Use of 8-Substituted-FAD Analogues To Investigate the Hydroxylation Mechanism of the Flavoprotein 2-Methyl-3-hydroxypyridine-5-carboxylic Acid Oxygenase[†,‡]

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ABSTRACT: 2-Methyl-3-hydroxypyridine-5-carboxylic acid (MHPC) oxygenase (MHPCO) is a flavoprotein that catalyzes the oxygenation of MHPC to form α-(*N*-acetylaminomethylene)-succinic acid. Although formally similar to the oxygenation reactions catalyzed by phenol hydroxylases, MHPCO catalyzes the oxygenation of a pyridyl derivative rather than a simple phenol. Therefore, in this study, the mechanism of the reaction was investigated by replacing the natural cofactor FAD with FAD analogues having various substituents (−Cl, −CN, $-NH_2$, $-OCH_3$) at the C8-position of the isoalloxazine. Thermodynamic and catalytic properties of the reconstituted enzyme were investigated and found to be similar to those of the native enzyme, validating that these FAD analogues are reasonable to be used as mechanistic probes. Dissociation constants for the binding of MHPC or the substrate analogue 5-hydroxynicotinate (5HN) to the reconstituted enzymes indicate that the reconstituted enzymes bind well with ligands. Redox potential values of the reconstituted enzymes were measured and found to be more positive than the values of free FAD analogues, which correlated well with the electronic effects of the 8-substituents. Studies of the reductive half-reaction of MHPCO have shown that the rates of flavin reduction by NADH could be described as a parabolic relationship with the redox potential values of the reconstituted enzymes, which is consistent with the Marcus electron transfer theory. Studies of the oxidative half-reaction of MHPCO revealed that the rate of hydroxylation depended upon the different analogues employed. The rate constants for the hydroxylation step correlated with the calculated pK_a values of the 8-substituted C(4a)-hydroxyflavin intermediates, which are the leaving groups in the oxygen transfer step. It was observed that the rates of hydroxylation were greater when the pK_a values of C(4a)-hydroxyflavins were lower. Although these results are not as dramatic as those from analogous studies with parahydroxybenzoate hydroxylase (Ortiz-Maldonado et al., (1999) *Biochemistry 38,* 8124−8137), they are consistent with the model that the oxygenation reaction of MHPCO occurs via an electrophilic aromatic substitution mechanism analogous to the mechanisms for parahydroxybenzoate and phenol hydroxylases.

2-Methyl-3-hydroxypyridine-5-carboxylic acid (MHPC)[1] oxygenase (MHPCO) is involved in the degradation of vitamin B_6 by the soil bacterium *Pseudomonas* MA-1 (*1*). MHPCO catalyzes an oxygenation and a ring-cleavage reaction of its substrate, MHPC, to yield α-(*N*-acetylaminomethylene)-succinic acid (AAMS) (Scheme 1). Previous studies have shown that MHPCO belongs to the aromatic flavoprotein hydroxylase class of enzymes (*2*−*5*). In addition to hydroxylation, however, MHPCO carries out the hydrolysis and cleavage of the product ring. Hydroxylation involves the FAD cofactor, while the ring-breaking reaction, which takes place after hydroxylation, is apparently catalyzed by protein residues (*3*). This enzyme and 5-pyridoxic acid oxygenase (*6*) are the only two enzymes thus far reported that catalyze ring-cleavage reactions in aromatic compounds without the use of metal cofactor (*3*, *5*). Recently, a protein sequence with 97% identity with MHPCO was reported in the genome of *Mesorhizobium loti* (*7*), and the protein sequence of an enzyme in the degradation pathway of quinoline was reported to have 36% identity with MHPCO (*8*).

There are a wide variety of enzymes in the class of aromatic flavoprotein hydroxylases that catalyze the monooxygenation of various aromatic substrates (*9*, *10*). These enzymes include several that have been studied in detail:

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[1] Abbreviations: MHPC, 2-methyl-3-hydroxypyridine-5-carboxylic acid; MHPCO, 2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase; 5HN, 5-hydroxynicotinic acid; $FADH_2$, reduced flavin adenine dinucleotide; FADHOOH, C(4a)-hydroperoxyflavin; FADHOH, C(4a)-hydroxyflavin; $FADHO^-$, deprotonated form of C(4a)-hydroxyflavin; 8-X-FAD, FAD with substituents at position C8 of isoalloxazine ring; 8-CN-FAD, FAD with cyano group replacing the 8-methyl group of the isoalloxazine; 8-Cl-FAD, FAD with chlorine replacing the 8-methyl group of isoalloxazine; 8-OCH_3-FAD, FAD with methoxy group replacing the 8-methyl group of the isoalloxazine; 8-NH_2-FAD, FAD with amino group replacing the 8-methyl group of the isoalloxazine; 8-X-MHPCO, MHPCO reconstituted with the corresponding FAD analogues; pOHB, *p*-hydroxybenzoate; PHBH, *p*-hydroxybenzoate hydroxylase; PCA, protocatechuic acid; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital.

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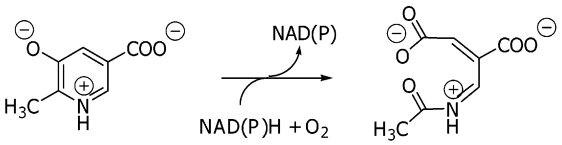
Scheme 1



p-hydroxybenzoate hydroxylase (PHBH) (*11*), phenol hydroxylase (*12*), salicylate hydroxylase (*13*), anthranilate hydroxylase (*14*), melilotate hydroxylase (*15*), hydroquinone hydroxylase (*16*), 2-hydroxybiphenyl-3-monooxygenase (*17*), and MHPCO. Recently, a flavin-dependent aromatic monooxygenation was reported to be carried out by a two-protein enzyme system, pyrrole-2-carboxylate monooxygenase (*18*), and three types of flavin-dependent *p*-hydroxyphenylacetate hydroxylases have been described that require two proteins (*19*−*21*). Note that the substrate of MHPCO is a derivative of a pyridine and is not a phenol like the other aromatic flavoprotein hydroxylases just mentioned. In addition to the flavin monooxygenases with aromatic substrates, there are related flavin-containing monooxygenases, such as cyclohexanone monooxygenase, that oxygenate electrophilic rather than electron-rich substrates (*22*, *23*) and the eukaryotic flavin-containing monooxygenase that oxygenates a wide variety of amines, thiols, and other compounds (*24*). Given the variety of substrates and reactions involved, it is conceivable that some of the mechanisms of oxygenation may also be substantially different within the flavoprotein oxygenase family.

Flavoproteins are particularly amenable to detailed mechanistic studies because the flavin, which is intimately involved in the reactions catalyzed, has distinct spectral properties in each of the states involved. Stopped-flow kinetic studies of the flavoprotein hydroxylases have been very important in elucidating the mechanisms and spectrally identifying intermediates in the reactions. Thus, C(4a)-hydroperoxyflavin (FADHOOH) has been shown to be the hydroxylating reagent for aromatic as well as other substrates (*9*, *10*), while the other intermediate, C(4a)-hydroxyflavin (FADHOH), is not an oxygenating species. The mechanism of how the oxygen atom is transferred from the FADHOOH intermediate to the aromatic substrate has been the subject of debate for more than 2 decades. FADHOOH has been proposed to transfer an oxygen atom by three different mechanisms: electrophilic aromatic substitution, radical formation, and a flavin ring-opening reaction (*10*). It has been shown that in the reaction of PHBH the mechanism involves electrophilic substitution for hydroxylations of aromatic substrates (*25*−*27*). By contrast, cyclohexanone monooxygenase is thought to utilize a nucleophilic substitution mechanism (*22*, *23*). With PHBH, it was possible to replace the FAD with a series of 8-substituted FADs, and the individual steps of the hydroxylation reaction could be monitored by stopped-flow techniques. Quantitative structure−activity relationship (QSAR) analysis of the results demonstrated that the monooxygenation catalyzed by PHBH occurred by electrophilic aromatic substitution (*26*). Because the MHPC substrate and the overall reaction of MHPCO appear to be somewhat different from those of the other monooxygenases in this class, it was decided to determine whether the oxygenation of nicotinate-related compounds by MHPCO also occurs by electrophilic aromatic substitution.

