Kinetic Mechanisms of the Oxygenase from a Two-component Enzyme, \( p \)-Hydroxyphenylacetate 3-Hydroxylase from \textit{Acinetobacter baumannii}**

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\( \textit{p} \)-Hydroxyphenylacetate hydroxylase (HPAH) from \textit{Acinetobacter baumannii} catalyzes the hydroxylation of \( \textit{p} \)-hydroxyphenylacetate (HPA) to form 3,4-dihydroxyphenylacetate (DHPA). The enzyme system is composed of two proteins: an FMN reductase (C1) and an oxygenase (C2) from \textit{Mahidol University}, Bangkok 10400, Thailand and the \textit{C2-FMNH}\(^ {2} \) from \textit{University of Michigan}, Ann Arbor, Michigan 48109-0600.

Hydroxylation of aromatic compounds in bacteria by single component flavoprotein hydroxylases has been studied extensively for 40 years (1–3). These enzymes are all classified in the \textit{catalytic class II} (1) and exhibit three kinetic properties (2). The first is that the small component enzyme binds FMN tightly to reduced FMN (\( K_\text{d} \approx 0.1 \text{ mM} \)), whereas the \( \textit{C2-FMNH}_{2} \) oxygenase complex reacts with oxygen to form (\( C(4a) \)-hydroperoxy-FMN at \( 1.1 \pm 0.1 \times 10^{5} \text{ M}^{-1} \text{ s}^{-1} \), whereas the \( \textit{C2-FMNH}_{2} \)–HPA complex reacts with oxygen to form (\( C(4a) \)-hydroperoxy-FMN–HPA more slowly (\( k = 4.8 \pm 0.2 \times 10^{3} \text{ M}^{-1} \text{ s}^{-1} \)). The kinetic mechanism of \( \textit{C2} \) was shown to be a preferential random order type, in which HPA or oxygen can initially bind to the \( \textit{C2-FMNH}_{2} \) complex, but the preferred path was oxygen reacting with \( \textit{C2-FMNH}_{2} \) to form (\( C(4a) \)-hydroperoxy-FMN intermediate prior to HPA binding. Hydroxylation occurs from the ternary complex with a rate constant of \( 20 \text{ s}^{-1} \) to form the (\( \textit{C2-C(4a)} \)-hydroperoxy–FMN-DHPA complex. At high HPA concentrations (>0.5 mM), HPA formed a dead end complex with the (\( \textit{C2-C(4a)} \)-hydroxy-FMN intermediate (similar to single component flavoprotein hydroxylases), thus inhibiting the bound flavin from returning to the oxidized form. When FAD\(^ {H+} \) was used, (\( \textit{C4a} \)-hydroperoxy–FAD, (\( \textit{C4a} \)-hydroxy–FAD, and product were formed at rates similar to those with FMNH\(^ {2} \)). Thus, \( \textit{C2} \) has the unusual ability to use both common flavin cofactors in catalysis.

Hydroxylation of aromatic compounds in bacteria by single component flavoprotein hydroxylases has been studied extensively for 40 years (1–3). Recently, several research groups, including ours, reported that hydroxylation of aromatic compounds can be catalyzed by two-protein or multiprotein monooxygenases. These include \( \textit{p} \)-hydroxyphenylacetate 3-hydroxylase (HPAH)\(^ {3} \) from \textit{Pseudomonas putida} (4), \textit{Escherichia coli} (5), and \textit{Acinetobacter baumannii} (6), phenol hydroxylase (PheA) from both \textit{Bacillus steatorrhoeus} BR219 and \textit{Bacillus thermococcosADIUS A7} (7, 8), chlorophenol-4-monoxygenase from \textit{Burkholderia cepacia} AC1100 (9), 2-A6-trichlorophenol monoxygenase from \textit{Ralstonia eutropha} JMP134 (10), pyrrole-2-carboxylate monoxygenase from \textit{Rhodococcus} sp (11), styrene monoxygenase from \textit{Pseudomonas} VB120 (12, 13), and \( \textit{p} \)-nitrophenol hydroxylase from \textit{Bacillus sphaericus} (14). Most of these enzyme systems consist of reductase and monooxygenase components, where the reductase component provides reduced flavin for the monooxygenase component to use for hydroxylating the aromatic substrate. The number of enzymes known in this class continues to increase and many more hypothetical proteins derived from genome projects have also been identified (15).

Hydroxylation of \( \textit{p} \)-hydroxyphenylacetate (HPA) to form 3,4-dihydroxyphenylacetate (DHPA) by HPAH is especially interesting because the same reaction is carried out by at least three types of two-component enzymes. The first HPAH purified was from \textit{P. putida}, and it was shown to have FAD tightly bound to the smaller protein, and the larger protein (at that time) was thought to be a coupling protein enabling hydroxylation (4, 16). A different HPAH system was later isolated from \textit{E. coli} \textit{W}, and studies have shown that the smaller component (HpaC) is a flavin reductase that generates reduced FAD to be transferred to the larger component (HpaB) to hydroxylate HPA (5, 17). A detailed analysis of the mechanism of the \textit{E. coli}–type HPAH is now in progress using the homologue from \textit{P. aeruginosa} (18). The oxygenase in this system exhibits complex dynamics in catalysis (19).

