Direction of the reactivity of vanillyl-alcohol oxidase with 4-alkylphenols

van den Heuvel, RHH; Fraaije, MW; van Berkel, WJH; Heuvel, Robert H.H. van den; Berkel, Willem J.H. van

Published in:
FEBS Letters

DOI:
10.1016/S0014-5793(00)01992-X

Publication date:
2000

Direction of the reactivity of vanillyl-alcohol oxidase with 4-alkylphenols

Robert H.H. van den Heuvel[a], Marco W. Fraaije[b], Willem J.H. van Berkel[a,*]

[a] *Laboratory of Biochemistry, Department of Biomolecular Sciences, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands*
[b] *Laboratory of Biochemistry, Department of Chemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands*

Received 8 August 2000; accepted 22 August 2000

Edited by Pierre Jolles

Abstract The covalent flavoprotein vanillyl-alcohol oxidase (VAO) predominantly converts short-chain 4-alkylphenols, like 4-ethylphenol, to (*R*)-1-(4′-hydroxyphenyl)alcohols and medium-chain 4-alkylphenols, like 4-butylphenol, to 1-(4′-hydroxyphenyl)alkenes. Crystallographic studies have indicated that the active site residue Asp170 is involved in determining the efficiency of substrate hydroxylation. To test this hypothesis, we have addressed the reactivity of Asp170 variants with 4-alkylphenols. The substrate preference of Asp170Glu was similar to wild type VAO. However, Asp170Ser was most active with branched-chain 4-alkylphenols. The hydroxylation efficiency of the Asp170 variants was dependent on the bulkiness of the newly introduced side chain. The Glu170 mutation favored the production of alkenes, whereas the Ser170 mutation stimulated the formation of alcohols.

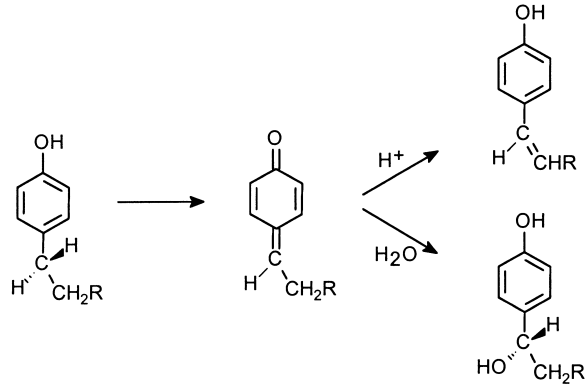
Key words: Vanillyl-alcohol oxidase; Covalent flavin; 4-Alkylphenol

1. Introduction

Vanillyl-alcohol oxidase (VAO; EC 1.1.3.38) from *Penicillium simplicissimum* is a flavin containing oxidoreductase involved in the biodegradation of 4-(methoxymethyl)phenol [1,2]. The enzyme is a homo-octamer of 509 kDa with each subunit comprising two domains [3,4]. The cap domain covers the active site, whereas the larger domain creates a binding site for the ADP part of the FAD prosthetic group. The flavin is covalently linked to His422 of the cap domain [3]. The phenolic substrate is bound almost parallel to the flavin ring and its hydroxyl group is hydrogen bonded to three basic residues (Tyr108, Arg503 and Arg504) [3].

VAO is active with a wide range of phenolic substrates, including 4-alkylphenols [5–7] (Scheme 1). The catalytic cycle of VAO consists of two half-reactions [8,9]. In the reductive half-reaction, the flavin is reduced by the substrate with the concomitant formation of a *p*-quinone methide intermediate. In the oxidative half-reaction, the flavin is reoxidized and the protein-bound quinone methide either reacts with water to yield the (*R*)-enantiomer of the alcohol, or is rearranged to yield the alkene [5,9].

