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Control of C4a-Hydroperoxyflavin Protonation in the Oxygenase Component of *p*-Hydroxyphenylacetate-3-hydroxylase

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Supporting Information

ABSTRACT: The protonation status of the peroxide moiety in C4a-(hydro)peroxyflavin of *p*-hydroxyphenylacetate-3-hydroxylase can be directly monitored using transient kinetics. The pK_a for the wild-type (WT) enzyme is 9.8 ± 0.2, while the values for the H396N, H396V, and H396A variants are 9.3 ± 0.1, 7.3 ± 0.2, and 7.1 ± 0.2, respectively. The hydroxylation efficiency of these mutants is lower than that of the WT enzyme. Solvent kinetic isotope effect studies indicate that proton transfer is not the rate-limiting step in the formation of C4a-OOH. All data suggest that His396 may act as an instantaneous proton provider for the proton-coupled electron transfer that occurs before the transition state of C4a-OOH formation.

C4a-(Hydro)peroxyflavin is a reactive intermediate that is essential for monooxygenation in flavin-dependent monooxygenases.[1,2] This enzyme family catalyzes a broad range of reactions. The versatility of these enzymes comes from the differences in their active site environments that control the reactivity of the intermediate. For example, the protonation status of the terminal -OOH group is important in controlling whether the intermediate can act as an electrophile in hydroxylation, dehalogenation, halogenation, and epoxidation reactions or as a nucleophile in Baeyer−Villiger oxygenation reactions and in the oxidations of amino acid sulfoxides, selenides, phosphate esters, and organoboron and in light-emitting reactions.[1−5] Understanding how different enzymes regulate the protonation status of C4a-(hydro)peroxyflavin should be useful for engineering them to fit the needs of specific biocatalytic applications in the future.

Formation of C4a-(hydro)peroxyflavin is initiated by the transfer of a single electron from the reduced flavin to oxygen to generate a caged radical pair of flavin semiquinone and superoxide anion.[6−8] Recently, density functional theory (DFT) calculations of pyranose 2-oxidase (P2O), an oxidase that can form C4a-hydroperoxyflavin,[9] indicated that the single-electron transfer process to generate the radical pair is tightly coupled with proton transfer. The proton-coupled electron transfer helps neutralize the negative charge that develops and aligns the resulting $^{\bullet}$OOH radical for formation of the C4a-hydroperoxyflavin adduct[9] (Figure S1 of the Supporting Information). In the P2O reaction, the catalytic residue His548, which is in the vicinity of the flavin C4a position, directly transfers a proton for this process. The findings from the P2O reaction prompted us to investigate the protonation status and the mechanism controlling this process in the reaction of the electrophilic monooxygenase that forms C4a-hydroperoxyflavin as an essential part of catalysis.

p-Hydroxyphenylacetate-3-hydroxylase (HPAH) from *Acinetobacter baumannii* is a flavin-dependent two-component monooxygenase that catalyzes the *ortho* hydroxylation of *p*-hydroxyphenylacetate (HPA). This enzyme consists of a smaller reductase component (C_1) that provides reduced flavin to a larger oxygenase component (C_2).[10−12] Previous studies of C_2 have shown that in the absence of HPA, the C4a-hydroperoxyflavin formed on this enzyme is quite stable,[13,14] presumably because of the H-bond interaction between the flavin N5 and Ser171.[14] Rapid-quench studies and product analysis indicated that the rate constant of hydroxylation is not significantly affected by pH and the hydroxylation ratio is around 90% over a pH range of 6.0−10.0.[15,16] These observations suggest that one or more amino acid residues are likely to be crucial for maintaining the C4a-hydroperoxide in its protonated form over this broad pH range so that it can promote efficient hydroxylation of its substrate (see the C_2 reaction in Figure S2 of the Supporting Information). In addition, the rate of C4a-hydroperoxyflavin formation in C_2 is very fast (~10^6 M^{-1} s^{-1} at 4 °C). Therefore, the active site of C_2 must contain a feature that ensures the effective formation of C4a-hydroperoxyflavin.

On the basis of the crystal structure of C_2, His396 is located at a strategic position (~4.6 Å from the C4a position of the flavin).[17] Previous investigations indicated that His396 is not directly involved with the elimination of H_2O_2 from C4a-hydroperoxyflavin; however, the mechanistic role of this residue is not yet known.[14] We speculated that this residue may be involved in the control of protonation of the -OOH moiety of C4a-hydroperoxyflavin (Figure 1A). In this report, we investigated (i) the protonation status of C4a-hydroperoxyflavin, (ii) the involvement of His396 in controlling the protonation of C4a-hydroperoxyflavin, and (iii) the involvement of a proton transfer process in the formation of C4a-hydroperoxyflavin.

