Switching the substrate preference of fungal aryl-alcohol oxidase: towards stereoselective oxidation of secondary benzyl alcohols†

Ana Serrano,‡a Ferran Sancho,‡b Javier Viña-González,c Juan Carro,a Miguel Alcalde,c Victor Guallarbd and Angel T. Martínez a,b

Oxidation of primary alcohols by aryl-alcohol oxidase (AAO), a flavoenzyme that provides H₂O₂ to fungal peroxidases for lignin degradation in nature, is achieved by concerted hydroxyl proton transfer and stereo-selective hydride abstraction from the pro-ᵣ benzyl position. In racemic secondary alcohols, the ᵣ-hydrogen abstraction would result in the selective oxidation of the ᵩ-enantiomer to the corresponding ketone. This stereoselectivity of AAO may be exploited for enzymatic deracemization of chiral mixtures and isolation of ᵩ-enantiomers of industrial interest by switching the enzyme activity from primary to secondary alcohols. A combination of computational simulations and mutagenesis has been used to produce AAO variants with increased activity on secondary alcohols, using the already available F501A variant of Pleurotus eryngii AAO as a starting point. Adaptive-PELE simulations for the diffusion of (S)-1-(p-methoxyphenyl)-ethanol in this variant allowed ile500 to be identified as one of the key residues with a higher number of contacts with the substrate during its transition from the solvent to the active site. Substitution of ile500 produced more efficient variants for the oxidation of several secondary alcohols, and the I500M/F501W double variant was able to fully oxidize (after 75 min) with high selectivity (ee >99%) the ᵩ-enantiomer of the model secondary aryl-alcohol (±)-1-(p-methoxyphenyl)-ethanol, while the ᵩ-enantiomer remained unreacted.

Introduction

There is an increasing interest in the production of pure enantiomers of a variety of chemical compounds for preparation of drugs and fine chemicals. The majority of chiral molecules of industrial interest are obtained by kinetic resolution from racemic mixtures in which two enantiomers react at different rates with a chiral catalyst resulting in a sample enanti-enriched with the less reactive isomer. For this purpose, the use of biological systems (microorganisms and enzymes) provides an alternative to chemical reagents due to the regio- and enantio-selectivity of many biocatalysts and their mild reaction conditions.

For oxidation of secondary alcohols in an enantioselective manner, both dehydrogenases and oxidases have been used. Due to the intrinsic asymmetry of these enzymes, their action on secondary alcohols often results in kinetic resolution with selectivity and enantiomeric excesses (ee) depending on the characteristics of their active sites. Several NAD(P)H-dependent alcohol dehydrogenases have been described for deracemization of secondary alcohols. However their use implies the need for auxiliary enzymes and stoichiometric amounts of reductants to recycle the NAD(P)H co-substrate. Thus, oxidases, which only need molecular oxygen as an oxidizing agent, are an interesting option for the stereoselective oxidation of secondary alcohols. Polyvinyl-alcohol oxidase is described as a secondary alcohol oxidase, although no information on its eventual selectivity is available. Other oxidases have been reported to oxidize secondary alcohols, including cholesterol oxidase, glycolate oxidase and alditol oxidase. Moreover, due to the importance of stereoselective oxidation of these substrates, several studies that widen the specificity of other oxidases on secondary alcohols have been reported in the last few years.

One potential candidate for oxidation of secondary alcohols is fungal aryl-alcohol oxidase from Pleurotus eryngii (AAO, E.C 1.1.3.7), whose biotechnological potential has been demonstrated. This flavooxidase catalyzes the oxidation of a range of primary alcohols conjugated to an aromatic
group (mainly phenyl but also naphthalenyl and furanyl) or even to an aliphatic-polyunsaturated system.\(^\text{18}\) The AAO catalytic mechanism consists of proton transfer from the hydroxyl group to a catalytic base, His502, taking place in a concerted (but asynchronous) way with hydride abstraction from the benzyl position by the oxidized flavin.\(^\text{19,20}\) Due to its active-site architecture and the concerted nature of the hydride and proton transfers, hydride abstraction in AAO is stereoselective, taking place only from the pro-R position.\(^\text{21}\) This stereoselectivity could be exploited for deracemization since it is maintained when secondary benzyl alcohols (with chiral centers) are assayed as substrates, although the AAO activity on these compounds is almost residual due to its narrow active site. In fact, it has been reported that the widening of the active site by substitution of the bulky Phe501 in variant F501A increases the enzyme activity on secondary alcohols, and improves its stereoselectivity.\(^\text{21}\)

In this work, we took advantage of computational simulations to design AAO variants with increased activity on secondary alcohols. These variants were kinetically characterized to evaluate their oxidation efficiency (on primary and secondary benzyl alcohols) and their selectivity for deracemization reactions (Scheme 1) was assessed. A switch in the substrate pattern of the variants was observed and rationalized at the atomic level.

