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

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

Robert H.H. van den Heuvela, Marco W. Fraaijeb, Willem J.H. van Berkelc,*

aLaboratory of Biochemistry, Department of Biomolecular Sciences, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands
bLaboratory of Biochemistry, Department of Chemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

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Abstract The covalent flavoprotein vanillyl-alcohol oxidase (VAO) predominantly converts short-chain 4-alkylphenols, like 4-ethylphenol, to (R)-1-(4′-hydroxyphenyl)alkenes and medium-chain 4-alkylphenols, like 4-butylphenol, to 1-(4′-hydroxyphenyl)alkanes. 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. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

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 reduced by the substrate with the formation of a p-quinone methide intermediate. The flavin is reoxidized and the protein-bound quinone methide either reacts with water to produce alcohols, or is rearranged to alkenes. 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 the 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].

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(-)
(Boehringer Mannheim) were used for expression of the vanA 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 × 150 mm Waters Novapak C18 column, essentially as described earlier [6]. Gas chromatography/mass spectroscopy analysis was performed on a Hewlett-Packard HP 6890 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 spectrophotometer.

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 shortchain 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].

Table 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Wild type(a)</th>
<th>Asp170Glu</th>
<th>Asp170Ser</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_m (\mu M))</td>
<td>(k_{cat} (s^{-1}))</td>
<td>(K_m (\mu M))</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>0.005</td>
<td>n.d.</td>
<td>0.0002</td>
</tr>
<tr>
<td>4-Ethylphenol</td>
<td>2.5</td>
<td>48</td>
<td>0.17</td>
</tr>
<tr>
<td>4-sec-Butylphenol</td>
<td>2</td>
<td>72</td>
<td>0.05</td>
</tr>
<tr>
<td>4-Propylphenol</td>
<td>4.2</td>
<td>10</td>
<td>0.26</td>
</tr>
<tr>
<td>4-Isopropylphenol</td>
<td>13</td>
<td>88</td>
<td>0.13</td>
</tr>
<tr>
<td>4-sec-Butylphenol</td>
<td>12</td>
<td>60</td>
<td>0.12</td>
</tr>
<tr>
<td>n.d.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n.d., not determined.

\(a\) Standard errors of kinetic parameters are less than 10%.

\(b\) Data from [6].

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-ethylphenol 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

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).
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 Cα-atom of isoeugenol. This figure was prepared with MOLSCRIPT \[20\].

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.

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

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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

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wild type</td>
</tr>
<tr>
<td></td>
<td>alcohol</td>
</tr>
<tr>
<td>4-Ethylphenol</td>
<td>76</td>
</tr>
<tr>
<td>4-Propylphenol</td>
<td>68</td>
</tr>
<tr>
<td>4-Isopropylphenol</td>
<td>20</td>
</tr>
<tr>
<td>4-Butylphenol</td>
<td>1</td>
</tr>
<tr>
<td>4-sec-Butylphenol</td>
<td>26</td>
</tr>
</tbody>
</table>

aStandard errors of relative product yields are less than 10%.
bData from [6].

References