In this paper, we present studies of the reaction mechanism of MHPCO with the use of FAD-analogues substituted at the 8-position of the isoalloxazine. The thermodynamic and kinetic properties of the modified enzymes and of the native enzyme are compared to gain insight into the details of the catalytic mechanism.

MATERIALS AND METHODS

Unless stated otherwise, studies were carried out in 50 mM phosphate, pH 7.0, at 4 °C.

Reagents. MHPC and 5HN were synthesized as previously described (*2*). NAD, NADH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and protocatechuic acid (PCA) were from Sigma. 8-Cl-riboflavin was obtained from Dr. J. P. Lamboy, University of Maryland, 8-CN-riboflavin was from Dr. Y. Murthy, University of Michigan, and 8-NH_2-riboflavin was from Dr. S. Ghisla, University of Konstanz (Germany). Conversions of riboflavin analogues to FAD analogues were carried out using the FAD synthetase from *Brevibacterium ammoniagenes* as previously described (*28*). The concentrations of the following compounds were determined using known extinction coefficients at pH 7.0: NADH, $\epsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$; MHPC, $\epsilon_{326} = 4.4\ mM^{-1}\ cm^{-1}$ (*29*); 5-hydroxynicotinic acid (5HN), $\epsilon_{315} = 4.19\ mM^{-1}\ cm^{-1}$ (0.1 N NaOH) (*3*). MHPCO used in this study was cloned, expressed, and prepared as previously described (*4*). The concentration of the purified enzyme was measured using $\epsilon_{452} = 13\ 110\ M^{-1}\ cm^{-1}$ per enzyme-bound FAD (*30*).

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 spectral instruments were equipped with thermostated cell compartments.

Rapid Reaction Experiments. The reactions were carried out in 50 mM sodium phosphate buffer, pH 7.0, at 4 °C, unless otherwise specified. Rapid kinetic measurements were performed with Hi-Tech Scientific model SF-61DX or model SF-61SX stopped-flow spectrophotometers in single mixing mode. The optical path lengths of the observation cells are 1 cm. The stopped-flow apparatus was made anaerobic by flushing the flow system with an anaerobic buffer solution consisting of 0.1 U/mL of protocatechuic acid dioxygenase and 400 μM protocatechuic acid at pH ~7 (*31*). This solution was allowed to stand in the flow system overnight. The flow unit was then thoroughly rinsed with anaerobic buffer before experiments. Enzyme and substrate solutions were placed in glass tonometers and made anaerobic by equilibration with oxygen-free argon that has been passed through an Oxyclear oxygen removal column (Labclear). 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 U/mL)). This reducing system reduced the FAD or FAD analogues of MHPCO sufficiently slowly that rereduction did not interfere with the studies of the oxidative half-reaction. A practical range of oxygen concentrations of about 60 to 1000 μM was achieved by equilibration of buffer solutions with certified nitrogen and oxygen gas mixtures. The highest concentration of oxygen was obtained by

equilibration of buffer solutions at 0 °C with 100% oxygen (1.9 mM before mixing in the stopped-flow instrument). Apparent rate constants from kinetic traces were calculated from exponential fits using software KinetAsyst3 (Hi-Tech Scientific, Salisbury, U.K.) or program A (written at the University of Michigan by Rong Chang, Jung-yen Chiu, Joel Dinverno, and D. P. Ballou). Determinations of maximal rate constants were calculated from plots of k_{obs} versus NADH using a Marquardt−Levenberg nonlinear fit algorithm that is included in the KaleidaGraph software (Synergy Software, Reading, PA). For studies of the reoxidation of 8-Cl-MHPCO, data were collected in the diode-array mode, and the global analysis software Specfit (R. A. Binstead) was used for data analysis using the built-in singular value decomposition methods.

Redox Potential Determinations. Redox potentials of the enzyme-bound FAD analogues, in the presence or absence of MHPC or 5HN, in 50 mM sodium phosphate buffer, pH 7.0, at 25 °C were determined by the method described by Massey (*32*). Xanthine and xanthine oxidase were used to catalytically reduce the enzyme with benzyl viologen as an electron mediator, and dyes with $E_m^{\circ\prime}$ values within 30 mV of the measured enzyme were used as a reference. The following redox potential values ($E^{\circ\prime}$) of reference dyes were used for 8-X-MHPCO: 2,6-dichlorophenol indophenol (219 mV) or *N*-methylphenazine methosulfate (80 mV) for ligand-bound 8-CN-MHPCO, phenazine ethosulfate (55 mV) for $E_l^{\circ\prime}$for ligand-free 8-CN-MHPCO, methylene blue (11 mV) for $E_m^{\circ\prime}$ of ligand-bound 8-Cl-MHPCO, indigo tetrasulfonate (−46 mV) for $E_l^{\circ\prime}$of ligand-free 8-Cl-MHPCO, navy blue (−210 mV) for $E_m^{\circ\prime}$ of ligand-bound and ligand-free 8-OCH_3-MHPCO, flavin mononucleotide (FMN, −219 mV) for ligand-bound 8-NH_2-MHPCO, and phenosafranine (−252 mV) for ligand-free 8-NH_2-MHPCO.

Apoenzyme Preparation and FAD Analogue Reconstitution. FAD was removed from the enzyme by precipitation with ammonium sulfate at pH 3.0 (*30*). All working buffers during the preparations contained 0.3 mM EDTA, 1 mM DTT, and 200 μM 5-hydroxynicotinate (5HN). The enzyme in 50 mM MOPS buffer, pH 7.0, was mixed with saturated ammonium sulfate solution (pH 3.0) with enzyme-to-ammonium sulfate solution ratio of 1:8 (v/v). The mixing process was carried out in a salt−ice bath and time of exposure of the enzyme to the acid ammonium sulfate was minimized. The suspension was centrifuged at 17 000 × *g* for 4 min, and the yellow supernatant was discarded. The pellet was resuspended in saturated ammonium sulfate solution (pH 8.0) and centrifuged at 17 000 × *g* for 4 min. The pellet, which contained apoenzyme, was resuspended in 30 mM MOPS buffer, pH 7.0, and the procedure for precipitation with ammonium sulfate at pH 3.0 was repeated for two additional cycles to convert all of the holoenzyme to the apoenzyme. Measurements by enzyme assays or spectrophotometric properties showed that the resultant apoenzyme contained less than 0.1% of the original FAD.

The excess ammonium sulfate was separated from the apoenzyme by passing through a Sephadex G-25 column. For reconstitution with high redox potential flavin analogues (8-Cl-FAD and 8-CN-FAD), the column was equilibrated with 50 mM sodium phosphate buffer, pH 7.0, in the absence of DTT. The solutions were shielded from room light to minimize unwanted photoreduction of the enzyme. An excess of flavin analogue (∼1.5-fold) was added to the desalted apoenzyme, and the mixture was passed through a Sephadex G-25 column. The reconstituted enzyme was stored at −80 °C in a freezer until used.