### Material and methods

#### Chemicals

\(p\)-Methoxybenzyl alcohol, (±)1-\((p\)-methoxyphenyl\)-ethanol (racemic mixture), (R)-1-\((p\)-fluorophenyl\)-ethanol, (S)-1-\((p\)-fluorophenyl\)-ethanol, (±)-1-phenylpropanol (racemic mixture), 2-phenyl-2-propanol and horseradish peroxidase (HRP) were purchased from Sigma-Aldrich. (±)-2-Methyl-1-phenyl-1-propanol and horseradish peroxidase (HRP) were used as substrates, although the AAO activity on these compounds is almost residual due to its narrow active site. In fact, it has been reported that the widening of the active site by substitution of the bulky Phe501 in variant F501A increases the enzyme activity on secondary alcohols, and improves its stereoselectivity.\(^\text{21}\)

#### Directed mutagenesis, enzyme production and purification

Wild-type recombinant (hereinafter native) AAO from *P. eryngii* was obtained by expressing the mature AAO cDNA (GenBank AF064069) in *Escherichia coli* followed by *in vitro* activation and purification, as previously described.\(^\text{22}\) Variants were produced by site-directed mutagenesis using the following synthetic primers (bold substituted nucleotides in underlined mutated triplets are indicated below): 5’-GGG TCT AGC TCT GTT CAC **ATG** GTC ATG ATG CG-3’ for Y92F, 5’-GAG AAC GCC AAC ACG GCT TTC CAC CCA GTT GG-3’ for I500A, 5’-GAC AAC GCC AAC ACG ATG TTC CAC CCA GTT GG-3’ for I500M, 5’-GAC AAC GCC AAC ACG **TGG** TTC CAC CCA GTT GG-3’ for F501W, 5’-C AAC GCC AAC ACG ATT GCC CAC CCA GTT GTA ACG-3’ for F501A and 5’-GAC AAC GCC AAC ACG **ATT** TGG CAC CCA GTT GG-3’ for F501W, using the plasmid containing the native AAO sequence as template. The double mutations were obtained using the I500A plasmid as a template and the primers 5’-CTA TCC GAC GTG CCT **GGC** CTT CCT GCT TCC TTG G-3’ for L315A/I500A and 5’-GCT CAT TGG GAG ACC GCC TTT CAC AAC TGA TGG-3’ for I391A/I500A and the F501W plasmid as a template and the primer 5’-GAC AAC GCC AAC ACG **ATG** TGG CAC CCA GTT GG-3’ for I500M/F501W including both mutations. Mutations were confirmed by gene sequencing, the variants were purified to electrophoretic homogeneity following the same protocol as for the native protein,\(^\text{23}\) and their electronic absorption spectra were recorded. Enzymes were quantified with a Cary-4000 spectrophotometer using extinction coefficients (Table S1) taken from the literature or calculated in the present work by heat denaturation,\(^\text{23}\) using \(e_{450} = 11 300 \text{ M}^{-1} \text{ cm}^{-1}\) for the free FAD.\(^\text{24}\)

#### Steady-state kinetics for alcohol oxidation

The kinetic parameters for oxidation of primary \(p\)-methoxybenzyl alcohol were calculated by following spectrophotometrically the oxidation initial rate (1 min reaction) of the alcohol to the corresponding aldehyde using the difference molar absorptivity (\(\Delta A_{285} = 16 950 \text{ M}^{-1} \text{ cm}^{-1}\)).\(^\text{25}\)

The kinetic parameters for oxidation of the secondary alcohol (±)-1-(\(p\)-methoxy-phenyl)-ethanol were calculated by monitoring the production of \(\text{H}_2\text{O}_2\) in the HRP-coupled assay with AmplexRed (\(\Delta A_{564} = 52 000 \text{ M}^{-1} \text{ cm}^{-1}\)) as an alternative to the spectrophotometric estimation of the ketone product.

