Reaction of 2-Methyl-3-hydroxypyridine-5-carboxylic acid (MHPC) Oxygenase with *N*-Methyl-5-hydroxynicotinic acid: Studies on the Mode of Binding, and Protonation Status of the Substrate†

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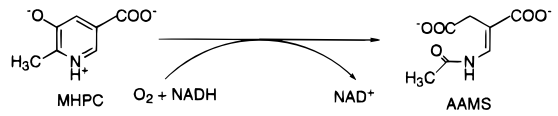
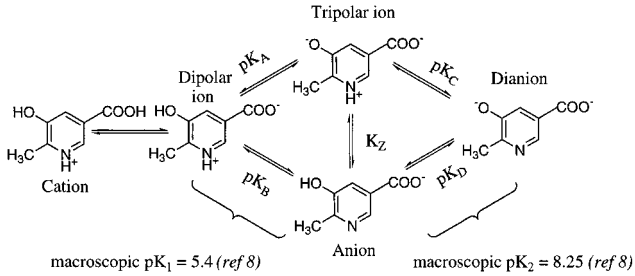
ABSTRACT: Titrations of 2-methyl-3-hydroxypyridine-5-carboxylic acid (MHPC) oxygenase with the substrate MHPC identified the MHPC species bound to the enzyme as the tripolar ionic species. This result was supported by studies of the binding to the enzyme of *N*-methyl-5-hydroxynicotinic acid (NMHN), an MHPC analog existing only in the tripolar ionic form. The K_d is 55 μM compared to a K_d of 9.2 μM for MHPC and 5.2 μM for 5-hydroxynicotinic acid. Kinetics studies of the binding of NMHN to MHPC oxygenase show that its binding, like that for MHPC and for 5HN, is also a two-step process. Since NMHN never exists as an anionic form, neither of the observed steps is due to the binding of an anionic species as an intermediate step. Investigations of the reduction and oxygenation half reactions demonstrate that the mechanism of catalysis with NMHN is basically the same as with MHPC or with 5-hydroxynicotinic acid. Product analysis from reactions using NMHN, a compound that possesses positive charge on the nitrogen atom, indicates that the product of NMHN is an aliphatic compound, similar to the products derived from MHPC and from another substrate analog, 5-hydroxynicotinic acid. These results indicate that the nitrogen atom of the substrate is invariably protonated during the catalytic reaction.

2-Methyl-3-hydroxypyridine-5-carboxylic acid (MHPC)[1] oxygenase (MHPCO) (EC 1.14.12.4) is an FAD-containing enzyme involved in the degradation of vitamin B_6 (pyridoxine) by the soil bacterium, *Pseudomonas* sp. MA-1 (P-MA1) (*1*). Degradation of vitamin B_6 proceeds via an oxidative pathway that is induced when these bacteria are grown on pyridoxine or pyridoxamine as their sole source of carbon and nitrogen (*2*−*3*). The pathway consists of a series of oxidative, hydrolytic, and decarboxylation reactions that convert pyridoxine to metabolites readily assimilated for growth. MHPCO catalyzes an oxygenation reaction and a ring cleavage of its substrate, MHPC, to yield α-(*N*-acetylaminomethylene)succinic acid (AAMS), as shown in Scheme 1 (*1*).

Scheme 1



Scheme 2



Although the reaction catalyzed by MHPCO is formally a dioxygenation, MHPCO belongs to the external aromatic flavoprotein monooxygenase (or aromatic flavoprotein hydroxylase) class (*4*−*5*). The reductive half-reaction (*4*) shows roles of the aromatic substrate and pyridine nucleotide in the reaction mechanism similar to those found with conventional aromatic flavoprotein hydroxylases, and the oxidative half-reaction (*5*) involves C(4a)-hydroperoxyflavin and C(4a)-hydroxyflavin intermediates, which are common to all other aromatic flavoprotein hydroxylases. The reaction with a substrate analog, 5-hydroxynicotinic acid, also shows that the oxygenation reaction of MHPCO consists of two parts: a hydroxylation and a subsequent ring cleavage reaction (*5*). Recently, the enzyme was cloned and expressed in *Escherichia coli* (*6*). Sequence comparison of MHPCO with other aromatic flavoprotein hydroxylases reveals significant homology to enzymes in this class (*6*).

Similar to vitamin B_6 and related 3-hydroxypyridine derivatives (*7*), MHPC can exist in solution at various pH values in five forms: cation, dipolar ion, tripolar ion, anion, and dianion (Scheme 2). In aqueous solution at pH 7.0, where most studies of MHPCO have been carried out (*4*−*8*), MHPC exists primarily in a tautomeric equilibrium between two ionic forms: the anion and the tripolar ion.

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[1] Abbreviations: MHPC, 2-methyl-3-hydroxypyridine-5-carboxylic acid; MHPCO, 2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase; NMHN, *N*-methyl-5-hydroxynicotinic acid; 5HN, 5-hydroxynicotinic acid; AAMS, α-(*N*-acetylaminomethylene)succinic acid; FMMS, α-(*N*-formyl-*N*-methyl-aminomethylene) succinic acid; FAMS, α-(*N*-formylaminomethylene)succinic acid; NADH, β-nicotinamide adenine dinucleotide reduced form; NAD^+, β-nicotinamide adenine dinucleotide oxidized form; FAD, flavin adenine dinucleotide; E_{ox} and E_{red}, oxidized and reduced forms of MHPC oxygenase; DTT, dithiothreitol; E_I (intermediate I), C-(4a)-hydroperoxy-flavin enzyme; E_{III} (intermediate III), C-(4a)-hydroxy-flavin enzyme; PHBH, *p*-hydroxybenzoate hydroxylase; pOHB, *p*-hydroxybenzoate.

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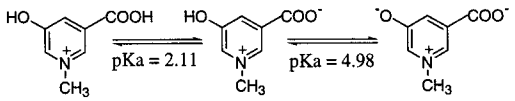
Scheme 3



Due to the specific environment of the enzyme active site, the enzyme is likely to preferentially bind to only one form of MHPC. The studies reported here show that the tripolar ionic form of MHPC binds to MHPCO.

N-Methyl-5-hydroxynicotinic acid (NMHN), an MHPC analog existing in only the tripolar ionic form at pH 7.0 (Scheme 3), was used in this binding study to substantiate the results with MHPC. Because NMHN is an MHPC analog that is always positively charged at the nitrogen atom, NMHN was also used to clarify the protonation status of substrate during catalysis. The mechanism of the reaction of NMHN was studied in detail and compared to the reactions of MHPC.

MATERIALS AND METHODS

Reagents. MHPC and 5HN were synthesized as previously described in ref 1. NAD^+, NADH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were from Sigma. 5HN methyl ester was from TCI. The concentrations of the following compounds were determined using known extinction coefficients at pH 7.0: NADH, $\epsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$ (*9*); MHPC, $\epsilon_{326} = 4.4\ mM^{-1}\ cm^{-1}$ (*10*). 5HN has an extinction coefficient of $4.19\ mM^{-1}\ cm^{-1}$ (0.1 N NaOH) at 315 nm (*4*). NMHN has an extinction coefficient of $5.34\ mM^{-1}\ cm^{-1}$ at 330 nm (0.1 M sodium phosphate, pH 8.0). MHPCO used in this study was the cloned MHPCO and was prepared as previously described (*6*). The concentration of the purified enzyme was measured using the molar absorption coefficient of $13\ 110\ M^{-1}\ cm^{-1}$ at 454 nm/enzyme-bound FAD (*8*).