Short-chain 4-alkylphenols are mainly hydroxylated to (*R*)-1-(4′-hydroxyphenyl)alcohols and medium-chain 4-alkylphenols are dehydrogenated to 1-(4′-hydroxyphenyl)alkenes [6]. Unlike the related flavocytochrome *p*-cresol methylhydroxylase (PCMH) [10], VAO is nearly inactive with *p*-cresol. Kinetic studies showed that this is due to the formation of an air-stable *p*-cresol-FAD-N5 adduct [9]. Crystallographic data have shown that the catalytic centers of PCMH and VAO are conserved except for the arrangement of the acidic residues [3,11]. VAO contains a single aspartate (Asp170) near the methylene group of the substrate [3], whereas PCMH contains two glutamates (Glu380 and Glu427) at opposite faces of bound substrate [11]. Recent studies have demonstrated that in VAO, Asp170 is crucial for keeping the high redox potential of the FAD cofactor. This high redox potential is important for efficient substrate oxidation and for stabilization of the complex between the reduced enzyme and the *p*-quinone methide intermediate of 4-(methoxymethyl)phenol [12]. Moreover, from site-directed mutagenesis it was established that the arrangement of acidic residues in the active site cavity tunes the stereospecificity of hydroxylation of 4-ethylphenol [13].



Scheme 1. Conversion of 4-alkylphenols by VAO.

In this paper we have investigated the role of Asp170 in directing the reactivity of VAO with 4-alkylphenols. For this purpose, we selected Asp170Glu and Asp170Ser as both these mutants contain covalently-bound FAD and are active with 4-(methoxymethyl)phenol [12]. Moreover, crystallographic analysis has revealed that the Asp170Ser replacement does not induce significant conformational changes compared to wild type enzyme [3,12]. Our studies clearly reveal that the replacement of Asp170 by Glu or Ser changes the efficiency of substrate hydroxylation of VAO in an opposite direction.

2. Materials and methods

2.1. Site-directed mutagenesis and enzyme purification

Escherichia coli strain TG2 [14] and the plasmid pEMBL19(-)

*Corresponding author. Fax: (31)-317-484 801.
E-mail: willem.vanberkel@fad.bc.wau.nl

Abbreviations: PCMH, *p*-cresol methylhydroxylase; VAO, vanillyl-alcohol oxidase

0014-5793/00/$20.00
PII: S0014-5793(00)01992-X

(Boehringer Mannheim) were used for expression of the *vao*A gene. All other chemicals and materials were as described previously [6,12].

pBC14 (Asp170Glu) and pBC15 (Asp170Ser) were constructed from pBC11 (wild type VAO) as reported before [12]. Transformed *E. coli* cells were grown in Luria–Bertani medium supplemented with 75 μg/ml ampicillin and 0.25 mM isopropyl β-D-thiogalactopyranoside [15]. The VAO mutant proteins were purified as described [12,15].

2.2. Analytical methods

All experiments were performed in 50 mM potassium phosphate buffer, pH 7.5 at 25°C unless stated otherwise. High-performance liquid chromatography (HPLC) experiments were conducted with an Applied Biosystems pump equipped with a Waters 996 photodiode array detector and a 3.9×100 mm or 3.9×150 mm Waters Novapak C18 column, essentially as described earlier [6]. Gas chromatography/mass spectroscopy analysis was performed on a Hewlett-Packard HP 6090 gas chromatograph equipped with a HP 5973 mass spectrometer and a HP-5 column [6]. Fluorescence emission spectra were recorded on an Aminco SPF-500C spectrofluorometer. The excitation wavelength was 360 nm [9].

VAO activity was routinely assayed by following absorption spectral changes of aromatic substrates or by oxygen consumption experiments using a Clark electrode [6]. The formation of 4-hydroxybenzaldehyde was measured at 340 nm ($\varepsilon_{340} = 10.0$ mM^{-1} cm^{-1}) and the formation of 4-vinylphenol was monitored at 255 nm ($\varepsilon_{255} = 14.3$ mM^{-1} cm^{-1}). The turnover rates of extremely slow enzymatic reactions (rate lower than 0.003 s^{-1}) were determined by HPLC. Enzyme-monitored turnover experiments were performed as described before [9] using a Hewlett Packard HP 8453 diode-array spectrophotometer.

