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The Nature of the Reaction Intermediates in the FAD-Dependent Epoxidation Mechanism of Styrene Monooxygenase*

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Abstract

Styrene monooxygenase (SMO) is a two-component flavoenzyme composed of an NADH-specific flavin reductase (SMOB) and FAD-specific styrene epoxidase (NSMOA). NSMOA binds tightly to reduced FAD and catalyzes the stereospecific addition of one atom of molecular oxygen to the vinyl side chain of styrene in the enantioselective synthesis of *S*-styrene oxide. In this mechanism, molecular oxygen first reacts with NSMOA(FADred) to yield an FAD C(4a)-peroxide intermediate. This species is non-fluorescent and has an absorbance maximum of 382 nm. Styrene then reacts with the peroxide intermediate with a second order rate constant of $2.6 \times 10^6 \pm 0.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ to yield a fluorescent intermediate with an absorbance maximum of 368 nm. We compute an activation free energy of $8.7 \text{ kcal.mol}^{-1}$ for the oxygenation step in good agreement with that expected for a peroxide-catalyzed epoxidation, and acid-quenched samples recovered at defined time points in the single-turnover reaction indicate that styrene oxide synthesis is coincident with the formation phase of the fluorescent intermediate. These findings support FAD C(4a)-peroxide as the oxygen atom donor and identity of the fluorescent intermediate as an FAD C(4a)-hydroxide product of the styrene epoxidation. Overall, four pH-dependent rate constants corresponding to peroxyflavin formation ($\text{pK}_a = 7.2$), styrene epoxidation ($\text{pK}_a = 7.7$), styrene oxide dissociation ($\text{pK}_a = 8.3$), and hydroxyflavin dehydration ($\text{pK}_a 7.6$) are needed to fit the single-turnover kinetics.

Pseudomonas bacteria are equipped with an efficient set of catabolic and detoxification enzymes that allow them to thrive in contaminated environments using a diverse array of aromatic hydrocarbons as sole sources of carbon and energy (1). Recently, a common pathway for the catabolism of styrene was identified (2). At the entry point of the catabolic pathway, styrene is oxidized through the sequential activities of three enzymes: styrene monooxygenase, which catalyzes the epoxidation of the vinyl side chain of styrene, styrene oxide isomerase, which transforms styrene oxide to phenylacetaldehyde and phenylacetaldehyde dehydrogenase, which catalyzes the oxidation of phenylacetaldehyde to phenylacetic acid (2). Phenylacetic acid is then transformed by enzymes shared in common with the phenylalanine catabolic pathway to yield intermediates of the tricarboxylic acid cycle (3,4). This pathway is a point of interest for researchers spanning disciplines of microbiology, biotechnology, and mechanistic enzymology (5–16).

The enzymatic synthesis of *S*-styrene oxide by styrene monooxygenase in the first committed step of this pathway is of particular interest in that it provides insight into the mechanism of a flavin-catalyzed epoxidation reaction, which occurs unassisted by metal

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cofactors and with exceptional enantioselectivity (2,17). Similar FAD-dependent epoxidation reactions are catalyzed by squalene and zeaxanthin epoxidases in the biosynthesis of cholesterol and plant pigments, but styrene monooxygenase has proven to be the best candidate for mechanistic studies due to technical challenges associated with working with these related systems (18,19). Styrene monooxygenase (SMO), is a two-component flavoenzyme composed of an NADH-specific reductase, SMOB, and FAD-specific epoxidase, NSMOA (9,14). SMO is most closely related to the two-component flavin-diffusible systems (20) and the epoxidase component is structurally very similar to that of 4-hydroxybenzoate-3-monooxygenase and related flavin monooxygenases (12,16).

In all studies of flavoprotein monooxygenases in which reaction intermediates have been kinetically resolved, it has been demonstrated that either a flavin C(4a)-peroxide or hydroperoxide is engaged as either a nucleophile (21,22) or electrophile (23,24) in its reaction with substrate. Oxygen atom transfer from the flavin peroxide to substrate results in the production of a C(4a)-hydroxyflavin intermediate, which dehydrates to yield oxidized flavin in a reaction that is dependent on both pH and ionic conditions (25,26).