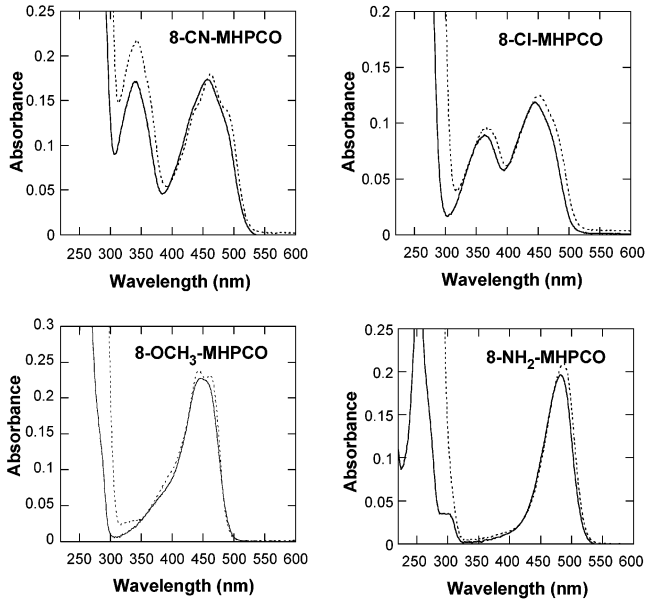


FIGURE 1: Absorption spectra of MHPCO reconstituted with FAD analogues. The absorption spectra of free FAD analogues are shown in solid lines, while dotted lines represent the spectra of the FAD analogues bound to apoenzyme. The concentrations of free FAD analogues used were 17.6 μM of 8-CN-FAD, 11.8 μM of 8-Cl-FAD, 10.6 μM of 8-OCH_3-FAD, and 4.5 μM of 8-NH_2-FAD.

Molecular Orbital Calculations. Molecular orbital calculations of FAD analogues were carried out as described by Ridder et al. (*33*) using semiempirical AM1 and PM3 algorithms included in the software Hyperchem (Hypercube). Only the isoalloxazine parts of the FAD were used for the calculation. The geometry optimization processes were carried out first by using the MM/AMBER method, and they were further optimized by the QM/semiempirical method using an AM1 or PM3 Hamiltonian with the restricted Hartree−Fock closed-shell method. The convergence limit of self-consistent field (SCF) was set at the value of 0.0001 (*33*).

RESULTS

Reconstitution of apoMHPCO with FAD Analogues. The apoenzyme of MHPCO binds tightly to all of the FAD analogues used with K_d values less than 1 μM (results not shown). This was the first indication that there were no major structural or chemical changes in the enzyme caused by the 8-substituents on the flavin. The binding resulted in perturbations of the flavin absorbance (Figure 1) and the fluorescence properties, implying that the isoalloxazine of FAD has close contact with the microscopic environment of the protein. The absorption spectra are more resolved when these flavin analogues bind to MHPCO (Figure 1), but the molar absorption coefficient values of these reconstituted enzymes are all about the same as those for the free FAD (Table 1).

Hydroxylation Stoichiometry. The conversion of the substrate MHPC or the substrate analogue 5HN to their corresponding products can be monitored by using the UV−visible absorbance properties of these compounds (*3*, *5*). MHPC or 5HN are aromatic compounds having absorption

Table 1: Molar Absorption Coefficients of Reconstituted Enzymes

MHPCO reconstituted with FAD analogues	molar absorption coefficients of free FAD analogues[a] ($mM^{-1}\,cm^{-1}$)	molar absorption coefficients of reconstituted enzyme ($mM^{-1}\,cm^{-1}$)[b]
8-CN-FAD	11.4 (450 nm)	11.7 ± 0.1 (462 nm)
8-Cl-FAD	11.6 (448 nm)	11.5 ± 0.3 (452 nm)
8-OCH_3-FAD	21.5 (445 nm)	23.9 ± 0.5 (443 nm)
8-NH_2-FAD	44.0 (482 nm)	50.5 ± 0.1 (487 nm)

[a] Values taken from ref *26*. [b] Determined as described in Materials and Methods.

Table 2: Hydroxylation Stoichiometry of MHPCO Reconstituted with FAD Analogs and Dissociation Constants for Substituted Enzymes Binding to the Substrate (MHPC) or a Substrate Analog (5HN)

FAD analogues	hydroxylation[b] (%)	K_d for MHPC binding[c] (μM)	K_d for 5HN binding[c] (μM)
8-CN-FAD	97	56 ± 4	3.8 ± 0.5
8-Cl-FAD	98	50 ± 3	9.3 ± 0.8
8-OCH_3-FAD	87	159 ± 7	16 ± 1
8-NH_2-FAD	89	82 ± 2	0.35 ± 0.07
native enzyme[a]	99	9.2 ± 0.6	5.2 ± 0.4

[a] Values taken from ref *2*. [b] Determined as described in ref *3*. [c] Determined as described in ref *2*.

between 280 and 330 nm, while their aliphatic products absorb maximally at 260 nm (*3*, *5*). Using this spectral difference to monitor the reactions, we found that all of the FAD analogues when bound to the enzyme were able to catalyze the hydroxylation and ring-cleavage hydrolysis reactions of both MHPC and 5HN to yield the same products as did enzyme with FAD (data not shown). The stoichiometries of the oxygenase-catalyzed reactions were also determined by measuring the amount of H_2O_2 formed during catalysis. Aromatic flavoprotein hydroxylases often produce a small amount of H_2O_2 during reactions with O_2, which results in less than stoichiometric quantities of oxygenated product being formed (*10*). The small quantity of H_2O_2 formed was determined by adding catalase at the end of the reaction to convert one-half of the resultant H_2O_2 to O_2, and this was monitored with the oxygen electrode (*3*). We measured the ratio of hydroxylated product formed per oxygen consumed for each of the reconstituted enzymes (Table 2). The ratio, (Oxygen consumed − H_2O_2 formed)/NADH oxidized, was determined for each derivative as described earlier (*3*). The results indicate that enzymes reconstituted with low redox potential flavins (8-NH_2-MHPCO and 8-OCH_3-MHPCO) had slightly lower hydroxylation efficiencies than those reconstituted with high redox potential flavins (8-Cl-MHPCO and 8-CN-MHPCO). Hydroxylation with the latter enzymes was similar to that with FAD bound (*3*).

Binding of Substrate or Substrate Analogues to the Reconstituted Enzyme. Incorporation of FAD analogues only slightly affected the binding affinity of the substrate (MHPC) or a substrate analogue (5HN) to the enzyme. The K_d values for binding of 5HN to 8-X-MHPCO forms vary from <1 to ~10 μM (Table 2), comparing closely to those for enzymes with FAD bound (5.2 μM). The binding constants for MHPC were somewhat weaker, ranging from 50 to 160 μM compared to 9.2 μM for FAD bound. These K_d values in the micromolar range indicate that there is good binding of the enzyme with the substrate or the substrate analogue and also suggest that no major changes in the substrate binding pocket of the enzyme had occurred on binding of the FAD analogues.

Table 3: Redox Potential Values of Ligand-Free and Ligand-Bound Forms of Reconstituted MHPCO

FAD analogue ($E_m°'$ in mV)	$E_m°'$ of ligand-free enzyme (mV) $E_1°'$	$E_2°'$	$E_m°'$	semiquinone stability (%)[e]	$E_m°'$ of ligand-bound enzyme (mV) +MHPC	+5HN
8-CN (−50[a])	90	40	65	57	80−219[f]	80−219[f]
8-Cl (−152[b])	−36	20	−8	14	+11	+6
8-OCH_3 (−260[c])			−210		−184	−204
8-NH_2 (−297[d])			−242		−233	−232
native[g] (−207[d])			−85	20	−78	−81

[a] From ref *36*. [b] From ref *63*. [c] From ref *64*. [d] From ref *32*. [e] Semiquinone accumulates during titrations but in three cases is dismuted over time to yield oxidized and reduced forms. The fraction remaining after >70 h is indicated by this column. [f] The redox potentials of ligand-bound 8-CN-MHPCO cannot be determined accurately by using available dyes. The values of the redox potentials were estimated to be in the range of +80 to +219 mV by comparing with *N*-methylphenazinium methosulfate and 2,6-dichlorophenol indolephenol, respectively. [g] Data for native enzyme were from ref *2*.

