Use of Free Energy Relationships To Probe the Individual Steps of Hydroxylation of *p*-Hydroxybenzoate Hydroxylase: Studies with a Series of 8-Substituted Flavins†

Mariliz Ortiz-Maldonado, David P. Ballou,* and Vincent Massey*

Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109-0606

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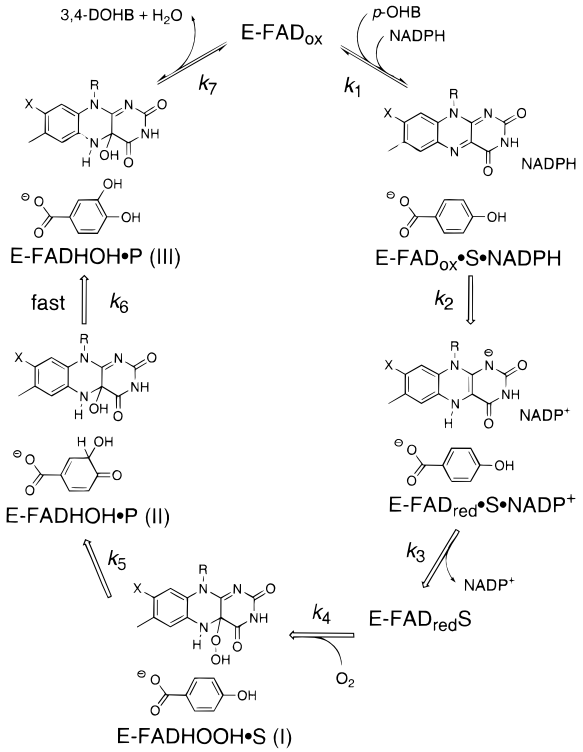
ABSTRACT: We report Hammett correlations, using 8-substituted flavins, to clarify the mechanism of hydroxylation by *p*-hydroxybenzoate hydroxylase (PHBH). The 8-position of the FAD isoalloxazine ring was chosen for modifications, because in PHBH it has minimal interactions with the protein, and it is accessible to solvent and away from the site of hydroxylation. Although two intermediates, a flavin-C4a-hydroperoxide and a flavin-C4a-hydroxide, are known to participate in hydroxylation, the mechanism of oxygen transfer remains controversial. Mechanisms as diverse as electrophilic aromatic substitution, diradical formation, and isoalloxazine ring opening have been proposed. In the studies reported here, it was possible to monitor spectrally each of the individual steps involved in hydroxylation, because the FAD cofactor acts as a reporter group. Thus, with PHBH, substituted separately with nine derivatives of FAD altered in the 8-position, quantitative structure−reactivity relationships (QSAR) have been applied to probe the mechanisms of formation of the flavin-C4a-hydroperoxide, the conversion to the flavin-C4a-hydroxide with concomitant oxygen transfer to the substrate, and the dehydration of the flavin-C4a-hydroxide to form oxidized FAD. The individual chemical steps in the mechanism of PHBH were not altered when using any of the modified flavins, and normal products were obtained; however, the rates of individual steps were affected, and depended on the electronic properties of the 8-substituent. Increased hydroxylation rates were observed when a more electrophilic flavin-C4a-hydroperoxide (i.e., with an electron-withdrawing substituent at the 8-position) is bound to PHBH. On the basis of QSAR analysis, we conclude that the mechanism of the hydroxylation step is best described by electrophilic aromatic substitution.

p-Hydroxybenzoate hydroxylase (PHBH)[1] (EC 1.14.13.2) from the soil microbe *Pseudomonas aeruginos*a is one of many external flavoprotein monooxygenases involved in the degradation of aromatic compounds, including numerous xenobiotics. This enzyme catalyzes the hydroxylation of *p*-hydroxybenzoate (*p*-OHB) to form 3,4-dihydroxybenzoate (3,4-DOHB) via a catalytic cycle that consists of reductive and oxidative phases (Scheme 1) (*1*). After NADPH and *p*-OHB bind randomly to the enzyme (k_1), a hydride equivalent is transferred from NADPH to FAD in the reductive half-reaction (k_2); the rate of reduction of FAD is

Scheme 1



10^5-fold greater in the presence of bound *p*-OHB. In the oxidative half-reaction, this reduced enzyme in complex with *p*-OHB reacts with oxygen to form the flavin-C4a-hydro-

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* To whom correspondence should be addressed. D.P.B.: Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109-0606; E-mail: dballou@umich.edu; Phone: 734-764-9582; Fax: 734-763-4581. V.M.: Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109-0606; E-mail: massey@umich.edu; Phone: 734-764-7196; Fax: 734-763-4581.

[1] Abbreviations: PHBH, *p*-hydroxybenzoate hydroxylase from *Pseudomonas aeruginosa*; *p*-OHB, *p*-hydroxybenzoate; 2,4-DOHB, 2,4-dihydroxybenzoate; 3,4-DOHB, 3,4-dihydroxybenzoate; FAD, flavin adenine dinucleotide; apoPHBH, the apoenzyme form of *p*-hydroxybenzoate hydroxylase; FADHOOH, flavin-C4a-hydroperoxide; FADHOH, flavin-C4a-hydroxide; $FADHO^-$, flavin-C4a-alkoxide; FMN, flavin mononucleotide; IPTG, isopropyl-1-thio-β-D-galactopyranoside; NADH, reduced nicotinamide adenine dinucleotide; NAD^+, oxidized nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; QSAR, quantitative structure−activity relationships; TNB, 5-thio-2-nitrobenzoic acid; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); P_i, phosphate; *R*, regression value from the curve fit; *F*, statistical *F*.

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peroxide (FADHOOH) (k_4), which then oxygenates *p*-OHB (k_5). After tautomerization to form the product, 3,4-DOHB (k_6) (*1*), product is released, and the flavin-C4a-hydroxide (FADHOH) intermediate eliminates water to re-form oxidized enzyme (k_7). The mechanism of oxygen transfer from the FADHOOH to the substrate has been proposed to involve electrophilic aromatic substitution (*2*, *3*), diradical formation (*4*, *5*), or isoalloxazine ring cleavage (*6*). If the reaction involves electrophilic aromatic substitution, the electrophilicity of the FADHOOH intermediate will affect the rate of hydroxylation. The electrophilicity of the FADHOOH can be varied by incorporating electron-donating or -withdrawing groups on the flavin.

Correlations of Hammett parameters with rate and equilibrium constants have contributed greatly to the understanding of many organic and bioorganic reactions (*7*−*10*). Such studies of tyrosine hydroxylase (*11*), monoamine oxidase (*12*−*15*), D-amino acid oxidase (*16*), L-lactate oxidase (*17*), bacterial luciferase (*18*, *19*), dopamine β-monooxygenase (*20*), phenol hydroxylase (*21*), and cytochrome P-450 (*22*−*25*) have all been useful in developing an understanding of their mechanisms. In the work presented here, the mechanism of oxygen transfer in the oxidative half-reaction of PHBH is analyzed to test whether hydroxylation proceeds via electrophilic aromatic substitution. The spectral properties of the flavins permitted determination of substituent effects on each step of the reaction, so that true QSAR can be applied.

Using a variety of 8-substituted flavin analogues free in solution, Schopfer et al. (*26*) showed a linear correlation between the redox potential of the flavin and the Hammett σ_p value of the 8-substituent. When 8-substituted flavins are incorporated into PHBH, the electronegativity of the various substituents influenced the kinetics of the oxidative half-reaction with 2,4-dihydroxybenzoate (2,4-DOHB) as substrate in the presence of 0.1 M NaN_3, but did not greatly affect the spectra of intermediates (*26*). Here we have probed the mechanism of oxygen transfer by measuring the rates of hydroxylation using a number of 8-substituted flavins. Utilizing the electronic effects of the 8-position of the flavin on the N1, C4a, and N5 positions, and the correlation with redox potential (*26*), inductive contributions from the substituent could be assessed. Chemical analysis (*27*) and X-ray crystal structures (*28*) of PHBH indicate that the 8-position of the flavin is accessible to solvent, is not constrained by protein contacts, and is away from the site of hydroxylation. 8-Substituted flavins, therefore, were chosen for probing the electronic contributions of FAD to catalysis in PHBH. The distinct spectral properties of PHBH in its various redox states enabled us to measure and study the influence of electronic effects of 8-substituents on each step of the oxidative half-reaction. The 8-substituent was found to affect not only the rate of hydroxylation but also the formation of the flavin-C4a-hydroperoxide and the elimination of water from the flavin-C4a-hydroxide intermediate. These studies provide strong support for the proposal that the oxygenation step proceeds via electrophilic aromatic substitution.

