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Kinetic Resolution of sec-Thiols by Enantioselective Oxidation with Rationally Engineered 5-(Hydroxymethyl)furfural Oxidase

Mathias Pickl, Alexander Swoboda, Elvira Romero, Christoph K. Winkler, Claudia Binda, Andrea Mattevi, Kurt Faber,* and Marco W. Fraaije*

Abstract: Various flavoprotein oxidases were recently shown to oxidize primary thiols. Herein, this reactivity is extended to sec-thiols by using structure-guided engineering of 5-(hydroxymethyl)furfural oxidase (HMFO). The variants obtained were employed for the oxidative kinetic resolution of racemic sec-thiols, thus yielding the corresponding thioesters and nonreacted R-configured thiols with excellent enantioselectivities (E ≥ 200). The engineering strategy applied went beyond the classic approach of replacing bulky amino acid residues with smaller ones, as the active site was additionally enlarged by a newly introduced Thr residue. This residue established a hydrogen-bonding interaction with the substrates, as verified in the crystal structure of the variant. These strategies unlocked HMFO variants for the enantioselective oxidation of a range of sec-thiols.

The demand for chiral building blocks for pharmaceutical applications is a driving force for the development of new methodologies in modern synthetic chemistry. In this context, biocatalysis, which excels in producing chiral molecules, represents an increasingly important tool for synthetic chemists. Organosulfur moieties constitute highly relevant structural elements with a broad range of bioactivities, and efficient strategies for their selective production are sought. Examples include the acyl-CoA: cholesterol acyltransferase inhibitor Efucilimibe, the leukotriene receptor antagonist Montelukast, or the calcium channel blocker Diltiazem (Figure 1). Recent approaches to the synthesis of non-racemic sec-thiols include the ring-opening of chiral thiazol-4-ones produced by organocatalytic Michael addition and S–H bond insertion cooperatively catalyzed by a rhodium catalyst and a chiral spirophosphoric acid and the formation of chiral vic-hydroxysthiols from cis-epoxides. However, these systems suffer from the requirement of complex starting materials and/or high loadings of sophisticated organocatalysts. For the kinetic resolution of racemic thiols by esterification, organo- and biocatalytic methods are known. Cinchona-alkaloid-based organocatalysts allowed the resolution of various benzylic sec-thiols, though excellent enantioselectivities were achieved only with a single substrate at high catalyst loadings (Scheme 1a). Biocatalytic methods, such as the kinetic resolution of thiols with baker’s yeast were only moderately successful. The prominent lipase B from Candida antarctica catalyzes the enantioselective hydrolysis of thioesters (Scheme 1a). In contrast, the kinetic resolution by esterification of thiols failed.

Flavoprotein oxidases, which are well established for the dioxygen-dependent oxidation of alcohols to carbonyl moieties, were recently shown to catalyze the transformation of primary thiols into the corresponding thiocarbonyl analogues. One of the enzymes exhibiting this activity is 5-(hydroxymethyl)furfural oxidase (HMFO; EC 1.1.3.47). In addition to its ability to produce the polymer building block 2,5-furandicarboxylic acid from 5-(hydroxymethyl)furfural, HFMO is able to oxidize a broad range of electronically activated benzylic and cinnamic primary alcohols, and its variants transform sec-alcohols in a stereo-preferred fashion. The enzyme oxidizes the alcohol (or the hydrated aldehyde) to the corresponding carbonyl/carboxy product by...
formal hydride abstraction via oxidized flavin (FAD). FADH₂, thus formed, reduces molecular oxygen and forms hydrogen peroxide as a byproduct.

The utility of HMFO variants for sec-alcohol oxidation, on one hand, and wild-type activity on primary thiols, on the other hand, triggered our interest in designing HMFO variants that would combine these two features and yield an enzyme active for sec-thiols. Such an biocatalyst would enable the production of nonracemic sec-thiols at the expense of molecular oxygen through enantioselective oxidation. According to the (S)-stereo-preference of HMFO variants for sec-alcohols, R-configured sec-thiols could be expected as unreacted enantiomers together with the corresponding thiolesters (Scheme 1b).