It was previously suggested that a flavin-oxaziridine could represent an alternative to the flavin-peroxide as an oxygen-atom donor in flavin-catalyzed monooxygenation reactions (27). Computational studies indicate oxaziridine-catalyzed epoxidations proceed through a more asynchronous transition state with greater radical character than computed for peracid-catalyzed alkene epoxidation reactions (28). Oxaziridines generally catalyze the epoxidation of substituted alkenes with greater enantioselectivity than is observed for corresponding reactions of organic peroxides or peracids (29), and the possibility that an FAD-oxaziridine could be the intermediate responsible for oxygen-atom transfer in the reaction mechanisms of flavoenzyme epoxidases could not be ruled out in our earlier studies of NSMOA (14).

Unique flavin products are predicted to result from the flavin-oxaziridine and flavin-peroxide catalyzed reactions: Oxygen-atom transfer from flavin C(4a)-peroxide results in C(4a)-hydroxyflavin, which then eliminates water to yield oxidized FAD. Oxygen-atom transfer from flavin-oxaziridine results in the production of oxidized flavin without a C(4a)-hydroxyflavin intermediate. Here we use rapid acid-quench in conjunction with stopped-flow absorbance and fluorescence studies to resolve the kinetic phase in which styrene oxide is synthesized and the nature of the intermediate responsible for the oxygen atom transfer step of the styrene epoxidation reaction. Activation parameters and pKa values defining the energetics of the transition states and pH-dependence of the kinetic steps in this reaction are presented.

MATERIALS AND METHODS

Expression and Recovery of Recombinant Protein

The N-terminally histidine tagged version of styrene monooxygenase used in this paper was expressed from the pET-28NSMOA vector in *E.coli* BL21(DE3) cells and purified by nickel-affinity chromatography. Purified protein was assayed for specific activity, flavin-binding, and redox potential as described previously (14).

Single-Turnover Absorbance and Fluorescence Studies

Stopped-flow absorbance and fluorescence studies were completed with a previously described single-mixing and double mixing stopped-flow instruments (14,16). Single wavelength absorbance and fluorescence measurements were recorded by photomultiplier interfaced with a Pico 216 ADC. Diode array spectra were recorded with a HR2000+ Ocean Optics diode array spectrophotometer interfaced with the stopped flow instrument.

FAD-fluorescence was excited in the double-mixing stopped-flow instrument along the 0.35 mm pathlength of the flow cell by using a Xenon-arc lamp filtered through an Edmund Optics U340 filter. In the single-mixing Applied Photophysics stopped-flow, FAD-fluorescence was excited by projecting 450 nm light with 5 nm spectral resolution across the 1 mm pathlength of the flow cell. In both instruments, fluorescence emission was monitored at a right angle to the excitation light across the 1 cm pathlength, and stray light was limited with an Edmund Scientific VG6 band pass filter placed directly in front of the emission photomultiplier tube. Exponential fits through kinetic data recorded in experiments using either stopped-flow fluorescence method return the same set of best fitting rate constants.

In experiments requiring anaerobiosis, solutions were made anaerobic in a glass tonometer equipped with a titration port and 1cm pathlength quartz cuvette by repeatedly evacuating and back filling with purified nitrogen gas on a Schlenk line. Solutions of reduced NSMOA(FAD_{red}) complex were generated by incremental addition of dithionite from a gas-tight Hamilton syringe attached to a tonometer.

Styrene, benzene, and flavin solutions were prepared as previously described (14). Solutions containing defined concentrations of dissolved oxygen were prepared by mixing appropriate volumes of air saturated and anaerobic buffers. Experimental data were fit and plotted by using the programs Kaliedagraph 4.02 and GraphPad Prism 4.0b.

pH-Dependence Studies

NSMOA was prepared in 5mM MOPSO pH 7 containing 5% glycerol and reacted in 0.1 M MOPSO pH=6.5, 0.1M MOPSO pH=7, 0.1M HEPES pH=7.5, 0.1M HEPES pH=8, 0.1M MOPSO pH=8.5, 0.1M CHES pH=9. Buffers included sodium chloride to give a final ionic strength of 0.1 M. The actual pH after 1:1 mixing was verified by measurement with a pH probe.