EXPERIMENTAL PROCEDURES

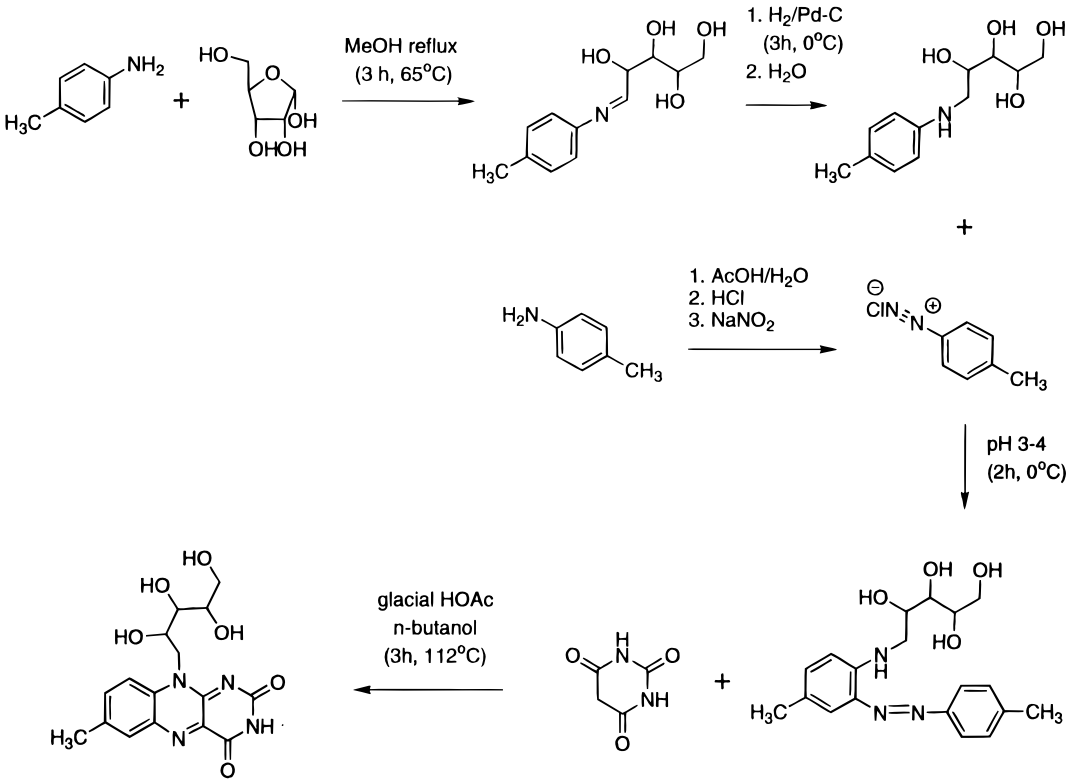
Materials. Substituted riboflavin analogues were obtained from various sources: 8-Cl-, Dr. J. P. Lamboy, University of Maryland; 8-OH-, 8-NH_2-, and 6-OH-, Dr. S. Ghisla, University of Konstanz (Germany); 8-$N(CH_3)_2$-, Drs. Matsui and Kasai, Osaka City University, Japan; and 8-CN-, Dr. Y. Murthy (this laboratory). 8-Mercapto-FAD and 8-SCH_3-FAD were prepared by reacting 8-Cl-FAD that was bound to PHBH with Na_2S (*27*) or $NaSCH_3$ (*29*) in 0.1 M sodium pyrophosphate buffer (pH 8.5) containing 0.1 mM EDTA; the reactions were monitored at 520 or 474 nm, respectively, for 8-mercapto- or 8-SCH_3-FAD. 8-Demethyl-FAD (8-H-FAD) was synthesized as reported (vide infra). Conversions of riboflavin analogues into the corresponding FAD analogues were achieved using FAD synthetase and flavokinase isolated from *Brevibacterium ammoniagenes* as previously reported (*30*). The extinction coefficients used for the various free 8-substituted FAD analogues (M^{-1} cm^{-1}) were as follows: ϵ_{448}(Cl) = 11 600, ϵ_{450}(H) = 11 300, ϵ_{450}(CN) = 11 400, $\epsilon_{450}(CH_3)$ = 11 300, $\epsilon_{474}(SCH_3)$ = 28 500, $\epsilon_{482}(NH_2)$ = 44 000, ϵ_{486}(hydroxy) = 32 200, $\epsilon_{505}((N(CH_3)_2)$ = 32 800, and ϵ_{523}(mercapto) = 30 000.

NADPH, NADH, NAD^+, riboflavin, FMN, FAD, glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, indigo-5,5′,7-trisulfonic acid, *p*-OHB, and 2,4-DOHB were from Sigma; *p*-OHB and 2,4-DOHB were recrystallized before use. IPTG and ampicillin were from Boehringer A. G.; *p*-toluidine, Na_2S, $NaSCH_3$, Pd/C, barbituric acid, anthraquinone-2-sulfonic acid, benzyl viologen dichloride, and safranine O were from Aldrich. Indigo carmine and brilliant cresyl blue were obtained from Pharmaceutical Laboratories, National Aniline Division; 1-hydroxyphenazine was from TCI America. Procion Red-A agarose was from Amicon, and Econo-Pac DG-10 columns and TNB (5-thio-2-nitrobenzoic acid)-thiol agarose were from Bio-Rad. The concentrations of solutions of the following were estimated spectrophotometrically using ϵ_{340} = 6220 M^{-1} cm^{-1} for NADPH, ϵ_{260} = 18 000 M^{-1} cm^{-1} for NAD^+, ϵ_{282} = 16 300 M^{-1} cm^{-1} for *p*-OHB (in 1 N NaOH), and ϵ_{258} = 13 600 M^{-1} cm^{-1} for 2,4-DOHB (in 1 N HCl).

Protocatechuate dioxygenase was purified from *Pseudomonas cepacia* DB01 (*31*), and xanthine oxidase was isolated from bovine milk as reported (*32*). Prepurified dry argon and analytical mixtures of oxygen and nitrogen gases were from Matheson Coleman and Bell. Dry argon was passed through a column of OxyClear (LabClear) to remove traces of oxygen.

PHBH Purification and Apoenzyme Preparation. PHBH was expressed and purified from extracts of JM105/pIE-130 using published procedures (*33*−*35*); 10 L of cell culture provides >500 mg of purified enzyme. The concentration of purified PHBH, which had an A_{280}/A_{450} ratio <9.0, was estimated using ϵ_{450} = 10 300 M^{-1} cm^{-1} (*34*). ApoPHBH was prepared by first chemically reacting an exposed protein thiol with a TNB-thiol agarose column, and then dissociating the flavin with high concentrations of salt as reported by Müller and van Berkel (*36*). The following modifications were implemented to increase the yield of apoenzyme. PHBH in a 70% ammonium sulfate suspension was collected by centrifugation, and the pellet was dissolved in a minimal volume of 0.1 M Tris−sulfate buffer (pH 8.0) containing 1 mM EDTA. To keep the accessible and conserved reactive thiol group of Cys 116 of the enzyme (*37*) in its reduced form during loading on the TNB-thiol agarose column, the enzyme preparation was incubated for 1 h at 25 °C with 5

Scheme 2



mM dithiothreitol. The dithiothreitol was removed by gel filtration using a DG-10 column equilibrated with coupling buffer [0.1 M KP_i buffer (pH 7.5) containing 0.5 M KBr and 1 mM *p*-OHB]. The resultant PHBH was applied to a column containing 10 mL of active TNB-thiol agarose that had been equilibrated at 25 °C with coupling buffer. When the column was washed with 40 mL of coupling buffer, no enzyme activity was detected in the wash fractions. The FAD released from resin-bound PHBH was eluted using 25 mL of 0.1 M phosphate buffer (pH 7.5) containing 3.0 M KBr, 4.0 M urea, and 1 mM *p*-OHB. The column was then rinsed with 100 mL of coupling buffer to remove excess KBr and urea. ApoPHBH was eluted using 50 mL of coupling buffer containing 5 mM dithioerythritol. The TNB-thiol agarose resin was regenerated by washing it with 0.1 M KP_i buffer (pH 7.5) containing 5 mM DTNB. The column was rinsed with water and then stored at 4 °C in a 0.02% sodium azide solution.

The level of enzymatic activity in eluted fractions was measured in reaction mixtures at 25 °C that contained 10 μM FAD, 0.33 mM *p*-OHB, 0.15 mM NADPH, 0.26 mM O_2, and 1 mM EDTA in 0.1 M Tris−sulfate buffer (pH 8.0). Fractions that contained apoPHBH were pooled and then concentrated using a Centricon-30 device (Amicon), and finally dialyzed for 24 h at 4 °C against two 4 L changes of 0.1 M KP_i buffer (pH 7.0) containing 1 mM *p*-OHB. ApoPHBH was precipitated with 70% saturated ammonium sulfate. The purified apoenzyme had no detectable activity in assay mixtures lacking FAD, and no absorbance at wavelengths above 310 nm. This procedure enables >99% of the enzyme applied to the column to be recovered as pure apoenzyme (as judged by the presence of a single band with a relative molecular mass of 42.2 kDa upon SDS−PAGE analysis). The catalytic activity is fully restored upon addition of stoichiometric amounts of FAD to apoPHBH. ApoPHBH can be stored as a 70% ammonium sulfate suspension at 4 °C for months in 0.1 M KP_i buffer (pH 7.0) containing 1 mM *p*-OHB without any loss of reconstitutable activity.

Apoenzyme Properties. The molar absorptivity at 280 nm of apoPHBH was determined to be 73 000 M^{-1} cm^{-1} using both amino acid analysis (carried out at The University of Michigan Protein Structure Core Facility) and flavin fluorescence to quantify the protein concentration. The fluorescence of free FAD (λ_{em} 525 nm and λ_{ex} 450 nm) was about 5-fold more intense than when bound to the enzyme. This made it possible to titrate the enzyme with FAD to determine the protein concentration. Similar behavior was observed when 8-Cl-FAD was used for reconstitution.

Synthesis of 8-H-FAD (Scheme 2). *(A) Condensation of p-Toluidine with* D*-Ribose To Form the Schiff's Base Imine (38). p*-Toluidine (10 g; 99.7% pure) and D-ribose (14 g) were refluxed in 100 mL of MeOH for 3 h. After the solution was reduced to a minimum volume using a rotary evaporator, it was cooled overnight in a refrigerator. The resultant white crystals were filtered and washed with cold ether and stored at −20 °C (97% yield).

(B) Reduction of the Schiff's Base by Hydrogenation (39). The imine (8 g) was dissolved in methanol and reduced at ice temperature by Pd/C under a hydrogen atmosphere for 3 h. After removal of the Pd/C, the filtrate was collected and concentrated by rotary evaporation. Crystals were allowed to form in this concentrate overnight at room temperature, and crystallization was continued for an additional day at −20 °C. The colorless crystals were filtered and washed with cold ether (36% yield).

(C) Diazotization (39). p-Toluidine (0.702 g) was dissolved in 10 mL of acetic acid and 2.5 mL of water. HCl (1.2 mL) was added dropwise with cooling, and the solution was

stirred on ice until it cleared. Then $NaNO_2$ (416 mg) was added slowly to the solution, with the temperature maintained below 5 °C. After the solution was stirred for 5 min at 0 °C, 1 g of the amine formed from the Schiff's base in the previous step was added over a period of 5−10 min with vigorous stirring. One milliliter of 4 N NaOH was added dropwise to the reaction mixture to maintain the pH at 3−4. The flask was kept in a cold water bath (8−10 °C) for 2 h, after which the mixture was diluted with 100 mL of water and extracted with three 150 mL portions of ether. The ether phase was collected after filtration and treated in a separation funnel with a saturated $NaHCO_3$ solution followed by a water wash. The ether solution was dried over solid Na_2SO_4, and after concentration, the product was obtained as a red−brown oil. This and subsequent steps were carried out in very low light intensity.