The geometry of the active site of wild-type HMFO explains the distinct selectivity for primary over secondary alcohols. The substrate positioning is mainly guided by hydrogen bonding of His467 with the substrate hydroxy group. This arrangement allows the abstraction of the pro-R hydrogen, at the substrate Cα atom, by the flavin N5 atom. In a previous study, additional space for the methyl group of sec-alcohols was provided by mutating Trp466. In the present work, Trp466 was replaced by smaller amino acids to create a more spacious active site for sec-thiols, that is, Trp466Phe, Trp466Ala, Trp466Tyr, and Trp466His mutants were created. The latter two mutations were chosen in view of the beneficial effect on dioxygen reactivity of an aromatic residue at the 466-position as found in the structurally related aryl alcohol oxidase (EC 1.1.3.7).

In addition, in silico models suggested that the mutation of Val465 to either Ser or Thr would provide additional space by rotating their side chain to establish a hydrogen bond with His467 and/or Glu306. To validate this hypothesis, we determined the crystal structure of the Val465Thr protein (Figure 2). The overall structure of the mutant enzyme is essentially unaltered with respect to wild-type protein (rmsd is 0.4 Å for the 529 Cα atoms of the two monomers present in the asymmetric unit). The only exception is represented by two loops which, in monomer B, lack electron density (residues 99–104 and 203–209). The active-site architecture is also highly conserved except for the mutation site (Figure 2b). Inspection of the electron density revealed that Thr465 adopts a double conformation, one being identical to that of Val465 in the wild-type enzyme and another one displaying a rotamer conformation that establishes a hydrogen bond with Glu306 (Figure 2c). Similarly to 1-phenylethanol, which was previously modeled in the crystal structure of the wild-type enzyme, 1-phenylethanethiol would fit perfectly into the mutant active site because of the alternative conformation of Thr465. These findings validated the in silico modeling prediction and prompted further analysis of Val465Thr and Val465Ser mutants.

In previous studies, commonly used oxidase assays could be used to determine HMFO activities. However, these assays were unsuitable for the slower reacting sec-thiols studied in the present work. As an alternative, the pseudo-

![Figure 2](image-url)

**Figure 2.** Crystal structure of the Val465Thr HMFO mutant. a) The overall structure is shown as a ribbon representation embedded in a semi-transparent surface in a clipped view to highlight the cleft space of the active site. The FAD cofactor is depicted using a stick representation with the carbon atoms in yellow. b) Zoomed view of the Val465Thr HMFO active site showing the Thr465 double conformation. The alternative conformation of Thr465 is shown in black (H-bond with Glu306 is represented as dashed line). The (S)-1-phenylethanethiol substrate was modelled in the enzyme active site (carbon atoms in magenta). The orientation of the molecule is approximately 180° rotated along a vertical axis with respect to (a). c) The weighted 2Fo-Fc electron density map, contoured at 1.2 σ level, highlighting the double conformation of Thr465.
first-order rate constants for anaerobic flavin reduction under saturation conditions ($k_{\text{red}}$) were determined using a stopped-flow apparatus. This data allowed us to estimate the overall activity because hydride transfer in the reductive half-reaction was demonstrated to be the rate-limiting step.\[12b,c\] Furthermore, the effect of mutations on substrate binding was simultaneously studied by determining the apparent macroscopic dissociation constant for binding of the substrate to the enzyme ($K_d$). For all stopped-flow measurements, the model substrate rac-1a was used (Scheme 2). Whereas wild-type HMFO, Trp466His HMFO, and Trp466Ala HMFO did not show any detectable activity with rac-1a, all other variants exhibited significant flavin reduction (Table 1). Remarkably, the activities and $K_d$ values were in a similar range. An attempt to combine both space-creating effects in a double mutant Trp466Phe/Val465Thr failed: no additional improvement over the corresponding single mutants could be generated. Overall, the strategy to introduce activity on sec-thiols was successful, as the $K_d$ value of rac-1a for all active variants was comparable to that of benzyl alcohol with wild-type HMFO ($K_d = 1.7 \text{mm}$).\[12b,c\]