NMHN. 5-Hydroxynicotinic acid methyl ester (3 g) was dissolved in a mixture of benzene (120 mL) and methanol (71 mL). Methyl iodide (50 mL) was added slowly to this solution. The reaction mixture was heated to 50 °C and was kept at this temperature for 5 h. It was then evaporated to about 30 mL when most of the *N*-methyl-5-hydroxynicotinic acid methyl ester crystallized from the solution. Recrystallization in hot benzene-methanol solution resulted in brilliant yellow crystals (yield 53%). The compound has a melting point of 172−175 °C and an $R_f = 0.46$ on silica-C_{18} thin-layer chromatography developed with methanol:water (40:60). The λ_{max} values (50 mM sodium phosphate, pH 7.0) were at 338 and 273 nm. ^{1}H NMR spectrum (d_6-DMSO): δ 3.96 (3H, s, $-COCH_3$), 4.36 (3H, s, $-NCH_3$), 8.20 (1H, s, ArH), 8.75 (1H, s, ArH), 9.04 (1H, s, ArH). This methylation procedure was adapted from ref 11, where it was shown to be an effective method for methylation of pyridine nitrogen, since there is no significant methylation of the phenolic group.

N-Methyl-5-hydroxynicotinic acid (NMHN) was obtained by acid hydrolysis of *N*-methyl-5-hydroxynicotinic acid methyl ester. Complete conversion to NMHN was attained after 4.5 h of gentle refluxing in 1 N HCl. The reaction mixture was then evaporated to dryness to expel the excess HCl. The residue was twice redissolved in 20 mL of water and evaporated to dryness in order to get rid of any remaining HCl. The residue was then washed with chloroform to remove a brown-colored contaminant and dried (yield 49%). The compound has an $R_f = 0.74$ on silica-C_{18} thin-layer chromatography developed with methanol:water (3:97). NMHN has a melting point of 270−271 °C (dec), and has λ_{max} (50 mM sodium phosphate, pH 7.0) at 330 and 264 nm. ^{1}H NMR spectrum (d_6-DMSO): δ 4.33 (3H, s, $-NCH_3$), 8.41 (1H, s, ArH), 8.77 (1H, s, ArH), 8.96 (1H, s, ArH).

Spectroscopic Studies. UV−visible absorbance spectra were recorded with a Hewlett-Packard diode array spectrophotometer (HP 8452A), a Cary model 219 double-beam spectrophotometer, or a Cary 3E spectrophotometer. All spectrophotometers were equipped with thermostated cell compartments. ^{1}H NMR spectra were recorded with a Bruker Avance DPX300, 300 MHz NMR instrument. Chemical shift values are reported in parts per million relative to tetramethylsilane.

Determination of MHPC Species Bound to MHPCO. The experiment was done in a two-sectored cell (Yankeloff cell), with a path length of 0.9 cm (0.45 + 0.45 cm). MHPCO (1 mL of 15 μM) was placed in one sector each of the sample and reference cells, and 1 mL of buffer was placed into the other sector of each cell. In the sample cell, when MHPC was added, the enzyme and buffer solutions in both sectors were mixed together. In the reference cell, the MHPC solution was added to the buffer side, and an equal volume of buffer was added to the enzyme side. The difference spectrum observed is mainly that of bound MHPC and of some of the free MHPC species. The spectrum of bound MHPC was obtained by correcting for the free species using the known K_d for MHPC.

Determination of NMHN pK_a. Spectra of NMHN (122 μM) were recorded in various buffers at different pH values. The range of pH was from 0.5 to 11, with increments of about 0.25 pH unit. The following buffers were employed: hydrochloric acid/potassium chloride, pH 0.50−2.50; sodium formate, pH 2.75−4.00; sodium acetate, pH 4.25−5.50; sodium phosphate pH 5.75−8.25; sodium carbonate, pH 8.50−12.00.

Rapid Reaction Experiments. Rapid kinetics procedures followed those described in ref 4. Enzyme and substrate solutions were placed in glass tonometers fitted with a cuvette that permitted absorbance spectra to be recorded in preparation for stopped-flow experiments. Enzyme samples were made anaerobic by equilibration with oxygen-free argon as described previously in ref 4. In studies of the oxidative half-reaction, enzyme was anaerobically reduced with an NADH regenerating system [NAD^+ (0.7 μM), glucose-6-phosphate (3 mM), glucose-6-phosphate dehydrogenase (1 unit/mL)]. This reducing system reduced the FAD of MHPCO sufficiently slowly such that rereduction did not interfere with the studies of the oxidative half-reaction. A practical range of oxygen concentrations of about 60−1000 μM was achieved by equilibration of buffer solutions with certified nitrogen and oxygen gas mixtures (Matheson). Dissolved oxygen concentration in buffer solution was calculated according to equations derived in ref 12. The highest concentration of oxygen was obtained by equilibration of buffer solutions at 0 °C with 100% oxygen (1.9 mM).

Preparation of the Product of NMHN and MHPCO Reaction. Preparation for structural identification of the product of the reaction of NMHN and MHPCO was carried out as follows. MHPCO (45 nmol), NMHN (48 μmol), and NADH (45 μmol) were slowly stirred into 10 mL of 0.1 M

ammonium bicarbonate buffer, pH 7.0, at room temperature. The reaction was monitored spectrophotometrically to observe consumption of NADH and NMHN and was complete in about 3 h. After completion of the reaction, the enzyme was removed immediately by ultrafiltration with a Centriprep-30 concentrator (Amicon). The filtrate was applied to a Sep-Pak C_{18} Vac cartridge (35 cc) (Waters) prewashed with water and methanol. This cartridge was then eluted with water. The product of the NMHN reaction eluted within 26 mL, while NAD^+ and NADH required further addition of water to elute. Fractions containing product from the reaction of NMHN were pooled and loaded onto a DEAE-Sepharose (fast flow) column (1.8 × 16 cm) prewashed with 300 mL of 1 M ammonium bicarbonate and then equilibrated in water. The column was washed with about 100 mL of water and 500 mL of a gradient of 0 to 0.2 M ammonium bicarbonate buffer. The NMHN product eluted at about 35−40% of the gradient. Fractions containing product were pooled and freeze-dried until all water was removed.

Steady State Kinetics of MHPCO and NMHN. MHPCO was assayed by monitoring the consumption of NADH and NMHN at 340 nm with $\Delta\epsilon_{340} = 10.43\ mM^{-1}\ cm^{-1}$ ($\Delta\epsilon_{340}$ of NADH = 6.22; $\Delta\epsilon_{340}$ of NMHN = 4.21 $mM^{-1}\ cm^{-1}$). Initial rate measurements were carried out at 4 °C, 50 mM sodium phosphate buffer, pH 7.0, using a stopped-flow spectrophotometer (Kinetic Instruments Inc.), similarly to the method described in ref 13. One reaction syringe of the stopped-flow spectrophotometer contained an anaerobic solution of MHPCO (3.4 μM). The other reaction syringe contained appropriate concentrations of NADH, NMHN, and oxygen. The concentrations of oxygen in the substrate syringe were established by equilibration of the solution with standard oxygen/nitrogen mixtures. At least four to five assays were performed at each concentration of substrate.