Our group has isolated HPAH from \textit{A. baumannii} and shown that the enzyme is quite different from the analogous HPAH enzymes from either \textit{P. putida} or \textit{E. coli} (6, 15, 20). The \textit{A. baumannii} HPAH is a two protein enzyme system consisting of a smaller reductase component (\( \textit{C1} \)) and a larger oxygenase component (\( \textit{C2} \)) (6). Sequence and several catalytic properties indicate that both components are different from others in the two protein class of aromatic hydroxylases (15, 20). Our recent investigations of the reaction mechanisms of \( \textit{C1} \) have shown that HPA controls the reduction of \( \textit{C1} \)-bound FMN by NADH by shifting the enzyme into a more active conformation (20). By contrast, HPA has no effect at all on the activity of the reductase from the \textit{E. coli}–type HPAH from \textit{P. aeruginosa} (18). The HPAH from \textit{P. putida} (above) (16) requires fresh examination based upon our current knowledge. It is possible that this enzyme system operates in a manner similar to the system from \textit{A. baumannii}, but the essential experiments to test this possibility have not been carried out.

\( \textit{C2} \) shows little sequence similarity to other oxygenases in the same class, and is unique for its ability to use reduced forms of riboflavin, FMN, or FAD to catalyze hydroxylations (6, 15). The overall reaction of \( \textit{C2} \) is described in Fig. 1. When \( \textit{C2} \) was mixed with reduced flavin and a
limited amount of oxygen, an intermediate spectrum resembling that of a C(4a)-oxygen adduct of flavin was observed (6). Similar observations were made in the analogous reactions of the oxygenase component involved in biosynthesis of actinorhodin in *Streptomyces coelicolor* (ActVA) (21, 22) and with chlorophenol 4-monoxygenase (9). Despite preliminary observations that C(4a)-oxygenated intermediates are likely to be involved in oxygenation reactions of these oxygenase components, investigations by presteady state methods to elucidate the enzyme reaction mechanism in detail have never been carried out. In this article, we report investigations on the reaction of oxygen with C2 and reduced flavin using single mixing and double mixing stopped-flow spectrophotometry. The results comprehensively elucidate the reaction mechanism of C2, the order of substrate binding, and the binding constants of FMNH2 and HPA to the enzyme.

**MATERIALS AND METHODS**

Reagents—NAD+, NADH, FAD, glucose, and glucose oxidase were from Sigma. FMN was prepared by conversion of FAD to FMN with snake venom from *Crotalus adamanteus* (23). In brief, FAD (2.5 mg/ml and venom (50 μg/ml) in 20 mM potassium phosphate buffer, pH 7.0 were incubated overnight in the dark. The reaction mixture was loaded onto a Sep-Pak cartridge (Waters), previously equilibrated with 20 mM potassium phosphate buffer, pH 7.0, and the cartridge was washed with 10 mM potassium phosphate buffer, pH 7.0. FMN was eluted with water, and the solution was freeze-dried. Concentrations of the following compounds were determined using known extinction coefficients at pH 7.0: NADH, ε260 = 6.22 mm–1 cm–1; FAD, ε450 = 11.3 mm–1 cm–1; FMN, ε340 = 12.2 mm–1 cm–1 and HPA, ε377 = 1.5 mm–1 cm–1 (6). C2 used in this study was cloned, expressed, and prepared as previously described (15). The concentration of C2 was estimated from the extinction coefficient (based on amino acid sequence) of ε280 = 56.7 mm–1 cm–1.

**Enzyme Activity**—Enzyme hydroxylation activity was detected in real time using a coupling reaction involving 3,4-dihydroxyphenylacetate dioxygenase (DHPAO) to convert the DHPA product of C2 to 5-carboxymethyl-2-hydroxy-muconate semi-aldehyde (CHS). This yellow compound has a maximum absorbance at 380 nm that is dependent upon pH (4, 6).

**Spectroscopic Studies**—UV-visible absorbance spectra were recorded with a Hewlett Packard diode array spectrophotometer (HP 8453A), or a Shimadzu 2501PC spectrophotometer. Fluorescence measurements were carried out with a Shimadzu RF5301PC spectrofluorometer. All these instruments were equipped with thermostatic cell compartments.

**Determination of the Kd for Binding Oxidized FMN to C2**—The measurements were performed by an ultrafiltration method using Centriprep® Y-M 10 from Amicon. Solutions were composed of 10 μM FMN in 50 mM sodium phosphate buffer, pH 7.0, and various concentrations of C2, (20, 40, 80, 160, and 200 μM) in a 10-ml total volume. Each solution was centrifuged at 3,200 rpm, 4 °C for 15 min to obtain a filtrate of ~1 ml (to minimize change in volume). The filtrate and retentate were analyzed for the amount of free and bound FMN, respectively. Ratios of the free and bound species were used to calculate the Kd value.

