Enantioselective oxidation of secondary alcohols by the flavoprotein alcohol oxidase from Phanerochaete chrysosporium

Tjallinks, Gwen; Martin, Caterina; Fraaije, Marco W

Enantioselective oxidation of secondary alcohols by the flavoprotein alcohol oxidase from *Phanerochaete chrysosporium*

Gwen Tjallinks, Caterina Martin, Marco W. Fraaije [*]

Molecular Enzymology Group, University of Groningen, Nijenborgh 4, Groningen, the Netherlands

ARTICLE INFO

ABSTRACT

The enantioselective oxidation of secondary alcohols represents a valuable approach for the synthesis of optically pure compounds. Flavoprotein oxidases can catalyse such selective transformations by merely using oxygen as electron acceptor. While many flavoprotein oxidases preferably act on primary alcohols, the FAD-containing alcohol oxidase from *Phanerochaete chrysosporium* was found to be able to perform kinetic resolutions of several secondary alcohols. By selective oxidation of the (*S*)-alcohols, the (*R*)-alcohols were obtained in high enantiopurity. *In silico* docking studies were carried out in order to substantiate the observed (*S*)-selectivity. Several hydrophobic and aromatic residues in the substrate binding site create a cavity in which the substrates can comfortably undergo van der Waals and pi-stacking interactions. Consequently, oxidation of the secondary alcohols is restricted to one of the two enantiomers. This study has uncovered the ability of an FAD-containing alcohol oxidase, that is known for oxidizing small primary alcohols, to perform enantioselective oxidations of various secondary alcohols.

1. Introduction

The outstanding selectivity of enzymes allows them to catalyse reactions that are difficult to accomplish in a chemocatalytic manner [1–3]. Hence, several strategies have been developed in order to obtain enantiopure alcohols using a biocatalytic approach, which include enantioselective reduction of prochiral ketones [4] and enzyme-catalysed kinetic resolutions of secondary alcohols [5]. A class of enzymes that has been widely employed for the selective oxidations of alcohols and amines are oxidases (EC 1.1), which are part of the oxidoreductases class [4]. Alcohol oxidases are a subclass of oxidases and contain either a flavin adenine dinucleotide (FAD) or copper as prosthetic group. An essential advantage of this class of enzymes is that they do not require the need to regenerate coenzymes and solely use molecular oxygen as electron acceptor [6]. Furthermore, this class is known for its broad substrate acceptance scope which includes primary and secondary alcohols, and aldehydes, converting them to their corresponding aldehydes, ketones and carboxylic acids [7]. In this paper, we investigated the selective oxidative abilities of a flavoprotein alcohol oxidase towards secondary alcohols.

Commonly, the substrate acceptance scope for alcohol oxidases is rather limited and they often exhibit higher activity towards primary alcohols [8–13]. However, Turner et al. [14] have been able to engineer and use a copper-dependent galactose oxidase for the deracemisation of secondary alcohols to ketones. Nevertheless, flavoprotein alcohol oxidases are still mainly employed for enantioselective oxidations of primary alcohols [15,16]. In 2018, our group has characterized and engineered an FAD-containing alcohol oxidase from the white-rot basidiomycete *Phanerochaete chrysosporium* (AOX) for glycerol oxidation [17]. The Phe101Ser-AOX variant was most potent towards glycerol and then further explored for its ability to perform selective double oxidations of diols towards hydroxy acids [4]. Furthermore, it was shown to enantioselectively oxidize the (*S*)-enantiomer of 1-phenylethanol. This is a striking discovery, as there have not yet been many flavin-dependent AOXs reported that are active on secondary alcohols. In this paper, various secondary alcohols and AOX variants were probed for their ability to perform kinetic resolutions. Furthermore, the molecular basis for the observed (*S*)-enantioselectivity was investigated.

2. Results and discussion

All studied AOX variants were expressed and purified as FAD-bound

Table 1
Conversions[a] and kinetic parameters[b] for the oxidation of secondary alcohols by AOX. [c].

substrate		Conversion[d] (%)	*e.e.*[e] (%)	E[f]	k_{obs} (s^{-1})
1-indanol (**1**)		50	>99	>200	n.d.[g]
1-phenylethanol (**2**)		43	72	93	0.13 ± 0.01
2-pentanol (**3**)		50	>99	>200	0.38 ± 0.02
3-butyn-2-ol (**4**)		42	57	15	0.12 ± 0.01

[a] Values obtained using 20 mM substrate and 20 μM AOX in 100 mM potassium phosphate pH 7.5, 24 h at 35 °C. Details can be found in the Supporting Information.