RESULTS

Determination of the Absorption Spectra of Anionic and Tripolar Ionic MHPC. It has previously been demonstrated that dioxane-water mixtures can be used to study the tautomeric equilibrium of vitamin B_6 and related 3-hydroxypyridine derivatives (*7*). We have used this principle to study the equilibria of anionic and tripolar ionic species of MHPC (Figure 1). The addition of dioxane to solutions of MHPC shifts the equilibrium toward the less polar tautomer, a species with an absorbance maximum at 296 nm. These results are consistent with a process in which the tripolar ionic species is converted to the anionic species (*7*). The absorbance spectrum of the tripolar ionic form can be approximated by estimating the tautomeric ratio of tripolar ionic and anionic forms in aqueous solution and subtracting the contribution of the anionic species from the total spectrum. Assuming that the anionic and tripolar ionic forms of MHPC exist in tautomeric equilibrium with respective molar fractions of 0.3 and 0.7 in 100% aqueous buffer,[2] the spectrum of the tripolar ionic species of MHPC was

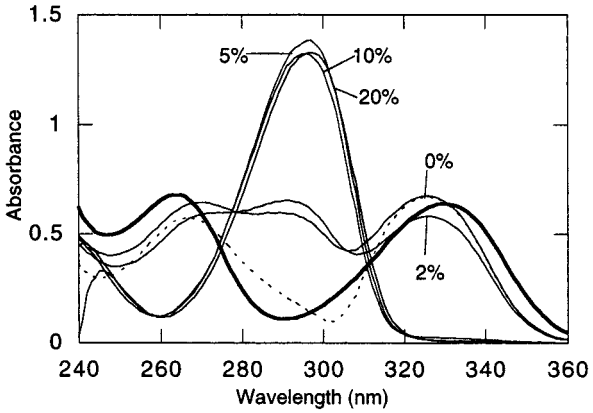


FIGURE 1: Absorbance spectra of MHPC in water-dioxane mixtures. Spectra of MHPC (158 μM) were recorded in 0, 2, 5, 10, and 20% dioxane in buffer (50 mM sodium phosphate, pH 7.0). Dioxane perturbs the tautomeric equilibrium by shifting the equilibrium in favor of the anionic form of MHPC. The spectrum (dashed line) representing that of the tripolar ionic form of MHPC was obtained by subtracting the spectral contribution of the anionic species. It was assumed that the tautomeric fractions at pH 7.0 were 0.7 for the tripolar ion and 0.3 for the anion so that the derived spectrum is of 111 μM. The spectrum of MHPC in 10% dioxane was assumed to be that of the anionic form. The spectrum of NMHN (122 μM), the model compound that exists only in tripolar ionic form, is shown at pH 7.0 in the bold line.

calculated and is shown in Figure 1. The derived spectrum corresponds closely to those for similar compounds determined previously (*7*).

Assuming the molar fractions of the tripolar ionic and anionic forms of MHPC (above), then $K_z = 2.33$ (K_z = the ratio of tripolar ion form to anion form). It was shown (*7*, *14*) that

$$K_1 = K_A + K_B \quad (1)$$

$$1/K_2 = 1/K_C + 1/K_D \quad (2)$$

$$K_z = K_A/K_B = K_C/K_D \quad (3)$$

[See the ionic species associated with the dissociation constants (K_{A-B}) in Scheme 2.] Using eqs 1−3, and the values of $pK_1 = 5.4$, $pK_2 = 8.25$, and $K_z = 2.33$, the values $pK_A = 5.56$, $pK_B = 5.92$, $pK_C = 8.09$, and $pK_D = 7.73$ were obtained.

Determination of the MHPC Species Bound in the MHPCO−MHPC Complex. Difference spectra obtained by titrating MHPCO with MHPC were used to identify the nature of the MHPC species present in the enzyme−substrate complex. The changes in absorbance in the near UV region (230−350 nm) are mainly associated with spectral contributions of the bound and unbound forms of MHPC. By using these titration results and the K_d at 25 °C for MHPCO−MHPC, which is 19 μM, we constructed the spectrum of the MHPC species bound to the oxidized enzyme (Figure 2).

Determination of NMHN pK_a. The spectrum of NMHN changes markedly with pH, similarly to those of 3-hydroxypyridine and vitamin B_6 derivatives (*7*, *15*). The spectra show clearly isosbestic points in the pH regions of both 0.5−3.5 and 4−8, a strong indication that mainly one dissociation step occurs in each of these regions, making it possible to calculate the ionization constants (pK_a) directly from the spectrophotometric data (*7*). No further spectral changes

[2] Several molar fraction ratios were used to estimate this tautomeric equilibrium. When molar fractions of the anionic form ≥0.4 were employed, the derived MHPC spectrum has negative absorbance near 300 nm. When molar fractions of the anionic form ≤0.2 are used, the derived tripolar ionic MHPC spectrum has a peak at about 290 nm. NMHN, a model for the tripolar ionic tautomer (Figure 1), shows that no peak near 290 nm should be present.

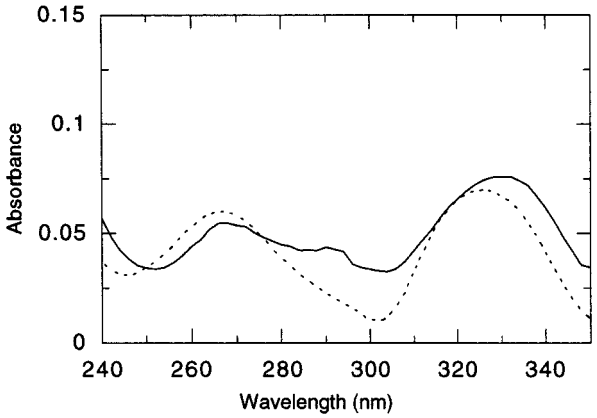


FIGURE 2: The absorbance spectrum of ionic species of MHPC in the MHPCO−MHPC complex. Difference titration of MHPCO with MHPC was done in 50 mM sodium phosphate buffer, pH 7.0, 0.3 mM EDTA, and 1 mM DTT, at 25 °C in a two-sectored cell (details described in Materials and Methods). The spectra in the sample cell represent spectra of the enzyme−substrate complex while the spectra in the reference cell represent the sum of spectra of free enzyme and of free MHPC. Therefore, the difference spectrum observed is mainly that of bound MHPC. The absorption spectrum of enzyme-bound MHPC was obtained and shown as the solid line. The spectrum of tripolar ionic MHPC (from Figure 1) is shown as a dashed line.

were observed up to pH 11.0, implying that there is no significant contamination with the unmethylated 5-hydroxynicotinic acid.

At pH 0.5−3.5, the NMHN absorption maximum changes from 300 to 296 nm with an isosbestic point at 301 nm. This change is associated with the titration of the carboxylic acid of NMHN (Scheme 3). A pK_a value of 2.11 ± 0.04 was obtained by plotting the absorbance at 278 and 308 nm as a function of pH.