(D) Condensation of the Diazo Compound with Barbituric Acid (39). Ten milliliters of 1-butanol plus 2.5 mL of acetic acid was added to the red−brown oil. Barbituric acid (250 mg) was then added, and the solution was refluxed for 3 h at 112 °C. After cooling, the solution was concentrated by rotary evaporation, and the solid obtained was dissolved with sonication in 2% dimethyl formamide in water and filtered by gravity. The yellow filtrate was loaded onto a 35 mL C-18 SepPak column (Waters, Millipore), which was first washed with 2.5 L of water and then eluted with a linear gradient of acetonitrile in water, 0−20%. The riboflavin fraction, which had green fluorescence and a spectrum similar to riboflavin (λ_{max} at 360 and 450 nm), eluted at 8% acetonitrile. The samples collected were dried using a SpeedVac (10% overall yield). Positive-ion FAB mass spectrometry data for this synthesized 8-H-riboflavin in water showed the expected ($M^+ + 1$) ion at 363 mu.

Conversion of 8-H-Riboflavin to 8-H-FAD. 8-H-Rf was converted to the FAD form using partially pure preparations of FAD synthetase and flavokinase isolated from *Brevibacterium ammoniagenes* as published (*30*). The enzymatic conversion of 8-H-Rf to 8-H-FAD was >90% efficient. The ^{1}H NMR spectrum of 8-H-FAD (200 MHz) in D_2O showed the expected signals: δ 8.3 (s, 1H, amide H from adenine), 7.88 (s, 1H, amide H from adenine), 7.78 (s, 1H, aromatic H), 7.68, 7.69, 7.72, 7.74 (q, 2H, aromatic H), 5.83, 5.84 (d, 1H, ribose H), 3.85−4.85 (ribityl side chain H), and 2.44 (s, 3H, aromatic Me).

HPLC Purification of 8-H-FAD. A Waters HPLC and a 0.46 × 25 cm Vydac C-18 reverse-phase 218TP54 column equilibrated with 10% MeOH in water were used to further purify 8-H-FAD. A 10−25% MeOH linear gradient over 15 min and a flow rate of 0.5 mL min^{-1} were used. 8-H-FAD absorbance was detected at both 280 and 450 nm. HPLC resolved the preparation into two peaks with retention times of 9.50 (8-H-FAD, 95%) and 12.77 min (contaminant, 4%). ApoPHBH reconstituted with this 8-H-FAD, and then denatured with SDS to liberate the bound FAD, showed only one peak in HPLC analysis. The contaminant may be a degradation product of 8-H-FAD. The absorbance spectrum of 8-H-FAD was identical to that of normal FAD with peaks at 375 and 450 nm and with an $\epsilon_{450} = 11.3$ mM^{-1} cm^{-1} determined by fluorescence titration of the flavin with known concentrations of apoPHBH. Using 1-hydroxyphenazine as a reference dye, the redox potential for free 8-H-FAD was determined to be −180 mV at pH 7.0, identical to that reported for 8-H-FMN (*18*).

Reconstitution of PHBH with 8-Substituted FAD Forms. ApoPHBH was reconstituted by incubating it in 0.1 M Tris−sulfate buffer (pH 8.0) containing 1 mM EDTA for 1 h at 25 °C with a 1.5-fold molar excess of 8-substituted FAD. Excess FAD was removed by gel filtration using a DG-10 column equilibrated with 50 mM KP_i buffer (pH 6.5) containing 1 mM EDTA. Although the binding affinity constants for the 8-substituted flavins were not determined, each binds tightly to PHBH; no flavin dissociates when the enzyme is passed through a gel-filtration column or an ultrafiltration unit. The methods for determination of extinction coefficients of the oxidized 8-substituted FAD enzyme, enzymatic hydroxylation efficiency, substrate dissociation constants, and the pK_a of the *p*-hydroxy group in enzyme-bound *p*-OHB have been described in detail in reference *34*.

Determination of Dissociation Constant with the Reduced Enzyme. The spectral perturbations of the reduced 8-hydroxy-FAD and 8-NH_2-FAD bound to PHBH were followed during titration with *p*-OHB using a Hi-Tech Scientific SF-61 stopped-flow spectrophotometer. Titrations were in 50 mM KP_i buffer (pH 6.5) at 4 °C using 10 μM enzyme that had been reduced anaerobically with 1 equiv of sodium dithionite. Changes in absorbance at 425 nm were used to determine the dissociation constant. Corrected difference spectra were calculated after each addition, and the change in absorbance was used to determine the dissociation constant. Dissociation constants were derived for a simple binding isotherm using a nonlinear least-squares fitting algorithm in the program KaleidaGraph, Synergy Software, Reading, PA.

Redox Potential. The redox potential of the 8-substituted FAD-reconstituted PHBH (20−30 μM), in the presence or absence of 0.5−1.0 mM *p*-OHB, was determined spectrophotometrically at pH 7.0 and 25 °C by using the xanthine/xanthine oxidase reducing system with benzyl viologen as a mediator (*40*). Reference dyes used for each 8-substituted FAD were selected to be within 30 mV of the redox potential of that FAD analogue when bound to PHBH. The following reference dyes ($E^{o\prime}$ value in mV) were used: anthraquinone-2-sulfonate (−225) for 8-N$(CH_3)_2$-FAD, 6-OH-riboflavin (−255) for 8-mercapto-FAD, safranine O (−289) for 8-NH_2-FAD, brilliant cresyl blue (+47) for 8-CN-FAD, indigo disulfonate (−125) for 8-SCH_3-FAD, 1-hydroxyphenazine (−172) for FAD and 8-H-FAD, and indigo trisulfonate (−81) for 8-Cl-FAD. The time-dependent reduction of the enzyme and dye was monitored by UV/vis absorbance spectra. The reduction of the enzyme was monitored at the isosbestic wavelength of the reference dye, and the reduction of the dye was monitored at a wavelength where the flavin does not absorb. The levels of oxidized and reduced enzyme or dye were calculated from each spectrum, and the midpoint potential ($E^{o\prime}$ at 25 °C, pH 7.0) was calculated using the method of Minneart (*41*).

Stopped-Flow Instrumentation. Kinetic Studies. Steady-state and rapid reaction studies were carried out with either a Kinetic Instruments, Inc., or a Hi-Tech Scientific SF-61 stopped-flow spectrophotometer as described in reference *42*. The Hi-Tech SF-61 instrument was also equipped with an M300 diode array detector to record spectra in 1.25 ms. For anaerobic studies, the instrument was flushed with an oxygen-scrubbing solution containing ~0.1 unit mL^{-1}

protocatechuate dioxygenase (*31*) and 200 μM protocatechuate (3,4-DOHB) in 0.1 M KP_i buffer (pH 7.0) and allowed to stand overnight. To remove any residual 3,4-DOHB, the system was thoroughly rinsed with anaerobic water prior to making measurements. All reactions and measurements, unless noted otherwise, were carried out at 4 °C in 50 mM KP_i buffer (pH 6.5).

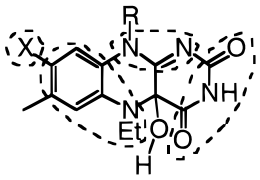
Steady-state kinetics were measured in a stopped-flow instrument by monitoring the disappearance of NADPH at 340 nm as described (*43, 44*). Initial rate measurements were initiated by mixing either anaerobic or air-saturated buffer containing 1 μM PHBH against various test solutions containing 10−320 μM *p*-OHB, 10−320 μM NADPH, and 30−738 μM O_2 in KP_i buffer in the presence of the corresponding *free*-FAD (1 μM).

Transient kinetics studies of the oxidative half-reaction were initiated by mixing reduced enzyme with a buffered solution containing O_2 (final concentrations of 0.062−0.98 mM) as described (*33, 34*). Anaerobic enzyme (∼30 μM) in a tonometer (alone or with substrate present) was reduced by one of three methods, depending on which FAD derivative was used. PHBH reconstituted with 8-H-, 8-mercapto-, 8-SCH_3-, and 8-$N(CH_3)_2$-FAD in solutions containing xanthine (0.18 mM) and benzyl viologen (20 μM) were reduced by adding xanthine oxidase (∼40 nM final) from a sidearm of the tonometer. Solutions of PHBH substituted with 8-Cl- and 8-CN-FAD containing 0.4−1.0 mM glucose-6-phosphate and 5 units of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* were reduced by adding 6 μM NAD^+ from the sidearm of the tonometer. PHBH reconstituted with 8-CH_3-, 8-hydroxy-, and 8-NH_2-FAD were reduced by titrating the enzyme with small aliquots of 6 mM sodium dithionite in 60 mM KP_i buffer (pH 7.0). Rate constants for the formation and decay of the transient intermediates were calculated from absorbance traces recorded at appropriate isosbestic wavelengths and by fluorescence emission traces (515 nm cut off filter) resulting from excitation at 400 nm. Rate constants were calculated from exponential fits by a procedure reported previously (*42*), using the programs KISS (Kinetic Instruments) and Program A which use the Marquardt algorithm (*45*). Spectra for the chemical intermediates were obtained from reaction traces recorded at multiple wavelengths 5−10 nm apart of the reaction of reduced, substrate-saturated enzyme with 0.65 mM oxygen. The SpecFit program, which fits data using a global least-squares method by factor analysis and Marquardt minimization (developed by R. A. Binstead and A. D. Zuberbuhler, Spectrum Software Associates), was used to obtain spectra from the data collected.

Simulation of the Oxygen Reaction for 8-Cl-FAD-Reconstituted PHBH. Program A, which uses a fourth-order Runge−Kutta numerical integration algorithm (*45*), was used for simulation analyses. Molar absorption coefficients for the reduced and oxidized enzyme, as well as for the flavin-C4a-hydroxide form of the enzyme, were obtained from the SpecFit analysis of the oxidation reaction as described earlier, while that for the flavin-C4a-hydroperoxide at 390 nm was previously determined for the native enzyme (8.9 mM^{-1} cm^{-1}) (*44*).

Calculation of the pK_a of the 8-Substituted Flavin-C4a-hydroperoxide. Due to the transient nature of the FADHOH species, the pK_a values of the C4a-OH group on the different flavins could not be determined experimentally, but were calculated as described by Bruice (*46*−*49*), with a slight modification—the 8-substituent was included in the calculation.[2] In this method, the isoalloxazine ring for the lumiflavin derivative was divided into four small domains (Scheme 3) so that the C4a-OH represents a substituted alcohol as described by Fox and Jencks (*50*). The domains X, Ph-N-Et, H_2NCO, HCONHCO, and C−OH were used to model the 8-X-FlEt-4a-OH, where X represents the 8-substituent.