Rapid Acid-Quench Studies

The double mixing stopped flow was reconfigured for use as a rapid quench instrument by modifying the internal flow path and adding a sample-aging loop, check valves, an electronically actuated flow-path-switching valve and sample-recovery syringe. In the first push, reduced enzyme is mixed with oxygen and styrene as it passes through a fluorescence flow cell and into a delay loop. As buffer is displaced from the aging loop by incoming reactants it enters and fills the stop syringe. Upon filling, the stop-syringe impacts the stop-block and closes a switch, which initiates fluorescence data collection and actuates the flow-path-switching valve to connect the aging loop and sample-recovery syringe. After user defined delay time, a second push directs the reacting sample in the aging loop to a second mixer where it is quenched with 2M HCl. In addition to stopping the progression of the single-turnover reaction, the acid instantly hydrolyzes the styrene oxide to yield α,β -dihydroxyethylbenzene. As the quenched sample exits from the mixer it passes through the flow-path switching valve and fills a removable 2.5 mL Hamilton gas-tight sample-recovery syringe.

Product Derivatization and Detection

Approximately 600 μ L of acid quenched reaction product was immediately injected through a septum into a gas-tight 10ml reaction vial containing 450mg of magnesium sulfate and 10mg of methyl boronic acid to form the methylboronic ester of α,β -dihydroxyethylbenzene. Two-minutes after vortexing the reaction vial, a 1 mL sample of headspace gas was drawn from the esterification reaction vial and immediately injected into a Varian Saturn 2000 GC/MS/MS equipped with a 30m Varian VF-5ms capillary column. Injector temperature was set to 80°C and separation was achieved with a temperature program, which initiated with a 5

minute isocratic step followed by a linear temperature gradient from 80 – 250 °C at a heating rate of 4°C per minute. Under these separation conditions, styrene detected at 4.8 min and the methylboronic ester of α,β -dihydroxyethylbenzene detected 11.7 min were quantified based on their respective total ion intensities monitored at m/z values of 105 and 161, respectively.

RESULTS

Temperature and pH Dependence of the Single-Turnover Reaction of NSMOA(FADred) with Oxygen and Styrene

The kinetics of the reaction of reduced NSMOA with oxygen and styrene were monitored by single-mixing absorbance and fluorescence stopped-flow spectroscopy over a range of pH values. Representative kinetic traces from experiments conducted at pH 6.4 and pH 8.8, are shown in Figure 1 A and B, respectively. A total of four exponentials were required to globally fit the fluorescence and absorbance data at each pH and each of the four resulting rate constants was found to be dependent on pH. Lines passing through the data in Figures 1A and 1B represent best sequential four exponential fits through these data as a function of pH (30).

pKa values corresponding to each of these rate constants were computed by fitting observed rate constants as functions of pH according to equation 1.

$$k_{obs} = \frac{k_{High}}{1 + 10^{(pKa - pH)}} + \frac{k_{Low}}{1 + 10^{(pH - pKa)}} \quad (1)$$

Best fits passing through the data over the temperature range of 15–25 °C are given in Figure 2. pKas and limiting values of the observed rate constants resulting from these fits are listed in Table 1. Activation parameters associated with the computed values of observed rate constants at limiting values of low and high pH (Table 2) were estimated from the best linear fits through Eyring plots (Figure 3A–D). Thermodynamic parameters associated with the kinetically-resolved macroscopic pKa values in the single-turnover reaction (Table 3) were estimated from the best linear fits through the van't Hoff plots (Figure 3E–H) (31).

pH-Dependence of the Spectra of Kinetic Intermediates in the Epoxidation Reaction

Time resolved absorbance spectra representative of the peroxide and hydroxide intermediates were recorded each with an 8 ms integration time by diode array spectroscopy at 100 ms after mixing. After subtracting the spectral contribution of free oxidized-FAD present in each sample, it was possible to obtain spectra representative of the intermediates occurring in the reaction of oxygen and styrene with the reduced enzyme (Figure 4). These spectra have absorbance maxima of 382 and 368 nm, respectively that are independent of pH in range 6–9.