Measurements were performed at 25 °C in air-saturated (0.256 mM \(\text{O}_2\) concentration)\(^\text{26}\) 50 mM phosphate, pH 6.0. Kinetic parameters were determined by fitting the initial reaction rates at different alcohol concentrations to the Michaelis–Menten equation (eqn (1)):

\[
\frac{v}{e} = \frac{k_{\text{cat}} [S]}{K_m + [S]}
\]

In the case of (±)-1-(\(p\)-methoxy-phenyl)-ethanol, the enantio-\(\text{mer}\) concentration was considered to be 50% of the racemic mixture concentration, and an apparent turnover number (\(\text{app} K_{\text{cat}}\)) was estimated in the presence of both enantiomers.

#### Analysis of stereoselective reactions

The time course of mid-term (up to 75 min) and long-term (up to 24 or 72 h) reactions of native AAO and variants with (±)-1-(\(p\)-methoxyphenyl)-ethanol, (±)-1-phenyl-1-propanol and (±)-2-methyl-1-phenyl propanol was followed at 25 °C in air saturated 50 mM phosphate, pH 6.0, under continuous shaking. The samples were taken at different times and, after
addition of an internal standard (Table S2†), were liquid–liq-
uid extracted with hexane, and analyzed by chiral HPLC.

The enantiomers were separated in a Chiralcel IB column
(4.6 × 250 mm, 5 µm; Daicel Chemical Industries, Ltd.) using
a pre-column of the same material, and 98 : 2 (v/v) n-hexane:
isopropanol (98 : 1 for 2-methyl-1-phenyl propanol) as a
mobile phase (at 1 mL min\(^{-1}\) and 25 °C). Detection was
performed at 206 nm (see spectra of the different compounds
in Fig. S1†). The retention times for the corresponding \(R\) and \(S\) enan-
tiomers (Table S2†) were obtained from the racemic
standards assuming the published elution order.\(^{21}\) Quantifi-
cation was performed using internal standards and calibr-
ation curves (Fig. S2†).

The ee was calculated using eqn (2):

\[
\text{ee} = 100 \times \left( \frac{R - S}{R + S} \right)
\]

where \(R\) and \(S\) are the amounts of each enantiomer cal-
culated from the calibration curves.

Enantiomeric ratios (\(E\)-values)\(^{27}\) were calculated from eqn (3):

\[
E = \frac{\ln[(1 - C)(1 - \text{ee})]}{\ln[(1 - C)(1 - \text{ee})]}
\]

where \(C\) is the conversion rate.

The oxidation of \((R)\)- and \((S)\)-1-(\(p\)-fluorophenyl)-ethanol to
\(p\)-fluoracetophenone by native AAO and variants was evalu-
ated in air-saturated 50 mM phosphate, pH 6.0, at 25 °C, af-
ter long-term incubations (up to 48 h). The amount of
\(p\)-fluoracetophenone formed was calculated using the dif-
fERENCE in the molar absorbance coefficients of the alcohol and
the ketone at 248 nm \((\varepsilon_{\text{ketone}} - \varepsilon_{\text{alcohol}}, \Delta\varepsilon_{248} = 12 606 \text{ M}^{-1}
\text{cm}^{-1}\) (Fig. S3†).

Computational analysis

The new adaptive-PELE (protein energy landscape explora-
tion) software\(^{28}\) was used to study \((R)\)- and \((S)\)-1-(\(p\)-methoxyphenyl)-ethanol diffusion and binding on native AAO
and four variants. The adaptive protocol improves sampling
in PELE by running multiple short simulations (epochs)
where the initial conditions in each of them are selected
through a reward function aiming at sampling non-visited
areas. Briefly, PELE uses a Monte Carlo (MC) procedure in-
cluding protein structure prediction algorithms and a ligand
rotamer library for sampling enhancement. Each MC iteration
includes three main steps: 1) ligand and protein (backbone)
perturbation; 2) side chain sampling; 3) overall minimization.
For this study, ligand perturbation involved the [0.5–1.5]
transformation and [0.05–0.1] rotation ranges, in \(\AA\) and rad,
respectively. Backbone flexibility was allowed using the lowest
6 modes in an anisotropic network model,\(^{29}\) while all side
chains within 6 Å of the ligand were predicted on each step.
The ligand was allowed to move enforcing (its center of mass)
a 5 Å radius sphere center on the FAD N5 coordinates. Each
simulation involved 192 trajectories with 40 epochs and 20
MC PELE iterations per epoch. To improve the sampling to-
wards the FAD cofactor, we used an epsilon value of 0.1,
meaning that 10% of the processors started each epoch from
the best ligand-FAD distance previously sampled. More details
and examples on running PELE can be found elsewhere.\(^{20}\)