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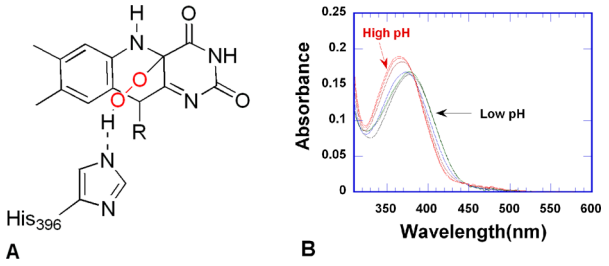

Figure 1. (A) Putative hydrogen bonding between the Nε2 atom of His396 and the -OOH moiety of C4a-hydroperoxyflavin. (B) Spectra of the C4a-(hydro)peroxyflavin of H396N at pH 7, 7.5, 8.2, 9.0, 9.6, 10.0, and 10.4.

Absorption spectra of the flavin intermediate formed in the reactions of wild-type C_2 and the His396 variants (H396N, H396A, and H396V) at various pHs and 4 °C were monitored using stopped-flow spectrophotometry with diode array detection to probe the effect of pH on the spectrum of the intermediate. In general, the λ_{max} of the intermediate shifted to shorter wavelengths at higher pH values (Figure 1B and Figure S3 of the Supporting Information). For wild-type C_2 at pH 7.5, the λ_{max} of the intermediate was 380 nm, while at pH 11.2, it was 370 nm. These results are consistent with the spectral changes being due to protonation and deprotonation of the C4a adduct or the protonation state of nearby residues as in the case of cyclohexanone monooxygenase.[18] For H396N at pH 7, the λ_{max} of the intermediate was 378 nm, while at pH 10.4, it was 365 nm. For H396V at pH 6, the λ_{max} of the intermediate was 380 nm, while at pH 9.8, it was 376 nm. For H396A at pH 6, the λ_{max} of the intermediate was 373 nm, while at pH 9.5, it was 368 nm. The results indicated that C4a-hydroperoxyflavin has λ_{max} values ~10, 13, 4, and 5 nm longer than C4a-peroxyflavin in the reactions of the wild type and H396N, H396V, and H396A variants, respectively. The fact that the C4a adduct was detected at all pHs employed also indicates that C_2 proteins are stable over this pH range.

To investigate whether the shift in the intermediate λ_{max} is indeed due to the deprotonation of C4a-hydroperoxyflavin to form C4a-peroxyflavin, a pH jump experiment to convert the two forms of the intermediate was performed. C4a-Hydroperoxyflavin at low or high pH was generated *in situ* by mixing the reduced enzyme and oxygen in the first mixing of the double-mixing stopped-flow spectrophotometer. The pH of the system was then quickly changed to a different value in the second mixing. The change in absorption characteristics was monitored by diode array and single-wavelength spectrophotometry. This experiment was performed with the H396N mutant because it showed the maximum difference in λ_{max} between the two forms of intermediates (previous paragraph and Figures S3 and S5 of the Supporting Information). The results indicate that when the intermediate that was formed at pH 7.0 (λ_{max} = 378 nm) was mixed with a buffer with a higher ionic strength to change the pH of the system to 10, an intermediate with a λ_{max} of 365 nm was immediately observed. Likewise, when the intermediate preformed at pH 9.5 (λ_{max} = 365 nm) was mixed with a buffer to change the final pH to 7.3, the intermediate with a λ_{max} of 378 nm was immediately observed (Figure S5 of the Supporting Information). This experiment clearly shows that the intermediate with a longer λ_{max} is indeed C4a-hydroperoxyflavin, while the intermediate with the shorter λ_{max} is C4a-peroxyflavin.

A similar shift in λ_{max} corresponding to the conversion of C4a-hydroperoxyflavin to C4a-peroxyflavin was also observed for cyclohexanone monooxygenase,[18] siderophore-associated flavin monooxygenase (SidA),[19] and ornithine hydroxylase (PvdA),[20] but it was different from the shift observed for 3-hydroxybenzoate-6-hydroxylase.[21] The rapid change of intermediate characteristics immediately after the stopped-flow second mixing for the C_2 reaction (Figures S5 and S6 of the Supporting Information) indicates that proton transfer between the two forms of the intermediate is very fast. These data are different from those for cyclohexanone monooxygenase[18] and pyranose 2-oxidase[22] in which the proton equilibration was slow enough that the kinetics of proton transfer could be measured.

The pK_a values of the deprotonation of C4a-hydroperoxyflavin in wild-type, H396N, H396V, and H396A C_2 were thus analyzed by plotting the increase in absorption at 370, 365, 376, and 368 nm versus pH, respectively. Results indicate that the pK_a values for the wild-type enzyme, H396N, H396V, and H396A are 9.8 ± 0.2, 9.3 ± 0.1, 7.3 ± 0.2, and 7.1 ± 0.2, respectively (Figure 2A

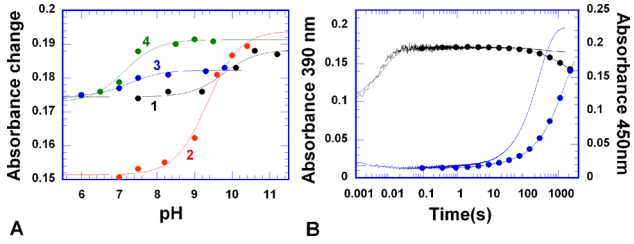

Figure 2. (A) Plots of the change in absorbance vs pH of WT, H396N, H396V, and H396A. The changes in the spectra of intermediates with a change in pH were used to determine the pK_a values of intermediates (Figures S3–S6 of the Supporting Information). Graphs 1–4 are plots of the change in absorbance at 370, 365, 376, and 368 nm vs pH of wild-type, H396N, H396V, and H396A C_2, respectively. (B) Comparison of the reactions of reduced wild-type C_2 with 0.13 mM O_2 in D_2O (filled circles) and H_2O (solid lines) buffers. The values of absorbance at 390 and 450 are colored black and blue, respectively.