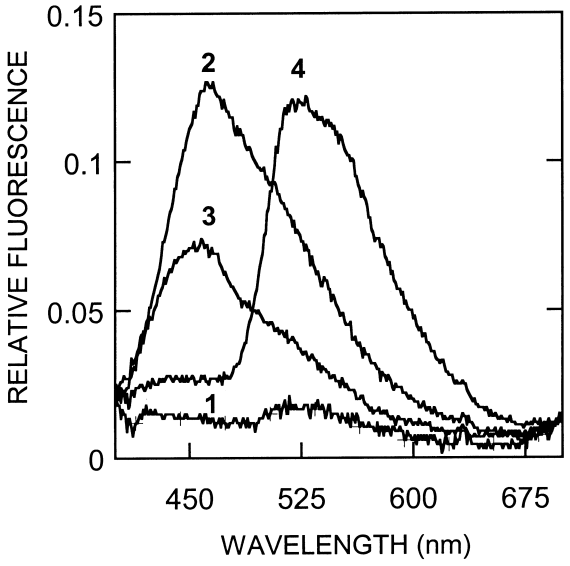


Fig. 1. Fluorescence emission properties of free Asp170Ser and after mixing with 4-alkylphenols. The excitation wavelength was 360 nm. 9 μM Asp170Ser in 50 mM potassium phosphate, pH 7.5 at 25°C (1), after mixing with 500 μM *p*-cresol (2), 4-ethylphenol (3) and 4-isopropylphenol (4).

3. Results

3.1. Catalytic properties

Table 1 summarizes the steady state kinetic parameters of wild type VAO, Asp170Glu and Asp170Ser with several 4-alkylphenols. With all these substrates, the turnover rate of Asp170Glu was about one order of magnitude lower than that of wild type enzyme and the Michaelis constant was increased up to five-fold. The substitution of Asp170 by Ser had a more severe effect on catalysis. All straight-chain 4-alkylphenols were extremely slowly converted, whereas branched-chain 4-alkylphenols were relatively good substrates for Asp170Ser. As found for wild type VAO, both mutant enzymes were nearly inactive with *p*-cresol [9].

When Asp170Ser was mixed aerobically with *p*-cresol, 4-ethylphenol or 4-propylphenol the flavin was nearly completely in the reduced state during turnover (95, 95 and 89%, respectively), suggesting that the reductive half-reaction does not limit the turnover rate [16]. Moreover, upon excitation at 360 nm, the aerobic complexes between Asp170Ser and short-chain 4-alkylphenols displayed a stable fluorescence emission with a maximum at 460 nm (Fig. 1), indicative for the formation of a covalent flavin N5 adduct with the substrate [3,9]. Similar flavin adducts have been reported for lactate oxidase [17,18] and nitroalkane oxidase [19]. When Asp170Ser was incubated with 4-isopropylphenol or 4-*sec*-butylphenol, the flavin was mainly in the oxidized state during turnover (79 and 82%, respectively). In accordance with this, the fluorescence emission of 4-isopropylphenol-mixed Asp170Ser showed a maximum at 530 nm, indicative for oxidized flavin. A similar flavin fluorescence was observed when oxidized Asp170Ser was incubated with 4-vinylphenol or 1-(4′-hydroxyphenyl)ethanol. In contrast, uncomplexed Asp170Ser displayed almost no flavin fluorescence (Fig. 1). This suggests that the fluorescence emission at 530 nm represents the complex between the oxidized enzyme and the aromatic product. The Asp170Glu mutant displayed a similar enzyme-monitored turnover behavior as wild type VAO. Thus, only upon mixing the enzyme with *p*-cresol was the flavin mainly in the reduced stated during turnover [9].

3.2. Conversion of 4-alkylphenols

The replacement of Asp170 by Glu and Ser considerably changed the product pattern of the VAO catalyzed reactions with 4-alkylphenols (Table 2). Like wild type enzyme [5,6], Asp170Ser was highly specific for the hydroxylation of

Table 1
Steady state kinetic parameters for wild type VAO, Asp170Glu and Asp170Ser in 50 mM potassium phosphate buffer, pH 7.5 at 25°C

Substrate[a]	Wild type[b]		Asp170Glu		Asp170Ser	
	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})
p-Cresol	n.d.	0.005	n.d.	0.0002	n.d.	n.d.
4-Ethylphenol	9	2.5	48	0.17	n.d.	0.0001
4-*n*-Propylphenol	4	4.2	10	0.26	n.d.	0.0002
4-Isopropylphenol	16	1.3	88	0.13	26	0.18
4-*n*-Butylphenol	2	1.2	6	0.12	n.d.	0.0001
4-*sec*-Butylphenol	72	0.5	75	0.05	62	0.09

n.d., not determined.
[a]Standard errors of kinetic parameters are less than 10%.
[b]Data from [6].