During the titration from pH 4.0 to 11.0, the spectrum of NMHN changes from a single peak at 296 nm (ϵ = 5.4 mM^{-1} cm^{-1}) to a spectrum with peaks at 264 nm (ϵ = 5.7 mM^{-1} cm^{-1}) and 330 nm (ϵ = 5.3 mM^{-1} cm^{-1}). This is due to the deprotonation of the β-hydroxy group of NMHN (Scheme 3). From the plot of absorbance at 260, 296, 301, and 330 nm as a function of pH, a pK_a value of 4.98 ± 0.02 was obtained for the β-hydroxy group of NMHN.

Results of this pK_a determination demonstrate that NMHN exists only in the tripolar ionic form at pH 7.0 and above. The spectrum of tripolar ionic NMHN closely resembles the spectrum of tripolar ionic MHPC derived from the dioxane-water experiments (Figure 1), confirming that the derived spectrum of tripolar ionic MHPC obtained is reasonable.

Binding of NMHN to MHPCO. NMHN is a substrate analog that exists solely in the tripolar ionic form at pH 7.0 (see above). Therefore, binding of NMHN to MHPCO would provide further evidence that MHPCO binds the tripolar ionic form of MHPC. Titration of E_{ox} with NMHN results in spectral perturbations in the 360−520 nm region with five isosbestic points, where the flavin chromophore absorbs (data are not shown). These spectral changes reflect the change in the flavin environment upon NMHN binding. A K_d of 55 ± 6 μM for binding of NMHN to E_{ox} was obtained by using the changes in absorbance at 382, 410, 440, and 492 nm. The K_d value of NMHN is somewhat higher than those of MHPC (9.2 ± 0.6 μM) and 5HN (5.2 ± 0.4 μM) (*4*).

Kinetics of Binding of NMHN to MHPCO. Previous studies of the binding of MHPC and of the substrate analog

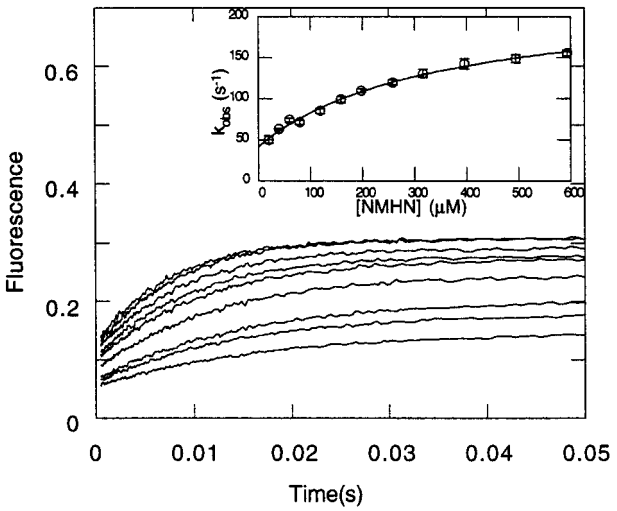


FIGURE 3: Kinetics of binding of NMHN to MHPCO. MHPCO (16 μM) was mixed with different concentrations of NMHN in 50 mM sodium phosphate, 0.3 mM EDTA, and 1 mM DTT, 4 °C. The reaction was monitored by stopped-flow fluorimetry with an excitation wavelength of 470 nm and emission at wavelengths greater than 515 nm. Binding of NMHN results in increased fluorescence of enzyme-bound flavin. The inset to the figure shows the graphical determination of the kinetic parameters for the binding of NMHN to the enzyme. Observed rate constants (k_{obs}) of the fluorescence change when NMHN was mixed with the enzyme are plotted versus NMHN concentration (after mixing).

Scheme 4

$$E_{ox} + NMHN \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_{ox}{\bullet}NMHN^* \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_{ox}{\bullet}NMHN$$

5HN to MHPCO have shown that substrate binding is a two-step equilibrium process in which an enzyme−substrate complex is initially formed, followed by an isomerization reaction (*4*). Since the enzyme binds only to the tripolar ionic form of MHPC at equilibrium, it was thought that E_{ox}−$MHPC_{(anion)}$ might be an intermediate preceding the formation of E_{ox}−$MHPC_{(tripolar\ ion)}$. If the second step of the binding process of MHPC is the conversion of E_{ox}−$MHPC_{(anion)}$ to E_{ox}−$MHPC_{(tripolar\ ion)}$, the binding of the tripolar NMHN should result in a one-step binding process.

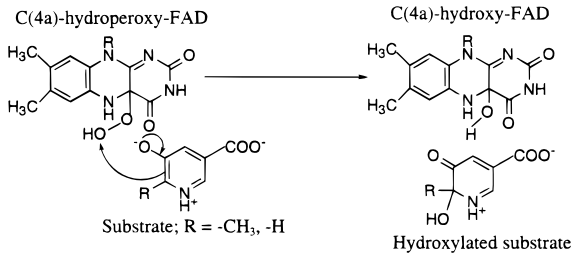
Kinetic traces for binding of NMHN to E_{ox} are shown in Figure 3. A solution of uncomplexed MHPCO was mixed with solutions containing various concentrations of NMHN, and the reaction was observed by stopped-flow fluorimetry. The binding of NMHN to E_{ox} results in an increase of flavin fluorescence and the observed rate contant for the formation of the E_{ox}−NMHN complex approaches a limiting value at high concentrations of NMHN (inset of Figure 3). This indicates that this binding process of NMHN is also a two-step equilibrium process as in the MHPC case (Scheme 4).

Data were fit to eq 4, which describes a two-step binding reaction (*16*), by using the Levenberg−Marquardt nonlinear fitting algorithm that is included in the KaleidaGraph software. This analysis method gives k_3 = 184 ± 14 s^{-1}, k_4 = 45 ± 2 s^{-1}, k_2/k_1 (microscopic K_d for primary binding) = 384 ± 69 μM; k_{obs} = the observed rate constant.

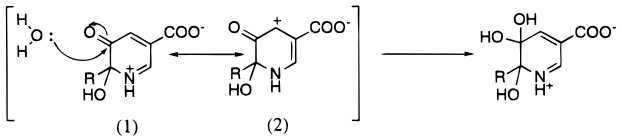
$$k_{obs} = \frac{k_3[NMHN]}{[NMHN] + (k_2/k_1)} + k_4 \qquad (4)$$

The overall K_d (74 μM) calculated from the above kinetic parameters (*4*) is in reasonable agreement with K_d value from static titration (55 μM).

Scheme 5



Scheme 6



Identification of the Product of the Reaction of NMHN and MHPCO. MHPCO is a flavoprotein that has two activities: hydroxylation of the substrate and subsequent ring-cleavage of the nascent product (*5*). The hydroxylation involves catalysis by the flavin cofactor and is thought to occur as shown in Scheme 5, while the ring-cleavage reaction is not likely to involve the enzyme-bound flavin. The first step of the ring cleavage reaction may involve an attacking water molecule as in Scheme 6 (*5*).