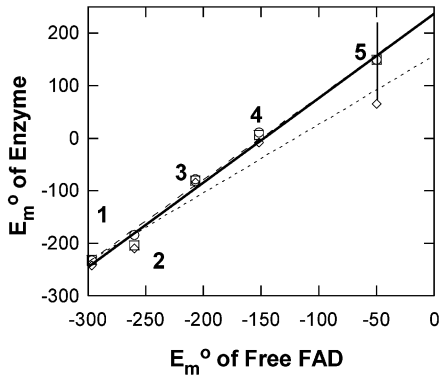


FIGURE 2: Correlation between redox potential values of free FAD and enzyme-bound FAD: (◇, ···) free enzyme ($y = 157.1 + 1.31x$; $R = 0.974$); (○, − − −) enzyme−MHPC ($y = 238.1 + 1.58x$; $R = 0.997$); (□, —) enzyme−5HN ($y = 238.2 + 1.61x$; $R = 0.994$). Measured redox values are listed in Table 3. Number 1 denotes 8-NH_2-MHPCO; 2, 8-OCH_3-MHPCO; 3, native MHPCO; 4, 8-Cl-MHPCO; and 5, 8-CN-MHPCO.

Redox Potential Values of the Reconstituted Enzyme. Redox potentials of FAD and its analogues were shifted to considerably more positive values when bound to MHPCO (Table 3). Plots of the redox potential values of the enzyme-bound 8-X-FADs in the form of ligand-free, MHPC-bound, and 5HN-bound, versus the values of free 8-X-FADs were linear with slopes of 1.31, 1.58, and 1.61 respectively (Figure 2). The uniform increases in redox potential values of different flavins when bound to the enzyme suggest that the enzyme interacts similarly with each of these flavin analogues and that the substituents at the 8-position do not produce significant changes in enzyme conformation or greatly perturb the enzyme active site. The mixing of 8-Cl-FAD−MHPCO with thiophenol rapidly yielded 8-phenylthio-FAD (data not shown), also indicating that the 8-position of isoalloxazine is accessible to solvent (*34*).

Flavin Semiquinone Formation during Reductive Titrations. When low concentrations of benzyl viologen are used to mediate electron transfer, native MHPCO develops a

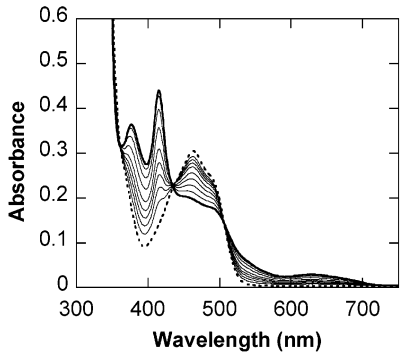


FIGURE 3: Reduction of 8-CN-MHPCO in the presence of MHPC by the xanthine/xanthine oxidase system. 8-CN-MHPCO (25.6 μM) and MHPC (448 μM) in 50 mM sodium phosphate buffer, pH 7.0, at 25 °C was catalytically reduced with xanthine (200 μM), benzyl viologen (5 μM), and xanthine oxidase (6 nM) in an anaerobic cuvette. The dashed line shows the spectrum of the fully oxidized enzyme. Other spectra were recorded after the initiation of the reduction process. The thick line shows the spectrum of the reaction after a period of 1 day, showing the maximum formation of 8-CN-flavin anionic semiquinone with a characteristic peak at 415 nm.

significant amount of flavin semiquinone during anaerobic reduction by reducing systems that can supply one electron at a time, such as dithionite or the xanthine oxidase system (*32*). In the absence of substrate, red anionic semiquinone is observed, while in the presence of substrate, blue neutral semiquinone appears. However, very little semiquinone was observed during determinations of redox potentials in which the enzyme was reduced in the presence of higher concentrations of indicator dyes and allowed to fully equilibrate (*2*). All of the ligand-bound 8-X-MHPCO forms of MHPCO also behaved similarly and the semiquinones were not stable. This indicates that the semiquinone is not thermodynamically favored, and although it will form transiently during reductions, it slowly attains equilibrium when mediator dyes are present. The ligand-free 8-NH_2-MHPCO and 8-CH_3O-MHPCO did not show significant amounts of semiquinone in similar reduction reactions. However, semiquinone formed and was stable during the determinations of the redox potentials of ligand-free 8-Cl-MHPCO and ligand-free 8-CN-MHPCO (Table 3). In absence of substrate, 8-CN-MHPCO formed the anionic semiquinone as shown by the intense peak at about 410 nm (*35*). By contrast to the native MHPCO, in the presence of MHPC (Figure 3), the semiquinone formed was approximately 70% in anionic form. The absorbance at wavelengths longer than 550 nm shows that about 30% of the semiquinone is the blue neutral form. Thus, it appears that with the strong electron-withdrawing cyano group, which delocalizes the negative charge, the red semiquinone is more stable even in the presence of the negatively charged substrate.

Reduction of 8-X-Substituted Enzyme−Substrate Complexes. The oxidized enzyme was mixed with various concentrations of NADH anaerobically in the stopped-flow spectrophotometer, and the reactions were followed over the range 440−480 nm, where the oxidized 8-X-MHPCO enzyme forms had maximum absorbance and reduced forms had very little (*2*, *5*). Charge-transfer species are often observed as long wavelength bands during reductions of flavoproteins by pyridine nucleotides. The intensity of these bands can indicate proximity and proper orientation for transferring a hydride from the nicotinamide to the flavin

Table 4: Rate Constants for the Reduction of 8-Substituted-MHPCO with NADH (k_{red}) and Rate Constants for the Reaction of Reduced 8-Substituted-MHPCO with Oxygen

substituents	k_{red} (s^{-1})	k_I (M^{-1} s^{-1})	k_{II} (s^{-1})	k_{III} (s^{-1})
8-CN	128 ± 18	512 ± 10	2.0 ± 0.3	0.43 ± 0.07
8-Cl	20.8 ± 0.2	$(9.6 \pm 0.5) \times 10^3$	1.53 ± 0.03	
8-OCH_3	0.57 ± 0.5	$(6.5 \pm 0.7) \times 10^4$	1.24 ± 0.05	
8-NH_2	0.17 ± 0.3	$(9 \pm 1) \times 10^4$	*a*	
native	12.7 ± 0.3	$(5.5 \pm 0.2) \times 10^4$	0.86 ± 0.2	

[a] Not determined.

