Studies of the Two-Component *p*-Hydroxyphenylacetate Hydroxylase from *Acinetobacter Baumannii.*

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Introduction

Hydroxylation of aromatic compounds at *ortho* position relative to the phenolic group are usually catalyzed by aromatic flavoprotein hydroxylases containing FAD as the prosthetic group and the reactions are mostly carried out on single polypeptide chains [1]. However, in the past decade, flavoenzymes catalyzing aromatic hydroxylation were found to consist of two proteins, such as the reaction of *p*-hydroxyphenylacetate (HPA) hydroxylase (HPAH) of *Pseudomonas putida* [2] and of *E. coli* W [3], and pyrrole-2-carboxylate monooxygenase [4]. Studies of *Pseudomonas putida* HPAH have shown that FAD is tightly bound to the small component and the large component is a coupling protein that enables hydroxylation to occur [2]. In contrast, studies of *E. coli* HPAH have shown that the small component of the enzyme is the flavin reductase generating free reduced FAD for the large component to hydroxylate HPA [3].

We have isolated another HPAH from *Acinetobacter baumannii* that quite distinct from *P. putida* HPAH or *E. coli* HPAH [5]. HPAH from *A. baumannii* also requires two-protein components for complete activity. The small component (C_1) by itself catalyzes the HPA-stimulated NADH oxidation without hydroxylating HPA. C_1 is a flavoprotein with FMN as a native cofactor but can also bind to FAD. The large component (C_2) is required in order to accomplish the hydroxylation reaction. Our studies have demonstrated the role of each protein component for this aromatic hydroxylation. The redox potential values of C_1-bound FMN both in the free enzyme and the enzyme-HPA complex were also investigated.

Materials and Methods

HPAH was isolated from *A. baumannii* by the procedure described in reference [5]. Methods for determination of redox potential values are described in reference[6].

Results and Discussion

Dependence of hydroxylation activity on the molar ratio of C_2/C_1.

The hydroxylation activity of HPAH requires both C_1 and C_2 components. In the reaction of C_1 alone with HPA and NADH, the oxidation of NADH results in the formation of H_2O_2 without hydroxylating the aromatic substrate. Increasing the concentration of C_2 in the reaction led to less H_2O_2 being formed and more DHPA being produced (Figure 1).

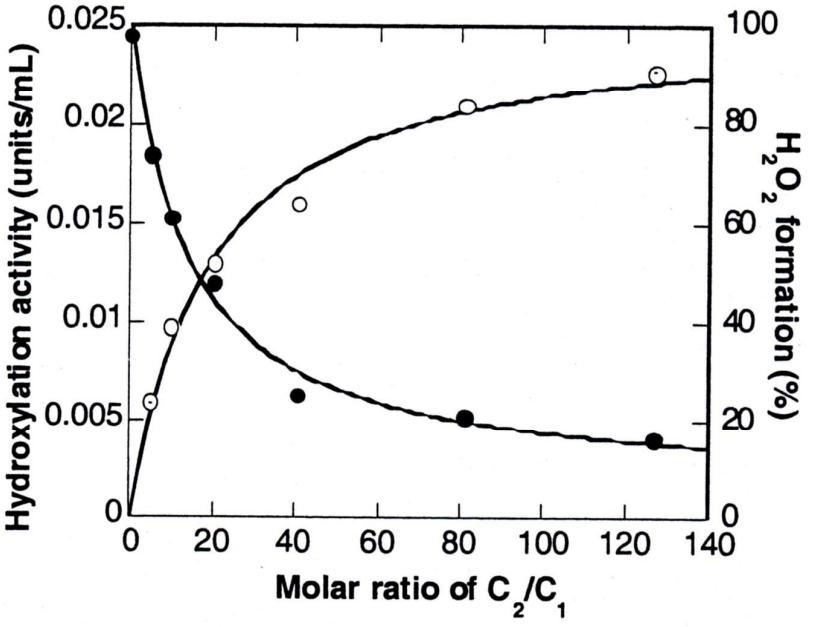


Figure1 Influence of the molar ratio of the two components of HPAH on hydroxylation activity and H_2O_2 formation. Initial rates in the DHPAO coupled assay were measured while keeping C_1 concentration constant at 10.9 nM and varying the concentration of C_2 (0-1.39 μM) in reaction mixtures containing 150 μM NADH, 15 μM FAD and 200 μM HPA. The plot of the initial rates versus C_2/C_1 ratio is shown with empty circles. The percentage of H_2O_2 formed (filled circle) was measured at the end of the reaction by adding the enzyme catalase and measuring the production of oxygen using an oxygen electrode.