Scheme 3



$$\Delta pK_a = \rho_I \sigma_I \quad (1)$$

$$pK_{a_{(calc)}} = pK_{a_{(MeOH)}} + \sum \Delta pK_a \quad (2)$$

The substituent constants used for the inductive effect (σ_I) were the following: σ_I(Ph-N-Et) = 0.17 (calculated from *51*); $\sigma_I(H_2NCO)$ = 0.27 (*52*); σ_I(HCONHCO) = 0.31 (calculated from *52*); σ_I values for the substituents at the 8-position (*10*) are listed in Table 2. The pK_a value used for MeOH was 15.5 (*53*), and the reactivity constant, ρ_I, for the ionization of MeOH was −8.2 (*50*). Equation 1 was used to determine the contribution of each small domain to the pK_a for C4a-OH. The calculated pK_a for the 8-substituted C4a-flavin-alkoxide was then obtained by substituting in eq 2.

RESULTS

Properties of PHBH Reconstituted with 8-Substituted Flavins. The molar extinction coefficients at λ_{max} of the UV−vis peaks of PHBH that had been reconstituted with 8-substituted flavins or with native FAD (*1*) are all about 1000 M^{-1} cm^{-1} less than those for the corresponding free flavins. The dissociation constants of PHBH for *p*-OHB (Table 1) range from 9.5 μM (native FAD) to 100 μM (8-CN-FAD), indicating that no major changes in binding properties occur. On binding to native PHBH, the pK_a of *p*-OHB is decreased from 9.3 to about 7.4 (*34*), which helps to activate it for hydroxylation. With the substituted flavins (except for those with negative charges), similar effects are observed. For example, with 8-Cl-FAD, the pK_a for bound *p*-OHB is lowered to 7.6. This observation of the similar lowering of the pK_a of the bound substrate, along with the data showing similar dissociation constants, indicates that the substituents on the flavins do not greatly affect the interaction of the enzyme with substrate.

Crystallographic studies with the native enzyme have shown that binding of 2,4-DOHB to oxidized PHBH causes

[2] The method used to estimate the pK_a values for the 8-substituted FADHOH is likely to be more reliable when the substituent X is directly attached to the carbon that contains the hydroxy group. Since the substituent X is not directly connected to this carbon, the pK_a values of the 8-substituted FADHOH may be affected less than the values we have calculated. This would lead to a greater slope; i.e., β_{lg} would be more negative.

Table 1: Thermodynamic Constants for Free and PHBH-Bound 8-Substituted Flavins

			$E^{o\prime}$(bound) (mV)[a]					
				+*p*-OHB			K_d(*p*-OHB) (μM)[c]	
	8-substituent	$E^{o\prime}$(free) (mV)	−*p*-OHB	−NaN_3	+NaN_3	% hydrox[b]	E_{ox}	E_{red}
1	CN	−50	+37	+60	+13	100	100	−
2	Cl	−152	−83	−81	−128	100	30	−
3	H	−180	−154	−151	−166	100	81	−
4	SCH_3	−204	−153	−152	−183	100	19	−
5	CH_3[d]	−207	−163	−165	−189	100	9.5	21
6	$N(CH_3)_2$	−254	−201	−210	−243	95	17	−
7	S^-	−290	−259	−267	−270	100	16	−
8	NH_2	−330	−292	−298	−305	87	31	89
9	O^-	−334	−304	−308	−307	99	31	77

[a] Redox potentials were determined in 50 mM KP_i buffer (pH 7.0) at 25 °C using the method from reference *40*. Individual measurements for the given flavin varied ≤4 mV. [b] Hydroxylation stoichiometry was determined by measuring NADPH consumed from 50 μM *p*-OHB in 100 mM Tris−SO_4 buffer (pH 8.0) at 25 °C. Individual measurements for the given flavin varied ≤2%. [c] Dissociation constants for *p*-OHB were determined from static titrations in 50 mM KP_i buffer (pH 6.5) at 4 °C. Individual measurements for the given flavin varied ≤1 μM. [d] Values taken from references *1* and *56*.

Table 2: Substituent Constants

	8-substituent	σ_p[a]	σ_I[a]	pK_a(FADHOH)[b]	pK_a(*p*-X−aniline)[c]
1	CN	0.66	0.73	4.76	1.74
2	Cl	0.23	0.46	5.58	4.15
3	H	0.0	0.0	9.36	4.60
4	SCH_3	0.0	0.23	7.47	4.35
5	CH_3	−0.17	−0.04	9.68	5.08
6	$N(CH_3)_2$	−0.83	0.06	8.86	6.59
7	S^-	−1.21	−0.12[d]	10.34	−
8	NH_2	−0.66	0.12	8.37	6.20
9	O^-	−0.81	−0.12	10.34	5.29

[a] Values taken from reference *10*. [b] Calculated using method from reference *46*. [c] Values taken from reference *63*. [d] Assumed to be similar to that for $-O^-$ since σ_I values for the protonated forms, −SH and −OH, are 0.26 and 0.29, respectively.

the flavin to swing out from the active site to where it has more contact with solvent. In contrast, when *p*-OHB binds to the enzyme, the flavin remains in the site where hydroxylation occurs (*54*−*57*). These two positions of the flavin can be monitored by characteristic changes in the spectrum of the flavin that occur on binding substrates (*54*−*57*). When PHBH was reconstituted with FAD analogues that have similar spectra to those of normal FAD, the binding of *p*-OHB or 2,4-DOHB generally caused spectral perturbations that are consistent with those of FAD. Several of the 8-substituted flavins [-X = mercapto, hydroxy, SCH_3, NH_2, and $N(CH_3)_2$] have significantly different spectral properties from those of FAD, so that it was difficult to assign the perturbations to a particular flavin position. However, the perturbations caused by binding *p*-OHB were different than those caused by binding 2,4-DOHB. Furthermore, crystallographic studies with PHBH reconstituted with 8-$N(CH_3)_2$-FAD in the presence of *p*-OHB have shown this flavin (the largest FAD analogue used in this study) to be in the in-conformation as in native enzyme (D. Gatti and M. Ortiz-Maldonado, unpublished results). It seems reasonable to suggest that the different spectral perturbations caused by 2,4-DOHB with these analogues were due to the flavin moving to the out position.

The hydroxylation of *p*-OHB by PHBH reconstituted with any of the 8-substituted flavins was essentially fully coupled; 87−100% of the enzyme-bound *p*-OHB was converted to the product, 3,4-DOHB (Table 1). In addition, the characteristic intermediates previously reported, both in reduction by NADPH and in the reaction of the reduced form with oxygen (*1*), were also observed with all of the 8-substituted flavins (when the rate constants for the various conversions permitted their detection, see below).

The redox potential of FAD can be modulated not only by substituent groups on the isoalloxazine moiety, especially on the benzene ring (*58*, *59*), but also by the protein environment. If the various substituents in the 8-position do not produce significantly different solvation, charge, steric, and conformational effects to the overall binding of the flavin to the apoprotein, the shifts in redox potentials that occur on binding each of the substituted flavins should be predictable by a simple function. Table 1 presents the relevant $E^{o\prime}$ values, and a linear relationship between $E^{o\prime}$(free) and $E^{o\prime}$(bound) is found with a slope of 1.2 ($R = 0.99$). For each of the flavins, the redox potential of the bound FAD is higher than for the free FAD due to the environment provided by the protein. The linear relationship between $E^{o\prime}$(free) and $E^{o\prime}$(bound) is consistent with the crystal structure, which shows that the 8-position is exposed to solvent and has minimal interaction with the protein.

Enzyme that had been reconstituted with substituted flavins generally exhibited simple two-electron reduction reactions (no observable semiquinone) during redox titrations, either in the presence or in the absence of substrate; the binding of substrate had very little effect on the redox potentials (Table 1). However, PHBH with 8-CN-FAD behaved differently; the redox potential increased from +37 mV when no substrate was present to +60 mV in the presence of *p*-OHB. In the presence of 0.1 M N_3^-, the redox potentials of the 8-substituted flavoenzymes in complex with *p*-OHB are decreased in a systematic fashion, and a linear relationship is observed between $E^{o\prime}$(free) and $E^{o\prime}$(bound) with a slope of 1.1 ($R = 0.99$) (Table 1). The binding of azide imparts negative charge to the protein, making reduction of the flavin more difficult.

Steady-State Kinetics. We carried out a steady-state analysis for the 8-Cl-FAD-substituted PHBH enzyme (as an example) to ascertain whether any significant mechanistic changes occurred with the altered flavins. Double reciprocal plots of the initial velocities for each substrate combination show intersecting lines when the concentrations of *p*-OHB and NADPH were varied at a constant oxygen concentration

Scheme 4

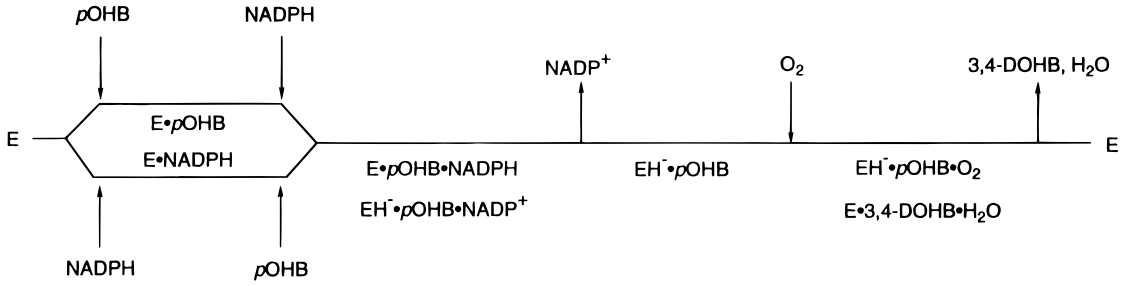


Table 3: Steady-State Kinetic Constants[a] for Native and 8-Cl-FAD PHBH

		K_m (μM)		
8-substituent	k_{cat} (s^{-1})	*p*-OHB	NADPH	O_2
CH_3	6.9	10.9	23	37
Cl	11	14	23	150

[a] Steady-state kinetic constants were determined by initial rate measurements in the stopped-flow instrument using 1 μM enzyme in the presence of 1 μM of the corresponding FAD in either anaerobic or air-saturated solutions mixed against solutions containing a wide range of *p*-OHB, NADPH, and O_2. 50 mM KP_i buffer (pH 6.5) at 4 °C was used for all solutions. Values of steady-state kinetic constants were the average of at least four measurements that varied 2−6% from one another.

of 738 μM. On the other hand, when the concentrations of oxygen and *p*-OHB were varied at a constant NADPH concentration, as well as when the concentration of *p*-OHB was held constant and oxygen and NADPH levels were varied, double reciprocal plots of initial rate data yielded parallel lines. Replots of slopes and intercepts in all cases were linear. The mechanism of reaction of PHBH reconstituted with 8-Cl-FAD is random bi uni uni uni ping pong as previously reported for native PHBH (*43*), and the mechanism is represented in Scheme 4. Thus, the order of addition of substrates and release of products remains unchanged by incorporating the 8-substituted flavin, although the magnitude of the constants changes slightly (Table 3).