If the nitrogen atom of compound 1 is protonated, compound 1, in principle, can exist in another resonance form (compound 2). This results in an electron deficient carbon atom next to the carbonyl group, which makes the carbonyl group more susceptible to the attacking water molecule. Therefore, protonation of the nitrogen atom could be the important factor governing the ring breakage reaction. We tested the importance of the positive charge on nitrogen by using NMHN, which is a compound with a nitrogen that is always positively charged. If the product of the reaction of MHPCO and NMHN is a ring-cleaved compound similar to the product of the normal substrate, it implies that the nitrogen atom of the regular substrate is also protonated throughout the reaction cycle.

The NMHN product was isolated as described in Materials and Methods. It has an absorption maximum at 254 nm that is similar to that of the product of MHPC (*1*) and to that of the product of 5HN (*5*). The ^{1}H NMR spectrum of the NMHN product in D_2O showed a composite spectrum of two compounds in approximately 3:1 ratio (Figure 4). The major compound has ^{1}H NMR peaks at δ (in ppm) 2.97 (3H, s, $-CH_3$), 3.27 (2H, s, $-CH_2-$), 7.17 (1H, s, $=CH-$), 8.17 (1H, s, $-HCO$). The ^{13}C NMR spectrum (decoupled) has peaks at δ (in ppm) 31.36, 34.29, 121.88, 137.75, 160.67, 166.37, 177.03. The minor compound has ^{1}H NMR peaks at δ (in ppm) 3.09 (3H, s, $-CH_3$), 3.18 (2H, s, $-CH_2-$), 7.08 (1H, s, $=CH-$), 7.96 (1H, s, $-HCO$). The ^{13}C NMR spectrum of the minor compound cannot be accurately assigned due to the low signal intensity.

The above NMR results can be assigned to the two configurations (a and b) of α-(*N*-formyl-*N*-methyl-aminomethylene)succinic acid (FMMS) (Figure 4). Thus, the methyl and methine hydrogens each have two different environments with respect to the carbonyl. This assignment is based on the experiment showing that *N*,*N*-dimethylformamide has two observable configurations due to the restricted rotation of the C−N bond (*17*) so that rotation is

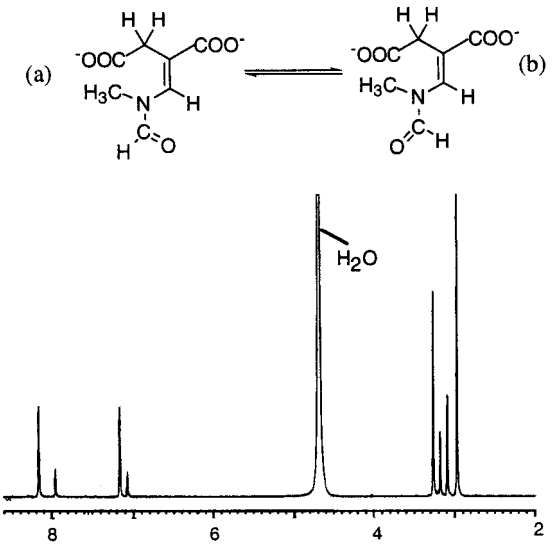


FIGURE 4: NMR spectrum of product of MHPCO and NMHN reaction. The ^{1}H NMR spectrum of NMHN product shows a mixture of spectra of the isomers of α-(*N*-formyl-*N*-methylaminomethylene)succinic acid.

slower than the NMR time scale. The NMR spectrum of FMMS is similar to two compounds with similar structures to FMMS: α-(*N*-formylaminomethylene)succinic acid (*5*) and 2-(*N*-formylamino)-2-pentenedioic acid (*18*). The mass spectrum of the NMHN product shows a molecular mass of 187, confirming that the compound is indeed FMMS.

This result shows that the product of the reaction of MHPCO with NMHN is a ring-cleaved compound similar to that from the reaction with MHPC or 5HN, implying that the nitrogen atom of any of the substrates (see Scheme 6) is invariably protonated throughout the ring cleavage reaction.

Reduction of E_{ox}−NMHN Complex. Kinetics for the reduction of E_{ox}−NMHN were examined by absorbance stopped-flow spectrophotometry under anaerobic conditions (data not shown). No transient long-wavelength absorbing species were observed during the reduction of the enzyme. Therefore, the kinetic data do not indicate the existence of either flavin semiquinones or pyridine nucleotide−enzyme charge-transfer complexes during the reduction.

Reduction monitored at 450 nm shows an initial lag period followed by the hydride transfer phase, which has a large change in absorbance. At high concentrations of NMHN, the observed rate of the hydride transfer phase approached a limiting value of 32 ± 1.5 s^{-1}. This can be compared with the slow second-order rate constant in the absence of substrate (4.1 M^{-1} s^{-1}). For example, at 2 mM NADH, the E_{ox}−NMHN complex is reduced by NADH at a rate of about 30 s^{-1} (i.e., essentially the limiting rate), while the reduction of free E_{ox} is about 8×10^{-3} s^{-1}. Indeed, this stimulated reduction rate constant for E_{ox}−NMHN (32 s^{-1}) is higher than when either MHPC (12.7 s^{-1}) or 5HN (10.0 s^{-1}) is used as a substrate (*4*).

Reaction of the Reduced Enzyme−NMHN Complex with Oxygen. The reduced enzyme−NMHN complex was mixed with oxygenated buffer in the stopped-flow spectrophotometer, and the reaction was monitored at multiple wavelengths between 350−500 nm (Figure 5A). This reoxidation of the enzyme consists of two phases, similar to the reaction of MHPCO with MHPC or with 5HN (*5*). The first phase is characterized by a large increase in absorbance at 370 and

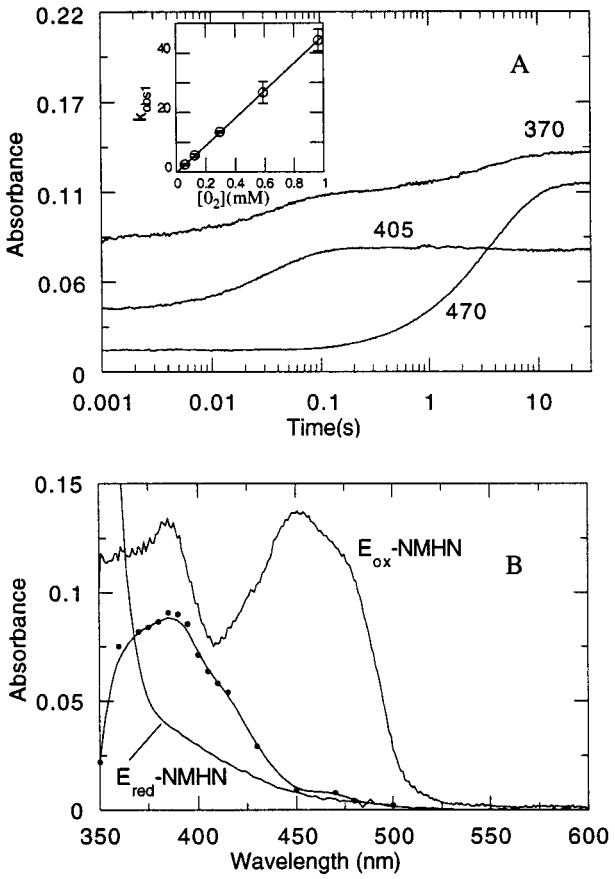


FIGURE 5: Reoxidation of reduced enzyme−NMHN complex. Reduced MHPCO (11.4 μM) was reacted in the stopped-flow spectrophotometer with oxygen (588 μM) in buffer containing NMHN (500 μM). (A) The reaction was followed by its absorbance at 370, 405, and at 470 nm. The experiments were performed in 95 mM sodium phosphate buffer, pH 7.0, 0.3 mM EDTA, 1 mM DTT at 4 °C. The inset figure shows the dependence on oxygen concentration of the observed rate contant for the formation of the first intermediate. (B) Calculated spectrum of the transient intermediate present during reoxidation of the reduced MHPCO−NMHN complex. The intermediate spectrum (filled circles) has a λ_{max} at 385 nm with an extinction coefficient of 9080 M^{-1} cm^{-1}. For reference, the spectra of the reduced enzyme−NMHN complex (E_{red}−NMHN) and of the final enzyme species after reoxidation (E_{ox}−NMHN) are shown.