[b] Values obtained using 200 mM substrate and 0.2 μM AOX in 100 mM potassium phosphate pH 7.5.

[c] Commercial enantiopure substrates were used as standards to establish the absolute configuration.

[d] Conversion is based on the amount of formed ketone, except for 3-butyn-2-ol which was based on racemic substrate depletion.

[e] Enantiomeric excess of substrate.

[f] E value calculated using http://biocatalysis.uni-graz.at/enantio/.

[g] Value could not be obtained due to insolubility of the substrate at high concentrations, this was indicated as n.d. (not determined).

holo enzymes. No addition of FAD during purification was required which indicates a tight binding of the flavin cofactor (Fig. S1). A typical UV–Vis absorption spectrum was obtained for the purified AOX variants with an absorption maximum at 455 nm, suggesting that the flavin is in the oxidized state. The substrate acceptance profile and enantioselectivity of wild-type AOX for secondary alcohols was analyzed using chiral GC analysis and a set of 30 different aromatic and aliphatic secondary alcohols (see Table S2 for the complete overview of screened substrates). This revealed that wild-type AOX was able to convert four secondary alcohols: 1-indanol, 1-phenylethanol, 2-pentanol, and 3-butyn-2-ol. Activity of AOX on these apolar aromatic and aliphatic secondary alcohols had not yet been reported before (Table 1). Three AOX mutants (Thr315Ser, Leu317Phe and Trp560Phe AOX), which are expected to have an altered substrate binding pocket, were tested for their conversion of 1-indanol, 1-phenylethanol, and 2-pentanol. However, all AOX mutants displayed low or no significant activity and/or lower enantioselectivity (Fig. S4-S6). Ultimately, wild-type AOX was

found to be most potent and was the only AOX variant used for further studies.

Conversions with all identified secondary alcohols were performed for 24 h and it was observed that AOX selectively oxidizes the (*S*)-alcohols to the corresponding ketones. Fig. 1 illustrates the enantioselective conversion of the (*S*)-enantiomer when AOX is incubated with racemic 1-phenylethanol. The (*R*)-alcohols were left almost untouched. As a consequence, high E values of >90 were obtained for 1-indanol, 1-phenylethanol and 2-pentanol (Table 1). Only for 3-butyn-2-ol a somewhat lower enantioselectivity was observed (E value = 9). AOX was found to be most active towards 2-pentanol (Table 1). 2-Pentanol is a relatively small compound with a flexible aliphatic chain which could allow for facile entry into the active site and for obtaining an optimal conformation for subsequent alcohol oxidation. The reaction rate of 1-phenylethanol and 3-butyn-2-ol were lower but in the same range as 2-pentanol. Overall, the reaction rates were relatively low, however almost full conversions could be obtained for the secondary alcohols within 24 h.

The underlying reason for the observed (*S*)-enantioselectivity of AOX was examined by inspecting the crystal structure and performing *in silico* substrate docking. The selectivity can be explained by investigating the role of active-site residues and residues that form the substrate binding pocket. Just as for other members of the GMC-oxidoreductase superfamily, AOX contains a strictly conserved catalytic base His561 [18–21]. In addition, it holds a spatially conserved Asn604 residue that, as postulated previously, most likely functions as a hydrogen bond donor during catalysis. Both residues structurally occupy the *Re*-face of the flavin cofactor, hence the substrate binding domain is also located on the *Re*-face. In the oxidation reaction, proton abstraction of the α-hydroxyl group by the histidine catalytic base His561 with a simultaneous or stepwise with hydride transfer from the substrate Cα atom to flavin N5 atom occurs [19,21–23]. Hence, the specific orientation of the substrate towards the FAD cofactor, His561 and Asn604 is decisive in the enantioselective outcome of the reaction. Docking of 1-phenylethanol revealed that residues Phe101, Phe399, Phe422, Tyr424 and Trp560 create an apolar cavity in which the substrate can comfortably undergo van der Waals and pi-stacking interactions (Fig. 2). Consequently, only (*S*)-1-phenylethanol can bind in the active site in a productive manner: the hydride can only be transferred to the *Re*-face of the flavin cofactor. The aromatic ring of 1-phenylethanol prevents the formation of any other conformation necessary to convert the (*R*)-enantiomer. Docking of the other three substrates resulted in analogous optimal binding poses, in which the hydrophobic environment drives the observed (*S*)-enantioselectivity (Figs. S10-S12).