Identification of the Kinetic Phase Associated with Styrene Oxide Synthesis

A time course for the evolution of kinetic intermediates in the reaction NSMOA with oxygen and styrene is given in Figure 5. Species concentrations computed by using best fitting rate constants from kinetic fitting at pH 7 and 25 °C and the integrated rate expression for a sequential 4-exponential reaction (30) are shown as solid lines. The solid line marked by a solid circle shows the formation and decay kinetics of the fluorescent intermediate. Dashed lines contrast the computed time courses expected if styrene oxide synthesis is coupled to the formation (marked by a solid triangle) or to the decay (marked by a hollow

triangle) of the fluorescent intermediate. Hollow circles with error bars show the relative yield of styrene oxide at 0.1 second and 1 second after mixing detected by GC-MS as the methylboronic ester of α,β -dihydroxyethylbenzene.

Binding of Benzene to the *FAD C(4a)-peroxide Intermediate of NSMOA*. When NSMOA(FADred) is rapidly mixed in the stopped-flow with oxygen, a rapid rise in absorbance at 375 nm corresponding to the formation of flavin peroxide is observed. In the 100 ms – 10 s time range, the 450 nm absorbance increases as hydrogen peroxide is eliminated slowly to regenerate the spectrum of oxidized flavin. The substrate analog benzene has no significant effect on the kinetics of FAD C(4a)-peroxide formation, but has a significant stabilizing effect that greatly increases the half-life of the peroxide intermediate (Figure 6A). Since the observed rate constant of formation of NSMOA(FAD_{OOH}) is much larger than the decay rate constant, the total enzyme and benzene concentrations involved in the decay reaction are well approximated by equations (2 and 3).

$$[NSMOA]_T = [NSMOA(FAD_{OOH})]_{Free} + [NSMOA(FAD_{OOH}):Benzene] \quad (2)$$

$$[Benzene]_T = [Benzene]_{Free} + [NSMOA(FAD_{OOH}):Benzene] \quad (3)$$

The absorbance data plotted in Figure 6B correspond to the values recorded in Figure 6A at 100 seconds after mixing. Equations 2 and 3 were combined to allow derivation of equation (4), which makes the approximation that benzene (B) binds with the enzyme peroxide intermediate of NSMOA (A) in a rapid equilibrium (K_{d8}) with respect to the decomposition rate constant of the peroxide intermediate, and that benzene-bound NSMOA(FAD_{OOH}) represents a dead-end complex.

$$A_{450} = A_{450}^{FAD} = \frac{(K_{d8} + [A]_T + [B]_T) - \sqrt{(K_{d8} + [A]_T + [B]_T)^2 - 4[A]_T[B]_T}}{2} \epsilon l \quad (4)$$

The complete set of species involved in this transformation are given in Figure 7 and the subset of direct significance to the NSMOA(FAD_{OOH}) decomposition reaction are enclosed within the dashed rectangle. K_d values 1–4 (16) represent rapid equilibria with respect to the NSMOA(FAD_{OOH}) decomposition rate constant. Thus, immediately following decomposition of the peroxide intermediate, the enzyme can be described by the subset of states related through K_{d1} – K_{d4} . (Figure 7) The extinction coefficient (ϵ) in equation (4) does not discriminate between the free and bound forms of oxidized FAD presented in this model. The best fitting values of K_{d8} and ϵ are $62.7 \pm 4.7 \mu\text{M}$ and $9.6 \pm 0.1 \text{ mM}^{-1}\text{cm}^{-1}$, respectively.

Chloride Effect in the Epoxidation Mechanism of NSMOA

Observed rate constants (k_2) and (k_3) (Table 1) corresponding to the kinetics of formation and decay of the fluorescent intermediate observed in the epoxidation reaction are dependent on ionic conditions (Figure 8A). As the sodium chloride concentration is increased from 0–1M, the formation rate constant decreases linearly and the rate constant for the decay of the fluorescent intermediate decreases hyperbolically. The best hyperbolic fit through the data indicates the decay rate constant is decreases from 2.83 s^{-1} in the absence of added salt to a

limiting value of 0.013 s^{-1} . A salt concentration of $162 \pm 13 \text{ mM}$ results in half of the maximum inhibitory effect.

