMFO expression, an overnight culture of *Escherichia coli* BL21 (DE3) cells was diluted 1:100 in 1 L Terrific Broth containing 50 \( \mu \text{g} \cdot \text{mL}^{-1} \) kanamycin and grown at 37 °C to an \( \text{OD}_{600} \) density of 0.5. Cells were induced with 1.0 \( \mu \text{M} \) isopropyl \( \beta\)-D-1-thiogalactopyranoside (IPTG) and grown for 68 hours at 17 °C. Cells were harvested by centrifugation at 37,300 g for 15 minutes (JLA 10,500 rotor, 4 °C) and resuspended in 80 mL 100 \( \text{mM} \) Tris/HCl pH 8.0 supplemented with 10% v/v glycerol, 150 \( \text{mM} \) NaCl and 10 \( \mu \text{M} \) FAD. The cell-free extract was obtained after cell disruption using sonication and subsequently centrifuged for 1 hour at 29,100 g (JA 17 rotor, 4 °C). The soluble fraction was mixed with 2 mL pre-equilibrated Ni-Sepharose resin (GE-Healthcare) for 1.5 hour. The flow through was removed and the column was washed with 5 CV of 50 \( \text{mM} \) Tris/HCl with 150 \( \text{mM} \) NaCl and subsequently washed with 3 CV of 50 \( \text{mM} \) Tris/HCl with 150 \( \text{mM} \) NaCl containing 5 \( \text{mM} \) imidazole. The protein was eluted with 4 CV of 50 \( \text{mM} \) Tris/HCl with 150 \( \text{mM} \) NaCl containing 500 \( \text{mM} \) imidazole. The eluate was desalted on a 10 DG Biorad column.

**Steady state kinetics**

The steady state parameters for HMFO on phenylmethanol (benzyl alcohol) and dideuterio(phenyl)methanol (\( \text{D}_2 \)-benzyl alcohol, 98 atom % D) were determined using 50 \( \text{nm} \) purified His\(_6\)-SUMO-HMFO. The substrates were in the concentration range of 9.8 \( \mu \text{M} \) to 15 \( \text{mM} \). All reactions were performed at 25 °C, in a 50 \( \text{mM} \) potassium phosphate buffer of pH 8.0. The reaction was monitored by the formation of product at 240 nm (\( \varepsilon_{240} = 7.8 \text{ mM}^{-1} \text{ cm}^{-1} \)); the reaction was not monitored at the absorbance maximum of benzaldehyde (\( \varepsilon_{259} = 10.4 \text{ mM}^{-1} \text{ cm}^{-1} \)) due to the higher absorbance of benzyl alcohol at this wavelength. The kinetic parameters were obtained at three different concentrations of oxygen: ambient (258 \( \mu \text{M} \)), 536 \( \mu \text{M} \) and saturated (1280) \( \mu \text{M} \) molecular oxygen. The buffer containing 536 \( \mu \text{M} \) was prepared by mixing oxygen saturated buffer with buffer of ambient oxygen concentration. The steady state parameters were calculated by fitting the observed rates to \( v = k_{\text{cat}} [S]/K_M + [S] \).

**Oxygen affinity**

The affinity for oxygen was assayed using 0.20 \( \mu \text{M} \) HMFO and 15 \( \mu \text{M} \) benzyl alcohol or 0.72 \( \mu \text{M} \)
HMFO and 15 mM D₂-benzyl alcohol in an air tight cuvette. The oxygen concentration was measured during catalysis with REDFLASH sensor spots and a Firesting O₂ detector and light source (Pyroscience, Aachen, Germany). The slope of oxygen decrease in time was plotted against the concentration of oxygen. The curve was fitted using a hyperbolic function: $v = \frac{k_{cat} [O_2]}{K_{ox} + [O_2]}$.

Enzyme monitored turnover
The oxidation state of FAD during steady state was determined on an Applied Photophysics SX20 stopped-flow apparatus. The substrate, either benzyl alcohol or D₂-benzyl alcohol (30 mM), was mixed 1 to 1 with 10 μM HMFO or the mutant enzyme HMFO H333M-F352Y-V367R-W466F. The experiments were performed in a 50 mM potassium phosphate buffer of pH 8.0 at 25 °C. The absorbance at 456 nm was followed in time. Data was collected in a logarithmic way, for 10 or 20 seconds. All experiments were performed in triplicate. The absorbance of HMFO in the absence of substrate was recorded using buffer and served as a reference.