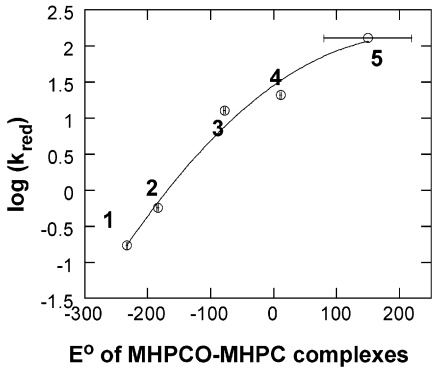
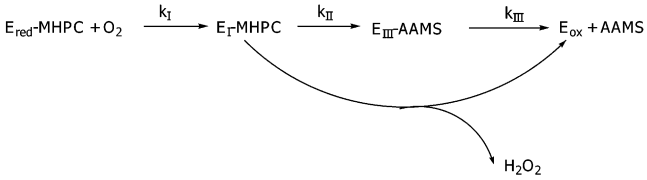


FIGURE 4: Free energy relationship of the reduction rate of the reconstituted enzyme by NADH. The logarithms of the maximum reduction rates of reduction by NADH (k_{red}) of the reconstituted MHPCO were plotted against the redox potential values of enzyme−MHPC complexes. Number 1 denotes 8-NH_2-MHPCO; 2, 8-OCH_3-MHPCO; 3, native MHPCO; 4, 8-Cl-MHPCO; and 5, 8-CN-MHPCO.

(*36*−*38*). However, as with native MHPCO, the reconstituted enzymes did not show any formation of long-wavelength charge-transfer species. Nevertheless, before the hydride transfer phase, where the absorbance in the visible region of the flavin decreased, there was an initial lag period that was due to the binding of NADH. Apparently with this enzyme, orbital overlap appropriate for hydride transfer to the flavin can occur without the two rings stacking to yield a charge-transfer interaction. The rates for the flavin reduction (hydride transfer phase) were obtained by reacting NADH with enzyme anaerobically in the stopped-flow instrument, as employed in the study of the native enzyme (*2*). The apparent rate constants approached maxima at high concentrations of NADH, and these maxima were interpreted to represent the intrinsic rates of the hydride transfer for each 8-X-MHPCO (Table 4). It is clear that FAD analogues with higher redox potentials are reduced more rapidly than those with lower redox potentials. A plot of the log of the hydride transfer rates versus the two-electron redox potential values ($E_m^{\circ\prime}$) of MHPC-bound 8-X-MHPCO could be fit with a parabolic function (Figure 4). The rates of hydride transfer increased proportionally according to the flavin $E_m^{\circ\prime}$ values when 8-X-MHPCO with low $E_m^{\circ\prime}$ values were used but approached a limiting value in the reaction of 8-CN-MHPCO, the highest potential enzyme form studied (Figure 4).

Reaction of the Reduced 8-X-FAD Enzymes with Oxygen. Stopped-flow spectrophotometric studies were carried out to determine the rate constants of the individual steps in the reactions of the MHPC-bound reduced 8-X-MHPCO species with oxygen. It has been known from previous studies that the oxidative half-reaction of MHPCO in the presence of

Scheme 2



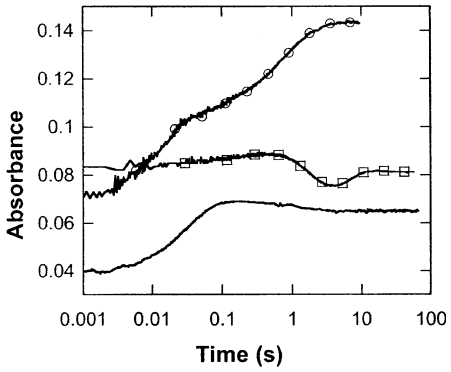


FIGURE 5: Kinetic traces of the reaction of various reduced 8-X-FAD-reconstituted MHPCOs with O_2. Reduced MHPCO was mixed with oxygen in 50 mM sodium phosphate buffer, pH 7.0, and the reaction was monitored by stopped-flow spectrophotometry at 4 °C. Line without symbols (bottom trace) represents the reaction of reduced native MHPCO (7 μM) and MHPC (344 μM) with oxygen (130 μM), as monitored at 405 nm (data were taken from ref *3* and were offset downward by 0.02 absorbance units). Line with squares (middle trace) is of the reaction of reduced 8-CN-MHPCO (12.8 μM) and MHPC (400 μM) and oxygen (960 μM), and the reaction was monitored at 375 nm. Line with circles (top trace) is of the reaction of reduced 8-OCH_3-MHPCO (10.5 μM) and MHPC (300 μM) with oxygen (960 μM), and the reaction was monitored at 400 nm.

substrate involves the formation of C(4a)-hydroperoxyflavin (FADHOOH) and C(4a)-hydroxyflavin (FADHOH) intermediates. This reaction was treated in simplified form as shown in Scheme 2. In this study, the reactions of 8-CN-MHPCO and 8-OCH_3-MHPCO were monitored at several wavelengths where it was possible to distinguish the formation and disappearance of enzyme intermediates, while the reaction of 8-Cl-MHPCO was recorded with the diode array detector from 300 to 750 nm.

The reaction of reduced 8-CN-MHPCO with oxygen resulted in triphasic traces when monitored at wavelengths between 370 and 420 nm. The kinetic trace at 375 nm, shown in Figure 5, obtained with an oxygen concentration of 960 μM, indicated the existence of two flavin intermediates. The fastest phase is indicated by a small increase in absorbance and is described by an apparent rate constant of 2.0 ± 0.3 s^{-1}, which was independent of oxygen concentration, while the second phase (decrease in absorbance) became faster with larger oxygen concentrations and is described by an apparent second-order rate constant of 512 M^{-1} s^{-1} (this occurs between ~0.9 and 5 s.). The third phase is characterized by an increase in absorbance with an apparent rate constant of 0.43 s^{-1} (Table 4). The absorbance change of the first phase is due to the formation of the C(4a)-hydroperoxyflavin, that of the second phase to the formation of the C(4a)-hydroxyflavin, and that of the third phase to the dehydration of FADHOH to form oxidized MHPCO. Because it is a bimolecular reaction between MHPCO and oxygen, one might expect that the rate of formation of the FADHOOH in the first phase would vary with oxygen concentration. However, because the flavin in 8-CN-MHPCO has such a high $E^{\circ\prime}$, the reaction of reduced enzyme with oxygen is quite slow, and the rate constant for the formation of FADHOH in the second phase (2.0 s^{-1}) is greater than the apparent rate constant for the formation of the prior species FADHOOH at any oxygen concentration that could be examined. Thus, in contrast to the absorbance changes, the first rate constant represents the formation of FADHOH and the second rate constant represents the formation of FADHOOH (*39*). Such a condition leads to only small fractions of the enzyme being in the FADHOOH form at any one time. The absolute absorbance of the two transient spectra corresponding to FADHOOH and FADHOH intermediates were calculated from data obtained from reacting reduced enzyme with 960 μM oxygen and monitoring at multiple wavelengths from 360 to 520 nm. Intermediates were calculated according to procedures previously described (*40*, *41*) using the apparent reaction rates $1/\tau_1 = 0.52$ s^{-1}, $1/\tau_2 = 2.0$ s^{-1}, and $1/\tau_3 = 0.43$ s^{-1} (Figure 6A). Results showed that the C(4a)-hydroperoxyflavin of 8-CN-MHPCO has a λ_{max} near 365 nm and the C(4a)-hydroxyflavin of 8-CN-MHPCO has λ_{max} at ~350 nm. The spectra of both intermediates resemble those of native enzyme, but the λ_{max} is shifted to shorter wavelength.