Evidence for C_2 as the hydroxylating component.

In order to elucidate the role of C_2 in this HPAH system, HPA and reduced FMN were mixed with C_2 under limiting amounts of oxygen in an anaerobic cuvette. The reaction showed the appearance of an intermediate species with λ_{max} at 370 nm (Figure 2A). The absorption characteristics of this species resembled those of a C(4a)-substituted flavin (i.e. C(4a)-hydroperoxyflavin or C(4a)-hydroxyflavin) [7] and was distinctly different from oxidized and reduced flavin. When reduced FMN was mixed with C_2 in the absence of HPA, some intermediate absorption was found similar to that found in the presence of HPA but with more oxidized flavin characteristics (Figure 2B). When the reaction in Figure 2A (with HPA) was completely opened to air, the formation of DHPA was detected by uisng HPLC and the DHPAO assay (Inset to Figure 2A), clearly indicating that C_2 alone can hydroxylate HPA by using reduced FMN.

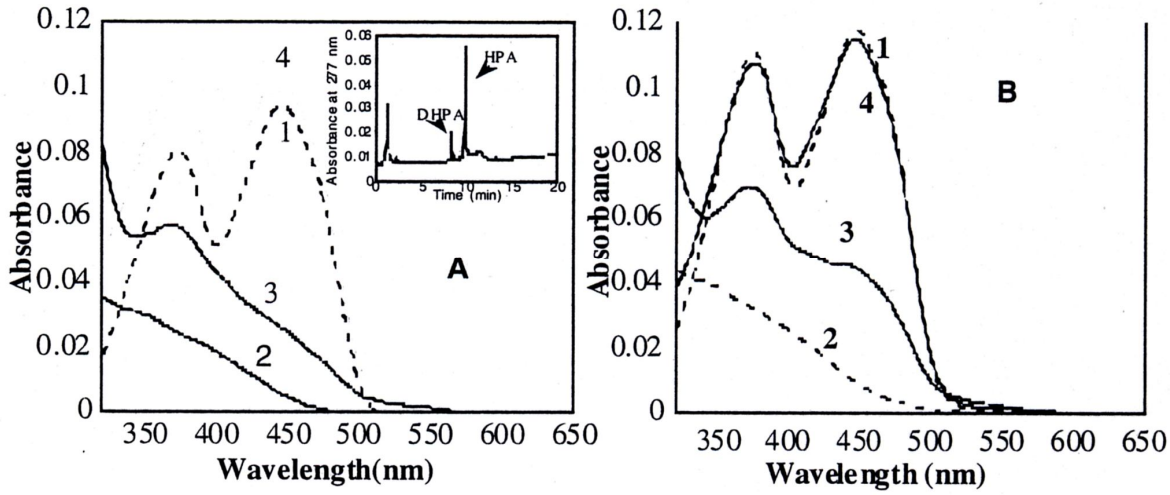


Figure 2. Reaction of C_2 with reduced FMN and a limited amount of oxygen. A: The UV-visible absorption spectrum of oxidized FMN (8 μM) and HPA (106 μM) in 50 mM sodium phosphate buffer, pH 7.0 in an anaerobic cuvette, is shown with a dashed line (1). The fully reduced FMN spectrum is shown in (2). After C_2 in the side-arm was tipped into the main body of the cuvette, the resulting spectrum of the "intermediate" is shown in (3). (4) shows the spectrum of the reaction when the cuvette was fully opened to air. The inset to the figure shows HPLC analysis of the reaction, clearly indicating the formation of DHPA. **B:** All spectra of (1-4) are the same as in **A** except that no HPA was added to the reaction.

Determination of redox potential values for C_1-FMN and the C_1-FMN•HPA complex. The redox potential of flavin in C_1-FMN and C_1-FMN•HPA complex was determined by using phenosafranine as the standard dye (E^o = -252 mV) and xanthine/xanthine oxidase system as a reductant (Figure 3). The midpoint potentials (E^o_m) obtained from the plot of $\log(E_{red}/E_{ox})$ and $\log(Dye_{red}/Dye_{ox})$ are -236 mV for C_1-FMN and -245 mV for the C_1-FMN•HPA complex (Inset to Figure 3Aand 3B). The slopes of both plots have values close to unity indicating that this reduction reaction is a two-electron process in both species.