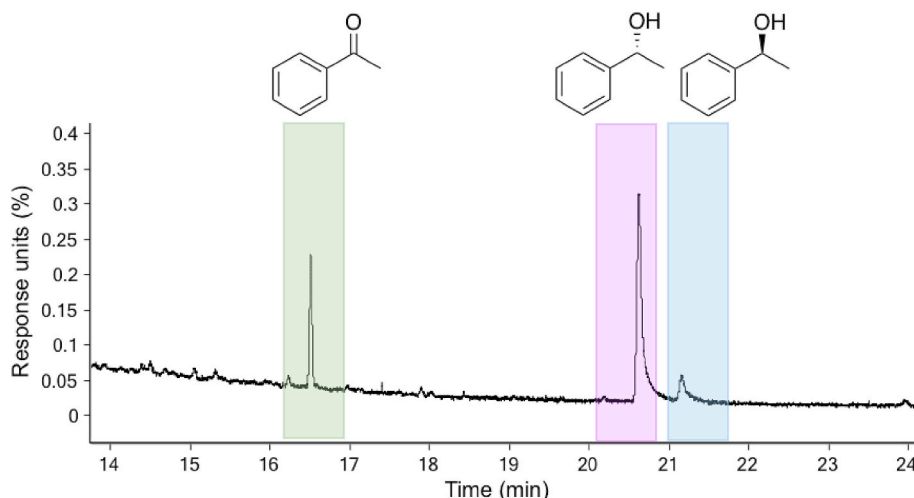The enantioselectivity of wild-type AOX was then compared to the

Fig. 1. GC chromatogram after 24 h conversion of 20 mM racemic 1-phenylethanol (**2**) by 20 μM AOX in 100 mM potassium phosphate buffer pH 7.5.
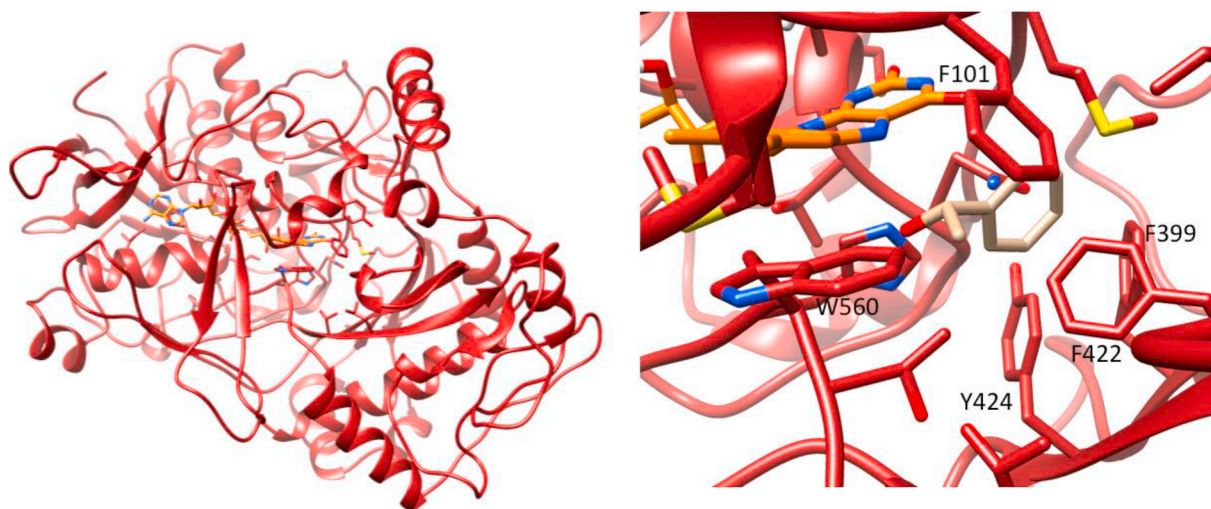
Fig. 2. Binding pose of (*S*)-1-phenylethanol (**2**) in AOX (PDB:6H3G). Left: overall structure of AOX. Right: close-up of binding of (*S*)-1-phenylethanol (gray sticks) binding pose.