PELE uses an all atom OPLS2005 force field,\(^{31}\) with an im-
plicit generalized born model. All ligands and FAD charges,
however, were extracted from quantum mechanical (QM) cal-
culations. The FAD cofactor (in its quinone state) was opti-
mized with mixed quantum mechanics/molecular mechanics
(QM/MM) calculations at the M06-2X/6-31G\(^*\)/OPLS2005 level of
theory using Qsite.\(^{32}\) The initial model was derived from the
3FIM crystal structure, solvated with an 8 Å layer of water
molecules and prepared with Maestros’s protein wizard.\(^{33}\)
Only the FAD, which does not present any covalent inter-
action with the rest of the protein, was included in the QM re-
region. Ligands were optimized at the same QM level of theory
with an implicit PBF solvent using Jaguar.\(^{34}\) Ligands were
then parameterized in accordance to OPLS2005, keeping
the electrostatic QM charges and a rotamer library was built with
Macromodel.\(^{35}\)

Results and discussion

Although the activity of AAO on secondary alcohols is nearly
residual, a variant with increased activity was obtained by
substituting the bulky Phe501 by an alanine.\(^{21}\) Taking this
variant as a starting point, we combined computational simu-
lations and site-directed mutagenesis to obtain new variants
with higher activity on secondary alcohols of interest in
deracemization reactions.

Computational approach for AAO engineering

To identify mutations along the access path to the AAO active
site (Fig. 1A) that could improve the secondary alcohol access
and binding, PELE simulations of \((S)\)-1-(\(p\)-methoxyphenyl)en-
thanol diffusion in the F501A variant (molecular structure
from \textit{in silico} mutation of PDB 3FIM) were carried out. The
goal was to identify residues at the active-site access channel
potentially-involved in the substrate diffusion and oxidation.
The strategy included exploring the energy profiles for the en-
trance of this secondary alcohol and determining which
amino acids can be limiting the ligand’s access or
constraining it in an incorrect orientation for oxidation at the
active site.

During the ligand transition from the solvent to the active
site, several residues showed signiﬁcantly higher number of
interaction contacts with the alcohol (Fig. 2) being the main di-
rect obstacles that the substrate has to bypass. Among them,
Tyr92 and Phe397 together with Phe501 in native AAO consti-
tute a hydrophobic bottleneck (Fig. 1A) for the access of sub-
strates, being also involved in their stabilization at the active
site.\(^{35–38}\) According to these computational data, residues with
contact numbers higher than 35 000—such as Pro79, Tyr92,
Leu315, Ile391, Phe397, Pro399 and Ile500, together with
previously mutated Ala501, all of them located at less than 4 Å of the active-site channel—were selected for site-directed mutagenesis. In vitro folding of the mutated proteins was performed after replacing the above residues with alanines, and alternative mutations were introduced in several cases.

Proper incorporation of the FAD cofactor was shown by the presence of typical bands I and II in their electronic absorption spectra (with only slight displacements due to mutations in the flavin environment; Table S1†). The ability of these variants to oxidize secondary alcohols was tested by incubating them with (±)-1-(p-methoxyphenyl)-ethanol, as a model chiral substrate, and analyzing the resulting ketone and the remaining R and/or S substrate enantiomers (Fig. S2†). Quantification of both isomers by chiral HPLC after 24 h of reaction indicated that, with the exception of I500W, the variants oxidized (S)-1-(p-methoxyphenyl)-ethanol to the corresponding ketone with different rates, without any activity on (R)-1-(p-methoxyphenyl)-ethanol. I500A showed 15-times higher activity than the native enzyme, with 50% conversion of the racemic substrate in the first 4 h of reaction (Fig. 3 and Table S3†) due to the almost total oxidation of the S-enantiomer.