Reoxidation of reduced 8-Cl-MHPCO in the presence of MHPC was measured with a diode-array stopped-flow spectrophotometer scanning from 350 to 600 nm. The C(4a)-hydroxyflavin species could not be visually discerned because the rate constant for its hydrolysis is significantly greater than that for its formation from the hydroperoxyflavin. Thus, we applied global analysis using the program SpecFit and the model A → B → C to yield spectra of intermediate B, corresponding to the flavin−C4a-hydroperoxide (data not shown). The rate for formation of this intermediate is dependent on oxygen concentration (9.63×10^3 M^{-1} s^{-1}), while its decay rate (1.53 s^{-1}) is independent of oxygen concentration (Table 4). When the same reaction was carried out in the presence of 50 mM sodium azide, the kinetic traces were resolved into three phases. Global analysis based on Scheme 2 was employed to resolve the spectra of the two intermediates shown in Figure 6B. The first rate constant, which was dependent on oxygen concentration, was 1.01×10^4 M^{-1} s^{-1}. The first intermediate has the same spectrum found in the reaction without azide and is assigned as the flavin−C4a-hydroperoxide intermediate. The second intermediate in Figure 6B with a λ_{max} of 370 nm is assigned as the flavin−C4a-hydroxide, and it is formed with an apparent rate of 2.1 s^{-1}. It should be noted that the small shoulder around 450 nm on the spectrum of this intermediate (Figure 6B) indicates that about 15% of the flavin−C4a-hydroperoxide oxidizes directly in the uncoupling pathway (Scheme 2) to the oxidized flavin species and H_2O_2 without resulting in hydroxylation (*3*). Therefore, the actual rate for the hydroxylation step in the presence of azide is 1.78 s^{-1}, which is greater than the value for the reaction in absence of azide, and the formation of H_2O_2 is ~0.31 s^{-1}. Finally, the flavin−C4a-hydroxide lost H_2O to form the oxidized flavin species at a rate of 0.8 s^{-1}. Azide has also been useful to help resolve flavin intermediates in the analogous reaction of the native enzyme (*3*, *5*). In the absence of azide, the reaction of reduced native enzyme with oxygen resolves into

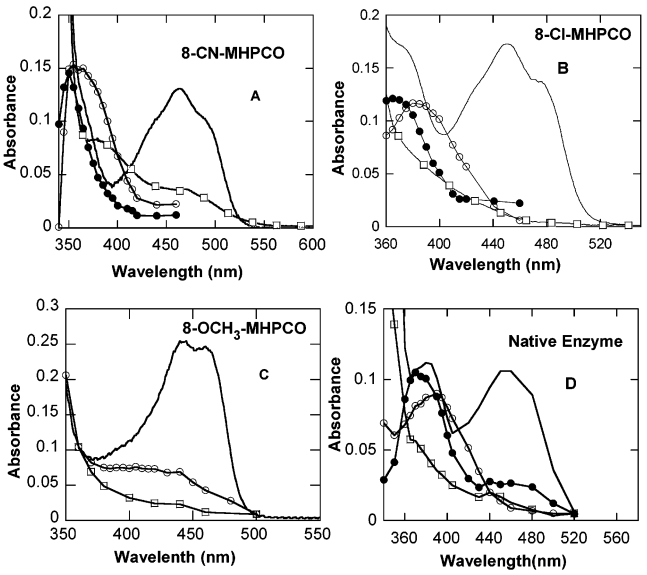


FIGURE 6: Spectra of transient intermediates of various flavin analogues formed during reoxidation of reduced MHPCO−MHPC complexes. The spectra were obtained from analysis of various kinetic traces at different wavelengths (panels A, C and D) or global analysis of multiwavelength data (panel B). All experiments were carried out in 50 mM sodium phosphate buffer, pH 7.0, at 4 °C. The solid lines with squares show the spectra of reduced enzymes, and the lines without symbols show the spectra of the oxidized enzymes. In panel A, reduced 8-CN-MHPCO (12.8 μM) and MHPC (400 μM) were reacted with oxygen (960 μM). The spectrum with empty circles represented the first intermediate obtained, and the spectrum with filled circles represents the second intermediate. The spectrum of the substrate was subtracted from the observed spectrum. In panel B, reduced 8-Cl-MHPCO (15.6 μM) and MHPC (400 μM) were reacted with oxygen (960 μM) in the presence of 50 mM sodium azide. The spectrum with empty circles represents the first intermediate obtained, and the spectrum with filled circles represents the second intermediate. Both spectra were corrected for the absorbance of the MHPC substrate. In panel C, reduced 8-OCH_3-MHPCO (10.5 μM) and MHPC (300 μM) were mixed with oxygen (960 μM). The spectrum with empty circles represents the calculated spectrum of the C(4a)-hydroperoxyflavin for 8-OCH_3-MHPCO (see text). In panel D, reduced native MHPCO (7 μM) and MHPC (344 μM) were reacted with oxygen (960 μM). The spectrum with empty circles represents the first intermediate obtained, and the spectrum with filled circles represents the second intermediate. Both spectra were corrected for the absorbance of MHPC substrate. Data of panel D were from ref *3*.

two phases with the formation of flavin−C4a-hydroperoxide and no detectable amount of flavin−C4a-hydroxide. It was reasoned that the dehydration step of the flavin−C4a-hydroxide has a larger rate constant than that for its formation, so very little flavin−C4a-hydroxide intermediate accumulated. When azide was included in the reaction mixture, the dehydration of flavin−C4a-hydroxide was slower, while the previous two steps were not greatly affected (*3*, *5*). Thus, more accurate spectra of transient intermediates in the reaction of native enzyme could be calculated (Figure 6). These results show that the reoxidation of reduced 8-Cl-MHPCO is similar to the reaction of the native enzyme but with different values of rate constants.

The reaction of reduced 8-OCH_3-MHPCO with oxygen was monitored at several wavelengths similarly to that with 8-CN-MHPCO. The reaction resolved into two phases at wavelengths between 360 and 520 nm (Figure 5). The rate of the first phase depended on oxygen concentration (6.5×10^4 M^{-1} s^{-1}), which is consistent with it being due to the formation of the flavin−C4a-hydroperoxide. The second phase (1.24 s^{-1}) was independent of oxygen concentration, consistent with it being due to the decay of the flavin−C4a-hydroperoxide. When azide was included, the reaction did not resolve into three phases as did the reaction of native and 8-Cl-FAD enzymes. This result indicates that azide did not decrease k_{III} to less than k_{II}, the formation of the FAD−C4a-hydroxide, as it did for the native enzyme, and it also implied that the dehydration step in 8-OCH_3-MHPCO was much faster than that in the reaction of native or 8-Cl-FAD enzymes. Data obtained from monitoring the reaction at several wavelengths at an oxygen concentration of 960 μM were used to calculate the absolute spectrum of the FAD−C4a-hydroperoxide intermediate using the apparent rate constants of 68 s^{-1} and 1.24 s^{-1} for the first and second phase, respectively. It could be calculated that at 70 ms <1% of the reduced MHPCO remained and that only about 7% of oxidized would have formed. Thus, the spectrum at 70 ms was about 92% that of the hydroperoxide. The calculated spectrum of this intermediate is shown in Figure 6C. This spectrum resembles the spectrum of the flavin−C4a-hydroperoxide intermediate of parahydroxybenzoate hydroxylase with 6-hydroxy-FAD replacing the normal FAD (*42*).

