Tuning of the product spectrum of vanillyl-alcohol oxidase by medium engineering

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Abstract The flavoenzyme vanillyl-alcohol oxidase (VAO) catalyzes the conversion of 4-alkylphenols through the initial formation of *p*-quinone methide intermediates. These electrophilic species are stereospecifically attacked by water to yield (*R*)-1-(4′-hydroxyphenyl)alcohols or rearranged in a competing reaction to 1-(4′-hydroxyphenyl)alkenes. Here, we show that the product spectrum of VAO can be controlled by medium engineering. When the enzymatic conversion of 4-propylphenol was performed in organic solvent, the concentration of the alcohol decreased and the concentration of the *cis*-alkene, but not the *trans*-alkene, increased. This change in selectivity occurred in both toluene and acetonitrile and was dependent on the water activity of the reaction medium. A similar shift in alcohol/*cis*-alkene product ratio was observed when the VAO-mediated conversion of 4-propylphenol was performed in the presence of monovalent anions that bind specifically near the enzyme active site. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Vanillyl-alcohol oxidase (VAO; EC 1.1.3.38) is a flavoenzyme from the ascomycete *Penicillium simplicissimum* containing an 8α-(*N*[3]-histidyl)-FAD as the covalently bound prosthetic group [1]. The biological function of VAO is unclear, but induction experiments have indicated that the enzyme is involved in the biodegradation of 4-(methoxymethyl)phenol [2]. The scope of reactions catalyzed by VAO is rather broad. Besides converting 4-(methoxymethyl)phenol to 4-hydroxybenzaldehyde, the enzyme is active with a wide range of *p*-substituted phenols, including 4-alkylphenols [3].

The catalytic mechanism of VAO with 4-propylphenol (**1**) involves the initial transfer of a hydride from the substrate to the flavin, resulting in the formation of a complex between the reduced enzyme and the *p*-quinone methide of 4-propylphenol (**2**) (Scheme 1) [4]. Next, the reduced enzyme is reoxidized by molecular oxygen and the enzyme-bound product intermediate reacts in a stereospecific manner with water to yield (*R*)-1-(4′-hydroxyphenyl)propanol (**3**) (*e.e.* = 95 ± 1%) or rearranges in a competing reaction to 1-(4′-hydroxyphenyl)propene (**4**) [5]. Studies with a range of substrate analogs have shown that the efficiency of alcohol formation and the ratio between the *cis* and *trans* forms of the alkene products are affected by the type of 4-alkylphenol [3]. The outcome of the enzymatic conversion of 4-alkylphenols is of biotechnological relevance since the alkene products can serve as flavors, whereas the enantiomerically pure alcohols are suited as building blocks for the synthesis of complex chiral compounds [6,7].

Crystallographic studies of VAO in complex with substrate analogs have provided insight into the binding mode of 4-alkylphenols (Fig. 1) [8,9]. Recent studies of protein engineering have shown that Asp170, located in the proximity of the flavin N5 atom (3.6 Å) and the substrate Cα atom (3.0 Å) [8], is involved in regulating the water attack on the electrophilic *p*-quinone methide intermediate [10,11]. Moreover, by relocating the acidic residue to the opposite face of the substrate (Asp170Ser/Thr457Glu double mutation), the enantioselectivity of the enzyme could be inverted [12].

It has been well established that medium engineering represents a convenient alternative to protein engineering to tune the enzyme selectivity [13–16]. Most of these studies have been performed with hydrolases and oxynitrilases but relatively little is known about the behavior of redox enzymes. For mushroom tyrosinase it was shown that a quantitative conversion of phenols to catechols could be achieved when the reaction was performed in chloroform [17] and that the initial formation of *ortho*-quinones could be efficiently coupled to the non-enzymatic synthesis of bicyclo[2,2,2]octene-diones [18]. For horseradish peroxidase, it was reported that polymers of *para*-phenylphenol could be produced in aqueous dioxane and that the size of the polymers increased with decreasing water content [19]. Here, we report on the medium engineering of the reaction of VAO with 4-propylphenol. It is shown for the first time that the hydration of *p*-quinone methides in the enzyme active site can be effectively controlled in low water media or by the addition of specific monovalent anions.