**Results**

Reaction of C2-FMNH2 with O2 in the Absence of HPA—A solution of FMN (16 μM) plus C2 (25 μM) was placed in a glass tonometer equipped with a quartz cuvette, and made anaerobic as described under “Materials and Methods.” An anaerobic solution of sodium dithionite was delivered into the tonometer to stoichiometrically reduce the FMN (see “Materials and Methods”). The resulting C2-FMNH2 complex was loaded onto the stopped-flow spectrophotometer, where its reaction with oxygen was monitored at 380 and 446 nm (Fig. 2). A significant
fraction of the first phase, which is shown by an increase in absorbance at 380 nm and no change at 446 nm, occurred during the dead time of the instrument (0.002 s) and was complete by 0.006–0.02 s (high to low oxygen concentration, Fig. 2). The plot of $k_{obs}$ of this phase versus oxygen concentration was linear, yielding a second-order rate constant of 1.1 ± 0.1 × $10^7$ M$^{-1}$ s$^{-1}$ (inset in Fig. 2). When absorbance values at the end of the first phase (reaction time of 0.01 s of highest oxygen concentration) at various wavelengths were plotted, the spectrum B in Fig. 3 was obtained. This spectrum has characteristics typical for a flavin-C(4a)-adduct with maximum absorbance at 380 nm. Based upon analogy to the reactions catalyzed by one component hydroxylases and the condition that HPA is absent, the spectrum B in Fig. 3 is likely to be C(4a)-hydroperoxy-FMN (1–3). Spectrum B is also similar to that of C(4a)-hydroperoxyflavins generally found in the class of single component aromatic hydroxylases (1, 3, 24–26), as well as for luciferase (the C(4a)-hydroperoxyflavins described in the next paragraph),∼1.6 μM free FMNH$^-$ is present under these reaction conditions. Therefore, this small absorbance change is likely to be because of free FMNH$^-$ reacting with oxygen.

When the same reaction of the C$_2$-FMNH$^-$ complex was investigated with fluorescence detection using the excitation wavelengths of 360–446 nm and emission at wavelengths of greater than 500 nm, fluorescence increases were only observed with formation of the final species, oxidized FMN (data not shown). This result indicated that the C$_2$-C(4a)-hydroperoxy-FMN was non-fluorescent.

**Determination of Binding Constants of Reduced and Oxidized Flavin to C$_2$**—Free reduced FMN was mixed with air-saturated C$_2$ solution in the stopped-flow apparatus, resulting in a reaction with kinetic traces nearly identical to those obtained when preformed C$_2$-FMNH$^-$ was mixed with oxygen, as shown in Fig. 2. This result implies that binding of FMNH$^-$ to C$_2$ is much faster than the oxidation of free FMNH$^-$ by oxygen (32), and also greater than the rate of formation of the C(4a) flavin hydroperoxide at 0.13 mM oxygen, 185 ± 9 s$^{-1}$ (Fig. 2). Thus, the rate constant for C$_2$ binding to FMNH$^-$ is likely to be 10$^7$ M$^{-1}$ s$^{-1}$ ($k_1$, in Fig. 10).

Therefore, when FMNH$^-$ (16 μM) was mixed with various concentrations of C$_2$ in air-saturated buffer in the stopped-flow spectrophotometer, the absorbance increase at 380 nm during the first phase (Fig. 4), because of the C(4a)-hydroperoxy FMN formed, was also directly dependent on the amount of C$_2$-FMNH$^-$ complex initially present. In the absence of C$_2$ (the lowest trace), the absorbance increased with a $k_{obs}$ of 0.7 s$^{-1}$ as free FMNH$^-$ oxidized to FMN in a complex autocatalytic reaction (32). As the concentration of C$_2$ increased, less auto-oxidation of FMNH$^-$ is observed. Therefore, the increase in absorbance observed at 0.04 s represents the amount of C$_2$-FMNH$^-$ present at the start of the reaction, and the plot of this change in absorbance versus the free C$_2$ concentration represents the binding isotherm for FMNH$^-$.

The plot (inset in Fig. 4) shows that the absorbance increase is hyperbolically dependent on C$_2$ concentration. A $K_d$, referred to as $K_d^{C_2}$ in the kinetic scheme in Fig. 10) value for the complex was calculated to be 1.2 ± 0.2 μM.
The $K_p$ for the binding of oxidized FMN to C2 was determined by an ultrafiltration method described under “Materials and Methods.” This experiment indicated that the $K_p$ of C2-FMN was 250 ± 50 μM. The large uncertainty occurred because values could not be obtained at appropriately high C2 concentrations.

**Reaction of the C2-FMNH⁻-HPA Complex with Oxygen**—The reaction of C2 in the presence of substrate was investigated by mixing an aerobic solution of FMNH⁻ (16 μM), C2 (25 μM), and HPA (2 mM) (final concentrations) with 2 mM HPA with buffer containing various oxygen concentrations in the stopped-flow spectrophotometer (Fig. 5). The rate of formation of C2 intermediate in presence of HPA was second order with respect to oxygen; however, the reaction is considerably slower than when no HPA is present (compare the increases at 380 nm in Fig. 2 to those in Fig. 5). An obvious interpretation of this result is that the reaction with oxygen is slower when HPA present). The second phase (20 s⁻¹ for the second intermediate, and 0.35 s⁻¹ for the final step in which oxidized FMN is formed. The analysis shows that spectra of the two intermediates are very similar (inset of Fig. 5), and have absorption characteristics similar to C(4a)-intermediates found for the single component flavoprotein hydroxylases (1, 24–26, 33). This also implies that the first and second intermediates are likely to be C2-C(4a)-hydroperoxy-FMN-HPA complex and C2-C(4a)-hydroxy-FMN-product complex, respectively (inset of Fig. 5). The slight increased absorbance in the region of 450 nm of the second intermediate in the inset to Fig. 5 is unlikely to belong to absorption of C2-C(4a)-hydroxy-FMN, but rather to a small amount of oxidized FMN resulting from an uncoupling pathway that does not result in hydroxylation (2, 24–26).