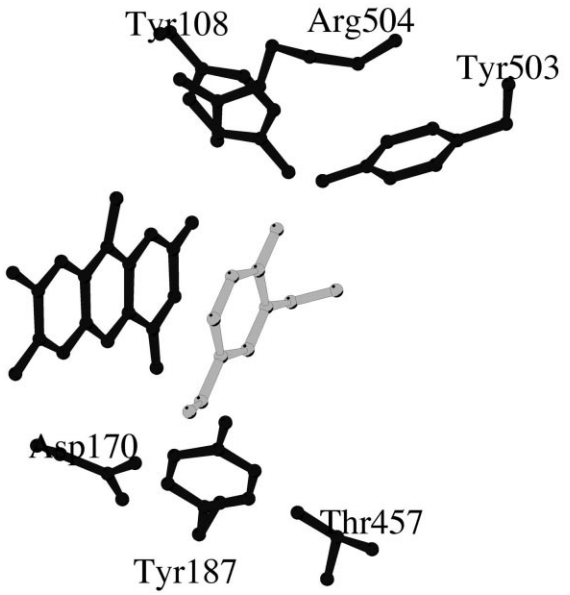


Fig. 2. Drawing of the active site cavity of VAO in complex with isoeugenol. Asp170 is positioned at 3.5 Å from flavin N5 and the Cα-atom of isoeugenol. This figure was prepared with MOLSCRIPT [20].

short-chain alkylphenols. Previous studies have shown that both wild type VAO and Asp170Ser are selective for the production of the (*R*)-enantiomer of 4-ethylphenol [13]. In contrast, the hydroxylation reaction in Asp170Glu was nearly completely blocked. As a result, this mutant converted short-chain alkylphenols to the corresponding alkenes.

With medium-chain and branched-chain alkylphenols, the change in product pattern was even more pronounced. Unlike wild type enzyme [6], Asp170Ser was highly specific for the hydroxylation of these compounds. However, Asp170Glu resembled wild type enzyme and mainly produced aromatic alkenes.

4. Discussion

The active site cavity of VAO contains a single acidic residue, Asp170, whose side chain is close to flavin N5 and the reactive Cα-atom of the substrate (Fig. 2). Recent studies from site-directed mutants have shown that this residue is important for catalysis by raising the redox potential of the flavin and stabilizing the enzyme-bound *p*-quinone methide of 4-(methoxymethyl)phenol [12]. Furthermore, Asp170 appeared to be important for the stereospecificity of hydroxylation of 4-ethylphenol [13]. This raised the question as to whether the replacement of Asp170 will also influence the efficiency of substrate hydroxylation.

The Asp170Glu variant had a similar substrate preference as wild type VAO, but the catalytic efficiency (k_{cat}/K_m) was considerably decreased. The low turnover rate is in line with the reduced redox potential of the mutant ($E_m = +11$ mV) compared to wild type VAO ($E_m = +55$ mV) [12]. In contrast to the wild type enzyme, Asp170Glu converted both short-chain and medium-chain 4-alkylphenols almost exclusively to the corresponding alkenes. The inability of Asp170Glu to hydroxylate these substrates suggests that the bulkiness of the side chain of Glu170 limits the accessibility of water to the planar quinone methide intermediate.

