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Hydroxylation of 4-hydroxyphenylethylamine derivatives by R263 variants of the oxygenase component of *p*-hydroxyphenylacetate-3-hydroxylase

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ABSTRACT

p-hydroxyphenylacetate 3-hydroxylase from *Acinetobacter baumannii* catalyzes the hydroxylation of *p*-hydroxyphenylacetate (HPA) to yield 3,4-dihydroxyphenylacetate (DHPA). In this study, we investigated whether variants of the oxygenase component (C_2) could catalyze hydroxylation of 4-hydroxyphenylethylamines to synthesize catecholamine derivatives. Single turnover product analysis showed that the R263D variant can catalyze hydroxylation of tyramine to form dopamine with the highest yield (57%). The enzyme was also found to have dual substrate charge specificity because it can also maintain reasonable hydroxylation efficiency of HPA (86%). This property is different from the R263E variant, which can hydroxylate HPA (73%) but not tyramine. The R263A variant can hydroxylate HPA (72%) and tyramine to a small extent (7%). Stopped-flow experiments indicated that tyramine and HPA prefer binding to R263D after C4a-hydroperoxy-FMN formation, while tyramine cannot bind to the wild-type or R263E enzymes. Data also indicate that the hydroxylation rate constant is the rate-limiting step. The R263D variant was used as a starting enzyme for further mutation to obtain other variants for the synthesis of additional catecholamine drugs. The R263D/Y398D double mutant enzyme showed interesting results in that it was able to catalyze the hydroxylation of octopamine to form norepinephrine. However, the enzyme still lacked stereo-selectivity in its reaction.

1. Introduction

Flavin-dependent monooxygenases catalyze the insertion of a single atom of molecular oxygen into organic molecules. These enzymes use NAD(P)H, O_2 and organic compounds as substrates [1,2]. They catalyze a broad range of reactions such as hydroxylation, epoxidation, Baeyer-Villiger monooxygenation, sulfoxidation, amine oxidation and halogenation [2]. These reactions are involved in a wide variety of biological processes such as in the biodegradation of aromatic compounds in the environment, in the biosynthesis of antibiotics, and in drug detoxification processes [3–5]. Many of these enzymes catalyze reactions with high regio- and/or stereo-specificity [2,3,6]. Some of them, such as Baeyer-Villiger monooxygenase (BVMO) [7–11], styrene monooxygenase from *Pseudomonase* sp. VLB120 [12,13], *p*-hydroxybenzoate hydroxylase [14], and *p*-hydroxyphenylacetate 3-hydroxylase from *Acinetobacter baumannii* [15] have been shown to be useful for the synthesis of high-value compounds.

p-hydroxyphenylacetate 3-hydroxylase (HPAH) is a flavin-dependent two-component monooxygenase that catalyzes the hydroxylation of *p*-hydroxyphenylacetate (HPA) to yield 3,4-dihydroxyphenylacetate (DHPA). The HPAH enzymes from *Pseudomonase putida* [16], *Escherichia coli* [17], *Klebsiella pneumonia* [18], *Pseudomonas aeruginosa* [19], *Acinetobacter baumannii* [20,21] have been isolated. Of these enzymes, the mechanistic details of

Abbreviations: HPAH, *p*-hydroxyphenylacetate 3-hydroxylase; C_2, the oxygenase component of *p*-hydroxyphenylacetate-3-hydroxylase; HPA, *p*-hydroxyphenylacetate; BVMO, Baeyer-Villiger monooxygenation; CHMO, cyclohexanone monooxygenase; DHPA, 3,4-dihydroxyphenylacetate; TPL, tyrosine phenol-lyase; TDC, tyrosine decarboxylase.

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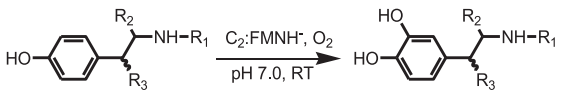
HPAH from *Acinetobacter baumannii* [22–27] remains the most extensively studied. HPAH from *A. baumannii* consists of a smaller reductase component (C_1) and a larger oxygenase component (C_2) [21]. C_1 generates reduced FMN ($FMNH^-$) to be used as a substrate for C_2 in the hydroxylation of HPA. C_2 binds to $FMNH^-$ and the resulting binary complex reacts with oxygen to form C4a-hydroperoxy-FMN, a reactive intermediate that hydroxylates HPA [22]. The enzyme has been shown to be an efficient and robust biocatalyst because it can catalyze 90% hydroxylation of HPA over a wide range of pHs 6.2–9.9 [23]. A few C_2 variants useful for biocatalysis were obtained by rational engineering. By introducing more space at the ligand binding site in the Y398S variant, the enzyme that is more efficient than the wild-type enzyme in catalyzing the bioconversion of lignin-derived *p*-coumaric acid into bioactive trihydroxyphenolic acids could be obtained [15]. The S146A variant was also found to be more efficient in catalyzing the hydroxylation of an aniline derivative at pH 6.0 [28]. Ser146 is a key catalytic residue involved in optimizing C2 reactivity toward a phenolic compound. Removing this hydroxyl group expands C2 activity toward an aniline substrate [28].