Determination of the Second-Order Rate Constant for the Reaction of NSMOA(FAD_{OOH}) with Styrene

The kinetics of the reaction of the FAD C(4a)-peroxide intermediate with styrene was examined by double mixing stopped-flow fluorescence spectroscopy. In these studies, NSMOA(FAD_{red}) was rapidly mixed with aerobic buffer to generate the peroxide intermediate. After a 100 ms delay time, the peroxide intermediate generated in the first push was rapidly mixed with aerobic buffer containing various amounts of styrene (Figure 9). Two kinetic reaction phases were observed corresponding to the styrene-dependent formation of the NSMOA(FAD_{OH}) intermediate followed by the styrene-independent dehydration of this intermediate to yield oxidized FAD. Reaction conditions were second order considering the similar concentrations of the styrene and NSMOA(FAD_{OOH}) reacted in this study and the overall reaction was fit by using equation 5, which represents the sum of equations corresponding to a second order styrene-dependent formation and first order, styrene-independent decay of fluorescence.

$$F_t = \frac{[FAD_{OOH}]_0 - [Styrene]_0 e^{([FAD_{OOH}]_0 - [Styrene]_0)k_{sty}t}}{1 - e^{([FAD_{OOH}]_0 - [Styrene]_0)k_{sty}t}} \epsilon_1 l + f e^{-k_3 t} + const. \quad (5)$$

The four data sets presented in Figure 9 were fit simultaneously at all styrene concentrations with this equation by using the non-linear curve fitting program GraphPad Prism 4.0b. In the fit, the rate constants for the formation and decay of fluorescence, k_{sty} and k_3 , respectively, and the fluorescence extinction coefficient of the FAD ϵ_1 were globally constrained to return the same best-fitting values for each of the data sets. The pathlength (l) was 0.5 cm for fluorescence emission. The best fit through the data returned values of $k_{sty} = 2.6 \times 10^6 \pm 0.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $k_3 = 1.7 \pm 0.01 \text{ s}^{-1}$.

DISCUSSION

Reaction of NSMOA with Reduced Flavin, Oxygen, and Styrene

The binding affinity of NSMOA is very high for reduced FAD (210 nM) and very weak for the oxidized FAD (1.6 mM) Scheme 1 (16). This thermodynamic linkage of ligand-binding and redox equilibria is critical to efficient catalysis by this system in that it allows the enzyme to efficiently scavenge reduced flavin from solution and minimize the non-productive reaction of dissolved oxygen with free-flavin and to release oxidized FAD at the end of the catalytic cycle.

When NSMOA(FAD_{red}) is rapidly mixed with oxygen in the presence of 5 mM benzene, the kinetics of flavin peroxide formation are unaffected as shown in the inset in Figure 6A. Our previous ligand-binding studies (16) indicate the equilibrium dissociation constant of benzene and NSMOA(FAD_{red}) is quite weak ($K_{d7} = 2.3 \text{ mM}$). In the sub-millimolar benzene-concentration range only a small fraction of available NSMOA(FAD_{red}) active sites are expected to be occupied by benzene. However, at 5 mM benzene we would expect ~ 68% occupancy. The fact that we see no inhibition of the peroxide formation kinetics under these conditions suggests that bound benzene does not block access of oxygen to the reduced enzyme or significantly influence the kinetics of the peroxide formation reaction. Given the close structural relationship of benzene and styrene it is reasonable to conclude that styrene and oxygen similarly react with NSMOA(FAD_{red}) in a random order.

We find that the benzene binds to the NSMOA(FAD_{OOH}) intermediate with $K_d = 62.7 \mu\text{M}$ (Figure 6B). This represents an increase in affinity of nearly 40-fold over the binding affinity of the reduced enzyme for substrate analog, benzene. This increase in active site affinity affords the NSMOA(FAD_{OOH})-state of the enzyme a significantly enhanced ability bind and react with substrate. We previously estimated the K_i of benzene to be $\sim 173 \mu\text{M}$ by steady-state kinetic inhibition studies (14). This value is quite similar to the value of K_{d8} determined here and suggests that in the steady-state kinetic studies styrene and benzene were probably competing primarily for the NSMOA(FAD_{OOH}) state of the enzyme.