Asp170Ser had a drastically changed substrate preference compared to wild type VAO. This mutant was nearly inactive with straight-chain 4-alkylphenols due to the formation of air-stable covalent flavin N5 adducts. With wild type VAO, such an adduct is only observed with *p*-cresol [9]. The crystal structure of isoeugenol-complexed Asp170Ser does not give a rationale for the easy formation of flavin adducts since there are no significant structural differences compared to wild type VAO [3,12]. We have proposed before that in wild type VAO, Asp170 might be involved in covalent adduct formation by abstracting a proton from flavin N5, thereby facilitating the nucleophilic attack of the *p*-quinone methide intermediate. However, the formation of flavin N5 adducts in Asp170Ser suggests that the flavin N5-atom can also be activated by a water molecule (Fig. 3). Asp170Ser was rather active with branched-chain 4-alkylphenols. This indicates that with these substrates, adduct formation may well be prevented by steric constraints. In this respect it is interesting to note that the flavin redox potential of Asp170Ser is only −91 mV [12].

The Asp170Ser variant was more specific for the hydroxylation of 4-alkylphenols than wild type VAO. This supports an earlier observation that the hydration of the *p*-quinone methide intermediate can also occur in the absence of an active site base [12]. Furthermore, it suggests that the quinone methides of 4-alkylphenols react rapidly with unactivated water, which is in line with the reactivity of analogous quinone methides [21,22].

The differences in the efficiency of substrate hydroxylation between wild type VAO, Asp170Ser and Asp170Glu show

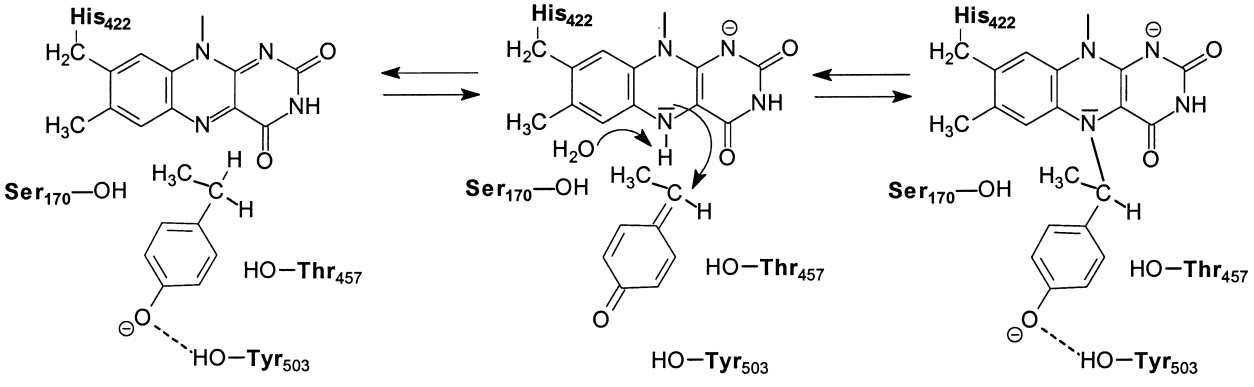


Fig. 3. Schematic drawing of the proposed mechanism for covalent adduct formation in Asp170Ser.

Table 2
Conversion of 4-alkylphenols by wild type VAO, Asp170Glu and Asp170Ser in 50 mM potassium phosphate buffer, pH 7.5 at 25°C

Substrate[a]	Product (%)					
	wild type[b]		Asp170Glu		Asp170Ser	
	alcohol	alkene	alcohol	alkene	alcohol	alkene
4-Ethylphenol	76	24	8	92	92	8
4-Propylphenol	68	32	7	93	96	4
4-Isopropylphenol	20	80	8	92	80	20
4-Butylphenol	1	99	0	100	82	18
4-*sec*-Butylphenol	26	74	1	99	78	22

[a]Standard errors of relative product yields are less than 10%.
[b]Data from [6].

that the bulkiness of the side chain of residue 170 determines the outcome of the VAO catalyzed reaction. The small side chain of Ser170 increases the accessibility of water to the *p*-quinone methide intermediate in the active site and, therefore, the hydroxylation efficiency. This increased accessibility of water to the quinone methide in Asp170Ser might also explain the decreased stereospecificity of hydroxylation of 4-ethylphenol [13]. On the other hand, the large side chain of Glu170 clearly prevents the attack of water to the quinone methide. These results reinforce the idea that a single amino acid substitution can be sufficient to change the enzyme selectivity [23,24].