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2. Materials and methods

2.1. Enzyme purification

Wild-type VAO was overexpressed and purified as described before [10,20].

2.2. Enzyme catalysis in organic solvents

VAO 0.32 mg in 50 mM potassium phosphate buffer, pH 7.5, was freeze-dried in a 3 ml glass vial for 12 h. The reaction was started by adding 1 ml of 4-propylphenol (10 mM) dissolved in the appropriate solvent at a preset water activity. The reaction mixture was then incubated at 20°C with constant reciprocal shaking (200 min^{-1}). The water contents of the reaction mixtures were determined by Karl Fischer coulometric titration (Metrohm 684). Samples of 200 µl were taken at regular time intervals and analyzed by HPLC (Gilson 715 system) equipped with a reversed phase 4.6×250 mm HiChrom HIRBP column. In all reaction media, except for the acetonitrile media with water activities (a_w) between 0.99 and 0.86, VAO was present as a suspension. The a_w in acetonitrile was set by adding water [21] and in toluene by adding salt hydrates ($a_w = 0.80$, $Na_2SO_4.10/0$; $a_w = 0.61$, $Na_2HPO_4.7/2$; $a_w = 0.49$, $Na_4P_2O_7.10/0$ at 25°C) [22].

2.3. Enzyme catalysis in the presence of monovalent anions

VAO 0.13 mg in 50 mM potassium phosphate buffer, pH 7.5, was mixed with 1 mM 4-propylphenol and varying concentrations of the anions (4–500 mM) in 50 mM potassium phosphate buffer, pH 7.5, in a total volume of 1 ml and 25°C. Samples of 200 µl were taken at regular time intervals and analyzed by HPLC. The inhibition of VAO by monovalent anions was measured using vanillyl alcohol as the variable substrate. The data were treated according to Lineweaver and Burk.

2.4. Analytical methods

Dissociation constants of enzyme–inhibitor complexes were determined from flavin perturbation spectra by titrating a known concentration of enzyme with the inhibitor using an Aminco DW-2000 spectrophotometer in 50 mM potassium phosphate buffer, pH 7.5, at 25°C.

The stereospecificity of hydroxylation of 4-propylphenol by VAO was measured by HPLC using an Applied Biosystems pump equipped with a 4.6×250 mm Chiralcel OB column (Daicel Chemical Industries).

3. Results and discussion

3.1. VAO catalysis in organic solvents

When using acetonitrile or toluene media, we found that the activity of lyophilized VAO with 4-propylphenol was highly dependent on the a_w of the reaction medium, but independent of the solvent used. In both toluene and acetonitrile, the activity of VAO at $a_w = 0.8$ was about one order of magnitude lower compared to that in aqueous medium ($k' = 0.3$ s^{-1} vs. $k' = 4.2$ s^{-1}) [23]. Upon lowering a_w, the turnover rate of VAO decreased and the enzyme became nearly inactive at $a_w = 0.49$. In an aqueous medium ($a_w = 1$), VAO converts 4-propylphenol mainly to (R)-1-(4′-hydroxyphenyl)propanol and low but equal amounts of cis- and trans-1-(4′-hydroxyphenyl)propene (Fig. 2). When the VAO-catalyzed conversion
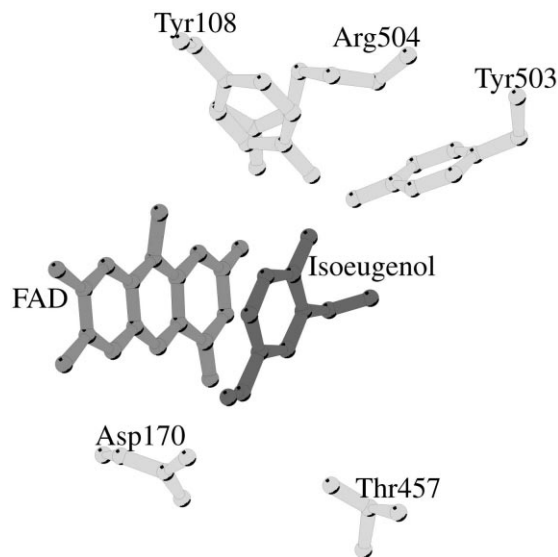