In the double-mixing stopped-flow studies of the reaction of styrene with the peroxide intermediate we first prepared NSMOA(FAD_{OOH}) by mixing NSMOA(FAD_{red}) with aerobic buffer in the absence of styrene. Once formed, the NSMOA(FAD_{OOH}) was rapidly mixed with styrene and the kinetics of the reaction monitored. The single-mixing stopped-flow studies involved a different order of substrate addition. Reduced enzyme was pre-mixed with styrene under anaerobic conditions and then rapidly mixed with oxygen. Based on the fits presented in Figure 2C we calculate an observed pseudo-first order rate constant $k_2 = 104\text{s}^{-1}$ for the epoxidation reaction. Dividing this rate constant by the $125 \mu\text{M}$ styrene concentration used in this study gives an estimate of second order rate constant for the reaction of styrene with the peroxide intermediate equal to $8.3 \times 10^5 \text{M}^{-1}\text{s}^{-1}$, which is about a factor of three lower than the value we obtained for k_{sty} in the double-mixing stopped-flow investigation. This is a very slight difference in terms of the free energy of activation, but suggests that the peroxide intermediate formed in the absence of bound styrene in the double mixing stopped-flow may be slightly more reactive than the corresponding intermediate, which forms in the presence of styrene in the single-mixing stopped flow studies.

Identification of FAD C(4a)-peroxide as the Oxygen Atom Donor in the Epoxidation Reaction

Flavin C(4a)-peroxides have been identified as the key reaction intermediate responsible for substrate oxygenation by flavin monooxygenases. Oxygen atom-transfer to substrate is coupled to the synthesis of C(4a)-flavin hydroxide intermediate, which eliminates water to regenerate oxidized flavin (32). In early theoretical investigations a flavin oxaziridine was proposed as an alternate candidate oxygen atom-donor in the reaction mechanism of flavoprotein monooxygenases (27). This intermediate could form through protonation of a flavin C(4a)-peroxide intermediate followed by the elimination of the oxygen atom distal to the flavin as water and cyclization to form an oxaziridine with the proximal oxygen atom bridging the N-5 and C(4a)-positions of the flavin. Oxaziridines have demonstrated value as reagents in epoxidation reactions where they have superior enantioselectivity when compared to organic peroxides (29). However, flavin-oxaziridines have not been prepared synthetically and based on computational studies, the transition state of an oxaziridine-catalyzed epoxidation is expected to require significantly more activation energy than the corresponding transition state of an organic peroxide-catalyzed epoxidation (28). By using the second order rate constant $k_{\text{sty}} = 2.6 \times 10^6 \pm 0.1 \text{M}^{-1}\text{s}^{-1}$ and standard state reactant concentrations of 1 M at 25° C, the free energy of activation for the styrene epoxidation reaction is computed to be $\sim 8.7 \text{kcal. mol}^{-1}$. This value is in closer agreement with the calculated transition state free energy of the epoxidation of propylene by performic acid ($12.0 \text{kcal. mol}^{-1}$) compared with the oxaziridine-mediated epoxidation reaction ($31.4 \text{kcal. mol}^{-1}$) as estimated by computational methods (28).

The NSMOA(FAD_{OH}) intermediate is expected to be synthesized only in a flavin-peroxide-catalyzed epoxidation of styrene, so its presence can be used to definitively rule out flavin oxaziridine as a reaction intermediate. In considering the defining spectral features reported in the literature for flavin-oxygen intermediates, flavin C(4a)-peroxide is generally reported to be red shifted ($380 \pm 6 \text{nm}$) relative to flavin hydroxide ($375 \pm 10 \text{nm}$) spectra

(21,23,24,33–39). Intermediates identified as flavin C-(4a) hydroperoxides may be fluorescent (23) or non-fluorescent depending on the active site environment, whereas flavin hydroxides are often reported to be highly fluorescent (39). In addition, the elimination of water from hydroxyflavin to yield oxidized flavin occurs in a reaction that is quite sensitive to both pH and ionic conditions (24–26,40). Since no model compound information is available regarding the absorbance and fluorescence properties of flavin oxaziridine, or how pH or ionic conditions might affect the reactivity of flavin oxaziridine, we could not use these properties alone to argue for or against the possible role of flavin oxaziridine in the epoxidation mechanism of NSMOA.