The turnover number for native PHBH at 4 °C and pH 6.5 is 6.9 s^{-1} as measured either by steady-state kinetics or by oxygen consumption during enzyme-monitored turnover (*44*). By changing the methyl group of the flavin at the 8-position to an electron-withdrawing Cl group, the steady-state maximum turnover number increases slightly to 11.0 s^{-1} (Table 3).

General Description of the Oxidative Half-Reaction. The reactions of oxygen with PHBH containing reduced forms of 8-substituted flavins were measured in the absence of substrate. All of the substituted reduced enzymes convert in a single phase to oxidized enzyme with concomitant formation of H_2O_2. Plots of the observed rates of oxidation vs oxygen concentration are linear, and the second-order rate constants (k_{4a}) for the modified enzymes depend on the substituent at the 8-position of the FAD (Table 4). The putative flavin-C4a-hydroperoxide formed without substrate present (*1*) decays quickly, so that none is detected in these experiments.

In the presence of *p*-OHB, the reactions with oxygen proceed about 10-fold faster than without substrate (analogously to WT). For all of the FAD analogues, except the cyano- and Cl- derivatives, this reaction results in the formation of observable C4a-hydroperoxides, consistent with previous studies of native PHBH (*1*, *44*). The kinetics can be described by one phase (for 8-CN-FAD), two phases (for 8-Cl-FAD), or three phases (for the remaining flavin analogues tested) (Figure 1), indicating, respectively, the observation of no chemical intermediates, the detection of one intermediate, or the detection of two intermediates. By comparison, native PHBH exhibits three phases (*1*). Ex-

Table 4: Rate Constants[a] for the Reaction of Oxygen with Reduced Forms of 8-Substituted FAD-PHBH, with and without *p*-OHB and 0.1 M Sodium Azide

			+*p*-OHB					
			−NaN_3			+NaN_3		
	8-substituent	k_{4a} (M^{-1} s^{-1})	k_4 (M^{-1} s^{-1})	k_5 (s^{-1})	k_7 (s^{-1})	k_4 (M^{-1} s^{-1})	k_5 (s^{-1})	k_7 (s^{-1})
1	CN	5.9×10^1	3.4×10^2	nd[c]	nd[e]	3.8×10^2	nd	nd
2	Cl	2.4×10^3	3.2×10^4	≥300[d]	45	4.0×10^4	18.5	5.0
3	H	1.3×10^4	1.4×10^5	40	7.84	1.5×10^5	6.54	1.53
4	SCH_3	1.3×10^4	1.4×10^5	151	41	1.0×10^5	13.2	4.2
5	CH_3[b]	2.6×10^4	2.6×10^5	48	14.5	2.7×10^5	6.5	1.5
6	$N(CH_3)_2$	1.9×10^4	1.2×10^5	34	22	1.2×10^5	4.7	3.6
7	S^-	8.2×10^3	1.2×10^5	14.3	5.65	5.8×10^4	4.0	nd
8	NH_2	3.4×10^4	3.6×10^5	156	34.1	2.5×10^5	5.6	nd
9	O^-	2.0×10^4	4.2×10^5	7.6	nd	3.4×10^5	3.9	nd

[a] Values of rate constants were the average of at least four determinations. Rate constants varied ≤5% from one another. Definition of the rate constants: See Scheme 1 for all except k_{4a}:

$$E_{red} + O_2 \xrightarrow{k_{4a}} I \xrightarrow{fast} E_{ox}$$

[b] Values taken from references *1* and *33*. [c] Expected to be ∼3000 s^{-1} based in Figure 4. However, the estimation of this rate constant is limited by the formation of the FADHOOH intermediate (k_4 = 0.33 s^{-1} at 0.98 mM oxygen, final concentration); thus, k_5 should be ≥3.3 s^{-1}. [d] Value from simulation experiments. [e] nd = not detected.

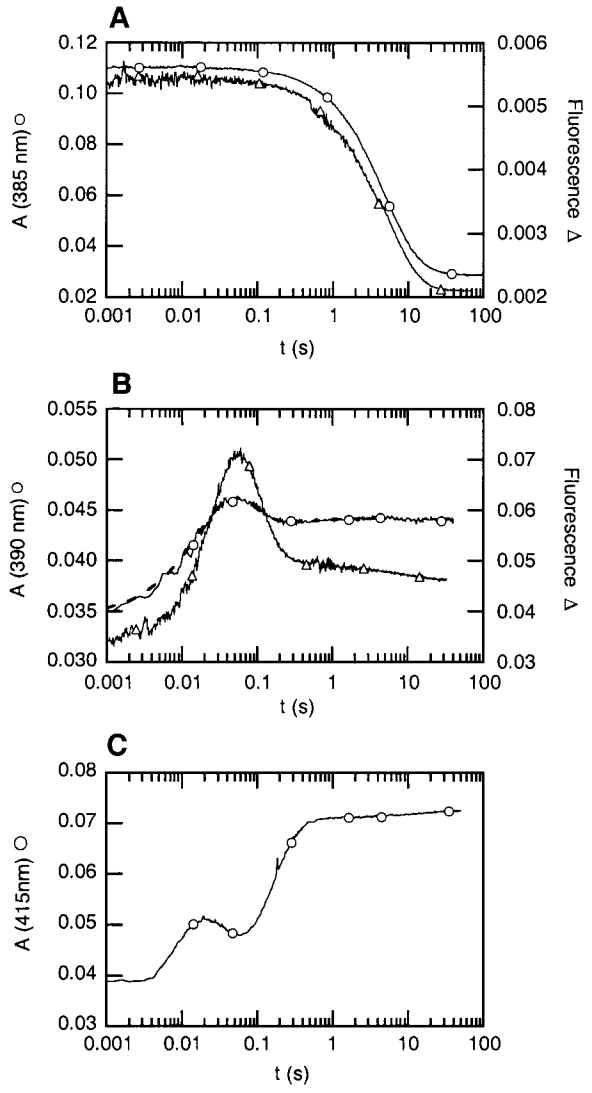


FIGURE 1: Kinetic traces from the oxidative half-reaction of (A) 8-CN-, (B) 8-Cl-, and (C) 8-H-FAD PHBH in the presence of *p*-OHB. Reduced PHBH (15 μM) with bound *p*-OHB (0.5 mM) in 50 mM KP_i (pH 6.5) was reacted with 0.62 mM oxygen at 4 °C in the same buffer solution using a stopped-flow spectrophotometer (concentrations after mixing). Absorbance (○) at indicated wavelengths and fluorescence (△) with $\lambda_{ex} = 400$ nm and $\lambda_{ex} > 510$ nm are shown. Note that in contrast to most flavins the reduced form of the 8-CN-FAD-substituted PHBH is more fluorescent when excited at 400 nm than is the oxidized form. The reaction of 8-Cl-FAD PHBH (panel B) was simulated at 390, 440, and 470 nm [the 390 nm trace is shown: experimental kinetic trace (—), simulated data (- - -)]. The rate constants used in the simulations (constants are numbered to be consistent with Scheme 1) for the reaction of 8-Cl-FAD-enzyme were $k_4 = 3.2 \times 10^4$ M^{-1} s^{-1} (the reaction with oxygen) and $k_7 = 45$ s^{-1} (dehydration of the pseudobase). Values for k_5 were varied between 50 and 3000 s^{-1}. The extinction coefficients used in the simulation reaction were obtained from reference *44* for normal enzyme: ϵ_{390} (mM^{-1} cm^{-1}) = 5.44 (E_{red}), 8.90 (I), 8.15 (III), 6.19 (E_{ox}); ϵ_{440} (mM^{-1} cm^{-1}) = 1.85 (E_{red}), 0.50 (I), 0.50 (III), 10.00 (E_{ox}); ϵ_{470} (mM^{-1} cm^{-1}) = 0.55 (E_{red}), 0.01 (I), 0.01 (III), 8.40 (E_{ox}). To enable better fitting, the slow extra phase occurring between 0.2 and 10 s, indicating the association of *p*-OHB to intermediate III, was also included in the simulation reaction: $k_{as} = 1.95$ s^{-1}; $\epsilon_{390} = 6.24$ mM^{-1} cm^{-1}, $\epsilon_{440} = 10.28$ mM^{-1} cm^{-1} and $\epsilon_{470} = 8.60$ mM^{-1} cm^{-1}.

amples of spectra calculated from the absorbance data recorded at individual wavelengths are shown in Figure 2. For most of the FAD analogues, an additional slow phase involving a small fraction (<5%) of the enzyme is observed.