Motivated by the promising kinetic properties observed for the above HMFO variants, their biocatalytic applicability was investigated with substrate rac-1a. DL-dithiothreitol (DTT) was added as an anti-oxidant to the reaction mixture (see Figure S1 in the Supporting Information).\[13\] This addition suppressed the formation of disulfide adducts and traces of the elimination product styrene for all tested substrates. Wild-type HMFO was almost unreactive with rac-1a (ca. 3% conversion), thus confirming the kinetic data. In contrast, all variants gave good conversions in the oxidation of rac-1a (up to 42%, Figure 3) and an enantiomeric excess of substrate (ee) of up to 91% (Table 2). The degrees of conversion correlate well with the determined kinetic parameters (Figure 3 and Table 1). The highest conversions were obtained with the two Val465 mutants which also displayed the lowest $K_d$ values. No conversion of the substrate was observed in control reactions without the enzyme. Overall, Val465Ser was identified as the most potent variant and was used for follow-up experiments.

![Scheme 2. Biocatalytic enantioselective oxidation of the (S)-1-phenylethanethiols 1a–h with HMFO variants.](image)

![Figure 3. Conversion of rac-1a by HMFO variants. Reactions were performed in phosphate buffer (100 mM, pH 7.0) containing isopropanol (1%, v/v), DTT (50 mM), 5 mM substrate, and HMFO variants (3.5 mM), at 30°C for 24 h in air-saturated solutions (235 mM dioxygen). Disulfide formation less than 3%.](image)

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**Table 1: Pre-steady-state kinetic parameters of HMFO variants on rac-1-phenylethanethiol (1a).**

<table>
<thead>
<tr>
<th>HMFO</th>
<th>$k_{\text{red}}$ [s$^{-1}$]</th>
<th>$K_d$ [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>&lt; 0.001</td>
<td>n.d.</td>
</tr>
<tr>
<td>Trp466His</td>
<td>&lt; 0.001</td>
<td>n.d.</td>
</tr>
<tr>
<td>Trp466Ala</td>
<td>&lt; 0.001</td>
<td>n.d.</td>
</tr>
<tr>
<td>Trp466Phe</td>
<td>0.034 ± 0.007</td>
<td>1.3 ± 0.5</td>
</tr>
<tr>
<td>Trp466Tyr</td>
<td>0.010 ± 0.001</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Val465Ser</td>
<td>0.020 ± 0.002</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Val465Thr</td>
<td>0.021 ± 0.003</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Val465Thr/Trp466Phe</td>
<td>0.009 ± 0.003</td>
<td>1.3 ± 0.9</td>
</tr>
</tbody>
</table>

[a] Experiments were performed at 30°C in phosphate buffer (100 mM, pH 7.0) under anaerobic conditions. Reduction was measured at $A_{\text{max}}$ of HMFO-variant (see Table S2). n.d. = not detectable.
of hydride transfer in HMFO.[15] The m- and p-substituted substrates rac-1b-g and the naphthyl analogue 1h were tested (Scheme 2). The latter were prepared from the corresponding alcohols by mesylation through a Mitsunobu protocol, with subsequent nuclophilic substitution with thioacetate and thioether reduction over lithium aluminium hydride (see the Supporting Information).[5]

HMFO-Val465Ser accepted all 1-phenylethanethiol analogues (Table 2). Rac-1f and rac-1g, bearing activating electron-donating substituents were converted in up to 50% with ee values of greater than 97% for R-configured enantiomers, and corresponds to perfect E values of greater than 200. On the contrary, the conversions of the bromo-substituted substrates rac-1d and rac-1e were slower, albeit with excellent enantioselectivities. Overall, unreacted R-configured enantiomers were recovered for all substrates in yields of 15–61% after column chromatography. The absolute configurations of 1a, 1c, 1g, and 1h were determined to be R by comparison of optical rotation values with those in the literature.[9,14] The absolute configurations of 1b and 1d-f were concluded to be R because of the contiguous (+)-sense of optical rotation of 1a-h, as it is in line with an identical elution order on chiral-phase HPLC [(R) first, (S) second; see the Supporting Information]. The absolute configuration for 1e was additionally confirmed by CD spectroscopy (see Figure S8). Overall, the Val465Ser HMFO variant proved to be a reliable biocatalyst for the kinetic resolution of benzyl sec-thiols.

