Kinetic Mechanisms of the Oxygenase from a Two-component Enzyme, p-Hydroxyphenylacetate 3-Hydroxylase from Acinetobacter baumannii*

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p-Hydroxyphenylacetate hydroxylase (HPAH) from Acinetobacter baumannii catalyzes the hydroxylation of p-hydroxyphenylacetate (HPA) to form 3,4-dihydroxyphenylacetate (DHPA). The enzyme system is composed of two proteins: an FMN reductase (C1) and an oxygenase component to use for hydroxylation 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 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 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 E. coli 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 E. coli-type HPAH is now in progress using the homologue from P. aeruginosa (18). The oxygenase in this system exhibits complex dynamics in catalysis (19).

Our group has isolated HPAH from A. baumannii and shown that the enzyme is quite different from the analogous HPAH enzymes from either P. putida or E. coli (6, 15, 20). The A. baumannii HPAH is a two protein enzyme system consisting of a smaller reductase component (C1) and a larger oxygenase component (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 C1 have shown that HPA controls the reduction of 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 E. coli-type HPAH from P. aeruginosa (18). The HPAH from 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 A. baumannii, but the essential experiments to test this possibility have not been carried out.

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 C2 is described in Fig. 1. When 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**—NADH, NADPH, 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 C18 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, ε340 = 6.22 mM⁻¹ cm⁻¹; FAD, ε3900 = 11.3 mM⁻¹ cm⁻¹; FMN, ε450 = 12.2 mM⁻¹ cm⁻¹; and HPA, ε277 = 1.5 mM⁻¹ cm⁻¹ (6). C2 was 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.

**Rapid Reaction Experiments**—Reactions were carried out in 50 mM sodium phosphate buffer, pH 7.0, 4 °C unless otherwise specified. Rapid kinetics measurements were performed with a Hi-Tech Scientific Model SF-61DX in double mixing mode, or with either a model SF-61SX or a SF-61DX stopped-flow spectrophotometer in single mixing mode. The optical pathlengths of the observation cells were 1 cm. The stopped-flow apparatus was made anaerobic by flushing the flow system with an oxygen-scrubbing solution consisting of 400 µM glucose, 1 mg/ml glucose oxidase (15.5 unit/ml), and 4.8 µg/ml catalase in 50 mM sodium phosphate buffer, pH 7.0. The oxygen-scrubbing solution was allowed to stand in the flow system overnight and was then thoroughly rinsed with anaerobic buffer before experiments.

To study the oxidative half-reaction of C2 enzyme, or enzyme plus substrate and oxidized FMN in 50 mM sodium phosphate buffer, pH 7.0, were made anaerobic in glass tonometers by several cycles of evacuation followed by equilibration with argon that had been passed through an Oxyclear oxygen removal column (Labclear). Enzyme was anaerobically reduced with a solution of sodium dithionite (~5 mg/ml in 100 mM potassium phosphate buffer, pH 7.0) delivered from a syringe attached to the tonometer, and the reduction was monitored by UV-visible spectrophotometry. Solutions with various concentrations of oxygen were prepared by equilibrating buffer with air or with certified oxygen/nitrogen gas mixtures. Determinations of rate constants were obtained by fitting plots of apparent rate constants (kobs) versus concentration of substrate with a Marquardt-Levenberg non-linear fit algorithm that is included in the KaleidaGraph software (Synergy Software). The kobs from kinetic traces were calculated from exponential fits using KinetA-syst3 software (Hi-Tech Scientific, Salisbury, UK) or program A (written at the University of Michigan by Rong Chang, Jung-yen Chiu, Joel Dinverno, and D. P. Ballou).