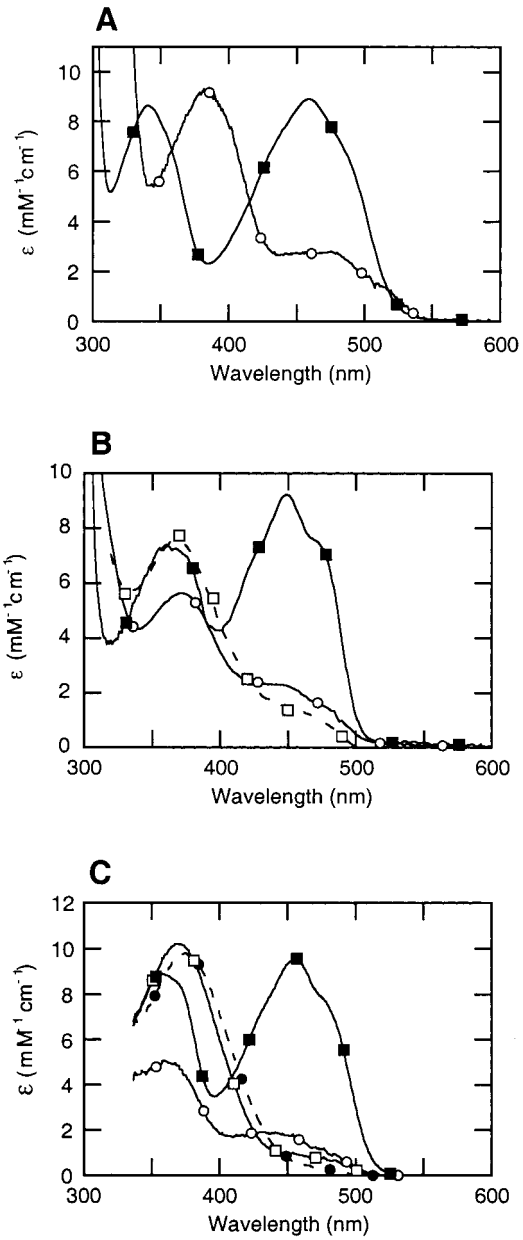


FIGURE 2: Spectra of the chemical species detected during the oxidative half-reaction with various 8-substituted flavins. (A) 8-CN-, (B) 8-Cl-, and (C) 8-H-FAD PHBH in the presence of *p*-OHB: (○) reduced 8-X-FAD PHBH·*p*-OHB complex, (●) 8-X-flavin-C4a-hydroperoxide, (□) 8-X-flavin-C4a-hydroxide, and (■) oxidized 8-X-FAD·*p*-OHB complex. Reaction conditions are the same as in Figure 1.

At higher concentrations of substrate, this fraction increased, indicating that it was due to nonproductive binding of *p*-OHB to the FADHOH intermediate. This phenomenon has also been observed with phenol hydroxylase (*60*). With 8-mercapto-FAD or 8-hydroxy-FAD bound to PHBH, a prominent slow phase is observed, but for these, it is insensitive to changes in either substrate concentration or pH. It is not known what causes this phase with these negatively charged substituents.

Formation of the Flavin-C4a-hydroperoxide (k_4, Scheme 1). The reactions of the 8-substituted FAD enzymes with oxygen were examined both without substrate (k_{4a}) and in the presence of saturating concentrations of *p*-OHB (0.5−1 mM) (k_4). The second-order rate constants for these reactions (Table 4) follow a trend with dependence on the electronic

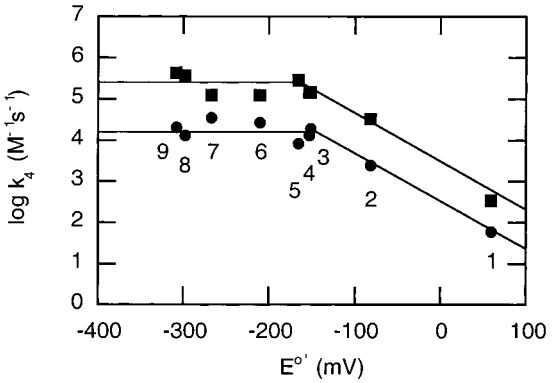


FIGURE 3: Free energy relationship of the bimolecular reaction of reduced enzyme with oxygen. The log of the second-order rate constants for the oxygen reaction of PHBH reconstituted with 8-substituted FAD species in the absence (●) or presence (■) of *p*-OHB are plotted vs the $E^{o\prime}$ for the 8-substituted FAD bound to PHBH in the absence or presence of *p*-OHB, respectively. See Table 1 for identification of the flavins.

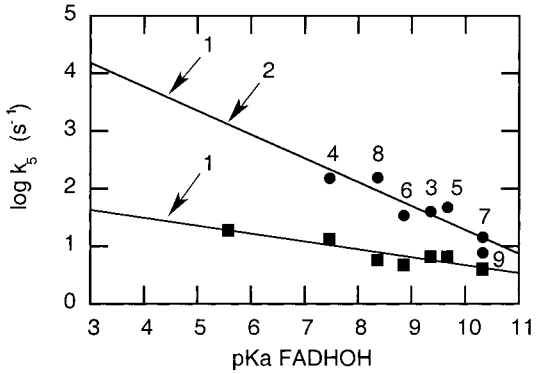


FIGURE 4: Hydroxylation rate constants (k_5) as a function of the pK_a of the 8-substituted flavin-C4a-hydroxide. The pK_a values of the nascent 8-X-flavin-C4a-hydroxide were calculated as described under Experimental Procedures. The log of the first-order rate constants (k_5) for the hydroxylation reaction of *p*-OHB by 8-substituted FADHOOH species, in the absence (●) or presence of 0.1 M NaN_3 (■). The arrows denote predicted values of the rate constants. See Table 1 for identification of the flavins.

characteristics of the 8-substituent, such as the redox potential, as shown in Figure 3. The same general trends are observed both in the presence and in the absence of *p*-OHB, although the rate constants without substrate are about 10-fold smaller (Figure 3). Electron-withdrawing groups decrease the reactivity with oxygen. For example, the 8-CN group decreases the reactivity with oxygen by ~1000-fold relative to native PHBH, and this step is rate limiting in the hydroxylation reaction. As with native enzyme, azide had very little effect on the rates of the reaction of the reduced 8-substituted enzymes with oxygen, and plots of *k* vs $E^{o\prime}$ are similar to that of Figure 3.

Hydroxylation Reaction (k_5, Scheme 1). Based on previous experimental observations with PHBH (*2*, *3*), the hydroxylation reaction has been suggested to proceed by electrophilic aromatic substitution. Bruice and colleagues have shown that the log *k* values for the rates of electrophilic oxygenations by hydroperoxides are linearly related to the pK_a of the leaving group, which is the alkoxide (*46*−*49*). Included in their studies was N5-ethyl-C4a-hydroperoxide. Their work prompted us to carry out the experiments described below that use a series of substituted flavins in a defined protein environment. Thus, if the reaction proceeds via an electrophilic substitution mechanism, an electron-withdrawing group will lower the pK_a of the flavin-C4a-hydroxide, making it a better leaving group, which should increase the hydroxylation rate. The transient nature of the various FADHOH species prevented the direct determination of their pK_a values. Therefore, pK_a values were calculated using empirical methods based on σ_I values of the substituents (*10*, *46*−*53*) as described under Experimental Procedures. The rates of hydroxylation of *p*-OHB effected by PHBH that had been reconstituted with a series of 8-substituted flavins were obtained from stopped-flow kinetic traces as previously described (*34*). The data for hydroxylation (k_5 in Table 4) are plotted logarithmically vs the calculated pK_a values of the substituted hydroxyflavins (Figure 4). The linear relationship demonstrates the importance of the leaving group in this reaction. Electron-donating groups at the 8-position of the flavin increase the pK_a of the flavin-C4a-hydroxide, which decreases the rate of hydroxylation.

The linear plot of log k_5 vs the calculated pK_a for the free 8-substituted FADHOH species (eq 3) in Figure 4 has a slope (β_{lg}) of −0.42 ($R = 0.90$ and $F = 21.21$).

$$\log k_5 = \beta_{lg} pK_a + C \quad (3)$$

This β_{lg} value is an indication of charge development on the leaving group in the transition state, which is typically taken as a measure of the degree of bond breaking in the transition state. Although the pK_a values plotted are those calculated for the free flavins rather that those of the bound flavins, any change in the pK_a values on binding to the enzyme can be expected to be very nearly the same for each FADHOH analogue as observed in other systems (*61*). Note, for example, that the redox potentials of this same series of flavins are affected in a constant fashion (Table 1). Thus, the β_{lg} value (slope), which reflects the sensitivity of the reaction to the pK_a of the leaving group, should not be significantly affected.

Hydroxylation steps involving 8-Cl- and 8-CN-FADHOOH were obscured because formation of the FADHOOH for these analogues was the slowest step in the oxidative half-reaction (21.4 and 0.33 s^{-1}, respectively), even at the highest concentration of O_2 used (0.98 mM). The hydroxylation rate constants for 8-Cl-FAD or for 8-CN-FAD PHBH extrapolated from Figure 4 (arrows) are ≥1000 and ≥3000 s^{-1}, respectively. These steps are too fast to measure by normal stopped-flow methods and, because of the slow reaction with oxygen, prevent the observation of any flavin-C4a-hydroperoxide. Thus, with the 8-Cl-FAD analogue, the first observable intermediate was the C4a-hydroxide, which dehydrated at 45 s^{-1}. With 8-CN-FAD, no intermediates are observed in the reaction with oxygen, because the formation of the hydroperoxide is slow and, by comparison, both the hydroxylation and dehydration steps are very fast. Thus, only reduced and oxidized forms of 8-CN-FAD PHBH are seen (Figure 2A).

The reaction of oxygen (0.62 mM) with reduced 8-Cl-FAD PHBH in the presence of *p*-OHB was studied at several wavelengths between 300 and 500 nm. The SpecFit program was used to derive spectra of intermediates, and a spectrum typical of a flavin-C4a-hydroxide intermediate was obtained

Table 5: Rate Constants[a] for the Reaction of Oxygen with Reduced Forms of Native and 8-CN-FAD-PHBH Determined in the Presence of 2,4-DOHB

	−NaN_3				+NaN_3			
8-substituent	k_4 (M^{-1} s^{-1})	k_5 (s^{-1})[c]	k_6 (s^{-1})	k_7 (s^{-1})	k_4 (M^{-1} s^{-1})	k_5 (s^{-1})[c]	k_6 (s^{-1})	k_7 (s^{-1})
CN	1×10^4	nd[d]	0.50	0.151	5.9×10^3	10.9	0.22	0.0011
CH_3[b]	5.9×10^5	16.3	0.7	0.124	2.9×10^5	5.6	0.032	0.0009

[a] Values of rate constants were the average of at least four determinations. Rate constants varied ≤5% from one another. [b] Values in the absence of NaN_3 are calculated from reference *44*, whereas those in the presence of NaN_3 are those from PHBH isolated from *P. fluorescens* (*1*). PHBH isolated from *P. aeruginosa* and *P. fluorescens* behaves very similarly. [c] The flavin-C4a-hydroperoxide decayed by two divergent pathways when 2,4-DOHB was the substrate (76−80% formed hydroxylated product and 20−24% eliminated H_2O_2). [d] nd = not detected.

