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On the origin of vanillyl alcohol oxidases

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ABSTRACT

Vanillyl alcohol oxidase (VAO) is a fungal flavoenzyme that converts a wide range of para-substituted phenols. The products of these conversions, e.g. vanillin, coniferyl alcohol and chiral aryl alcohols, are of interest for several industries. VAO is the only known fungal member of the 4-phenol oxidising (4PO) subgroup of the VAO/PCMH flavoprotein family. While the enzyme has been biochemically characterised in great detail, little is known about its physiological role and distribution in fungi.

We have identified and analysed novel, fungal candidate VAOs and found them to be mostly present in Pezizomycotina and Agaricomycotina. The VAOs group into three clades, of which two clades do not have any characterised member. Interestingly, bacterial relatives of VAO do not form a single outgroup, but rather split up into two separate clades.

We have analysed the distribution of candidate VAOs in fungi, as well as their genomic environment. VAOs are present in low frequency in species of varying degrees of relatedness and in regions of low synteny. These findings suggest that fungal VAOs may have originated from bacterial ancestors, obtained by fungi through horizontal gene transfer.

Because the overall conservation of fungal VAOs varies between 60 and 30% sequence identity, we argue for a more reliable functional prediction using critical amino acid residues. We have defined a sequence motif P-x-x-x-x-S-x-G-[RK]-N-x-G-Y-G-[GS] that specifically recognizes 4PO enzymes of the VAO/PCMH family, as well as additional motifs that can help to further narrow down putative functions. We also provide an overview of fingerprint residues that are specific to VAOs.

1. Introduction

Vanillyl alcohol oxidase (VAO, EC 1.1.3.38) is a covalent flavoenzyme first isolated from the ascomycetous fungus *Penicillium simplicissimum* (de Jong et al., 1992) . VAO is active with a wide range of para-substituted phenols (Fraaije et al., 1995; van den Heuvel et al., 1998). Several VAO reactions produce high-quality aromatic compounds, e.g. vanillin and coniferyl alcohol (see Fig. 1A for an overview). These molecules are of interest for the food, flavour and fragrance industry, exemplified by several patents, e.g. from Mane (Lambert et al., 2007), Rhodia (Gayet et al., 2014) and Unilever (van Berkel et al., 1993).

VAO can also produce chiral aryl alcohols. For instance, the VAO-mediated conversion of 4-ethylphenol results in the formation of (*R*)-1-(4-hydroxyphenyl)ethanol with an enantiomeric excess of 94% (Drijfhout et al., 1998). Interestingly, an engineered variant of VAO was shown to be capable of producing the (*S*)-isomer of 1-(4-hydroxyphenyl)ethanol with an enantiomeric excess of 80% (van den Heuvel et al., 2000a). It has been proposed that the methyl ether 4-(methoxymethyl)phenol is the physiological substrate of VAO, as it is the only known substrate that induces expression of the *vao* gene in *P. simplicissimum* (de Jong et al., 1992). However, little is known about the origin of 4-(methoxymethyl)phenol and the physiological role of VAO. Subcellular localisation studies showed that the *P. simplicissimum* enzyme (*Ps*VAO), together with a co-inducible catalase-peroxidase, is distributed throughout the cytosol and peroxisomes (Fraaije et al., 1998a).

*Ps*VAO is the prototype of a large flavoprotein family, the VAO/PCMH family, together with *p*-cresol methylhydroxylase (*Pp*PCMH, from *Pseudomonas putida* NCIMB 9866, recommended name: 4-methylphenol dehydrogenase (hydroxylating), EC 1.17.99.1). The FAD-binding domains of all enzymes within this family share a common fold (Fraaije et al., 1998b). Within the VAO/PCMH family, VAO and PCMH belong to the 4-phenol oxidising (4PO) subgroup (Ewing et al., 2017a). All 4PO enzymes contain a Tyr-Tyr-Arg triad, which is crucially involved in substrate binding, and therefore is the cause of the selectivity