**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
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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 $1.1 \pm 0.1 \times 10^6 \text{M}^{-1} \text{s}^{-1}$ (inset in Fig. 2). When absorbance values at the end of the first phase (reaction time of 0.01 s of highest oxygen reaction) 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)-hydroperoxyFLavin generally found in the class of single component aromatic hydroxylases (1, 3, 24–26), as well as for luciferase (the first two-component flavin-dependent oxygenase studied in detail) (27, 28), cyclohexanone monoxygenase (29) and model systems (30, 31). The hydroperoxide intermediate decayed slowly to yield oxidized FMN and probably H$_2$O$_2$ (at 0.037 $\pm$ 0.002 s$^{-1}$, see traces at 446 nm from 1 to 200 s in Fig. 2). A small increase in absorbance at 380 nm was also observed between 0.01 and 1 s, and the $k_{obs}$ describing this phase was also dependent on oxygen concentration. Based on the $K_d$ value of C$_2$-FMNH$^-$ of 1.2 $\pm$ 0.2 M$^{-1}$ (described in the next paragraph), $\sim$1.6 M$^{-1}$ free FMNH$^-$ is present under these reaction conditions. Therefore, this small absorption 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 curvature 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 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 $\pm$ 9 s$^{-1}$ (Fig. 2). Thus, the rate constant for C$_2$ binding to FMNH$^-$ is likely to be $\approx 10^7 M^{-1} s^{-1}$ ($k_1$ in Fig. 10).

Therefore, when FMNH$^-$ (16 M) was mixed with various concentrations of C$_2$ in air-saturated buffer in the stopped-flow spectrophotometer, the absorbance increased 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 $k_{obs}$ $\sim$0.7 s$^{-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 $K_d^0$ in the kinetic scheme in Fig. 10) value for the complex was calculated to be $1.2 \pm 0.2 \mu M$.

FIGURE 2. Kinetics of reoxidation of C$_2$-FMNH$^-$ complex. FMNH$^-$ (16 M) plus C$_2$ (25 M) were mixed with buffer containing oxygen (0.13, 0.31, 0.61, and 1.03 mM from the lowest to the upper trace) in the stopped-flow spectrophotometer. All concentrations given are after mixing. The reactions were monitored at 380 and 446 nm (all traces at 446 nm superimposed). The inset shows the plot of $k_{obs}$ of the first phase against oxygen concentrations. The second order rate constant was calculated from the slope of the plot to be $1.1 \pm 0.1 \times 10^6 \text{M}^{-1} \text{s}^{-1}$.

FIGURE 3. Absorbance spectra observed during the reoxidation of C$_2$-FMNH$^-$.

Results from the experiment described in the legend to Fig. 2 monitored at various wavelengths ranging from 310 to 550 nm, were used for plotting the intermediate spectrum. The dashed line (A) represents the beginning C2-FMNH complex, and the solid line (C) represents the spectrum of the final species, oxidized FMN with C$_2$. The intermediate spectrum (B) was obtained by plotting absorbance values occurring at the reaction time of 0.01 s. This is valid because the decomposition of the intermediate is very slow compared with its rate of formation.

FIGURE 4. Determination of the dissociation constant for C$_2$ binding with reduced FMN. FMNH$^-$ (16 M) was mixed with air-saturated buffer containing various concentrations of C$_2$ (0, 2, 6, 10, 16, 20, 30, and 40 M from lower to upper traces). The reactions were monitored at 380 nm, and all concentrations given are after mixing. The inset is a plot of the absorbance change that had occurred at 0.04 s versus the concentration of free C$_2$. The free C$_2$ concentration was calculated by subtraction of the concentration of C(4a)-hydroperoxy-FMN formed from the total amount of C$_2$ present. The $K_d$ for the C$_2$-FMNH$^-$ complex was calculated to be $1.2 \pm 0.2 \mu M$.\[\text{[49x564]/H9262}\]
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FIGURE 5. Reaction of oxygen with the C2-FMNH2-HPA complex. A solution containing FMNH2 (16 μM), C2 (25 μM), and HPA (2 mM) (final concentrations) was mixed with various concentrations of oxygen containing HPA (2 mM) in the stopped-flow spectrophotometer. Reactions were monitored by absorbance at 380 nm. The arrow indicates enzyme absorbance (0.076 AU) before starting the reaction. Absorbance traces with final oxygen concentrations of 0.13, 0.31, 0.61, and 1.03 mM are shown from right to left. The dotted line represents multiphasic exponential fits to the experimental data. The inset shows spectra of flavin intermediates formed in the oxygen reaction of the C2-FMNH2-HPA complex. Traces of reactions monitored at multiple wavelengths from 310–550 nm were used for calculating the spectra of flavin intermediates. The dotted line is a spectrum of the C2-FMNH2-HPA complex, whereas the upper solid line is the spectrum of oxidized FMN at completion of the reaction. Absorbance at 60 ms after the start of the reaction is plotted in the line with filled circles, and this represents predominately the spectrum of C2-C(4a)-hydroperoxy-FMN-HPA complex. Absorbance at the reaction time of 150 ms was plotted in the empty circle line, and this represents predominately the spectrum of C2-C(4a)-hydroxy-FMN. Times were chosen on the basis of the rate constants in the reaction.