Fig. 1. Drawing of the isoeugenol-bound active site cavity of VAO. This figure was prepared with MOLSCRIPT [24].

was performed in acetonitrile or toluene the concentration of alcohol product decreased and the concentration of the cis-alkene product, but not the trans-alkene product, increased. This change in selectivity occurred in both solvents and was dependent on a_w (Fig. 2A,B). These results show that the availability of water for the enzyme determines the reactivity of the enzyme-bound p-quinone methide and that the hydration and rearrangement of this intermediate species are competing processes.

A previous study has revealed that in an aqueous medium, the cis/trans-alkene product ratio is dependent on the type of 4-alkylphenol [3]. This suggests that the strong and specific increase in cis-alkene formation in acetonitrile and toluene is related to the relative energies of the enzyme-bound p-quinone methides and not to the higher energy of the cis-conformation. With 4-propylphenol as a substrate, the p-quinone methide intermediate can, hypothetically, take up three different conformations (Scheme 2). **5** has the methyl attached to the Cα atom already in the trans position. This conformation can only proceed to the trans-alkene product. The two other conformations (**6**, **7**) have the methyl group attached to Cα in the cis position and are likely to give only the cis product. These latter two quinone methide conformations are subject to competitive rearrangement or hydration at Cα. However, we cannot exclude an alternative mechanism in which there is only a single quinone methide intermediate, and attack by water competes with removal of just one of the two protons attached to the Cα. But it is difficult to see how selection between these protons could control the alkene geometry pro-
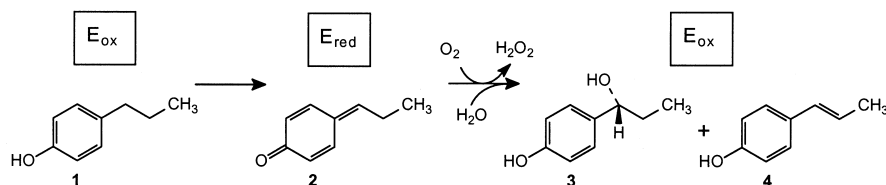
Scheme 1. Reaction scheme for the conversion of 4-propylphenol by VAO. The redox state of VAO during the enzymatic reaction is depicted at the top. E_{ox}, oxidized enzyme; E_{red}, reduced enzyme; **1**: 4-propylphenol; **2**: p-quinone methide of 4-propylphenol; **3**: 1-(4′-hydroxyphenyl)propanol; **4**: 1-(4′-hydroxyphenyl)propene.
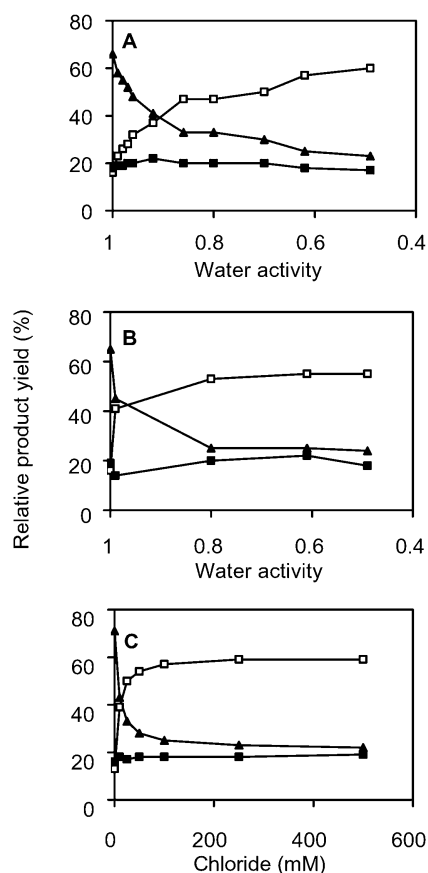
Scheme 2.