Scheme 7

$$E_{red}\text{-NMHN} + O_2 \xrightarrow[k_I]{4.6 \times 10^4\ M^{-1}\ s^{-1}} E_I\text{-NMHN} \xrightarrow[k_{II}]{0.27\ s^{-1}} E_{ox} + \text{FMMS}$$

405 nm and a lag phase at 470 nm. This phase is dependent on oxygen concentration (inset of Figure 5A) with a second-order rate constant of 4.6 ± 0.2 × 10^4 M^{-1} s^{-1}, while the second phase (the increase in absorbance at 370 and 470 and small decrease in absorbance at 405 nm) is independent of oxygen concentration (0.27 ± 0.12 s^{-1}).

An absorbance spectrum of the intermediate was calculated (Figure 5B) as previously described (*5*, *23*) from data recorded at several wavelengths using 588 μM oxygen, and assuming a two-step irreversible mechanism (Scheme 7) for the reoxidation [k_I = 27 s^{-1} (4.6 × 10^4 M^{-1} s^{-1} × 588 μM), k_{II} = 0.27 s^{-1}]. The intermediate spectrum has an absorption maximum at 385 nm (ϵ_{385} = 9.1 mM^{-1} cm^{-1}) and is assigned as a C(4a)-hydroperoxy-FAD enzyme (*19*, *20*). This species was also observed in the reaction of MHPCO with MHPC and with 5HN (*5*) and in the oxygen reaction with all known flavin-dependent monooxygenases (*21*−*31*).

Scheme 7 describes the reoxidation of the E_{red}−NMHN complex as investigated by rapid kinetic techniques. In this reaction scheme, oxygen reacts with the reduced enzyme−NMHN complex to form the C(4a)-hydroperoxy-FAD enzyme (E_I−NMHN). Oxygen transfer from the transient intermediate to NMHN yields oxidized enzyme and the oxygenated product, FMMS. No C(4a)-hydroxy-FAD enzyme can be observed, presummably because the rate constant for dehydration is larger than that for its formation.

Reoxidation of the Reduced Enzyme−NMHN Complex in Presence of Azide. Similar to the study of MHPCO with MHPC and with 5HN (*5*), sodium azide was included in the reoxidation reaction for better kinetic resolution of the intermediates. It is known that, for most flavoprotein hydroxylases, the presence of monovalent anions (such as azide) affects the rate constants of the oxygenation reaction so that the intermediates can be more fully distinguished. When 50 mM sodium azide was included in the reaction mixture, the return to oxidized enzyme was resolved into three phases. The first phase in the reoxidation process results in increases in absorbance at 370 and 410 nm and a lag period at 470 nm (Figure 6A). This phase is dependent on oxygen concentration (inset of Figure 6A) with a rate constant of 5.0 ± 0.5 × 10^4 M^{-1} s^{-1}. The second kinetic phase of the reoxidation is associated with a decrease in absorbance at 410 nm and an increase in absorbance at 370 and 470 nm with a rate constant of 0.42 ± 0.15 s^{-1}. The third phase leads to oxidized flavin and displays increases in absorbance at 370, 410, and 470 nm with a rate constant of 0.16 ± 0.04 s^{-1}.

The absolute absorbance spectra of the two transient enzyme intermediates were calculated (Figure 6B) from data recorded at several wavelengths using 588 μM oxygen and assuming a three-step irreversible mechanism for the reoxidation [k_I = 27.4 s^{-1} (5.0 × 10^4 M^{-1} s^{-1} × 588 μM), k_{II} = 0.42 s^{-1}, k_{III} = 0.16 s^{-1}]. The first intermediate (filled circles) has an absorption maximum at 390 nm (ϵ_{390} = 9.4 mM^{-1} cm^{-1}), and the second intermediate (empty circles) has λ_{max} at 370 nm (ϵ_{370} = 11 mM^{-1} cm^{-1}) as well as some absorbance in the 440−490 nm range. The intermediate spectra were assigned by analogy to the reaction of MHPCO with MHPC and with 5HN (*5*) and to the reaction of flavoprotein hydroxylases (*21*−*31*). The first intermediate is assigned as the C(4a)-hydroperoxy-FAD enzyme. The second intermediate is a summation of the spectra of C(4a)-hydroxy-FAD enzyme−FMMS (E_{III}−FMMS; λ_{max} = 370 nm) and about 30% of oxidized enzyme (440−490 nm region). This result suggests that the C(4a)-hydroperoxy-FAD enzyme converts to two products. Seventy percent leads to hydroxylation as it converts to the C(4a)-hydroxy-FAD, while 30% undergoes elimination of H_2O_2 (uncoupling pathway) and leads to oxidized flavin. Thus, rate constants for the formation of E_{III}−FMMS and the uncoupling pathway can be calculated from the rate of disappearance of E_I−NMHN (0.42 s^{-1}) to be 0.29 and 0.13 s^{-1}, respectively (Scheme 8). It should be mentioned that, in the normal reaction without azide, about 12% uncoupling is observed.

Steady State Kinetics of MHPCO and NMHN. Equation 5 is a general equation for describing the steady state kinetics of MHPCO (*4*, *5*, *32*, *33*).