The Kf for the binding of oxidized FMN to C2 was determined by an ultrafiltration method described under “Materials and Methods.” This experiment indicated that the Kf 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-FMNH2-HPA Complex with Oxygen—The reaction of C2 in the presence of substrate was investigated by mixing an anaerobic solution of FMNH2 (16 μM), C2 (25 μM), and 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 is bound to the enzyme. This interpretation was later verified (Figs. 7 and 8).

In experiments where the concentration of HPA was varied in double mixing stopped-flow experiments, the rate of formation of C(4a)-hydroperoxy-FMN decreased with increasing concentrations of HPA (data not shown).

Fig. 5 shows that the reaction monitored at 380 nm consists of 4 phases. For example, with 130 mM oxygen (concentration after mixing), the first phase consisted of an increase of absorbance of ~0.026 AU occurring during the dead time, and continuing until ~0.012 s. With the highest oxygen concentration used (1.03 mM), this phase was complete occurring during the dead time, and continuing until the first phase consisted of an increase of absorbance of ~0.079 AU at 380 nm was also dependent on oxygen concentration and was characterized by a second order rate constant of 4.8 ± 0.2 × 104 M⁻¹ s⁻¹ (data not shown). Therefore, the first phase is likely to be the reaction of oxygen with C2-FMNH2 without HPA bound, whereas the second phase is due to the ternary complex C2-FMNH2-HPA reacting with oxygen. The third phase is a lag in absorbance at 380 nm and corresponds to a process with a rate of ~17–22 s⁻¹ (k2 in Fig. 10). This phase can only be resolved clearly in the reaction with 1.03 mM oxygen. The decrease in absorbance because of the fourth phase (0.7–20 s) was coupled with a large increase at 446 nm; this phase was fitted with a rate constant of 0.35 ± 0.02 s⁻¹ that was independent of oxygen concentration.

The absorption spectra of intermediates were calculated from the traces over a range of wavelengths of the reaction with 1.03 mM oxygen using the following rate constants: 54 s⁻¹ for formation of the first intermediate, 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-FMNH2 (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-HPA complex 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-FMNH2-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 FMNH2 (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 collected for analysis. The collected solutions were ultrafiltered using Centricon to remove the enzyme, and the samples were analyzed for DHPA by HPLC methods described previously (6). 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, FMNH2, 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-FMNH2-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

<table>
<thead>
<tr>
<th>Type of reaction</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syringe A / Syringe B</td>
<td>%</td>
</tr>
<tr>
<td>C2-FMNH → HPA / O2</td>
<td>73 ± 4</td>
</tr>
<tr>
<td>C2 + HPA + O2 / FMNH</td>
<td>82 ± 3</td>
</tr>
<tr>
<td>C2-FADH → HPA / O2</td>
<td>74 ± 4</td>
</tr>
<tr>
<td>C2 + HPA + O2 / FADH</td>
<td>68 ± 3</td>
</tr>
</tbody>
</table>

* 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 or FADH. All concentrations are described as after mixing. Under these conditions, the reaction follows Path B in Fig. 10.