and Figures S3–S6 of the Supporting Information). The decrease in the pK_a values of C4a-hydroperoxyflavin observed for the His396 variants, especially in H396A and H396V, where a hydrogen-bonding group is removed, suggests that His396 is important for keeping C4a-hydroperoxyflavin in the protonated form (Figure 1A).

On the basis of the crystal structure of C_2, the distance between the Nε2 atom of His396 and the carbon atom at the C4a position of FMN is ~4.6 Å (Figure S7 of the Supporting Information).[17] His396 may be involved in stabilizing the FMN-C4a-OOH group by acting as a hydrogen bond acceptor (FMN-OOH···Nε2-His396) (Figure 1A). The putative hydrogen bonding interaction between the Nε2 atom of His396 and the -OOH group of C4a-hydroperoxyflavin may be key for maintaining its high pK_a value (9.8 ± 0.2) in the wild-type enzyme. In the H396A and H396V mutants from which a hydrogen bond acceptor was removed, much lower pK_a values (7.1 ± 0.2 and 7.3 ± 0.2) were observed (Figure 2A). For the H396N variant in which the hydrogen bonding interaction can still be maintained, the pK_a value of C4a-OOH is less perturbed (9.3 ± 0.1). Altogether, these data suggest that His396 may act as a hydrogen bond acceptor for the C4a-OOH group. The ability of C_2 to maintain a high pK_a value for the C4a-OOH group is advantageous for catalytic function, as the C4a-OOH group acts as an electrophile in the hydroxylation of HPA.

To explore whether the ability of C_2 to maintain a high pK_a value for C4a-OOH is correlated with the hydroxylation

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efficiency, product analysis of wild-type C_2 and the H396 variants at various pH values was conducted. For wild-type C_2, product formation was ~90% over the pH range of 6−10,[15] while for the His396 variants, product formation significantly decreased at higher pHs (Table S1 of the Supporting Information). The results indicate that His396 is indeed important for maintaining protonation of C4a-OOH, and subsequently the efficiency of hydroxylation.

We used solvent kinetic isotope effects (SKIE) to probe whether the proton transfer occurs prior to or is a part of the transition state for the formation of C4a-OOH. If the proton transfer occurs before the transition state for C4a-OOH formation (proton-coupled electron transfer to form a cage radical pair) as in P2O,[9,23,24] no SKIE should be observed. The kinetic traces indicated that the reactions in D_2O are biphasic, similar to those in H_2O (Figure 2B and Figure S8 of the Supporting Information). The increase in absorbance at 390 nm (black) over the first 0.02 s is due to formation of the C4a adduct.[13] There is no measurable difference in the rate constants in H_2O and D_2O, with both yielding second-order rate constants of $\sim 1 \times 10^6\ M^{-1}\ s^{-1}$. However, the formation of oxidized FMN as shown in traces recorded at 450 nm (blue) shows a SKIE of ~4 with rate constants of $7.7 \times 10^{-4}\ s^{-1}$ (in D_2O) and $0.003\ s^{-1}$ (in H_2O).[15] A significant value observed for the SKIE for the hydrogen peroxide elimination step implies that breakage of the N5−H bond in C4a-hydroperoxyflavin plays a significant role in the H_2O_2 elimination process similar to the reaction of P2O[23] and SidA.[25]

The lack of a SKIE for the C4a-hydroperoxyflavin formation step indicates that the proton transfer process is rapid and is not the rate-limiting step for C4a-hydroperoxyflavin formation, similar to the results observed for P2O and SidA.[19,24] According to the DFT analysis of P2O, this phenomenon is due to the prompt donation of a proton from His548 (in P2O) that is ~4.5 Å from position C4a of flavin[26] before the transition state for formation of the C4a intermediate. The results in Figure 2B indicate that His396 may function like His548 in P2O by donating a proton for the proton-coupled electron transfer process that occurs before the transition state (Figure S9 of the Supporting Information). DFT analysis of the C_2 reaction should allow the future confirmation of this hypothesis.

In summary, our findings indicate that His396 is important for maintaining the protonation of C4a-OOH by acting as a hydrogen bond acceptor. A high pK_a of C4a-hydroperoxyflavin is required for efficient hydroxylation of HPA. Proton transfer is not the rate-limiting step for the process of C4a-hydroperoxyflavin formation, possibly because the concerted proton-coupled electron transfer occurs prior to the transition state for formation of the C4a-OOH.

■ ASSOCIATED CONTENT

Supporting Information

Experimental methods, stopped-flow kinetic traces, and SKIE results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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