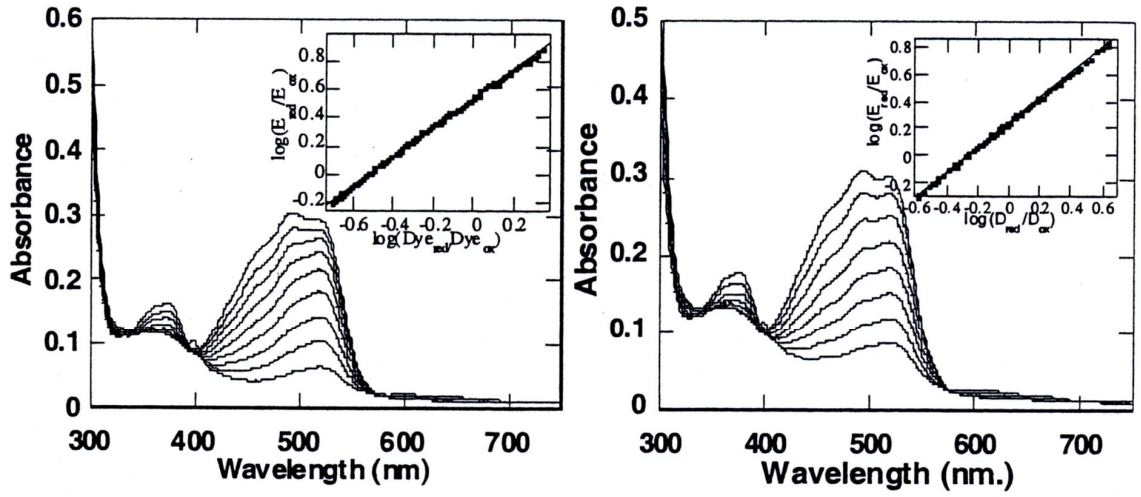
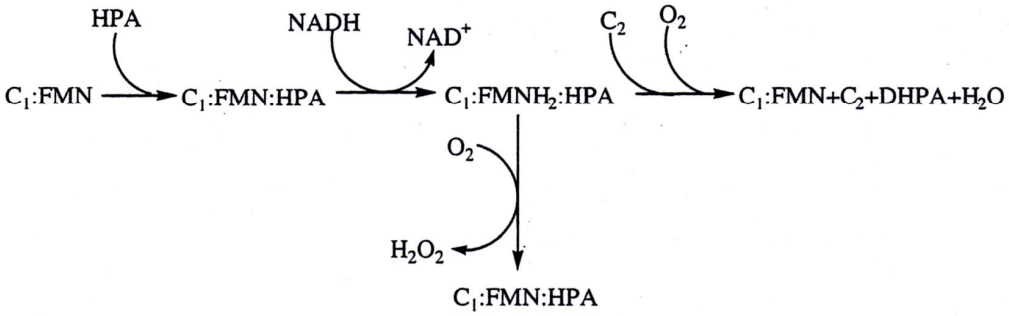


Figure 4 Determination of the redox potential value of the C_1-FMN•HPA complex. C_1-FMN (14.3 μM), HPA 300 μM, phenosafranine (8.8 μM), and benzyl viologen (5 μM) in 1 mL of 50 mM sodium phosphate, pH 7.0 were slowly reduced by xanthine (200 μM) and xanthine oxidase (88 nM) under anaerobic condition. Reactions were followed spectroscopically in over a period of 8 hr. The absorbance at 520 nm was used to monitor the concentration of Dye_{OX}, and the absorbance at 458 nm was used to monitor E_{OX}. The inset shows that the plot of log (E_{red}/E_{ox}) vs. log (D_{red}/D_{ox}) has a slope of 1.0.

Conclusion

Based on the available evidence, we postulate the model for the reaction of HPAH from *A. baumannii* as shown in Scheme 1. In this model, C_1 is a reductase component sending reduced flavin to the hydroxylase component, C_2. HPA binds first to C_1-FMN to form the C_1-FMN•HPA complex and the complex is subsequently reduced by NADH. The resulting reduced flavin is then transferred to C_2 and the re-oxidation of reduced FMN occur concomitantly with hydroxylation of HPAH.



Scheme 1

Acknowledgement

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