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. If such a complex does form, it would be expected that this complex in the presence of O2 would react with FMNH 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 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 (trace B) as well as with mixing C2 in air-saturated solution with FMNH (trace C). These results suggest that C2 alone does not bind effectively to HPA, and that FMNH is the first species binding to the enzyme during catalysis.

**Binding of HPA to C2-FMNH**—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 + HPA 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 prior to reacting with oxygen. The binding kinetics of HPA to C2-FMNH were investigated by double mixing stopped-flow spectrophotometry, where the first mixing added HPA to C2-FMNH under anaerobic conditions to initiate the formation of the C2-FMNH–HPA complex, and after various times of aging, the second mixing 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 (16 μM), and oxygen (1.03 mM). This resulted in ~16 μM C2-FMNH in the solution after double mixing. The reaction was monitored by absorbance at 380 nm to detect formation of C2-C(4a)-hydroperoxy-FMN. The dotted line represents the reaction of C2-FMNH 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.

**FIGURE 6.** Reduced FMN is the first ligand to bind to C2. Trace A is from a reaction in which C2-FMNH–HPA complex (16 μM FMNH, 25 μM C2, and 2 mM HPA final) was mixed with a solution of HPA (2 mM) containing 130 μM oxygen. Trace B is from the reaction in which the C2-FMNH complex was mixed with a solution containing HPA and oxygen. Trace C was obtained from reacting a solution of C2 containing oxygen with a solution of FMNH. Trace D is the result of premixing C2 with HPA in buffer containing oxygen, and then mixing with a solution of FMNH.

**FIGURE 7.** Kinetics of C2-FMNH complex formation with HPA. Formation of the complex was investigated by double mixing stopped-flow spectrophotometry. In the first mix, HPA was added anaerobically to C2-FMNH⁻, and after various age times (0, 0.05, 0.1, 2, 4, 6, 8, and 10 s, upper to lower traces), oxygen was mixed with the aged solution. The final concentrations after double mixing were 16 μM C2-FMNH⁻ (25 μM C2 plus 16 μM FMNH⁻), 2 mM HPA and 1.03 mM oxygen. Reactions were 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⁻ 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⁻.
The Reaction of C₂-C(4a)-hydroperoxy-FMN with HPA—Experiments from the previous section show that hydroxylation can occur via Path A in Fig. 10 where C₂-FMNH⁻ first binds to HPA and then reacts with oxygen to form C₂-C(4a)-hydroperoxy-FMN-HPA or through Path B of Fig. 10, where the enzyme first forms C₂-C(4a)-hydroperoxy-FMN-HPA, more of this intermediate was trapped as the C₂-C(4a)-hydroperoxy-FMN during the first phase, 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 concentrations (Inset B in Fig. 8).

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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 (kᵣ in Fig. 10), whereas the third phase was a small increase in absorbance with a rate constant of ~6–9 s⁻¹ (kᵣ in Fig. 10). The absorbance at 370 nm decreased again in the fourth phase with the kₜᵣ 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 C₂-C(4a)-hydroperoxy-FMN during the first phase, HPA was hydroxylated with the formation of C₂-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 C₂-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 C₂-C(4a)-hydroxy-FMN-HPA species, so that the dehydration to form the oxidized FMN was retarded (Inset B in Fig. 8). Similar trapped C(4a)-hydroxyflavin-substrate species have also been observed in the oxidative half-reactions of several single component flavoprotein oxygenase enzymes (24–26, 34–35).

The intermediate C₂-C(4a)-hydroperoxy-FMN was generated by initially mixing C₂-FMNH⁻ (16 μM) with 0.31 mM oxygen in the double mixing stopped-flow spectrophotometer, and aging for 0.1 s. The resultant intermediate was then mixed with buffer containing various HPA concentrations of 80, 160, 400, 800, 2000, 4000, 8000 μM (lower to upper traces, respectively). Reactions were monitored by absorbance at 370 nm. All concentrations are given as final reaction conditions. The increase of absorbance during the first phase was dependent on HPA concentration and represents binding of HPA to C₂-C(4a)-hydroperoxy-FMN. Inset A shows the plot of kₜᵣ for the first phase versus HPA concentrations. Inset B is a plot of kₒᵣ for the final step, dehydration of C₂-C(4a)-hydroxy-FMN to the oxidized FMN versus HPA concentration.