The reduced 8-NH_2-MHPCO in complex with MHPC used in reactions with oxygen had noticeable absorbance in the region of 550 to 700 nm, suggesting that there was semiquinone present in solution. This semiquinone might have been formed by the reaction of reduced enzyme with trace amounts of H_2O_2 that formed from residual oxygen in the solution. Consistent with this being semiquinone, the reaction traces of the reaction of reduced enzyme with oxygen were biphasic in the range of 350−550 nm and monophasic at longer wavelengths. The reaction observed at long wavelengths was second-order with respect to oxygen ($k = 1.4 \times 10^3$ M^{-1} s^{-1}) and was kinetically very similar to the slower phase observed at shorter wavelengths. It was surmised that the observations at longer wavelength and the slower reaction in the shorter wavelength region were due to the presence of a fraction ($\sim^1/_4$−$^1/_3$) of the enzyme in the semiquinone oxidation state of 8-NH_2-FAD. The fast phase observed in the range of 350−550 nm was also second order with respect to oxygen ($k = 9.1 \times 10^4$ M^{-1} s^{-1}, Table 4) and resulted in the conversion of reduced enzyme to oxidized enzyme with no indication of intermediates. This was surprising because of the coupling ratio (~89%, Table 2), as well as because results from an experiment that measured product formation indicated that the 8-NH_2-MHPCO can hydroxylate MHPC to form AAMS (data not shown). The faster second-order reaction with oxygen is likely due to the reaction with oxygen to form the flavin−C4a-hydroperoxide. Because it was slow compared to subsequent steps in this half-reaction, no intermediates were observed. Nevertheless, the fast phase is likely to be part of the normal catalytic pathway for the modified enzyme. This step must be rate-limiting in the oxygen half-reaction of catalysis so that the rate of hydroxylation and the rate for loss of water from the hydroxyflavin intermediate (return to oxidized enzyme) could not be measured; they were masked by the slow initial reaction with oxygen. 8-NH_2-MHPCO is the enzyme with lowest redox potential value in this study. When the rates of the dehydration steps among 8-X-MHPCO enzyme forms are compared, one can predict that the rate of dehydration

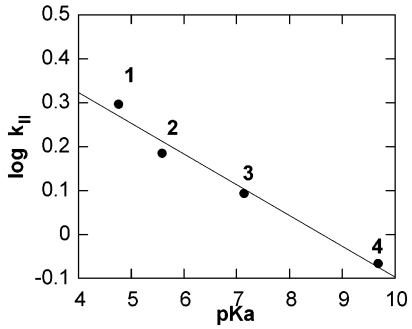


FIGURE 7: Correlation of logarithms of the rates of hydroxylation (k_{II}) with the calculated pK_a values of 8-X-FADHOH (a Bronsted plot). The linear fit gives a slope (β-value) of -0.07 and $R = 0.99$: (1) 8-CN-MHPCO; (2) 8-Cl-MHPCO; (3) 8-OCH_3-MHPCO; (4) native MHPCO.

in 8-NH_2-MHPCO would be much faster than that of 8-OCH_3-MHPCO. The high preference to dehydrate FADHOH to form FAD might be reflected in a large rate for the last step, so there would be no detectable enzyme intermediates. Therefore, the kinetic constants for the reoxidation of the reduced 8-NH_2-MHPCO could not be compared with the values of other 8-X-MHPCO species.

Correlation of the Rate of Hydroxylation with Thermodynamic and Electronic Constants. The rates of the hydroxylation step for the various 8-X-MHPCO forms were compared with several thermodynamic and electronic constants to develop a sharper view of the mechanism of the hydroxylation. The rates of hydroxylation in PHBH have been shown to be dependent on the theoretical pK_a values of the 8-X-FADHOH used (*26*). In an electrophilic substitution reaction, the pK_a values are related to the facility of the leaving group $FADHO^-$ (the flavin−C4a-alkoxide) to stabilize its negative charge. Thus, because 8-X-FADHOH species with lower pK_a values have more stable $FADHO^-$ species (they are better acids), they promote faster hydroxylation rates. A plot of the log of the k_{II} values of 8-X-PHBH versus the calculated pK_a values of the 8-X-FADHOH species (a Bronsted plot) was linear. A similar Bronsted plot for 8-X-MHPCO was also linear (Figure 7), suggesting that the oxygenation step catalyzed by MHPCO, like that of PHBH, is also an electrophilic aromatic substitution mechanism. The plot of Figure 7 yielded a small β value of -0.07, indicating that only a small negative charge was developed at the flavin−C4a-alkoxide at the transition state. The β value is often taken as the degree of charge development on the leaving group at the transition state. Previous studies of PHBH obtained a β value of -0.42 (*26*), indicating that a larger negative charge develops at the transition state than in the reaction of 8-X-MHPCO. Therefore, the range of the rate constants determined using the 8-X-FAD MHPCO enzymes was only about 2.5-fold versus a few 100-fold in PHBH. The pK_a values used in this plot (Figure 7) were from ref *26* and are based on the pK_a values of substituted methanols, assuming that the inductive effects of the other substituents (the other parts of FADHOH) are additive (*43*). This method of calculation was shown to be reasonably valid in the studies of flavin model compounds (*43*, *44*) and in the reaction of PHBH (*26*).

Although the results in Figure 7 suggested that the oxygenation catalyzed by MHPCO is an electrophilic aromatic substitution mechanism, we explored whether other parameters also correlated with this conclusion. The pK_a values of compounds are usually linearly dependent on the HOMO values of the conjugate base (*45*, *46*). Therefore, the HOMO energy of the base ($FADHO^-$) is also a measure of the pK_a value of the 8-X-FADHOH. Molecular orbital calculations (see Materials and Methods) were used to estimate electronic energies of 8-X-FAD intermediates (Supplementary Information). Only the isoalloxazine portions of the FAD derivatives were considered in these calculations because the adenine and ribose moieties are not intimately involved in the chemistry of the reaction (*9*, *47*). The natural logarithms of the hydroxylation rates were linearly correlated with HOMO values of 8-X-$FADHO^-$ species with $R = 0.93$ (data not shown). This result is also consistent with the notion that the rate of hydroxylation is dependent on the ability of the $FADHO^-$ leaving group to stabilize a negative charge. In this mechanism, where MHPC is the nucleophile and FADHOOH is the electrophile, the reactivities of the reactants, in theory, can be quantified by the HOMO value of the nucleophile and the LUMO value of the electrophile (*48*). Previous studies on the reaction of PHBH have shown that smaller differences in energy between the HOMO and LUMO (see Supporting Information) can be correlated with faster oxygen transfer (*27*). For this study of MHPCO, the logarithms of the rates of oxygen transfer from the flavin−4a-hydroperoxide also correlated reasonably well with the difference in these energies ($R = 0.91$) but, again, not as well as with the pK_a values.

DISCUSSION

This study investigated thermodynamic and kinetic properties of the enzyme MHPCO when various 8-X-FAD species were incorporated into the enzyme. Comparisons of the results for the different 8-X-FADs have been useful for developing a better understanding of both the reductive and the oxidative half-reactions of MHPCO. FAD analogues have previously been used to probe general mechanistic and structural details in studies of flavoproteins (*47*, *49*). Specific questions regarding mechanism can be addressed by using a suitable series of flavin analogues having different electron-donating or -withdrawing character, providing that interactions of the X-group with the protein are not significant. FAD analogues with 8-substituents having varying degrees of electron-withdrawing abilities were chosen for these studies because the 8-position is likely to be some distance from the site for hydroxylation so its effects should be mainly inductive. In addition, we have observed that 8-Cl-MHPCO reacts rapidly with thiophenol, which indicates that the 8-substituent is exposed to solvent and that it may not have strong interactions with protein residues. The X-ray structures of PHBH and phenol hydroxylase, enzymes in the same class as MHPCO, showed that the 8-position is accessible to solvent in these enzymes (*50*, *51*). Therefore, replacing native FAD with a series of 8-X-FADs was considered likely to have minimal effects on the global structure of MHPCO but nevertheless would have significant inductive effects on the reactivity of the FAD cofactor. The thermodynamic and catalytic properties of MHPCO reported in this paper imply that the enzyme probably did not undergo major structural or chemical changes upon incorporating the 8-X-FAD analogues used. The reconstituted enzymes still bind well with the substrate MHPC and with the substrate analogue 5HN, and they catalyze the same enzymatic reactions as does

native MPHCO (Table 2). Protein environments can manipulate the $E_m^{\circ\prime}$ of flavins by affecting the solvation, charge interaction, and conformation of the flavin (*47*). Thus, it is important to check whether effects specific for particular substituents might be involved. The linear correlations between the $E_m^{\circ\prime}$ of free FAD and the enzyme-bound $E_m^{\circ\prime}$ found in the studies of FAD/FMN analogues with PHBH (*26*) and lactate oxidase (*52*) were interpreted to imply that there were no significant substituent-dependent effects. The linear relationship of $E_m^{\circ\prime}$ values of the enzyme-bound 8-X-FADs to those of free 8-X-FADs (Figure 2) likewise suggests that protein residues in MHPCO have similar interactions with all of the FAD analogues studied. The small increase in redox potentials upon binding of MHPC or 5HN to reconstituted MHPCO species is also seen with the native MHPCO (*2*).