previously generated Phe101Ser-AOX variant, used for glycerol and selective diol oxidations. This variant was previously found to enantioselectively oxidize 1-phenylethanol, however only with 15% conversion of the (*S*)-enantiomer [4]. It is hypothesized that the apolar and aromatic pocket encompassed by Phe101, Phe399, Phe422, Tyr424 and Trp560 is the limiting factor in substrate acceptance. This apolar cavity tightly fits small aliphatic chains or planar aromatic rings but does not accept longer chained molecules or substituted benzene rings. The Ser101 variant is less capable of converting the (*S*)-alcohols and does not convert any of the (*R*)-alcohols. A potential explanation could be that Phe at position 101 is necessary to form the apolar cavity and without this residue the substrate is not able to correctly bind in the active site. Additionally, Ser101 is a hydrogen-bond donor and acceptor residue, which can therefore interact with the hydroxyl-group of the substrate and block it from reacting with the catalytic base His561. Ultimately, this aromatic and apolar pocket is necessary to obtain high conversion, substantiating the results obtained from the docking experiment.

To conclude, the FAD-containing alcohol oxidase from the white-rot basidiomycete *P. chrysosporium* was shown to be able to oxidize four secondary alcohols in an (*S*)-enantioselective manner. The secondary alcohols identified as substrate contained apolar aliphatic and aromatic moieties. *In silico* docking studies were carried out to understand the molecular basis for the (*S*)-enantioselectivity of AOX. It was hypothesized that the hydrophobic cavity comprised of residues Phe101, Phe399, Phe422, Tyr424 and Trp560 create a pocket in which the substrates can comfortably undergo van der Waals and pi-stacking interactions. Thereby, the substrate can obtain an optimal conformation for proton abstraction and subsequent hydride transfer to the flavin-cofactor. The discovery of the potential of AOX in kinetic resolutions of secondary alcohols together with the structural insights may pave the way for developing future AOX-based applications for the synthesis of enantiopure compounds.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.abb.2021.108888.

References

[1] J. Tao, R. Kazlauskas, Biocatalysis for Green Chemistry and Chemical Process Development, Wiley-VCH, 2011.

[2] R.N. Patel, in: R.N. Patel (Ed.), Biocatalysis in the Pharmaceutical and Biotechnology Industries, Taylor & Francis Group, Boca Raton, 2007.

[3] R.A. Sheldon, D. Brady, Broadening the scope of biocatalysis in sustainable organic synthesis, ChemSusChem 12 (2019) 2859–2881.

[4] C. Martin, M. Trajkovic, M.W. Fraaije, Production of hydroxy acids through selective double oxidation of diols by a flavoprotein alcohol oxidase, Angew. Chem. Int. Ed. 59 (2020) 4869–4872.

[5] B. Heinze, R. Kourist, L. Fransson, K. Hult, U.T. Bornscheuer, Highly enantioselective kinetic resolution of two tertiary alcohols using mutants of an esterase from *Bacillus subtilis*, Protein Eng. Des. Sel. 20 (3) (2007) 125–131.

[6] P.N.R. Vennestrøm, C.H. Christensen, S. Pedersen, J.D. Grunwaldt, J.M. Woodley, Next-generation catalysis for renewables: combining enzymatic with inorganic heterogeneous catalysis for bulk chemical production, ChemCatChem 2 (3) (2010) 249–258.

[7] E. Romero, J.R. Gómez Castellanos, G. Gadda, M.W. Fraaije, A. Mattevi, Same substrate, many reactions: oxygen activation in flavoenzymes, Chem. Rev. 118 (4) (2018) 1742–1769.

[8] A. Siebum, A. van Wijk, R. Schoevaart, T. Kieboom, Galactose oxidase and alcohol oxidase: scope and limitations for the enzymatic synthesis of aldehydes, J. Mol. Catal. B Enzym. 41 (3–4) (2006) 141–145.