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, C₂-C(4a)-hydroperoxy-FMN-HPA converted to C₂-C(4a)-hydroxy-FMN-DHPA with a rate of 17–22 s⁻¹ (kᵣ 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 C₂-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 C₂-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.
Path A. Therefore, the kinetic mechanism for the oxygenation reaction stopped-flow instrument with a solution of C2 (80 μM) and HPA, implying that HPA binds to the enzyme to form a complex with FMN was calculated to be 41 s⁻¹. Analyses were also carried out with reaction mixtures in which the second and third substrates, and probably the second and third hydroxylases is its ability to use a variety of reduced flavin substrates. FADH⁻ is as effective as FMNH⁻ (6, 15). Therefore, we tested whether the mechanistic details for oxygenation by C2 with FADH⁻ are similar to those in the reaction of C2 with FMNH⁻. Experiments similar to those described in Figs. 2 and 5 were carried out, but using C2-FADH⁻ instead of C2-FMNH⁻. The reaction of C2-FADH⁻ with oxygen is very similar to the reaction of C2-FMNH⁻. The rate constant was 0.98 ± 0.05 × 10⁶ M⁻¹ s⁻¹ for formation of C2-C(4a)-hydroperoxy-FAD versus 1.1 ± 0.1 × 10⁶ M⁻¹ s⁻¹ for the reaction with FMNH⁻ (Table 2, data not shown). In the presence of HPA, the C2-FADH⁻-HPA complex reacted with oxygen more slowly than in the absence of HPA, similar to the reactions with FMNH⁻. The rate constant for formation of the C2-C(4a)-hydroperoxy-FAD-HPA complex is slightly smaller than that with FMN (3.7 ± 0.2 × 10⁶ M⁻¹ s⁻¹ for FAD versus 4.8 ± 0.2 × 10⁶ M⁻¹ 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⁻ instead of FMNH⁻, yielded results similar to those with FMNH⁻. The Kᵟ values for binding of HPA to C2-FADH⁻ or to C2-C(4a)-hydroperoxy-FAD are both similar to those with FMNH⁻ (shown in Table 2), again emphasizing that FADH⁻ can be used nearly as well as FMNH⁻ by C2 with respect to both specificity and reactivity.

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

**DISCUSSION**

Our studies here have elucidated the detailed kinetic mechanism for the reactions of O₂ with reduced flavin bound to the oxygenase component (C₂) of HPAH from *A. baumannii*. 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 C₂ 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 *p*-hydroxybenzoate hydroxylase (3, 33), phenol hydroxylase (37–39), melittol hydroxylase (40), anthranilate hydroxylase (41), 2-methyl-3-hydroxypropylidine-5-carboxylic acid oxygenase (24–26), and 2-hydroxyphenyl-3-monoxygenase (42). C(4a)-hydroperoxyflavins and C(4a)-hydroxyflavins were also detected in the oxygen reactions of HPAH from *P. putida* (16). The intermediates detected in the reaction of C₂ are spectrally similar to those of the enzymes mentioned. However, the less common feature of C₂ 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 *p*-hydroxybenzoate hydroxylase (43). Partial resolution of spectra similar to those of C₂-C(4a)-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 C₂ with O₂ is similar to the reaction of single