Therefore, we conclude that the first phase is the reaction of C2-FMNH⁻ (without HPA bound) whereas the second phase is the formation of C2-C(4a)-hydroperoxy-FMN-HPA complex. This also implies that the binding of HPA to the enzyme decreases the rate of formation of C2-C(4a)-hydroperoxy-FMN about 20-fold. We interpret the third phase to be the hydroxylation step where C2-C(4a)-hydroperoxy-FMN reacted with HPA to form the C2-C(4a)-hydroxy-FMN and DHPA. The C(4a)-hydroperoxy-FMN and C(4a)-hydroxy-FMN species have very similar spectra with this enzyme (see below), causing the absorbance change upon hydroxylation to be very small. Because of this small absorbance change, the rate constant for the hydroxylation step could not be determined accurately by this procedure. The fourth phase was caused by the dehydration of C2-C(4a)-hydroxy-FMN to yield the oxidized FMN species.

To verify further if the C2-FMNH⁻-HPA complex has indeed led to hydroxylation as described above, DHPA product formed under the conditions used in stopped-flow experiments was determined. Reaction samples were collected from the stopped-flow instrument and quantified by HPLC methods. Solutions of FMNH⁻ (50 μM), C2 (80 μM), and HPA (2 mM) were mixed with air-saturated buffer containing 2 mM HPA at 4 °C in the stopped-flow spectrophotometer. The reaction solutions were analyzed for DHPA by HPLC as described previously. The analysis showed that 73 ± 4% of HPA was hydroxylated to form the DHPA product from the ternary complex under these conditions (Table 1).

**HPA Is Not the First Substrate Binding to C2**—The reaction of C2 involves three substrates (Fig. 1), HPA, FMNH⁻, and oxygen. In this section we describe experiments to determine the sequence of binding of these compounds to C2. Fig. 6 shows experiments of the reaction to form the C2-C(4a)-hydroperoxy-FMN involving various premixing protocols. Trace A shows the reaction of the C2-FMNH⁻-HPA complex with O₂ (from Fig. 5), and trace B shows the reaction of the
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TABLE 1
Determination of hydroxylated product from single turnover reactions of C2

| Type of reaction | Product                        | %          |
|------------------|--------------------------------|------------|
| syringe A        | syringe B                       |            |
| C2-FMNH–HPA      | O2                             | 73 ± 4     |
| C2 + HPA + O2    | FMNHa                          | 82 ± 3     |
| C2-FADH–HPA      | O2                             | 74 ± 4     |
| C2 + HPA + O2    | FADHa                          | 68 ± 3     |

a A solution of C2 (25 μM), FMNH or FADH (16 μM), and HPA (2 mM) was mixed with buffer containing 0.13 mM of oxygen. All concentrations were described as after mixing. Under these conditions, the reaction follows path A in Fig. 10.

The solution of C2 (25 μM), HPA (2 mM), and oxygen (0.13 mM) was mixed with buffer containing 16 μM of FMNH–oxygen, and FMNH–oxygen formed at the same rate as the reaction with free C2-FMNH–oxygen. The reaction was monitored by absorbance at 380 nm to assess the amount of C2-C(4a)-hydroperoxy-FMN formed. The dotted line represents the reaction of C2-FMNH–oxygen with oxygen under the same conditions. A plot of absorbance amplitudes that occurred at 380 nm at ~54 s−1 versus the age time (inset) represents the binding kinetics of HPA to C2-FMNH–oxygen.

C2-FMNH–complex with O2 and HPA. These demonstrate that the C2-FMNH–HPA complex reacts with O2 much more slowly than does the C2-FMNH–complex. We used this information to examine whether C2 can effectively bind HPA in the absence of FMNH–oxygen. If such a complex does form, it would be expected that this complex in the presence of O2 would react with FMNH–oxygen to form C2-C(4a)-hydroperoxy-FMN at the slower rate, as seen in trace A. Upon mixing a solution containing C2, HPA, and oxygen with FMNH–oxygen in the stopped-flow spectrophotometer (trace D, Fig. 6), C2-C(4a)-hydroperoxy-FMN formed at the same rate as the reaction with free C2-FMNH–complex (trace B) as well as with mixing C2 in air-saturated solution with FMNH–oxygen (trace C). These results suggest that C2 alone does not bind effectively to HPA, and that FMNH–oxygen is the first species binding to the enzyme during catalysis.