We find in the reaction of NSMOA(FADred) with oxygen and styrene first yields a C(4a)-flavin peroxide, with a peak absorbance of 382 nm, which is then transformed to a second intermediate with a peak absorbance of 368 nm (Figure 4). In separate studies in which the reaction kinetics monitored by fluorescence in the absence of styrene and in the presence or absence of benzene as a substrate analog, it was established that the first intermediate is non-fluorescent, and that the second, fluorescent intermediate forms only in the presence of substrate, styrene. By analyzing acid quenched reaction mixtures and assaying for styrene oxide, we were able to establish that the synthesis of styrene oxide is coupled to the formation phase of this reaction and that the decay of fluorescence takes place after styrene oxide is synthesized (Figure 5). Based on this analysis, we conclude that the fluorescent intermediate we are observing is an FAD C(4a)-hydroxide rather than the FAD oxaziridine and conclude that epoxidation of styrene occurs through a direct-transfer of an oxygen atom from the FAD C(4a)-peroxide to styrene.

In the absence of styrene, the peroxide intermediate decomposes to yield hydrogen peroxide and oxidized flavin at a rate of about 0.1 s^{-1} at 25°C and pH 7. This decomposition rate constant is more than an order of magnitude smaller than the rate constant for the release of oxidized-flavin in the single turnover reaction of NSMOA with styrene suggesting the active site environment plays an important role in stabilizing the FAD C(4a)-peroxide intermediate in the absence of substrate. We have not observed the significant substrate-inhibition of the C(4a)-hydroxyflavin dehydration reaction observed in the mechanisms of parahydroxybenzoate hydroxylase (24) and 4-hydroxyphenylacetate-3-monooxygenase (37). However, we propose that the dissociation of styrene oxide from the hydroxyflavin intermediate state of NSMOA similarly allows flavin exposure to solvent in a step that promotes subsequent elimination of water to yield oxidized FAD. Dissociation of styrene oxide from the hydrophobic active site of NSMOA is expected to be coupled to a significant ordering of solvent molecules as both the styrene oxide and active site are hydrated. This may be in part responsible for the large negative entropy of activation associated with k_3 in Table 2. This sequence of events is presented in Scheme 1 together with observed rate constants corresponding each reaction step.

Dependence of Single-Turnover Reaction Kinetics on Ionic Conditions

Earlier studies of flavoprotein hydroxylases have demonstrated a significant dependence of the kinetics of monooxygenation on azide, iodide, and chloride ions (24,26,41). The C(4a)-hydroxyflavin dehydration reaction is particularly sensitive to interference by anions and it has been suggested that anions may inhibit this step by blocking the deprotonation of N-5 position of the flavin (26). We also find that the observed rate constant for hydroxyflavin dehydration reaction decreases hyperbolically as a function of increasing chloride concentration, which provides support for the existence of an anion-binding site in NSMOA (Figure 8). In addition we find that the observed rate constant for the flavin epoxidation reaction decreases with sodium chloride concentration. In this case the observed rate constant appears to decrease linearly with salt concentration. However, this may represent

the first part of a much weaker binding interaction in which the saturation component of the hyperbolic isotherm can not be detected in the 0–1M salt concentration range.

Activation Parameters of the Transition States in Reaction of NSMOA(FADred) with Styrene and Oxygen

Values of activation free energies listed in Table 2 were used together with estimated differences in energies of reaction intermediates calculated from equilibrium constants for the binding of oxidized and reduced flavin and substrate to NSMOA (16) and estimated changes in chemical bond energies (42) associated with the transformations relating each intermediate state to construct the approximate reaction coordinate presented in Scheme 2. Since the dependence of activation free energy on pH is relatively small compared to the overall energetic change in the epoxidation reaction (Table 2), plots corresponding the reaction proceeding at high and low pH appear to nearly superimpose. The largest change in free energy in this reaction is coupled styrene oxide dissociation in the transformation of the first intermediate to the second intermediate. This is followed by a significantly smaller change in free energy coupled to the elimination of water from the C(4a)-hydroxyflavin.