(Figure 2B). This was expected, because earlier studies had shown that when using 2,4-DOHB as the substrate, the spectra of flavin-C4a-transient intermediates for several different 8-substituted flavins are very similar to those obtained with FAD (*26*). To estimate minimum rates of hydroxylation consistent with these observations, simulations (Figure 1B) of the reaction in Scheme 5 were carried out using the Runge−Kutta algorithm in Program A. Simulations of data observed at wavelengths selected for being sensitive to observation of intermediates were in excellent agreement with the experimental traces when the rate constants for the hydroxylation reaction were ≥300 s^{-1} for 8-Cl-FAD. Because with 8-CN-FAD the formation of the C4a-hydroperoxide is so slow (0.33 s^{-1}), the simulation was not very sensitive to values chosen for k_5 and k_7 (Scheme 5).

Scheme 5

$$E_{red}\cdot p\text{OHB} + O_2 \xrightarrow{k_4} I \xrightarrow{k_5} \text{III} \xrightarrow{k_7} E_{ox}\cdot p\text{OHB}$$

The hydroxylation step (k_5) for 8-Cl-FAD PHBH could be detected in the presence of 0.1 M NaN_3, but even with azide present, could not be observed for 8-CN-FAD PHBH using either absorbance or fluorescence detection. As with native enzyme (*1*), NaN_3 decreases the rate constants for the formation and the decay of intermediate III for 8-Cl-FAD, in this case to values of 18.5 and 5.0 s^{-1}, respectively (see Table 4), whereas the second-order rate constant for the reaction with oxygen is hardly affected. PHBH reconstituted with each of the flavin analogues was studied in the presence of 0.1 M NaN_3, and the results for hydroxylation are also plotted in Figure 4. It can be seen that azide decreases the rate of hydroxylation for PHBH that had been reconstituted with FAD analogues, and the smaller β_{lg} value (−0.14 vs −0.42 without azide; $R = 0.91$ and $F = 30.34$) implies that azide makes the hydroxylation less sensitive to the pK_a of the leaving group.

Spectra of Transient 8-CN-Flavin-C4a-Oxygenated Intermediates. Intermediates formed in the reactions of reduced 8-substituted FAD PHBH with oxygen are shown both here and in references *1*, *26*, and *44* to be C4a-adducts, just as with native FAD. We reasoned that our inability to resolve such intermediates with 8-CN-FAD-substituted PHBH could be attributed to two possible conditions: (a) the spectral properties of the reduced form of this flavin were too similar to those of C4a-adducts; or (b) the kinetics prevented the accumulation of sufficient intermediate. Figure 2 shows that the spectrum of the reduced form of 8-CN-FAD is similar to that of flavin-C4a-adducts (λ_{max} ∼374 nm), so that it might indeed obscure any C4a-adduct. The fact that PHBH reconstituted with 8-CN-FAD fully hydroxylates the enzyme-bound *p*-OHB implies that the mechanism must nevertheless involve these intermediates. Below we describe studies that characterize the spectra of C4a-adducts of the 8-CN-FAD-substituted PHBH. These studies show that we could discern such intermediates if they formed in sufficient quantity. Thus, because no intermediates were observed with 8-CN-FAD, we can conclude that the rate constants for the decay of 8-CN-FAD-C4a-hydroperoxide and -hydroxide are considerably greater than the rate constants for their formation.

The spectral properties of flavin C4a-adducts in 8-CN-FAD PHBH could be determined directly from studies of the oxidative half-reaction using 2,4-DOHB as substrate. The rate constants in Scheme 1 for this reaction were determined from kinetic traces at 361, 390, 430, and 480 nm, as described in references *33* and *34* both in the absence and in the presence of 0.1 M azide (Table 5). In the absence of azide, the reaction is characterized by three phases, with only one phase being oxygen-dependent: the formation of the hydroperoxide ($k_4 = 1.0 \times 10^4$ M^{-1} s^{-1}). The rate constant for hydroxylation of 2,4-DOHB (k_5) was much greater than the following steps, so that it could not be determined. However, in the presence of 0.1 M azide, four phases were observed. The formation of the flavin-C4a-hydroperoxide was dependent on oxygen ($k_4 = 5.9 \times 10^3$ M^{-1} s^{-1}), and was 5.8 s^{-1} at the highest concentration of oxygen used (0.98 mM). The largest observed rate constant, 13.6 s^{-1}, is oxygen-independent, and correlates with the formation of 80% of intermediate II, as well as 20% each of H_2O_2 and oxidized enzyme. Thus $k_5 = 10.9$ s^{-1} and $k_{elim} = 2.7$ s^{-1}. This observed intermediate spectrum has been shown to be due to the sum of the spectra of the flavin-C4a-hydroxide and the dienone form of the product (*26, 62*). Figure 5A shows the results of an experiment using diode array detection; spectra of intermediates I, II, and III were determined using the rate constants in Table 5 and the multicomponent analysis routines in SpecFit. With native PHBH using the same conditions, the rate of hydroxylation, k_5, is 5.6 s^{-1}. Thus, in the presence of azide and 2,4-DOHB, the hydroxylation rate constant (10.9 s^{-1}) for the 8-CN-FAD enzyme is only about twice that for native enzyme. It is clear that the spectrum of intermediate II could be resolved if k_6 were not much greater than k_5.

The subtraction of the spectrum of 8-CN-intermediate III from that for species II reveals a difference spectrum (Figure 5C) that is consistent with the dienone form of the product, as previously observed with native and several other 8-substituted flavins (*26, 62*). Moreover, when this experiment was repeated at different pH values, the spectra of intermediate II and the calculated difference spectra (Figure 5B,C) are similar to those using native and 8-sulfonyl flavins

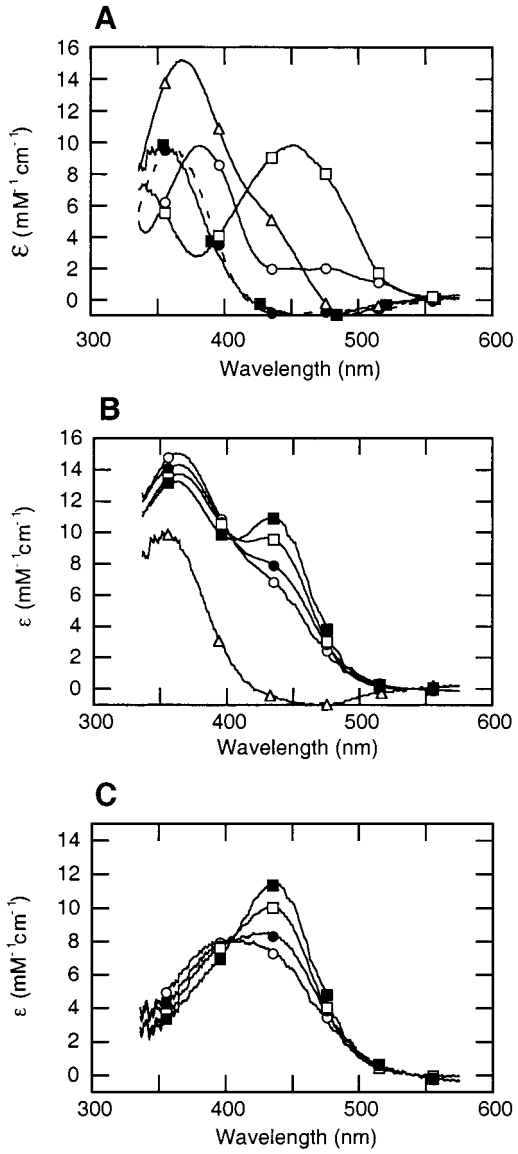


FIGURE 5: Spectra of the chemical species detected during the oxidative half-reaction of 8-CN-FAD PHBH in the presence of 2,4-DOHB and sodium azide. (A) Chemical species detected at pH 6.5 and 4 °C: (○) reduced 8-CN-FAD PHBH·2,4-DOHB complex, (●) 8-CN-flavin-C4a-hydroperoxide·2,4-DOHB complex (intermediate I), (△) 8-CN-flavin-C4a-hydroxide·nonaromatic 2,3,4-TOHB product complex (intermediate II), (■) 8-CN-flavin-C4a-hydroxide·aromatic 2,3,4-TOHB product complex (intermediate III), and (□) oxidized 8-CN-FAD·2,4-DOHB complex. (B) Spectral properties of intermediate II at pH 6.25 (○), 6.75 (●), 7.29 (□), and 7.85 (■). The spectrum of intermediate III (△) was taken from the experiment at pH 6.5. (C) Difference spectra calculated by subtracting the spectrum of intermediate III at pH 6.5 from spectra of species in panel B [pH 6.25 (○), 6.75 (●), 7.29 (□), and 7.85 (■)].

(*26*, *62*). These spectra show a pH dependence described by a pK_a between 7.1 and 7.5, very similar to that for native enzyme (7.4). Since the pK_a values calculated for the 8-substituted FADHOH analogues range from 4.76 to 10.34, the observed pK_a of intermediate II must be due to the dienone form of the product and not to the 8-X-C4a-FADHOH species.

Dehydration Reaction (k_7, Scheme 1). Following oxygen transfer to the substrate, the resulting enzyme-bound FADHOH intermediate loses water to regenerate oxidized FAD. The rate of dehydration for the FADHOH species might be expected to be dependent on the pK_a of its N5-proton, unless this step is controlled by a protein conformational change. Because the pK_a of the N5-proton in FADHOH is not measurable, due to the limited stability of the FADHOH intermediate, we chose *p*-substituted anilines as models for how 8-substituents might affect the pK_a of the flavins. The dehydration rate constants (k_7, Table 4) have no correlation with the pK_a of *p*-substituted anilines (Table 2). The presence of azide slows the dehydration step, but, again, there is no clear correlation with the predicted pK_a values of the N5-proton.

DISCUSSION

The mechanisms involved in the oxidative half-reaction of PHBH, with special attention to the hydroxylation step, have been probed by replacing the natural flavin with various 8-substituted flavins. The various substituents at the 8-position (normally 8-methyl) did not change enzyme conformation, substrate binding, hydroxylation stoichiometry, or the overall mechanism of catalysis. This is likely because the 8-position of the FAD is solvent accessible, away from protein contacts and distant from the binding site of *p*-OHB, so that it does not disrupt protein function. Thus, the 8-substituents primarily have electronic effects on the FAD, while their specific interactions with the protein are minimal. The substitutions at the 8-position of the flavin in PHBH have affected the reactivity of the enzyme in each step of the oxidative half-reaction. Depending on the flavin analogue used, the chemical steps in catalysis either are faster or are slower than in native PHBH.