Reductive half-reaction
The rate of reduction of HMFO was monitored by measuring the absorbance change of FAD at 456 nm at different concentration of benzyl alcohol and D₂-benzyl alcohol. Both the enzyme solution and the substrate solutions were made anaerobic by flushing with N₂. Residual oxygen was removed using final concentration of 10 μg/mL glucose oxidase and 20 mM glucose. The reduction rate was recorded after mixing 15 μM HMFO with seven different concentration of substrate in the range of 0.12 mM to 75 mM. These solutions were mixed 1 to 1 in an Applied Photophysics SX20 stopped-flow apparatus. All measurements were performed in triplicate, and the absorbance change in time was satisfactory fitted to a double exponential equation: $A = a \cdot e^{-k_A t} + b \cdot e^{-k_B t} + c$, where A is absorbance, t is time, $k_A$ and $k_B$ are the rates, a and b display the contribution of both exponential terms and c is a constant. The rates obtained from one of the two terms of the exponential equation were used to determine both the rate of reduction and the dissociation constant by fitting the following hyperbolic equation: $k_A = k_{red} [S] / K_d + [S]$. The rates of the other termed were also substrate dependent, and were fitted to a similar hyperbolic equation.
To determine whether the two rates observed can be addressed to two separated events, the reduction of HMFO by 15 mM benzyl alcohol was followed using a photo diode array (PDA) detector between 700 and 250 nm.
All experiments were performed at 25 °C in a 50 mM potassium phosphate buffer of pH 8.0.

Oxidative half-reaction
The reoxidation of reduced HMFO was determined at different oxygen concentration, in both the absence and the presence of product.
To reduce HMFO, the enzyme solution was made anaerobic by flushing with N₂ and traces of oxygen were removed using glucose and glucose oxidase as described before. In this anaerobic condition, 20 μM HMFO was reduced with 30 or 40 μM benzyl alcohol. The reoxidation was followed in an Applied Photophysics SX20 stopped-flow apparatus, where the reduced enzyme was mixed 1 to 1 with a buffer containing different concentrations of oxygen. In case of reoxidation in the presence of product, both the buffer and the enzyme solution contained 50 mM benzaldehyde. The absorbance change was measured at 456 nm and the traces were fitted to a single exponential curve: $A = a \cdot e^{-kt} + c$, where A is the absorbance, t is time, k is the rate
and a and c constants. The rate constant of reoxidation \((k_{\text{ox}})\) was obtained by fitting the rates at different oxygen concentrations to \(k = k_{\text{ox}} \cdot [O_2]\).

**Hydration of benzaldehyde**

The equilibrium between the hydrated (gem-diol) form and non-hydrated aldehydes forms was based on their nuclear magnetic resonance (NMR) spectra. \(^1\)H-NMR spectra of 5 mg aldehyde dissolved in 750 µL D₂O were recorded on a Varian Gemini spectrometer with an operating frequency of 300 MHz. The \(^1\)H-NMR chemical shifts are reported in ppm relative to TMS peak. The ratio gem-diol/aldehyde was calculated by dividing the area of the \(\text{H}-\text{C-(OH)}_2\) peak of the gem-diol by the area of the \(\text{H}-\text{C=O}\) peak of the aldehyde.

**Oxidation assay comparison**

The oxidation of 15 mM 4-hydroxy-3-methoxybenzyl alcohol (vanillyl alcohol) by 50 nM HMFO and eugenol oxidase was monitored using the absorbance increase at 340 nm (\(\varepsilon_{340} = 14 \text{ mm}^{-1} \text{ cm}^{-1}\)) or oxygen depletion using REDFLASH sensor spots and a Firesting O₂ detector and light source (Pyroscience, Aachen, Germany). The oxidation of 10 mM xylitol by alditol oxidase (EC 1.1.3.41) was measured using a coupled \(\text{H}_2\text{O}_2\) detection assay, where horseradish peroxidase (HRP (Sigma), 4 units/mL) reacts with 4-aminoantipyrine (0.1 mM) and 3,5-dichloro-2-hydroxybenzenesulfonic acid (1 mM) to form a pink product which was measured at 515 nm (\(\varepsilon_{515} = 26 \text{ mm}^{-1} \text{ cm}^{-1}\)). The same reaction was also measured using oxygen depletion as mentioned above. For all reactions, a 50 mM potassium phosphate buffer of pH 7.5 was used. All experiments were performed at 25 °C, at atmospheric oxygen concentrations.

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References

characterization, application and engineering of 5-(hydroxymethyl)furfural oxidase