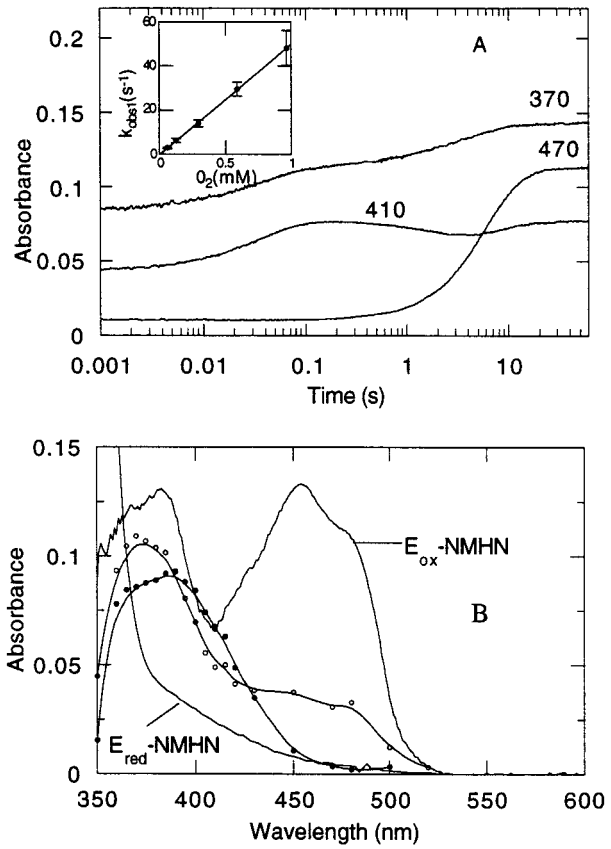
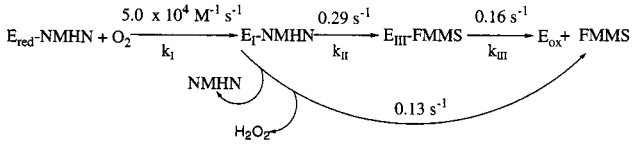


FIGURE 6: Reoxidation of reduced enzyme−NMHN complex in presence of azide. Reduced MHPCO (11.4 μM) was reacted with oxygen (588 μM) in buffer solution containing NMHN (500 μM), and 50 mM sodium azide in the stopped-flow spectrophotometer. (A) The reaction was followed by its absorbance at 370, 410, and at 470 nm. As in Figure 6, the experiments were performed in 95 mM sodium phosphate buffer, pH 7.0, 0.3 mM EDTA, and 1 mM DTT at 4 °C. The inset figure shows the dependence on oxygen concentration of the observed rate constant for the formation of the first intermediate. (B) Spectra of the transient intermediates formed during reoxidation of the reduced MHPCO−NMHN complex in the presence of azide. The reaction was monitored at several wavelengths in the stopped-flow apparatus. Reduced enzyme−NMHN complex (E_{red}−NMHN), first intermediate (filled circle), second intermediate (empty circles), oxidized enzyme−NMHN complex (E_{ox}−NMHN).

Scheme 8



$$e/v = \phi_o + \phi_{NMHN}/[NMHN] + \phi_{NADH}/[NADH] + \phi_{oxygen}/[oxygen] + \phi_{NMHN-NADH}/[NMHN][NADH] \quad (5)$$

The steady state parameters, k_{cat} and K_m values for NMHN, NADH, and O_2, were derived from the values of $1/\phi_o$, ϕ_{NMHN}/ϕ_o, ϕ_{NADH}/ϕ_o, and ϕ_{oxygen}/ϕ_o, respectively. The values of ϕ_o, ϕ_{NMHN}, ϕ_{NADH}, and ϕ_{oxygen} were evaluated according to the graphical method (*33*). The values for K_m and k_{cat} from steady state experiments were compared to values derived from stopped-flow data and are listed in Table 1. Values from both experiments are in reasonable agreement. Since k_{cat} (0.27 s^{-1} from stopped-flow data, 0.26 s^{-1} from steady state data) is the same value as the rate constant of the hydroxylating step (Scheme 7), the conversion of C(4a)-hydroperoxy-FAD to C(4a)-hydroxy-FAD enzyme is the rate-limiting step in the reaction.

Table 1: Kinetic Constants in the Reaction of MHPCO with NMHN

kinetic constants	from steady-state data	from stopped-flow data
k_{cat}	0.26 ± 0.1 s^{-1}	0.27 s^{-1} [b]
K_m (NMHN)	1.1 ± 0.6 μM [a]	0.56 μM [c]
K_m (NADH)	5.4 ± 3 μM [a]	*d*
K_m (O_2)	11.7 ± 5 μM [a]	5.9 μM [e]

[a] These K_m values should be considered only as estimations. Since K_m values are rather low, the lowest practical substrate concentrations that could be used were greater than 3-fold the estimated K_m values. [b] ϕ_o (s) = $1/k_{hydroxylation} + 1/k_{hydride\ transfer} + 1/k_3$ (see Scheme 4) = 1/0.27 s^{-1} + 1/33 s^{-1} + 1/183 s^{-1}. [c] ϕ_{NMHN} (μM s) = $k_2/k_1k_3 + 1/k_1$ (see Scheme 4) = 384 μM/184 s^{-1} + (≈0). [d] ϕ_{NADH} cannot be accurately derived since, by analogy to MHPC case, there may be more than one step of NADH binding (*4*). [e] ϕ_{oxygen} (μM s) = $1/k_{formation\ of\ C(4a)-hydroperoxy\ flavin}$ = 1/0.046 μM^{-1} s^{-1}.

DISCUSSION

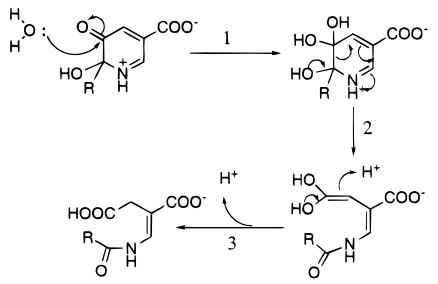
Spectral titrations of MHPC to MHPCO indicate that MHPCO binds to a single ionic form of MHPC identified as the tripolar ionic form. This result is substantiated by the finding that NMHN, an MHPC analog that exists only in the tripolar ionic form at neutral pH, readily binds to the enzyme. The kinetics studies indicate that, like the binding of MHPC, the binding of NMHN to MHPCO is a two-step process. Since NMHN cannot exist in the anionic form, these results imply that the intermediate is not the anionic form. The MHPC derivative 3-*O*-methyl-MHPC (*34*) that, by analogy to vitamin B_6 and 3-hydroxypyridine derivatives (*7*, *35*), exists in the anionic form was tested with MHPCO. The compound was found to be neither a substrate nor an inhibitor to the enzyme,[3] indicating that 3-*O*-methyl-MHPC does not bind to MHPCO and implying that MHPCO does not bind the anionic form of the substrate.

MHPCO is an enzyme with a high specificity for its substrate. It has been shown that only compounds with nicotinic acid structures are inhibitors (*1*). The enzyme clearly requires the positive charge of the nitrogen atom and the negative charge of the carboxylate group for binding. Considering that compounds without the 3-hydroxy group have only small inhibition effects, implying weak binding, and the fact that MHPC binds the tripolar form of MHPC, it is clear that the phenolate must also be an important determinant in binding.

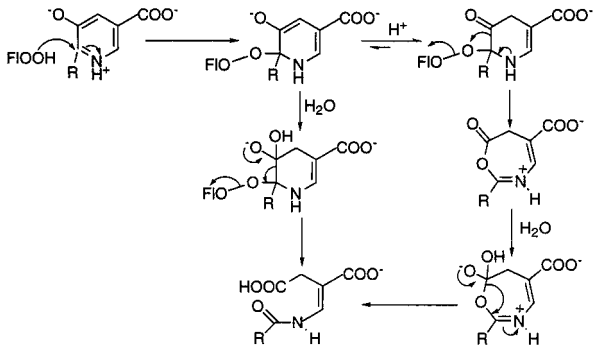
The binding of the tripolar ionic form of MHPC is also considered important in activating the substrate for an electrophilic aromatic substitution reaction (Scheme 5) of substrate with C(4a)-hydroperoxyflavin (*21*). It can be visualized that MHPCO selectively binds to the tripolar ionic, rather than to the anionic form (Scheme 2), in order to have the substrate already in the activated form to undergo the electrophilic aromatic substitution typical of flavoprotein hydroxylases. Specific groups in the enzyme active site may be responsible for holding the substrate in the proper ionic form. The reactivity of the substrate is an important factor for aromatic flavoprotein hydroxylase reactions (*22*). In the *p*-hydroxybenzoate hydroxylase (PHBH) reaction, although the substrate *p*-hydroxybenzoate (pOHB) exists mostly in

[3] Chaiyen, P., Ballou, D. P., & Massey, V., unpublished results.