Binding of HPA to C2-FMNH–complex—After binding to C2 to form the C2-FMNH–complex, the enzyme can in principle carry out the reaction through one of two paths (Fig. 10): A) C2-FMNH–complex is formed prior to reacting with oxygen, and B) C2-C(4a)-hydroperoxy-FMN is formed prior to binding HPA. In this section, we report investigations of the kinetics and thermodynamics of binding HPA to C2-FMNH–complex prior to reacting with oxygen. The binding kinetics of HPA to C2-FMNH–complex were investigated by double mixing stopped-flow spectrophotometry, where the first mixing added HPA to C2-FMNH–complex under anaerobic conditions to initiate the formation of the C2-FMNH–HPA complex, and after various times of aging, the second mix added oxygen to form the C2-C(4a)-hydroperoxy-FMN species, either in complex with HPA, or not. Final concentrations after double mixing were C2 (25 μM), FMNH–oxygen (16 μM), HPA (2 mM), and oxygen (1.03 mM). This resulted in ~16 μM of C2-FMNH–oxygen in the solution after double mixing. The reaction was monitored by absorbance at 380 nm to detect formation of C2-C(4a)-hydroperoxy-FMN. Any C2-FMNH–binary complex present reacted with this concentration of oxygen at >1000 s−1 and largely occurred in the dead time of the instrument, as shown by the dotted line in Fig. 7. Upon increasing the age time, formation of the C(4a)-hydroperoxy-FMN became slower as more C2-FMNH–HPA complex formed. Any C2-FMNH–HPA complex present reacted to form C2-C(4a)-hydroperoxy-FMN–HPA at ~54 s−1, and the reaction was completed by ~90 ms. This indicates that the more complete the formation of C2-FMNH–HPA, the slower was the formation of C2-C(4a)-hydroperoxy-FMN. This result is also consistent with our previous interpretation in Fig. 5 that C2-FMNH–HPA reacts with oxygen more slowly than does C2-FMNH–complex. Fig. 7 shows that the amount of C2-C(4a)-hydroperoxy-FMN formed between 7 and 80 ms (the slower reaction) was maximum when age times before mixing were ~1 s (inset in Fig. 7), indicating that binding of 2 mM HPA to C2-FMNH–complex was complete by 1 s. The apparent rate constant (kobs) for binding of HPA to C2-FMNH–complex, calculated from the slope of the absorbance increase observed at 380 nm versus the age times after the first mixing, was 9.6 ± 2 s−1.

The thermodynamics for binding of HPA to C2-FMNH–complex prior to reacting with oxygen were investigated by double mixing stopped-flow spectrophotometry, where various concentrations of HPA were mixed with C2-FMNH–complex anaerobically in the first mix, and oxygen was added to the preformed C2-FMNH–HPA complex in the second mix (Fig. 8). The age time was 10 s to ensure complete formation of the substrate complex, and the reaction was monitored at 370 and 446 nm. The final concentrations were 16 μM C2-FMNH–complex, 1.03 mM oxygen, and a range of HPA concentrations. Fig. 8 shows that the kinetic traces are composed of four phases. The first phase observed is an increase in absorbance at 370 nm with an observed rate constant of 54 s−1, and the amplitude of this phase is dependent on concentration of HPA. It is known from the single mixing experiments (Fig. 2) that the reaction of C2-FMNH–complex and 1.03 mM oxygen is fast enough to be largely completed.
Oxygenase Component of p-Hydroxyphenylacetate 3-Hydroxylase

**FIGURE 8.** Dissociation constant for binding HPA to C_2-FMNH^−_. This was measured by double mixing stopped-flow spectrophotometry. The first mix added various concentrations of HPA (80, 160, 400, 800, 2000, 4000, 8000 μM, from upper to lower traces) anaerobically to C_2-FMNH^−_ (16 μM), and the second mix added 1.03 mM oxygen to the resultant mixture. The age time between mixes was 10 s to ensure the complete formation of any C_2-FMNH^−_–HPA complex, and the reactions were monitored at 370 nm. All concentrations given are after final mixing. The amplitude changes in the phase occurring at 54 s⁻¹ (dead time to 80 ms) versus the HPA concentration are plotted in inset A and this was used to calculate the K_d^{upper}(180 ± 30 μM) for binding of HPA to C_2-FMNH^−_. Inset B shows the formation of oxidized FMN at 446 nm (see also Fig. 9).

The second phase in the reactions in Fig. 8 was a small decrease in absorbance with an observed rate constant of ~17–22 s⁻¹ (k_1 in Fig. 10), whereas the third phase was a small increase in absorbance with a rate constant of ~6–9 s⁻¹ (k_2 in Fig. 10). The absorbance at 370 nm decreased again in the fourth phase with the k_obs values inversely dependent on the concentration of HPA used. The fourth phase was identified as the formation of the final oxidized FMN species because the traces coincided with a large increase in absorbance at 446 nm (shown in inset B). These results suggest that after formation of C_2-C(4a)-hydroperoxy-FMN during the first phase, HPA was hydroxylated with the formation of C_2-C(4a)-hydroxy-FMN during the second phase, similar to the results of Fig. 5. However, it is clear from this experiment that excess HPA can also bind to the enzyme to trap the C_2-C(4a)-hydroxy-FMN–HPA species (Fig. 10) in the third phase, causing a slight increase in absorbance at 370 nm. With higher concentrations of HPA, more of this intermediate was trapped as the C_2-C(4a)-hydroxy-FMN–HPA complex, so that the dehydration to form the oxidized FMN was retarded (inset B in Fig. 8). Similar trapped C(4a)-hydroxy-FMN species were also observed in the oxidative half-reactions of several single component flavoprotein oxygenase enzymes (24–26, 34–35).