Fig. 2. Conversion of 10 mM 4-propylphenol by 0.32 mg VAO at 25°C in the organic solvents acetonitrile (A) and toluene (B), and (C) conversion of 1 mM 4-propylphenol by 0.13 mg VAO in 50 mM potassium phosphate buffer, pH 7.5, at 25°C in the presence of sodium chloride. The concentration of the products (R)-1-(4'-hydroxyphenyl)propanol (▲), cis-1-(4'-hydroxyphenyl)propene (□), and trans-1-(4'-hydroxyphenyl)propene (■) were determined by HPLC. All results are the average of three determinations with an error of less than 7%.

duced, from any of the possible intermediate conformations (Scheme 2).

3.2. VAO catalysis in the presence of monovalent anions

A second medium engineering-based approach of tuning the reactivity of the p-quinone methide in the catalytic center of VAO was the addition of specific monovalent anions to the reaction medium. Previous X-ray data have established that a chloride ion binds at the re face of the flavin cofactor, whereas the aromatic substrate binds at the si face of the flavin [8]. The binding of chloride, and also bromide and thiocyanate, near the flavin was confirmed by flavin absorption difference spectroscopy and inhibition studies with these anions revealed non-competitive inhibition (Table 1). In contrast, fluoride, sul-

Table 1
Dissociation constants of VAO–inhibitor complexes and non-competitive inhibition constants of monovalent anions in 50 mM potassium phosphate buffer, pH 7.5, at 25°C

	K_d (mM)	K_i (mM)
Chloride	130 ± 10	120 ± 15
Bromide	93 ± 9	102 ± 10
Thiocyanate	36 ± 3	70 ± 11
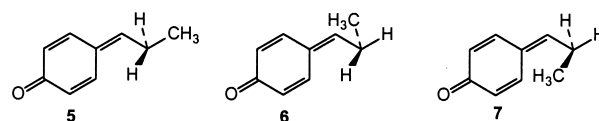
fate, and iodide ions did not perturb the optical properties of enzyme-bound flavin nor inhibit enzyme activity.

When the enzymatic conversion of 4-propylphenol was performed in aqueous medium in the presence of chloride, bromide, or thiocyanate, the efficiency of p-quinone methide hydration decreased and the efficiency of cis rearrangement increased (Fig. 2C). Thus, the addition of specific anions and a decreased water activity have similar outcomes. The anions are not likely to act via changes in hydrophobic interactions, because the effects do not follow the Hofmeister series (i.e. iodide would be expected to have a larger effect than chloride, thiocyanate, and bromide). On the basis of the crystallographic data [8] and the anion binding studies it seems likely that the decreased hydration efficiency of the p-quinone methide in the presence of chloride, bromide, or thiocyanate is a consequence of their specific binding near the enzyme active site. This binding does not completely block the hydration reaction, suggesting that the anions do not fully protect the p-quinone methide from water attack.

The low water activity of the medium and the presence of monovalent anions did not change the R-stereospecificity of the hydration reaction. Under both conditions, an e.e. of $95 \pm 1\%$ for the R-enantiomer of the 1-(4'-hydroxyphenyl)-alcohol product was observed. This is in accordance with the idea that Asp170 tunes the stereospecificity of VAO by activating the water molecule attacking the quinone methide [12].

3.3. Conclusions

In this paper we have shown that the hydration efficiency of the p-quinone methide of 4-propylphenol in VAO can be controlled by medium engineering. The presence of organic solvents or specific monovalent anions in the reaction medium decreases the water accessibility of the enzyme active site, thereby influencing the competition between the production of alcohol and alkene. Moreover, under the applied conditions, no quinone methide dissociation occurs, preventing the formation of racemic mixtures or unwanted by-products.

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