Rationalizing the effect of the I500A mutation

PELE simulations for (S)-1-(p-methoxyphenyl)-ethanol diffusion and positioning at the active site of the I500A variant, compared to native AAO, contributed to explaining its higher activity on secondary alcohols mentioned above.
In the case of native AAO, PELE diffusion leads to two main locations of the alcohol in the active site, in which its hydroxyl group is located near the catalytic His502 at an adequate distance (∼2.5 Å) for the proton abstraction to be produced (Fig. 4A). These two structures differ in the position of the benzylic hydrogen (in the R position) with regard to the flavin (hydrogen–FAD distances of 2.5 Å and 5.3 Å) only the first one being compatible with hydride transfer to flavin N5 and ketone formation.

By contrast, alcohol diffusion in I500A showed that in this variant the ligand is able to move more freely in the catalytic site (due to its increased size) with a range of positions (circles in Fig. 4B, top) at 2.0–2.5 Å from His502 and progressively shorter distances of the FAD, to finally attain a position compatible with catalysis (Fig. 4B, bottom, left).

Combinatorial saturation mutagenesis of AAO expressed in *Saccharomyces cerevisiae*, taking the above results into account, yielded the I500M/F501W variant with a noticeable increase in the oxidation of 1-(p-methoxyphenyl)-ethanol.

Alcohol oxidation by I500 and Phe501 variants

Therefore, to get insights into the AAO stereoselective oxidation of secondary benzyl alcohols, two additional variants (I500M and I500M/F501W) were expressed in *E. coli*, *in vitro* activated and purified to homogeneity, showing correct folding and FAD incorporation (with only slight displacements in bands I and II due to mutations in the flavin environment; Table S1†).

We first evaluated the effect of four selected single and double substitutions of Ile500 and Phe501 on the AAO activity towards its preferred substrate, p-methoxybenzyl alcohol, and mutant AAO variants on model primary (top) and secondary (bottom) benzyl alcohols.

<table>
<thead>
<tr>
<th>p-Methoxybenzyl alcohol</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$k_{\text{cat}}/K_m$ (mM$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAO</td>
<td>142 ± 5</td>
<td>27 ± 4</td>
<td>5230 ± 620</td>
</tr>
<tr>
<td>F501A</td>
<td>3.1 ± 0.03</td>
<td>12.3 ± 0.6</td>
<td>251 ± 12</td>
</tr>
<tr>
<td>I500A</td>
<td>1.5 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>1140 ± 45</td>
</tr>
<tr>
<td>I500M</td>
<td>5.3 ± 0.3</td>
<td>0.4 ± 0.1</td>
<td>13 100 ± 200</td>
</tr>
<tr>
<td>I500M/F501W</td>
<td>3.3 ± 0.1</td>
<td>0.4 ± 0.03</td>
<td>7930 ± 640</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(+)1-(p-Methoxyphenyl)-ethanol</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)$^b$</th>
<th>$K_m$ (mM)$^c$</th>
<th>$k_{\text{cat}}/K_m$ (mM$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAO</td>
<td>0.18 ± 0.002</td>
<td>25.0 ± 0.6</td>
<td>0.0072 ± 0.0002</td>
</tr>
<tr>
<td>F501A</td>
<td>0.05 ± 0.003</td>
<td>10.0 ± 1.5</td>
<td>0.0051 ± 0.0008</td>
</tr>
<tr>
<td>I500A</td>
<td>0.22 ± 0.01</td>
<td>2.9 ± 0.3</td>
<td>0.079 ± 0.009</td>
</tr>
<tr>
<td>I500M</td>
<td>0.42 ± 0.01</td>
<td>1.4 ± 0.1</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>I500M/F501W</td>
<td>2.2 ± 0.04</td>
<td>3.1 ± 0.2</td>
<td>0.71 ± 0.05</td>
</tr>
</tbody>
</table>

$^a$ Determined at 25 °C in air-saturated (0.256 mM O$_2$) 50 mM phosphate, pH 6.0. $^b$ Apparent $k_{\text{cat}}$ estimated in the racemic mixture. $^c$ Referring to the S-enantiomer, representing 50% of the racemic mixture.

By contrast, alcohol diffusion in I500A showed that in this variant the ligand is able to move more freely in the catalytic site (due to its increased size) with a range of positions (circles in Fig. 4B, top) at 2.0–2.5 Å from His502 and progressively shorter distances of the FAD, to finally attain a position compatible with catalysis (Fig. 4B, bottom, left).