In order to further explore the biocatalysis capabilities of C_2, we investigated R263 variants that may catalyze the hydroxylation of aromatic amines to synthesize catecholamine derivatives (Table 1). Catecholamines are endogenous neurotransmitters that are widely used as sympathomimetic drugs [29]. Dopamine and norepinephrine are used in the treatment of septic shock [30], and epinephrine is used for the treatment of anaphylaxis and as a local anesthetic drug [31–33]. The structures of these catecholamine derivatives are similar to DHPA, the product formed by C_2 using HPA, the native substrate, except these derivatives have an amine instead of a carboxylic group. As the crystal structures of C_2 [34] showed that the positive charge of the guanidinium side chain of R263 is positioned close to the negative charge of the carboxyl group of HPA (Fig. 1), the R263 residue was speculated to be important for directing the specificity of C_2 towards a substrate containing a negatively charged carboxylic acid moiety [34].

In this work, the residue R263 was replaced with Glu, Asp or Ala to substitute the positive charge at the R263 position with either a negative or neutral charge. The R263D variant was found to catalyze the conversion of tyramine to dopamine and HPA to DHPA with similar efficiency. Transient kinetics investigations using stopped-flow and rapid-quench flow experiments were performed to gain mechanistic insights into the nature of the R263 variants to compare with the wild-type enzyme. Double mutations of R263 and Y398 were also created in order to expand the active site space in addition to changing the charge at R263. These C_2 variants were explored for their ability to catalyze the synthesis of catecholamine derivatives such as dopamine, norepinephrine (NE) and epinephrine (EP).

Table 1
Single turnover reactions of C_2 catalyzed hydroxylation of 4-hydroxyphenylethylamine derivatives to generate 3,4-dihydroxyphenylethylamine (catecholamine) derivatives.



4-Hydroxyphenylethylamine derivatives

3,4-Dihydroxyphenylethylamine (catecholamine) derivatives

Substrate (4-Hydroxyphenylethylamines)	Product (Catecholamines)	R_1	R_2	R_3
Tyramine	Dopamine	-H	-H	-H
Octopamine	Norepinephrine	-H	-H	-OH
Synephrine	Epinephrine	$-CH_3$	-H	-OH

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents used in this study were purchased from commercial sources (analytical grad). Pure FMN was prepared from FAD by using snake venom from *Crotalus adamanteus* to catalyze the hydrolysis of FAD to FMN [35]. Concentrations of the following compounds were determined using known extinction coefficients at pH 7.0: FMN, $\varepsilon_{446} = 12.2\ mM^{-1}cm^{-1}$; HPA, $\varepsilon_{277} = 1.55\ mM^{-1}cm^{-1}$; tyramine, $\varepsilon_{274.5} = 1.31\ mM^{-1}cm^{-1}$; C_2 and the C_2 mutants, $\varepsilon_{280} = 56.7\ mM^{-1}cm^{-1}$. During protein purification, protein concentrations were measured using the Bradford method [36] and using BSA as a protein standard. Concentrations of C_2 and C_2 variants after purification were determined according to the extinction coefficient at 280 nm.

2.2. Site-directed mutagenesis

Single mutation of the R263 residue to alanine, aspartate and glutamate were performed according to the instructions in the QuickChange® II Site-Directed Mutagenesis Kit manual from Stratagene (La Jolla, CA). The oligonucleotide primers containing the appropriate codons for each C_2 mutant are showed in the supplementary material (Table S1). The pET-11a plasmid containing the C_2-hpah gene was used as a template for generating mutants using PCR reactions as described previously [24].

2.3. Protein expression and purification

Plasmids of the R263 mutants were transformed into *E.coli* BL21 (DE3) cells using a heat-shock transformation method. *E.coli* BL21 (DE3) cells containing the R263 mutant plasmid were grown in 3.6 L of Luria-Bertani broth medium (LB) containing 50 μg/ml of ampicillin at 37 °C with shaking at 250 rpm. When the OD_{600} of the cell culture reached ~1.0, the temperature was adjusted to 25 °C for 30 min and IPTG was added into the cell culture with a final concentration of 1 mM to induce protein expression. The cell culture was continuously grown at 25 °C with shaking at 250 rpm. When the OD_{600} of the cell cultures reached 4, cells were harvested by centrifugation at 5500 rpm for 20 min. The cell paste was stored

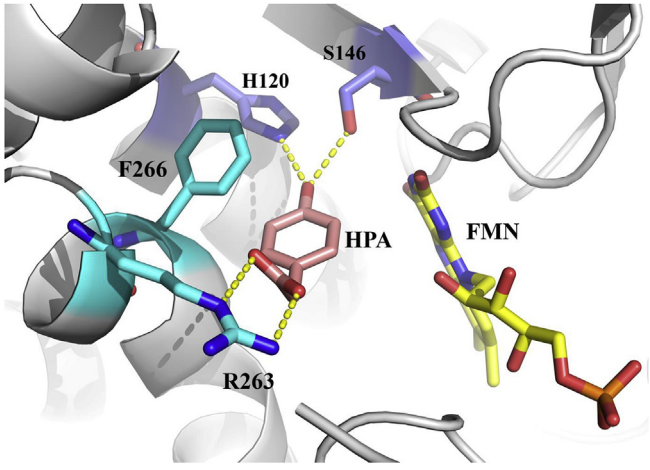


Fig. 1. Structure of the C_2 active site [34]. A guanidinium side chain of R263 directly interacts with a carboxyl group of HPA.