[9] S. Gandomkar, E. Jost, D. Loidolt, A. Swoboda, M. Pickl, W. Elaily, B. Daniel, M. W. Fraaije, P. Macheroux, W. Kroutil, Biocatalytic enantioselective oxidation of sec-allylic alcohols with flavin-dependent oxidases, Adv. Synth. Catal. 361 (22) (2019) 5264–5271.

[10] Q.T. Nguyen, G. de Gonzalo, C. Binda, A. Rioz-Martínez, A. Mattevi, M.W. Fraaije, Biocatalytic properties and structural analysis of eugenol oxidase from *rhodococcus jostii* RHA1: a versatile oxidative biocatalyst, ChemBioChem (2016) 1359–1366.

[11] S. Pils, K. Schnabl, S. Wallner, M. Kljajic, N. Kupresanin, R. Breinbauer, M. Fuchs, R. Rocha, J.H. Schrittwieser, W. Kroutil, et al., Characterization of the monolignol oxidoreductase AtBBE-like protein 15 L182V for biocatalytic applications, J. Mol. Catal. B Enzym. 133 (2016) S6–S14.

[12] A. Hernández-Ortega, P. Ferreira, P. Merino, M. Medina, V. Guallar, A.T. Martínez, Stereoselective hydride transfer by aryl-alcohol oxidase, a member of the GMC superfamily, ChemBioChem 13 (3) (2012) 427–435.

[13] B. Daniel, B. Konrad, M. Toplak, M. Lahham, J. Messenlehner, A. Winkler, P. Macheroux, The family of berberine bridge enzyme-like enzymes: a treasure-trove of oxidative reactions, Arch. Biochem. Biophys. 632 (2017) 88–103.

[14] F. Escalettes, N.J. Turner, Directed evolution of galactose oxidase: generation of enantioselective secondary alcohol oxidases, ChemBioChem 9 (2008) 857–860.

[15] W.P. Dijkman, M.W. Fraaije, Discovery and characterization of a 5-hydroxymethylfurfural oxidase from *methylovorus* sp. Strain MP688, Appl. Environ. Microbiol. 80 (3) (2014) 1082–1090.

[16] W.P. Dijkman, D.E. Groothuis, M.W. Fraaije, Enzyme-catalyzed oxidation of 5-hydroxymethylfurfural to furan-2,5-dicarboxylic acid, Angew. Chem. Int. Ed. 53 (25) (2014) 6515–6518.

[17] Q.-T. Nguen, E. Romero, W. Dijkman, D. Vasconcellos, S. Pantaroto, C. Binda, A. Mattevi, W.F. Marco, Structure-based engineering of *Phanerochaete chrysosporium* alcohol oxidase for enhanced oxidative power toward glycerol, Biochemistry 57 (2018) 6209–6218.

[18] M. Ghanem, G. Gadda, On the catalytic role of the conserved active site residue His 466 of choline oxidase, Biochemistry 44 (2005) 893–904.

[19] A. Hernández-Ortega, K. Borrelli, P. Ferreira, M. Medina, A.T. Mart, V. Guallar, Substrate diffusion and oxidation in GMC Oxidoreductases : an experimental and computational study on fungal aryl-alcohol oxidase, Biochem. J. 436 (2011) 341–350.

[20] G. Gadda, C. Smitherman, K. Rungsrisuriyachai, M.W. Germann, Identification of the catalytic base for alcohol activation in choline oxidase, Biochemistry 54 (2015) 413–421.

[21] G. Gadda, Hydride transfer made easy in the reaction of alcohol oxidation catalyzed by flavin-dependent oxidases, Biochemistry 47 (2008) 13745–13753.

[22] T. Wongnate, P. Chaiyen, The substrate oxidation mechanism of pyranose 2-oxidase and other related enzymes in the glucose – methanol – choline superfamily, FEBS J. 280 (2013) 3009–3027.

[23] A. Hernández-Ortega, P. Ferreira, A.T. Martínez, Fungal aryl-alcohol oxidase: a peroxide-producing flavoenzyme involved in lignin degradation, Appl. Microbiol. Biotechnol. 93 (2012) 1395–1410.