Combinatorial saturation mutagenesis of AAO expressed in *Saccharomyces cerevisiae*, taking the above results into account, yielded the I500M/F501W variant with a noticeable increase in the oxidation of 1-(p-methoxyphenyl)-ethanol.

Alcohol oxidation by I500 and Phe501 variants

Therefore, to get insights into the AAO stereoselective oxidation of secondary benzyl alcohols, two additional variants (I500M and I500M/F501W) were expressed in *E. coli*, *in vitro* activated and purified to homogeneity, showing correct folding and FAD incorporation (with only slight displacements in bands I and II due to mutations in the flavin environment; Table S1†).

We first evaluated the effect of four selected single and double substitutions of Ile500 and Phe501 on the AAO activity towards its preferred substrate, p-methoxybenzyl alcohol, and mutant AAO variants on model primary (top) and secondary (bottom) benzyl alcohols.

<table>
<thead>
<tr>
<th></th>
<th>Secondary (sec; s$^{-1}$)</th>
<th>Primary (pri; s$^{-1}$)</th>
<th>Relative sec/pri ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAO</td>
<td>0.18</td>
<td>142.0</td>
<td>1</td>
</tr>
<tr>
<td>F501A</td>
<td>0.05</td>
<td>3.1</td>
<td>13</td>
</tr>
<tr>
<td>I500A</td>
<td>0.22</td>
<td>1.5</td>
<td>116</td>
</tr>
<tr>
<td>I500M</td>
<td>0.42</td>
<td>5.3</td>
<td>63</td>
</tr>
<tr>
<td>I500M/F501W</td>
<td>2.2</td>
<td>3.3</td>
<td>526</td>
</tr>
</tbody>
</table>

Table 1: Kinetic constants of native AAO and four selected variants on model primary (top) and secondary (bottom) benzyl alcohols

Table 2: Changes in the relative secondary (1-(p-methoxyphenyl)-ethanol) to primary (p-methoxybenzyl alcohol) turnover ($s^1$) ratio of four selected variants referring to the native AAO (from Table 1 data)
by measuring the release of anisaldehyde (Table 1, top). Aldehyde estimation has been confirmed to provide similar kinetic constants to those obtained when the equimolecular

\[ \text{H}_2\text{O}_2 \text{ release is followed.}^{36} \]  

The variants showed lower turnover numbers \((k_{\text{cat}})\) but increased affinity, as shown by lower \(K_m\) values, with regard to the native enzyme. Thus, the I500M/F501W and I500M mutations resulted in 1.5-, and 2.5-fold more efficient oxidation of the primary alcohol, respectively.

Then, kinetic measurements of \((\pm)1-(p\text{-methoxyphenyl})\)-ethanol oxidation were followed by \(\text{H}_2\text{O}_2\) release (Table 1, bottom). Increased affinity for the secondary alcohol was reflected in the lower \(K_m\) values that the variants showed. As a consequence, I500A, I500M and I500M/F501W present 11-, 42- and 97-fold higher catalytic efficiencies \((k_{\text{cat}}/K_m)\) than native AAO, respectively, in agreement with the results from 24 h experiments (Table S3†). Comparison of the action of the variants on secondary and primary alcohols was based on their turnover \((k_{\text{cat}})\) values, since in deracemization reactions in an industrial context substrates are used under saturated conditions and the rates are therefore independent of the \(K_m\) value. \(^{41}\) Interestingly, the substitution of Ile500 produced an increase of the activity on the model secondary alcohol at the expense of reducing its activity on the primary \(p\text{-methoxybenzyl} \) alcohol, as shown by kinetic comparison (up to 500-fold higher secondary/primary ratio for I500M/F501W) (Table 2). Therefore, the more active a variant is on secondary alcohols, the less active it is on primary alcohols, revealing an interesting switch in the substrate preference of AAO. The higher activity of the double variant agrees with a broader active site (Fig. 1B) enabling

---

**Fig. 5** \((\pm)1-(p\text{-Methoxyphenyl})\)-ethanol oxidation to the corresponding ketone for 75 min by three selected AAO variants compared with the native enzyme (and control without enzyme): A) remaining \(R\)-enantiomer; B) remaining \(S\)-enantiomer; C) conversion yield (racemic mixture); and D) \(R\)-isomer ee. Reactions between alcohol (2.5 mM racemic mixture) and enzyme (2.5 \(\mu\)M) were performed in 50 mM phosphate, pH 6.0, at 25 °C, and the remaining substrates and product were analyzed by chiral HPLC.