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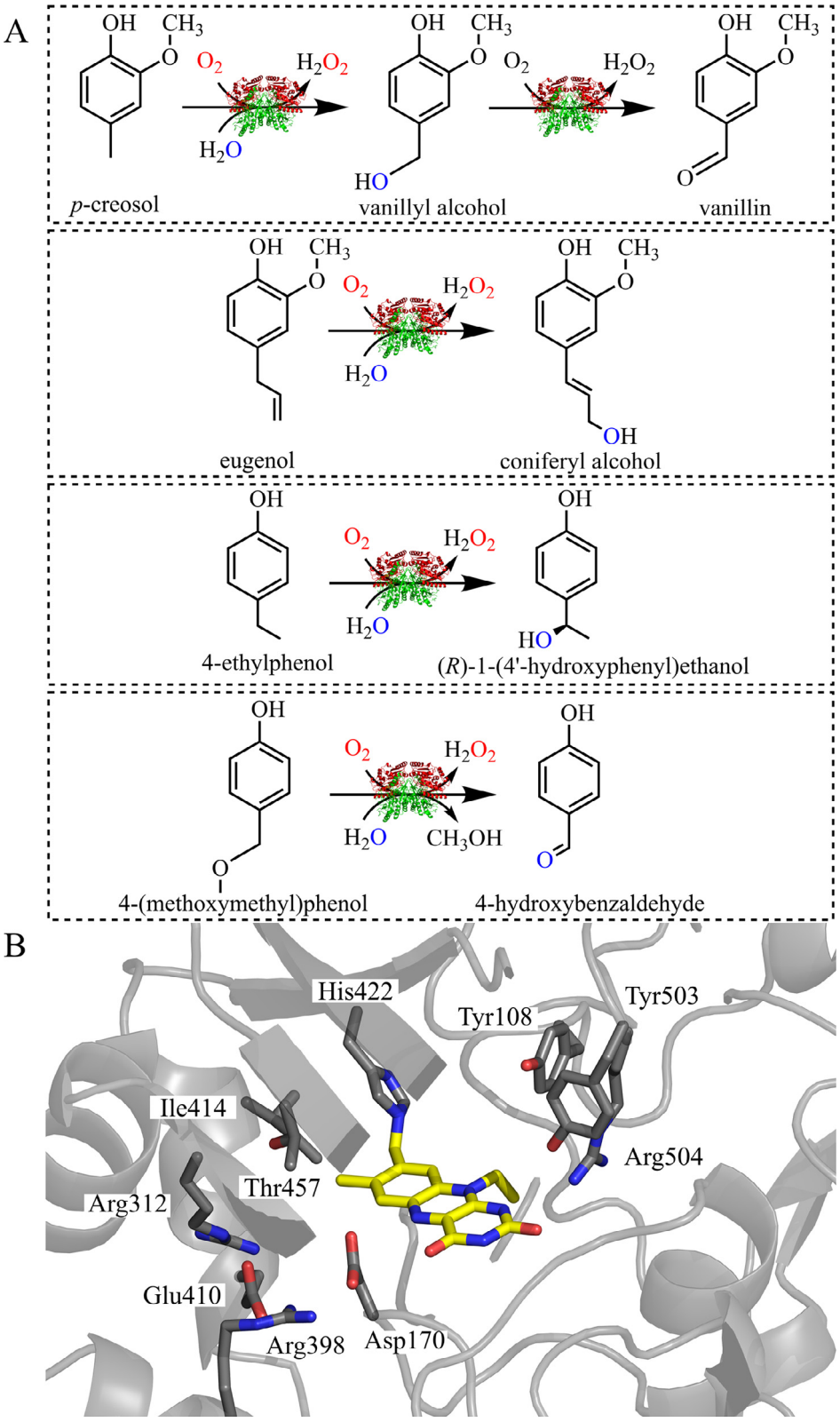

Fig. 1. Different reactions catalysed by *Ps*VAO (A) and active site of *Ps*VAO (B). A: *p*-Creosol (4-methylphenol) is converted via vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol) to vanillin (4-hydroxy-3-methoxybenzaldehyde). Coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol) is produced from eugenol (2-methoxy-4-allylphenol). 4-Ethylphenol is converted to (*R*)-1-(4-hydroxyphenyl)ethanol. The proposed physiological substrate 4-(methoxymethyl)phenol is oxidatively demethylated to 4-hydroxybenzaldehyde. B: Active site of *Ps*VAO (PDBID 1VAO). The FAD cofactor is coloured in yellow and critical amino acid residues are shown in dark grey. For clarity, only the isoalloxazine moiety of the FAD cofactor (which is covalently bound to His422) is shown. See Table 1 for more information about these critical amino acid residues.

of these enzymes for para-substituted phenols (Ewing et al., 2017b). VAO is the only known fungal member of this subgroup, whereas all other known members are bacterial enzymes (Brandt et al., 2001; Cunane et al., 2000; Ewing et al., 2017a; Jin et al., 2007; Leferink et al., 2008; Priefert et al., 1999; Reeve et al., 1989).

Among the 4PO enzymes, *Ps*VAO is characterised in greatest biochemical detail (Gygli et al., 2017). It has been established that the covalent binding of the FAD cofactor of *Ps*VAO is crucial for the redox properties of this enzyme, as it significantly increases its redox potential (Fraaije et al., 1999). This increase in redox potential speeds up the rate of reduction of the FAD by the substrate, and thus increases the overall reaction rate (Fraaije et al., 1999). The same effect has been observed in *Pp*PCMH (Efimov et al., 2004, 2001).

*Ps*VAO has most properties in common with eugenol oxidase from