Scheme 9



Scheme 10



the phenolic form at neutral pH in solution, it was shown to be in the phenolate form when bound to the enzyme (*36*). It thus appears that PHBH activates its substrate by lowering the phenolic pK_a (*36, 37*). Site-directed mutagenesis that changes tyrosine 201, a residue responsible for the lowering the pK_a of pOHB, to phenylalanine nearly abolishes the ability of the enzyme to hydroxylate the substrate (*36*).

It should be noted, however, that ionization of the phenol group of substrates is not a general property for all flavoprotein hydroxylases. Anthranilate hydroxylase, the enzyme catalyzing the hydroxylation and deamination of anthranilate, is unlikely to have a preference on the protonation state of its substrate, since anthranilate does not contain any phenolic group (*38*). Melilotate hydroxylase (*28*) and phenol hydroxylase (*29*) also bind their substrates in the phenolic form.

The formation of the product FMMS from the reaction of MHPCO and NMHN emphasizes the role of enzyme for controlling the chemical reaction in the catalysis. NMHN is a good substrate for MHPCO, inferring that the nitrogen atoms of substrates are protonated throughout the ring-cleavage reaction. This protonation of the nitrogen atom may be the important factor favoring the hydration step (the first step of the ring-breaking reaction). We propose the mechanism of the ring breaking reaction shown in Scheme 9. The formation of FMMS from NMHN also strongly supports the conclusion that the hydroxylation and the ring cleavage reactions are both enzymatic activities (*5*). MHPCO converts compounds with 5-hydroxynicotinic acid structures to aliphatic products. This argument has proven to be valid with three substrates: MHPC, 5HN, and NMHN.

Comparison of the steady state kinetics data with rate constants of individual steps indicates that the rate-limiting step of the MHPCO reaction with NMHN is the rate of hydroxylation, implying that the rate for the ring cleavage reaction is much faster than the hydroxylation. It is advantageous that the ring cleavage reaction is fast compared to hydroxylation. This assures that with MHPC the hydroxylation product will be cleaved as indicated in Scheme 9, step 2, rather than dissociate from the enzyme in the cyclic form. With 5HN and NMHN, which are not methylated at the 2-position, the rapid cleavage reaction prevents rearomatization of the initial hydroxylated species (Scheme 9).

Scheme 5 shows the hydroxylation as a "classical" aromatic mechanism involving nucleophilic attack of the ionized aromatic compound on the electrophilic flavin hydroperoxide. However, an interesting idea, suggested by a reviewer, is that the flavin hydroperoxide is the nucleophile and attacks the electrophilic substrate. This is based on the finding that the substrate is protonated throughout the reaction and therefore should be quite electrophilic, as are other pyridinium ions. The mechanism proposed (Scheme 10) involves nucleophilic attack on the pyridinium 2-position. The peroxide intermediate then undergoes a Baeyer−Villiger (*39*) rearrangement analogous to that thought to occur with cyclohexanone monooxygenase (*40, 41*). This mechanism is also consistent with the $H_2{}^{18}O$ labeling in the carboxylate and the $^{18}O_2$ labeling in the carbonyl as previously reported (*5*) and with the observation of the flavin hydroperoxide and flavin hydroxide intermediates.

Table 2

species	$E_{(HOMO)}$ (eV)[a]	$E_{(LUMO)}$ (eV)[a]
5-HN	−4.771	2.933
NMHN	−4.690	2.901
C(4a)-hydroperoxy flavin	−8.755	−1.000

[a] Energy for HOMO and LUMO [$E_{(HOMO)}$ and $E_{(LUMO)}$] were calculated by using Mopac/AM1 software.

It is difficult with the present evidence to distinguish which of these two mechanisms is correct. However, several points argue in favor of the flavin hydroperoxide acting as an electrophile rather than as a nucleophile. (1) sequence comparisons (*6*) show that MHPCO has significant homology with enzymes in the aromatic flavoprotein class, which have been shown to react as electrophilic flavin hydroperoxides (*21*−*23*, *28*−*30*). Sequences for enzymes with nucleophilic hydroperoxides including luciferase (*42*) and cyclohexanone monooxygenase (*43*) do not show significant homologies. (2) The tripolar ionic MHPC species shown in this work to be the form of the substrate in the enzymatic reaction has a net charge of −1, which could make it a better nucleophile than are typical pyridinium compounds. (3) Calculation of HOMO and LUMO energies for the flavin hydroperoxide and for the substrates shows that the most favorable reaction pair is the electrophilic hydroperoxide reacting with the nucleophilic substrate.[4] From the values in Table 2, it is clear that for either 5HN or NMHN the ΔE calculated using the HOMO of the substrate and the LUMO of the C(4a)-hydroperoxyflavin is much smaller than the ΔE calculated using the HOMO of the C(4a)-hydroperoxy flavin and the LUMO of the substrate. This implies that the substrate is the nucleophile (*44*). It might be noted that if the substrate were not to have the 5-carboxylate group, with the nitrogen protonated the HOMO would be −8.15, while the LUMO would be −0.5, so that neither pair of partners would have favorable interaction energies. (4) MHPCO shows many properties (order of substrate binding, stimulation of reduction on binding of substrate, and oxidative half-reaction) that are similar to those of the aromatic hydroxylases rather than to those of the nucleophilic monooxygenases (*21*).

[4] Palfey, B. A., unpublished results.

Studies of the binding, the reductive, and the oxidative reactions demonstrate that the enzyme uses the same reaction mechanism to catalyze the NMHN reaction as it does in the reactions with MHPC and with 5HN. Binding of NMHN appears to be a two-step process with a somewhat higher K_d than those for MHPC and 5HN (*4*). The higher K_d values for NMHN may be due to the bulkiness of the methyl group at the nitrogen atom making NMHN difficult to fit in the catalytic pocket. Reduction of MHPCO by NADH was somewhat more stimulated by binding of NMHN than by MHPC or 5HN (*4*). The reoxidation reaction for MHPCO with NMHN as a substrate also shows the same transient intermediates [C(4a)-hydroperoxyflavin and C(4a)-hydroxyflavin] as does the reaction with the regular substrate (*5*).

In conclusion, this work shows that MHPCO binds to the tripolar ionic form of its natural substrate and the nitrogen atom of the substrate remains protonated throughout the catalytic reaction. NMHN is a good substrate analog for MHPCO, and the reaction mechanism appears to be the same as the reaction of MHPCO with the regular substrate, MHPC.

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