**The Reaction of C_2-C(4a)-hydroperoxy-FMN with HPA**—Experiments from the previous section show that hydroxylation can occur via Path A in Fig. 10 where C_2-FMNH^−_ first binds to HPA and then reacts with oxygen to form C_2-C(4a)-hydroperoxy-FMN-HPA or through Path B of Fig. 10, where the enzyme first forms C_2-C(4a)-hydroperoxy-FMN and then binds to HPA in a following step. Therefore, the reaction of Path B was explored using a double mixing stopped-flow spectrophotometer, where the intermediate C_2-C(4a)-hydroperoxy-FMN was generated by reacting C_2-FMNH^−_ with oxygen in the initial mixing; after aging for 0.1 s to fully form the C(4a)-hydroperoxy-FMN, the resultant intermediate was mixed with buffer containing various HPA concentrations. Reactions were monitored at 370 nm (Fig. 9), and the results indicate that binding to HPA (the small increase in absorbance from 2–20 ms) gave a phase with amplitudes and rates that were dependent on the concentration of HPA. Kinetic analysis showed that the observed rate constants (k_obs) of this phase were hyperbolically dependent on HPA concentrations (inset A in Fig. 9). These results are consistent with binding being a two-step process, with a rapid equilibrium in the initial step and an isomerization in the following step (Path B in Fig. 10) (36). Data were analyzed according to Equation 1, yielding a K_d value for the initial binding of HPA of 0.35 ± 0.03 mM (K_d, Fig. 10), and the rate constant for the subsequent isomerization of 208 ± 4 s⁻¹ (k_4 in Fig. 10).

\[
    k_{\text{obs}} = \frac{k_a[\text{HPA}]}{K_d + [\text{HPA}]} \quad \text{(Eq. 1)}
\]

After HPA has bound, there are three more phases in the reaction similar to those seen in the double mixing experiments described in Fig. 8. In the second phase, C_2-C(4a)-hydroperoxy-FMN–HPA converted to C_2-C(4a)-hydroxy-FMN–DHPA with a rate of 17–22 s⁻¹ (k_1 in Fig. 10), and this was followed by a slight increase in absorbance during the third phase. The amplitude of the third phase is also dependent on HPA concentration. As before, the second phase is the hydroxylation step, and the third phase is the binding of HPA to C_2-C(4a)-hydroxy-FMN, coincident with the release of DHPA. The fourth phase is the decrease in absorbance 370 nm and a large increase of absorbance 446 similar to those of Fig. 8B (data not shown). The fourth phase was interpreted as the dehydration of C_2-C(4a)-hydroxy-FMN to form oxidized FMN, and this rate was inversely dependent on HPA concentration (as dis-
FIGURE 10. C₂ preferential random order reaction mechanism. Kinetic and thermodynamic constants relevant to each step are shown in the scheme.
C(4a)-hydroperoxy-FAD-HPA complex is slightly smaller than that with FMN (3.7 \pm 0.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1} for FAD versus 4.8 \pm 0.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1} for FMN (see Table 2). The spectra of C2-C(4a)-hydroperoxy-FAD, both in the absence and presence of HPA, calculated using the method described in the FMN experiments, are very similar to those for the C2-FMNH reactions (data not shown).

Double mixing experiments similar to those in Figs. 8 and 9, but using FADH\textsuperscript{−} instead of FMNH\textsuperscript{−}, yielded results similar to those with FMNH\textsuperscript{−}. The \( k_d \) values for binding of HPA to C2-FADH\textsuperscript{−} or to C2-C(4a)-hydroperoxy-FAD are both similar to those with FMNH\textsuperscript{−} (shown in Table 2), again emphasizing that FADH\textsuperscript{−} can be used nearly as well as FMNH\textsuperscript{−} by C2 with respect to both specificity and reactivity.

Single turnover reactions of C2 and FADH\textsuperscript{−} were analyzed for the amount of hydroxylated product (Table 1) using the same protocols described in previous sessions of C2 and FMNH\textsuperscript{−} reactions. Results in Table 1 indicate that FADH\textsuperscript{−} can be used by C2 nearly as efficiently as FMNH\textsuperscript{−}. The yields of DHPA obtained via Path A and B, 68 ± 3 and 74 ± 4\%, were comparable to those for the FMNH\textsuperscript{−} reaction (73 ± 4 and 82 ± 3\%).

### DISCUSSION

Our studies here have elucidated the detailed kinetic mechanism for the reactions of O\textsubscript{2} with reduced flavin bound to the oxygenase component (C\textsubscript{2}) of HPAH from \textit{A. baumanii}. The results and methods described can be used as prototypes for analyses of the two-component class of flavin-dependent oxygenases. These results clearly show that the oxygenation reaction of C\textsubscript{2} occurs via C(4a)-oxygenated flavin intermediates, similar to the reaction of the single component aromatic flavoprotein hydroxylases, where existence of C(4a)-flavin intermediates is well documented (1, 2). It was previously found that C(4a)-hydroperoxyflavins reacted with aromatic substrates to form hydroxylated products in the reactions of \textit{p}-hydroxybenzoate hydroxylase (3, 33), phenol hydroxylase (37–39), melittate hydroxylase (40), anthranilate hydroxylase (41), 2-methyl-3-hydroxyxpyridine-5-carboxylic acid oxygenase (24–26), and 2-hydroxybiphenyl-3-monooxygenase (42). C(4a)-hydroperoxyflavins and C(4a)-hydroxylflavins were also detected in the oxygen reactions of HPAH from \textit{P. putida} (16). The intermediates detected in the reaction of C\textsubscript{2} are spectrally similar to those of the enzymes mentioned. However, the less common feature of C\textsubscript{2} intermediates is that the C(4a)-hydroperoxyflavin and C(4a)-hydroxyflavin spectra are nearly identical; this characteristic has also been found in some mutant types of \textit{p}-hydroxybenzoate hydroxylase (43). Partial resolution of spectra similar to those of C\textsubscript{2}-(C4a)-oxygenated intermediates was also obtained in studies of the reactions of 4-chlorophenol hydroxylase (9), the monoxygenase in the actinorhodin biosynthetic pathway (21, 22), and styrene monoxygenase (13) when the enzymes were mixed with reduced flavin and limited quantities of oxygen in the absence of substrate.