The rates of hydride transfer from NADH to flavin (k_{red}) are parabolically correlated with $E_m^{\circ\prime}$. The rates of hydride transfer increased proportionally to the values of flavin $E_m^{\circ\prime}$ when 8-X-MHPCO with low $E_m^{\circ\prime}$ values were used but approach a limit, as shown in the reaction of 8-CN-MHPCO. According to Marcus electron transfer theory, the left part of the graph in Figure 4 could be due to the reaction being controlled largely by the thermodynamic driving force, while the curvature at the end of the graph could be principally due to the work component for bringing the reactants together, which becomes the dominant parameter in the electron-transfer reaction when higher $E_m^{\circ\prime}$ 8-X-MHPCOs were employed (*53*). Similar correlations with $E_m^{\circ\prime}$ were observed for the reduction rates of 8-X-FMN by mandelate in lactate oxidase (*52*) and in the turnover rate for the adrenodoxin reductase (*54*) that had been substituted with 8-X-FAD analogues. It was observed that the reaction rate decreased when an electron acceptor with higher $E_m^{\circ\prime}$ was employed in the reaction of formate dehydrogenase (*55*), and the result was interpreted as being due to a change of the transition state structure occurring in the reaction of substrate analogues (*55*). A simple linear correlation was not observed between k_{red} and $E_m^{\circ\prime}$ of 8-X-FAD in the studies of PHBH (*56*), but this was due to more complex behavior with PHBH. PHBH has at least three positions of the isoalloxazine of FAD within the protein structure (*50*, *57*−*58*). An "in" position is used for the hydroxylation reaction and an "out" position (solvent-exposed) is used for reduction by NADPH. In addition, an intermediate position appears to be involved with substrate binding and in the movement of the nicotinamide of NADPH to a position where it can transfer the hydride to the flavin (*50*, *57*−*58*). With PHBH, it was found that the reduction rate correlated inversely with the volume of the substituents rather than with the redox potentials of the 8-X-FAD analogues. It was reasoned that flavin movement to the out position was a major factor governing the reduction rate of PHBH (*56*). Therefore, the fact that the rates of reduction in MHPCO are directly dependent on the redox potential rather than on the volume of the substituents suggests that flavin movement might not play an important role in the reduction of flavin in MHCPO. Although the reductive half-reactions of the flavoprotein hydroxylases have many similarities, the determining factors in the rate can be quite different (*2*, *9*). Mainly, the thermodynamic driving force of the reaction controls the rate of hydride transfer in MHPCO. However, with PHBH a major determining factor in the reduction reaction is the movement of the flavin from "in" to "out" (*50*, *51*, *59*). In MHPCO, it appears that movement of the flavin may not be required.

We have tested whether MHPCO uses an electrophilic aromatic substitution mechanism for the oxygenation reaction. The enzyme hydroxylates a pyridyl derivative rather than a phenol. Other flavoproteins cannot oxygenate pyridyl derivatives of the parent substrates (*40*). The rates of the hydroxylation steps were correlated with the ability of particular 8-X-FADHO$^-$ species to stabilize the negative charge; in this mechanism, an alkoxide product is expected when FADHOOH is the electrophile. The calculated values of the pK_a of 8-X-FADHOH (*26*), the HOMO of 8-X-FADHO$^-$, which represents another method of estimating pK_a of FADHOH, and the difference in heat of formation of 8-X-FADHO$^-$ and 8-X-FADHOOH species were used to represent the relative stabilities of the 8-X-FADHO$^-$ leaving groups. These experiments and calculations have shown that the faster rates of hydroxylation are correlated with more stable forms of 8-X-FADHO$^-$. Previous studies of PHBH, the prototype model enzyme in the class of aromatic flavoprotein hydroxylases, have shown that the rate of hydroxylation depends on the values of pK_a of FADHOH (*26*). Moreover, studies of PHBH with a series of fluorinated pOHB substrate analogues has shown a linear correlation between k_{cat} and the difference in the heat of formation between the hydroxycyclohexadienone (the immediate hydroxylated product of the electrophilic hydroxylation step) and pOHB in the reaction of PHBH (*60*). These studies indicated that the more stable the product after the electrophilic aromatic substitution, the faster is the hydroxylation reaction (*60*).

In addition to our studies and previous studies (*26*, *27*, *56*), several investigations using flavin model compounds and theoretical methods have shown that flavin−C4a-hydroperoxides are good electrophiles in monooxygenation reactions. Studies using a series of organic hydroperoxides, including a flavin−C4a-hydroperoxide model compound, have shown that the rate of oxygenation of alkyl sulfides correlated with decreased pK_a values of the corresponding alkoxides (*43*, *44*). This indicated that in oxygenation of organic hydroperoxides, higher rates are observed when the leaving group alkoxides were better stabilized. In addition, it demonstrated the ability of flavin−C4a-hydroperoxide to act as an electrophile. Recently, theoretical calculations by ab initio methods have indicated the ability of flavin−C4a-hydroperoxides to act as electrophiles during the oxidation of dimethyl sulfide (*61*). In the reaction of phenol hydroxylase, activation energies were calculated according to the potential energy surface of the reaction coordinates, where the C(4a)-hydroperoxyflavin acted as an electrophile to hydroxylate the series of phenolate compounds (*62*). Results have shown close correlations between the experimental k_{cat} values and the theoretical activation energies of the electrophilic aromatic substitution reactions (*62*).

In conclusion, our results have shown that MHPCO has several similarities as well as differences with other enzymes in the aromatic flavoprotein hydroxylase family. The substrate MHPC and the final product AAMS are quite different from pOHB (substrate of PHBH) and phenol (substrate of phenol hydroxylase) and their respective products. Sequence analysis has shown only ∼20% identity among these

enzymes (*4*). This suggests that there are considerable differences in their structures. Yet these enzymes all use an electrophilic aromatic substitution mechanism for their monooxygenation reactions and proceed through similar chemical intermediates. It should be mentioned that another flavoprotein, cyclohexanone monooxygenase (*22*, *23*), as well as a few other similar flavoproteins (*9*, *10*), also utilize flavin−C4a-peroxide intermediates. Because these enzymes oxygenate electrophilic ketones, they must carry out a nucleophilic oxygenative attack. Thus, flavoprotein peroxides (hydroperoxides in the case of aromatic hydroxylases and peroxides in the case of enzymes such as cyclohexanone monooxygenase) are very versatile. The protein environment determines how flavin peroxides will react.

NOTE ADDED AFTER ASAP POSTING

This article was released ASAP on 3/2/2004. Subsequently, a change was made to the financial support acknowledgement. The article was reposted 3/4/2004.

SUPPORTING INFORMATION AVAILABLE

Calculated thermodynamic constants and electronic energy of 8-substituted FAD analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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