The formation of thioaceto phenone (2b) as an oxidation product of rac-1b was proven by GC-analysis using the reference material 2b, which was independently synthesized using Lawesson’s reagent (see Figure S4),[17] thus confirming previous observations.[9] However, the latter are rapidly hydrolyzed, via monothioketals, in aqueous medium to yield the corresponding carbonyl analogues along with the formation of hydrogen sulfide. Consequently, the corresponding acetophenone derivatives 3a-h were predominantly found in the reaction mixture after 24 hours (see Figure S5).[19]

Attempts to convert the kinetic resolution into a cyclic deracemization[19] through (chemical) in situ reduction of the thioke tone, formed in analogy to the cyclic deracemization of amines employing amino acid[20] or amine oxidases[21] and boron-based reducing agents, unfortunately failed. Neither borohydrides nor borane complexes were reactive enough to compete with the fast spontaneous hydrolysis of the thio ketone intermediate (see the Supporting Information).

In summary, rationally designed variants of HMFO were employed for the enantioselective oxidation of 1-phenylethanethiols, and allowed their kinetic resolution with unprecedented selectivities (E up to > 200). Variants were created by not only replacing bulky amino acid residues with smaller ones, but also by a space-creating rearrangement of amino acids within the active site enforced by additional hydrogen bonding, which was predicted by molecular modeling and confirmed by crystal structure analysis. This method provides a broadly applicable tool for the preparation of nonracemic sec-thiol building blocks.

General procedure for the kinetic resolution of thiols: Purified HMFO variants (3.5 $\mu$m) and DTT (50 mM) were dissolved in sodium phosphate buffer (500 $\mu$L, pH 7.0, 100 mM) containing isopropanol (1% v/v). Then the substrate (1a-h) was added (5 mM). The vials were shaken at 30°C with 750 rpm for 24 h in a horizontal position on a Heidolph Titramax 1000 plate shaker. The reaction was stopped by extraction with dichloromethane (2 $\times$ 0.5 mL). The combined organic phases were dried over anhydrous Na2SO4 and the conversion was measured by GC-MS after extraction with dichloromethane (2 $\times$ 0.5 mL).

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Conflict of interest
The authors declare no conflict of interest.

Keywords: biocatalysis · enzymes · kinetic resolution · oxidation · thiols

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References

Table 2: Enantioselective oxidation of 1-phenylethanethiol derivatives by Val465Ser HMFO variant.[6]

<table>
<thead>
<tr>
<th>rac-Substrate</th>
<th>Conversion [%]</th>
<th>ee, (%)</th>
<th>$E$ [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>48</td>
<td>91</td>
<td>&gt;200</td>
</tr>
<tr>
<td>1b</td>
<td>50</td>
<td>88</td>
<td>45</td>
</tr>
<tr>
<td>1c</td>
<td>47</td>
<td>87</td>
<td>&gt;200</td>
</tr>
<tr>
<td>1d</td>
<td>44 (50)[6]</td>
<td>78 (&gt;97) [6]</td>
<td>&gt;200</td>
</tr>
<tr>
<td>1e</td>
<td>30 (45)[6]</td>
<td>42 (80)[6]</td>
<td>150</td>
</tr>
<tr>
<td>1f</td>
<td>50</td>
<td>&gt;97</td>
<td>&gt;200</td>
</tr>
<tr>
<td>1g</td>
<td>49</td>
<td>&gt;97</td>
<td>&gt;200</td>
</tr>
<tr>
<td>1h</td>
<td>44 (49)[6]</td>
<td>78 (93) [6]</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

[a] Reactions were performed in phosphate buffer (100 mM, pH 7.0) containing isopropanol (1% v/v), DTT (50 mM), 5 mM substrate, and Val465Ser HMFO variant (3.5 $\mu$m), at 30°C for 24 h in air-saturated solutions. [b] Conversion was measured by GC-MS after extraction with dichloromethane. [c] Enantiomeric excess of the R-configured enantiomer was measured by HPLC after derivatization. [d] $E$ value determined from ee, value and conversion.[14] [e] Doubled concentration of HMFO variant (7 $\mu$m).