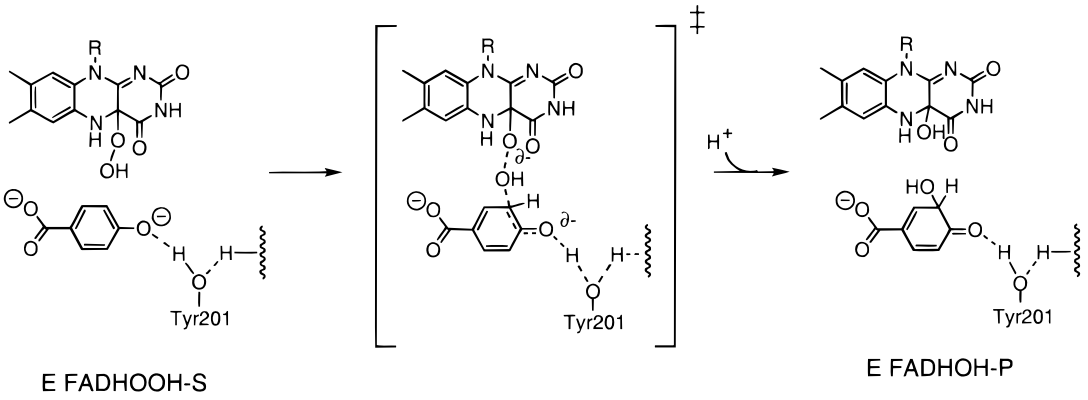
The redox potentials of the free substituted flavins are shifted to more positive values upon binding to PHBH, implying that the reduced forms of FAD bind tighter to the enzyme than do the oxidized forms. The reduced forms of flavins on enzymes are primarily anionic (N1 is ionized) (*64*) and would be attracted to electrostatically positive enzyme active sites such as found with PHBH (*56*), resulting from the presence of a positive dipole located at the end of α-helix H10, comprising residues 296−320, and several positively charged residues in the vicinity of the active site.

Although the hydroxylation step for PHBH has been well studied, its mechanism has generated considerable controversy and speculation. As expected from previous studies (*2*, *3*, *26*) and supported by this work, the hydroxylation reaction follows an electrophilic aromatic substitution mechanism. In an electrophilic aromatic substitution mechanism, the reaction should be driven partly by the reactivity of the electrophile, in this case the FADHOOH intermediate. For the flavin-C4a-hydroperoxide, the electrophilicity is largely determined by the pK_a of the leaving group, the flavin-C4a-alkoxide ($FADHO^-$) intermediate (*49*). Figure 4 clearly illustrates this principle. The contributions of the substituents to the pK_a values for 8-X-FADHOH are proportional to the ρ_I parameter as shown in eq 1. Another way of looking at the contribution of the substituent in the reaction is the extent of polarization of the oxygen−oxygen bond. This inductive polarization is also related to the Hammett ρ_I, and its relationship to the hydroxylation rate is given in eq 4.

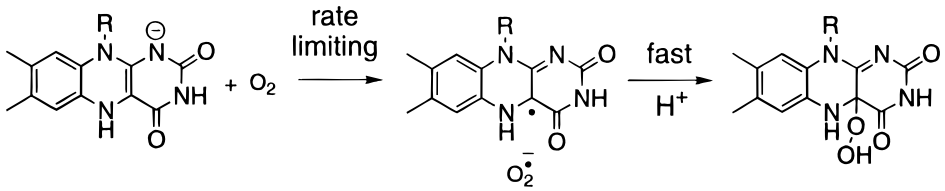
$$\log k_5 = \rho_I \sigma_I + C \qquad (4)$$

The inductive characteristic of the substituent is one of the most important electronic contributions to electrophilicity.

Scheme 6



Scheme 7



A Hammett plot (eq 4) of the data is also linear with a slope $\rho_I = 3.4$ (data not shown). Such large values of ρ_I are typical for electrophilic aromatic substitution reactions (*9*). If a diradical mechanism were involved, the ρ_I value would be <1 (*9*), emphasizing the modest effect substituents have on the stabilization of radicals. Thus, we conclude that the reaction proceeds by an electrophilic aromatic substitution rather than via a diradical mechanism. As determined by free energy correlations shown in Figure 4, the oxygen on the $FADHO^-$ leaving group contains a −0.42 charge (δ^- in Scheme 6) in the transition state, indicating a transition state nearly midway between the reactants and products, which is consistent with the flavin-C4a-hydroperoxide being a moderately reactive electrophile (*65*). A value of −1 in the leaving group would represent a product-like transition state. In solution, highly reactive electrophiles exhibit low activation energies and low substrate selectivity. In electrophilic substitution reactions, the characteristics of the aromatic substrate (charge density and the energy coefficients of the highest occupied molecular orbital, HOMO) determine the position of electrophilic attack (*65*). Vervoort et al. (*66*) and van der Bolt et al. (*67*) have calculated that electrophilic attack at the C3 position of the aromatic substrates *p*-OHB and F_4-*p*OHB is indeed guided by the distribution of the π-electrons in the HOMO. Although FADHOOH is a good electrophile, it can only react efficiently with activated, but not with deactivated, aromatic rings. The electronegativity of the ring nitrogens of FAD at positions 1, 5, and 10, and the electron-deficient carbonyl at position 4, as well as essential protein−FAD interactions all contribute to the electrophilicity of the C4a-position.

The findings reported here support an electrophilic aromatic substitution mechanism for PHBH in which the transfer of oxygen from FADHOOH is governed by the ability of the transition state to support a negative charge on the oxygen of the leaving group, the flavin-C4a-alkoxide ($FADHO^-$) (Scheme 6). These results agree with the important concepts developed by Bruice and co-workers (*46*−*49*), who showed that the rates of mono-oxygen transfer in reactions such as the sulfoxidation of thioxane, the *N*-oxidation of *N*,*N*-dimethylbenzylamine, and the oxidation of I^- to I_2 in solution are related to the pK_a of the alkoxide leaving group derived from the electrophilic hydroperoxide, R-OOH.

The hydroxylation rate constant for each of the reconstituted enzymes was decreased in the presence of azide. The binding of azide near the hydroxylation site might be expected to decrease the effective positive charge or electrostatic potential of the active site that normally stabilizes the transition state of the hydroxylation reaction. In addition, azide would raise the pK_a of the substrate phenolic group, so that the character of the nucleophile would be different than in the absence of azide. The protonated substrate would be a poor nucleophile so that the reaction would have an early transition state leading to a decrease in slope (Figure 4).

Bruice and colleagues have proposed that the mechanism for the reaction of oxygen with reduced enzyme-bound flavin consists of the formation of the superoxide−semiquinone pair, followed by collapse of the radical pair to form the C4a−O bond of FADHOOH (*68*, *69*) (Scheme 7). The formation of the superoxide−semiquinone pair is believed to be the rate-limiting step during the bimolecular reaction with oxygen. Thus, the rate of the reaction with oxygen is expected to correlate with the one-electron redox potential of the enzyme-bound flavin (Fl_{SQ}/Fl_{red}). However, this potential cannot be determined experimentally with PHBH because the enzyme does not stabilize the flavin semiquinone. In PHBH, no intermediates are observed prior to the formation of FADHOOH because the collapse of the radical pair is presumably too fast to be detected. Since the one-electron redox potentials of a series of 8-substituted flavins have been shown to be linearly correlated with the corresponding two-electron redox potentials (*18*), the oxygen reaction rates might also be expected to correlate approximately with the two-electron redox potential of the FAD. The dependence of the oxygen reactivity on the two-

electron redox potential of the enzyme-bound flavin is shown in Figure 3. The observed rate constants for the reaction of oxygen with PHBH reconstituted with the flavin analogues tested show a dependence on the two-electron redox potential only when it is more positive than −160 mV. When the redox potential was lower than −160 mV, the rate constant was almost invariant (Figure 3), suggesting that some other step such as a conformational change becomes rate limiting. Similar observations have been made with L-lactate oxidase containing the same series of artificial flavins at the FMN level (K. Yorita and V. Massey, unpublished results). If the second step of Scheme 7 were to become rate-limiting with flavins of low potential, it would be expected that flavin semiquinone intermediates would be detectable in the reaction; this has not been observed. It can be noted that the break-point in Figure 3 occurs at approximately −160 mV, close to that of the O_2/O_2^- couple in solution at pH 7 (−167 mV) (*70*).

The final step in the oxidative half-reaction is the elimination of water from the flavin-C4a-hydroxide and the release of product. This process might be expected to be dependent on the pK_a of the N5-proton of the FADHOH intermediate. Because the N5 pK_a values for FADHOH are not experimentally accessible, we tried a correlation with the pK_a values of *p*-substituted anilines. There is no correlation of the rate of dehydration and this pK_a (data in Tables 2 and 4). Thus, if the mechanism of elimination of water from the FADHOH intermediate follows a concerted process where the N5-proton is the donor of the reaction, it cannot be the rate-determining step. Instead, some other step (e.g., the release of product) could be rate-determining for some of the flavins. For example, if release of product precedes the dehydration step of FADHOH, it is likely that flavin movement is important in this process (*55*, *56*), just as it is in binding substrate (*42*). Further complications that might depend on flavin movement can arise from binding of substrate to the FADHOH species after product is released. Binding of substrate is known to substantially inhibit the dehydration of FADHOH (*33*).

This study has helped elucidate the mechanism of hydroxylation by flavoprotein monooxygenases that utilize reactive electrophilic flavin hydroperoxide intermediates. Information about the transition state structure for monooxygen transfer was obtained. An increased rate of hydroxylation is found when a good electrophilic flavin hydroperoxide is present on the enzyme, whereas a decreased rate of hydroxylation results from a less electrophilic hydroperoxide. The opposite relationship between the rate of hydroxylation and the inductive effect of the 8-substituent can be predicted for a nucleophilic flavin peroxide intermediate (e.g., the intermediate likely to be present in cyclohexanone monooxygenase). The work described here also contributes to our understanding of the formation of the flavin-C4a-hydroperoxide in the reaction with oxygen. The results are consistent with the dehydration reaction requiring flavin movement and release of product, in analogy to the binding of substrate (*42*).

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