**Fig. 6** Kinetic resolution of \((\pm)1-(p\text{-methoxyphenyl})\)-ethanol (A), \((\pm)1\)-phenyl-1-propanol (B) and \((\pm)1\)-phenyl-2-methyl-1-propanol (C) by the I500M/F501W variant: chromatograms before (left) and after incubation with the enzyme (for 1 h in A and 72 h in B and C) resulting in \(R\)-enriched samples (right).
entering and adequate positioning of the bulkier secondary alcohol.

**Stereoselective oxidation of (±)1-(p-methoxyphenyl)-ethanol**

The oxidation of the model secondary aryl alcohol by native AAO and its variants was monitored in time-course reactions using chiral HPLC. Reactions were performed at 25 °C under continuous shaking and aliquots were taken every 5 min for 2 h (Fig. S4†). As shown in Fig. 5, only 3% conversion (with 2% ee) was found after 2 h incubation with native AAO, and the results were only slightly improved with the F501A variant.

However, other variants were considerably more active on the secondary alcohol, with up to 18- and 66-fold higher conversion rates in the cases of I500M and I500M/F501W, respectively (Fig. 5B and C). The substitution of Ile500 by either methionine or alanine considerably increases both conversion and ee with regard to native AAO. After 75 min, the conversion reached 24% and 30% for I500A and I500M (with ee of 30% and 42%), respectively. These values increased up to 50% conversion of the total racemic mixture (with ee 100%) for I500M/F501W, indicating a high enantioselectivity of this double variant (Fig. 5C and D, 6A and Table 3).

**Reaction with other secondary alcohols**

To extend the deracemization potential of AAO, revealed by the (±)-1-(p-methoxyphenyl)-ethanol reactions, to other secondary benzyl alcohols, the I500A, I500M and I500M/F501W variants (10 μM) were incubated with (±)-1-phenyl-1-propanol and (±)-1-phenyl-2-methylpropanol (2.5 mM) up to 72 h.

Chiral HPLC analyses indicated that I500M and I500M/F501W were able to oxidize the two alcohols to their corresponding ketones (Fig. 6B and C) although with low conversion yields (4% and 22% of the above racemic compounds, respectively, by I500M; and 13% and 31%, respectively, by I500M/F501W) leading to an enantiomer ee up to 62% in the case of I500M/F501W (Table 4). These results suggest that the active site in these two variants has been sufficiently enlarged (as illustrated in Fig. 1B for I500M/F501W) to accommodate not only the methyl group of the model secondary alcohol but also larger groups, such as the ethyl and isopropyl groups of the two other secondary alcohols assayed.

The enantioselectivity of the Ile500 variants was confirmed by incubating them with pure R- and S-enantiomers of 1-(p-fluorophenyl)ethanol. After 48 h of reaction, the formation of p-fluorocacetophenone (up to 30%) was shown by difference spectra when (S)-1-(p-fluorophenyl)ethanol was treated with the single and double Ile500 variants (Fig. 7A). By contrast, hardly any reaction was observed for (R)-1-(p-fluorophenyl)ethanol (Fig. 7B) confirming the enantioselectivity shown using racemic mixtures.

**Stereoselectivity explained by R and S simulations**

The AAO selectivity for the S-enantiomers, during secondary alcohol oxidation, is consistent with the catalytic mechanism

<table>
<thead>
<tr>
<th>Conversionb (%)</th>
<th>ee (%)</th>
<th>E-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAO</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>F501A</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>I500A</td>
<td>24</td>
<td>30</td>
</tr>
<tr>
<td>I500M</td>
<td>30</td>
<td>42</td>
</tr>
<tr>
<td>I500M/F501W</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

*Mid-term (75 min) reaction of 2.5 mM alcohol and 2.5 μM enzyme in air-saturated 50 mM phosphate, pH 6.0, at 25 °C. a Referring to the racemic mixture. c —, too low E-values (<15) for practical purposes.*

<table>
<thead>
<tr>
<th>Conversionb (%)</th>
<th>ee (%)</th>
<th>Conversionb (%)</th>
<th>ee (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAO</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>I500A</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>I500M</td>
<td>4</td>
<td>9</td>
<td>22</td>
</tr>
<tr>
<td>I500M/F501 W</td>
<td>13</td>
<td>26</td>
<td>31</td>
</tr>
</tbody>
</table>