Although the reaction of C\textsubscript{2} with O\textsubscript{2} is similar to the reaction of single
component aromatic hydroxylases with respect to using C(4a)-hydroperoxylavlin to hydroxylate the aromatic substrate, the overall kinetic mechanism of C2 is quite different (1–3). The first step of the reaction is binding of FMNH\textsuperscript{-} to C2, followed by the reaction of the C2-FMNH\textsuperscript{-} complex with oxygen to form a quite stable C2-C(4a)-hydroperoxylavin. Under conditions of catalytic turnover, an aromatic substrate binds to the preformed C2-C(4a)-hydroperoxylavin intermediate (Path B in Fig. 10). This contrasts with the reactions of the single component aromatic hydroxylases where the aromatic compound must be bound to the enzyme prior to reduction and reaction with oxygen. The kinetic mechanism of C2 is remarkably similar to that for bacterial luciferases (Lux) in which the reaction of Lux-FMNH\textsuperscript{-} with oxygen to form C(4a)-hydroperoxy-FMN occurs prior to binding of an aldehyde substrate (28). Although both C2 and Lux bind more tightly to the reduced than to the oxidized flavin, the \( K_d \) for the Lux-FMNH\textsuperscript{-} complex is 80 nM (44), an order of magnitude smaller than that for C2-FMNH\textsuperscript{-} (1.2 \mu M). It is possible, however, that the C2-flavin complex becomes tighter after the reduced flavin is oxidized into C2-C(4a)-hydroperoxy FMN. The mechanism of C2 is also similar to that for the oxygenation half-reaction of cyclohexanone monoxygenase (CHMO), where cyclohexane binds to the enzyme after formation of the FAD-C(4a)-peroxide (29).

The kinetic mechanism of C2 has similarities to the reaction of HPAH from \( P. \) putida. It was reported that in the reaction of O\textsubscript{2} with the reduced flavoprotein plus the coupling protein of the \( P. \) putida HPAH, the rate of FAD-C(4a)-hydroperoxide formation is the same whether or not HPA was included in the oxygen-containing solution (1.1 \times 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1}) (16). However, as shown above, the rate for formation of the C2-C(4a)-hydroperoxylavin decreased from 1.1 \times 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1} to 4.8 \times 10\textsuperscript{5} M\textsuperscript{-1} s\textsuperscript{-1} when HPA was pre-bound to the C2-FMNH\textsuperscript{-} complex from \( A. \) baumannii (compare Figs. 3, 5, and 10). In the \( P. \) putida enzyme, it was also proposed that the reaction occurred via a pathway in which HPA bound to the oxygenase after the formation of C(4a)-hydroperoxy-FAD, similar to the reaction of C2 (Path B in Fig. 10). This was consistent with the rate of HPA binding to the reduced enzyme being rather slow (16). It is possible, however, that the \( P. \) putida enzyme is actually like the \( A. \) baumannii enzyme. The reported flavoprotein of \( P. \) putida might actually be a reductase regulated by HPA, similar to that from \( A. \) baumannii (20), whereas the coupling protein could be the oxygenase that receives reduced FAD from the reductase. Experiments to test this hypothesis have never been carried out. Thus, the lack of effect of HPA on the formation of the C(4a)-hydroperoxylavin from \( P. \) putida HPAH could be caused by HPA not binding to the oxygenase until FADH\textsuperscript{-} has bound.

Reduced flavin is reactive with oxygen. Therefore, to be effective, reduced flavin-utilizing enzymes such as C2 need to rapidly bind reduced flavin before auto-oxidation occurs. \( C_2 \) was shown in this report to bind FMNH\textsuperscript{-} very rapidly (Fig. 4) with an observed rate \( \approx 200 \) s\textsuperscript{-1} (compare traces B and C in Fig. 6). Such a rate corresponds to a second order rate constant of at least \( 10^n \) M\textsuperscript{-1} s\textsuperscript{-1}, and this binding is quite tight (\( K_d \) of 1.2 \mu M under conditions studied). Therefore, the ability of C2 to catalyze reactions without being constantly bound to the cofactor like other flavoproteins can be explained by the preferential binding of the enzyme to the reduced rather than to the oxidized flavin. Similar binding properties were also observed for the oxygenase component (HpaB) of HPAH from \( E. \) coli; HpaB binds to FADH\textsuperscript{-} with a \( K_d \) of 70 nM, whereas it binds to oxidized FAD with a \( K_d \) of 6 \mu M (45). Recently, a study of actinomadin monoxygenase has shown that the oxygenase component, ActVA, binds to FMNH\textsuperscript{-} with a \( K_d \) of 0.4 \mu M and to oxidized FMN with a \( K_d \) of 26 \mu M (21).