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**TABLE 2**

Kinetic constants for reactions of C₂-FMNH⁻ and C₂-FADH⁻

<table>
<thead>
<tr>
<th>Kinetic constants</th>
<th>C₂-FMNH⁻</th>
<th>C₂-FADH⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>kₚ₀</td>
<td>1.1 ± 0.1 × 10⁶ M⁻¹ s⁻¹</td>
<td>0.98 ± 0.05 × 10⁶ M⁻¹ s⁻¹</td>
</tr>
<tr>
<td>kₚ₁</td>
<td>4.8 ± 0.2 × 10⁴ M⁻¹ s⁻¹</td>
<td>3.7 ± 0.2 × 10⁴ M⁻¹ s⁻¹</td>
</tr>
<tr>
<td>Kₚ₀Hₑ₁₀</td>
<td>180 ± 3 μM</td>
<td>114 ± 3 μM</td>
</tr>
<tr>
<td>Kₚ₁Hₐ</td>
<td>0.35 ± 0.03 mM</td>
<td>0.46 ± 0.04 mM</td>
</tr>
<tr>
<td>kₛ₀</td>
<td>208 ± 4 s⁻¹</td>
<td>256 ± 5 s⁻¹</td>
</tr>
<tr>
<td>kₛ₁</td>
<td>17–22 s⁻¹</td>
<td>17–22 s⁻¹</td>
</tr>
</tbody>
</table>

* The k values are from Fig. 10.
* Values from single-mixing stopped-flow spectrophotometry.
* Values from double-mixing stopped-flow spectrophotometry.
Oxygenase Component of p-Hydroxyphenylacetate 3-Hydroxylase

component aromatic hydroxylases with respect to using C(4a)-hydroperoxyflavin 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⁻ to C₂, followed by the reaction of the C₂-FMN⁻ complex with oxygen to form a quite stable C₂-C(4a)-hydroperoxyflavin. Under conditions of catalytic turnover, an aromatic substrate binds to the preformed C₂-C(4a)-hydroperoxyflavin 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-FMN⁻ 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 oxidized substrate (28), although both C₂ and Lux bind more tightly to the oxidized flavin, the Kᵣ for the Lux-FMN⁻ complex is 80 nM (44), an order of magnitude smaller than that for C₂-FMN⁻ (1.2 μM). 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 monooxygenase (CHMO), where cyclohexanone 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 O₂ 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 HPAH was included in the oxygen-containing solution (1.1 × 10⁸ M⁻¹ s⁻¹) (16). However, as shown above, the rate for formation of the C₂-C(4a)-hydroperoxyflavin decreased from 1.1 × 10⁸ M⁻¹ s⁻¹ to 4.8 × 10⁷ M⁻¹ s⁻¹ when HPAH was pre-bound to the C₂-FMN⁻ 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 HPAH 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 HPAH 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 reduceate regulated by HPAH, 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 HPAH on the formation of the C(4a)-hydroperoxyflavin from P. putida HPAH could be caused by HPAH not binding to the oxygenase until FADH⁻ 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. C₂ was shown in this report to bind FMNH⁻ very rapidly (Fig. 4) with an observed rate of 200 s⁻¹ (compare traces B and C in Fig. 6). Such a rate corresponds to a second order rate constant of at least 10⁷ M⁻¹ s⁻¹, and this binding is quite tight (Kᵣ of 1.2 μM 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⁻ with a Kᵣ of 70 nm, whereas it binds to oxidized FAD with a Kᵣ of 6 μM (45). Recently, a study of actinomycin monooxygenase has shown that the oxygenase component, ActVA, binds to FMNH⁻ with a Kᵣ of 0.4 μM and to oxidized FMN with a Kᵣ of 26 μM (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 monooxygenase (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⁻ or FMNH⁻, although less efficiently with reduced riboflavin (6, 15). Here we report that both C(4a)-hydroperoxy-FAD and C(4a)-hydroxy-FAD accumulated during the reaction of FADH⁻ 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⁻ and FMNH⁻ 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 HPAH from A. baumannii (C₂) comes from the reductase, which binds more specifically and tightly to FMN (6, 20). This property contrasts to most other two-component monooxygenases, where the reductase is often less specific for the flavin whereas the oxygenase is specific for either FMNH⁻ or for FADH⁻.

In conclusion, this study has elucidated the reaction mechanism of the oxygenase component (C₂) 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 C₁) in the initial step, reacts with oxygen to form the C₂-C(4a)-hydroperoxyflavin, 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.

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