*Long-term (72 h) reaction of 2.5 mM alcohol and 10 μM enzyme in air-saturated 50 mM phosphate, pH 6.0, at 25 °C. a Referring to the racemic mixture.*

---

**Table 3 Conversion yield and selectivity of the (R-enantiomer) in mid-term reactions of racemic (±)-1-(p-methoxyphenyl)-ethanol with native AAO and four selected variants**

**Table 4 Conversion yield and selectivity (R-enantiomer ee) in long-term reaction of two additional secondary alcohols with native AAO and three selected variants**

**Fig. 7 Molar absorptivity difference spectra (final – initial) after 48 h of reaction of (S)-1-(p-fluorophenyl)-ethanol (A) and (R)-1-(p-fluorophenyl)-ethanol (B) with native AAO and three variants. The inset shows the p-fluorocacetophenone production. Reactions between alcohol (100 μM) and enzyme (~0.2 μM) were performed in 50 mM phosphate, pH 6.0, at 25 °C.**

**Catalysis Science & Technology Paper**
reported for the oxidation of \( p \)-methoxybenzyl alcohol, in which the hydrogen in the pro-\( R \) position is selectively abstracted by the flavin.\(^{21}\) To further investigate the stereo-selectivity in secondary (\( \pm \))-1-(\( p \)-methoxyphenyl)-ethanol oxidation, the energy profiles of the diffusion of both enantiomers, from the solvent to the active site of AAO and the \( F501A \), \( 1500A \), \( I500M \), \( F501M \) and \( I500M/F501W \) variants, were analyzed in PELE simulations.

According to these simulations, the \( S \)-enantiomer is capable of reaching the active site of the selected variants with good binding energies and adequate distances from both the FAD and the catalytic His502 for hydroxyl oxidation (Fig. 8, left). This means that the oxidation of the \( S \)-enantiomer will be more favorable for these variants than for the native enzyme, being consistent with the experimental kinetic parameters (Table 1). Moreover, the catalytic efficiency (\( k_{\text{cat}}/K_m \)) values – which cluster into three main groups: native AAO and \( F501A \) (low efficiency), \( 1500A \) (intermediate efficiency) and \( 1500M \) and \( 1500M/F501W \) (high efficiency) (Table 1) – qualitatively correlate with the catalytic population observed for the \( S \)-enantiomer (Fig. 8, left).

In contrast to the above calculations for the \( S \)-enantiomer, when simulations were performed with (\( R \))-1-(\( p \)-methoxyphenyl)-ethanol in the native enzyme and variants (Fig. 8, right), the alcohol did not reach the active site at distances that would allow the simultaneous abstraction of the proton from the alcohol and the hydride transfer (which in this case should be from the \( S \) position) in agreement with the experimental data (Fig. 5A).

**Conclusions**

The ability to oxidize secondary alcohols has been introduced in AAO by site-directed mutagenesis guided by computational simulations with the adaptive PELE software. The \( 1500M/F501W \) double variant appears as a biocatalyst of biotechnological interest since it produces enantiomerically-enriched secondary alcohols (up to ee > 99%), only at the expense of molecular oxygen, during the kinetic resolution of racemic mixtures. According to the \( S \) stereo-preference of AAO, the \( R \)-isomers remain unreacted, together with the ketone formed during oxidation of the \( S \)-isomers (Scheme 1). This stereo-selectivity, which agrees with the binding energy at catalytically-relevant positions (near the active-site catalytic base and the flavin cofactor) in the computational diffusion of the \( R \)- and \( S \)-isomers, can be exploited for deracemization of chiral secondary alcohols. In summary, we show how computational simulations can guide protein engineering to switch the oxidase preference on primary benzyl alcohols towards enantioselective oxidation of secondary alcohols. Analysis of the resulting variants reveals that the mutations introduced facilitate the entrance and accommodation of bulkier secondary alcohols at the active site of AAO.

**Conflicts of interest**

There are no conflicts to declare.

**Acknowledgements**

This work was supported by the INDOX (KBBE-2013-7-613549) EU project and by the BIO2017-86559-R (GenoBioref), CTQ2016-79138-R and BIO2016-79106-R projects of the Spanish Ministry of Economy, Industry and Competitiveness, cofinanced by FEDER funds. Pedro Merino (University of Zaragoza, Spain) is acknowledged for his suggestions on chiral HPLC analyses.
References