At high concentrations, HPA was found to form a dead-end complex with C2-C(4a)-hydroxyflavin and impede it from dehydrating to form the oxidized flavin (Figs. 8 and 9). Aromatic substrates were also found to bind to the C(4a)-hydroxy-FAD and inhibit the return to oxidized FAD in the reactions of several single component aromatic hydroxylases including phenol hydroxylase (34), 2-methyl-3-hydroxypridine-5-carboxylic acid monoxygenase (24), and \( p \)-hydroxybenzoate hydroxylase (PHBH) (46). This type of substrate inhibition was also found in the reaction of \( P. \) putida HPAH (16). In the case of PHBH, it has been proposed that this inhibition is the natural consequence of the need for a conformational change from a solvent-free active site (for hydroxylation) to an “open” conformation for product and substrate exchange (3). Perhaps this inhibition is not important in cells, because cells are not likely to accumulate high concentrations of substrate that could cause such inhibition.

A unique property of C2 is the ability to use a variety of reduced flavin substrates; the enzyme works well with either FADH\textsuperscript{-} or FMNH\textsuperscript{-}, although less efficiently with reduced riboflavin (6, 15). Here we report that both C(4a)-hydroxy-FAD and C(4a)-hydroxy-FAD accumulated during the reaction of FADH\textsuperscript{-} and C2 with oxygen (data not shown), implying that the reaction undergoes the same pathway as that of reduced FMN. Moreover, the kinetic constants for the reaction of FADH\textsuperscript{-} and FMNH\textsuperscript{-} are similar (Table 2), indicating that the reactivity of reduced FMN and FAD in each step of the C2 reaction is nearly the same. This also implies that C2 interacts with the reduced flavin primarily around the isoalloxazine where FAD and FMN share the same common structure. The flavin specificity of the HPA from \( A. \) baumannii (\( C_2 \)) comes from the reductase, which binds more specifically and tightly to FMN (6, 20). This property contrasts to most other two-component monoxygenases, where the reductase is often less specific for the flavin whereas the oxygenase is specific for either FMNH\textsuperscript{-} or for FADH\textsuperscript{-}.

In conclusion, this study has elucidated the reaction mechanism of the oxygenase component (\( C_2 \)) of the enzyme HPAH from \( A. \) baumannii. The results clearly illustrate that C(4a)-oxygcnated flavin intermediates are directly involved in the hydroxylation reaction. C2 binds to the reduced flavin (delivered from \( C_1 \)) in the initial step, reacts with oxygen to form the C2-C(4a)-hydroperoxylavin, and finally binds HPA before hydroxylation occurs. This knowledge is needed to understand catalysis by the enzymes in this two-component class. This report will be followed by a subsequent article that explains in detail the transfer of the flavin between the two protein components of the enzyme.

REFERENCES
1. Palley, B. A., Ballou, D. P., and Massey, V. (1995) Active Oxygen in Biochemistry, pp. 37–83, Chapman & Hall, Glasgow, Scotland
2. Palley, B. A., and Massey, V. (1998) Comprehensive Biological Catalysis, Vol. 3, pp. 83–134, Academic Press, San Diego, CA
3. Entsch, B., Cole, L. J., and Ballou, D. P. (2005) Arch. Biochem. Biophys. 433, 297–311
4. Arunachalam, U., Massey, V., and Vaidyanathan, C. S. (1992) J. Biol. Chem. 267, 25848–25855
5. Galan, B., Diaz, E., Prieto, M. A., and Garcia, J. L. (2000) J. Bacteriol. 182, 627–636
6. Chayen, P., Suadee, C., and Wiliarit, P. (2001) Eur. J. Biochem. 268, 5550–5561
7. Kim, J. C., and Oriel, P. I. (1995) Appl. Environ. Microbiol. 61, 1252–1256
8. Kirchner, U., Westphal, A. H., Muller, R., and von Berkel, W. J. H. (2003) J. Biol. Chem. 278, 47545–47553
9. Gisi, M. R., and Xun, L. (2003) J. Bacteriol. 185, 2786–2792
10. Louie, T. M., Webster, C. M., and Xun, L. (2002) J. Bacteriol. 184, 3492–3500
11. Becker, D., Schrader, T., and Andreessen, J. R. (1997) Eur. J. Biochem. 249, 739–747
12. Otto, K., Hufstetter, K., Rothlisberger, M., Wibulle, B., and Schmid, A. (2004) J. Bacteriol. 186, 5292–5302
13. Kantz, A., Chin, F., Nallamothu, N., Nguyen, T., and Gasser, G. T. (2005) Arch. Biochem. Biophys. 442, 102–116
14. Kadiyala, V., and Spain, J. C. (1998) Appl. Environ. Microbiol. 64, 2479–2484
15. Thotsaporn, K., Sucharitkul, J., Wongratana, J., Suadee, C., and Chaiyen, P. (2004) J. Biol. Chem. 279, 10213–10220