Acknowledgements: We thank Andrea Mattevi (University of Pavia) for fruitful discussions and Colja Laane (Wageningen University) for continuous interest and support. This work was performed within the framework of the Innovation Oriented Research Program (IOP) Catalysis of the Dutch Ministry of Economy Affairs (project IKA 96005).

References

[1] de Jong, E., van Berkel, W.J.H., van der Zwan, R.P. and de Bont, J.A.M. (1992) Eur. J. Biochem. 208, 651–657.
[2] Fraaije, M.W., Pikkemaat, M. and van Berkel, W.J.H. (1997) Appl. Environ. Microbiol. 63, 435–439.
[3] Mattevi, A., Fraaije, M.W., Mozzarelli, A., Olivi, L., Coda, A. and van Berkel, W.J.H. (1997) Structure 5, 907–920.
[4] van Berkel, W.J.H., van den Heuvel, R.H.H., Versluis, C. and Heck, A.J.R. (2000) Protein Sci. 9, 435–439.
[5] Drijfhout, F.P., Fraaije, M.W., Jongejan, H., van Berkel, W.J.H. and Franssen, M.C.R. (1998) Biotechnol. Bioeng. 59, 171–177.
[6] van den Heuvel, R.H.H., Fraaije, M.W., Laane, C. and van Berkel, W.J.H. (1998) J. Bacteriol. 180, 5646–5651.
[7] Fraaije, M.W., Veeger, C. and van Berkel, W.J.H. (1995) Eur. J. Biochem. 234, 271–277.
[8] Fraaije, M.W. and van Berkel, W.J.H. (1997) J. Biol. Chem. 272, 18111–18116.
[9] Fraaije, M.W., van den Heuvel, R.H.H., Roelofs, J.C.A.A. and van Berkel, W.J.H. (1998) Eur. J. Biochem. 253, 712–719.
[10] McIntire, W.S. and Hopper, D.J. (1985) Biochem. J. 228, 325–335.
[11] Cunane, L.M., Chen, Z.-W., Shamala, N., Mathews, F.S., Cronin, C.N. and McIntire, W.S. (2000) J. Mol. Biol. 295, 357–374.
[12] van den Heuvel, R.H.H., Fraaije, M.W., Mattevi, A. and van Berkel, W.J.H. (2000) J. Biol. Chem. 275, 14799–14808.
[13] van den Heuvel, R.H.H., Fraaije, M.W., Ferrer, M., Mattevi, A. and van Berkel, W.J.H. (2000) Proc. Natl. Acad. Sci. USA, in press.
[14] Gibson, T.G. (1984) Studies on the Epstein-Barr Virus Genome. Ph.D. thesis, University of Cambridge, UK.
[15] Benen, J.A.E., Sánchez-Torres, P., Wagemaker, M., Fraaije, M.W., van Berkel, W.J.H. and Visser, J. (1998) J. Biol. Chem. 273, 7865–7872.
[16] Gibson, Q.H., Swoboda, B.E.P. and Massey, V. (1964) J. Biol. Chem. 239, 3927–3934.
[17] Ghisla, S., Massey, V. and Choong, Y.S. (1979) J. Biol. Chem. 254, 10662–10669.
[18] Ghisla, S. and Massey, V. (1980) J. Biol. Chem. 255, 5688–5696.
[19] Gadda, G., Edmondson, R.D., Russell, D.H. and Fitzpatrick, P.F. (1997) J. Biol. Chem. 272, 5563–5570.
[20] Kraulis, P.J. (1991) J. Appl. Crystallogr. 24, 946–950.
[21] Bolton, J.L., Turnipseed, S.B. and Thompson, J.A. (1997) Chem. Biol. Interact. 107, 185–200.
[22] Leary, G. (1972) J. Chem. Soc. Perkin Trans. 2, 640–642.
[23] Sampson, N.S. and Kass, I.J. (1997) J. Am. Chem. Soc. 119, 855–862.
[24] Yamashita, M., Toyama, M., Ono, H., Fujii, I., Hirayama, N. and Murooka, Y. (1998) Protein Eng. 11, 1075–1081.