Kinetically Resolved pKa Values in the Reaction Mechanism of NSMOA

The macroscopic pKa values in this mechanism are quite similar and in the basic pH range. Van't Hoff analysis (Table 3) indicates ionization enthalpies for deprotonation ranging from 8–28 kcal.mol.⁻¹, which are large and suggest the existence of multiple linked protic equilibria contributing to each of the observed macroscopic pKa values (43). Van't Hoff plots of Ka₁ and Ka₂ are not very linear, and determined over a narrow temperature range. More data recorded over a broader temperature range are required to evaluate the significance of this observation. The exact chemical species contributing to the kinetically resolved macroscopic pKa values can not be rigorously addressed at this time, but in considering dependence of the observed rate constants on these pKa's, it can be concluded that the epoxidation reaction is acid catalyzed whereas formation flavin peroxide intermediate, elimination of water from the flavin hydroxide intermediate, and the dissociation of oxidized FAD from the enzyme at the end of the single turnover reaction are all base catalyzed (Figure 3).

It is likely that the macroscopic pKa for C(4a)-flavinperoxide formation (pKa₁ = 7.2) is linked to the protonation state of the isoalloxazine ring nitrogen (N-1) or an ionizable active site base that interacts with this ring position (Scheme 1). Deprotonation of this position will increase electron density at the C(4a)-position and increase the reactivity of the flavin with oxygen.

The macroscopic pKa of the epoxidation reaction (pKa₂ = 7.7) may be related to functional groups in the active site that tune the reactivity of the FAD C(4a)-peroxide. As shown in Scheme 1. The pKa of C(4a)-flavin peroxide is estimated to be ~10 (44), but the active sites of flavin monooxygenases must be able to tune this value considerably to meet mechanistic demands. In the reactions of aromatic ring hydroxylases, such as 4-hydroxybenzoate-3-monooxygenase (24,45), phenol hydroxylase (23), the aromatic ring of substrate is thought to make a nucleophilic attack on the protonated oxygen atom of the peroxide. Alternatively, bacterial luciferase (46), cyclohexanone, (21), and alkane sulfonate (47) monooxygenases, appear to effectively decrease the pKa to yield a reactive anionic flavin peroxide intermediate that attacks substrate in a Bayer-Villiger-type mechanism.

In the case of cyclohexanone monooxygenase, deprotonation of the flavin hydroperoxide yields an anionic flavin peroxide that is coupled to a red shift in the absorbance maximum (21). The spectrum of the flavin peroxide from of NSMOA generated in the absence of

styrene remains unchanged as a function of pH (Figure 4). Since we do not observe this spectral shift in the case of NSMOA, and it is possible that the peroxide anion does not form at all in the active site environment provided by NSMOA, and the observed decrease in epoxidation rate constant as a function of pH supports the FAD C(4a)-hydroperoxide as the reactive intermediate. The nature of this reaction intermediate may be better resolved through studies with nucleophilic and electrophilic styrene analogs and FAD analogs designed to react and generate C(4a)-peroxides spanning a range of electrophilicity (45).

The rate of elimination of water from the hydroxyflavin intermediate of the single-component phenol hydroxylase from *T. cutanum* is thought to be triggered by proton abstraction from the N-5 ring position by hydroxide ion in the active site (25). We hypothesize that $pK_{a3} = 8.3$ and $pK_{a4} = 7.7$ similarly represent pK_a values associated with the N-5 position of the C(4a)-FAD hydroxide intermediate of NSMOA in the presence and absence of bound styrene oxide. Deprotonation of an active site residue in proximity of the N-5 position of the flavin in the presence of styrene oxide ($pK_{a3} = 8.3$) would generate negative charge in the vicinity of the oxide ring decreasing the affinity of bound styrene oxide through electrostatic repulsion. We further propose that the product dissociation equilibrium constant may be linked to pK_{a3} such that the product dissociation is coupled to a decrease in this pK_a from 8.3 to $pK_{a4} = 7.6$. Following the dissociation of styrene oxide product, the rate constant for the elimination of water from the FAD C(4a)-hydroxide in the regeneration of oxidized FAD may then be controlled by pK_{a4} (Scheme 1).

Abbreviations

SMO	styrene monooxygenase
NSMOA	N-terminally histidine-tagged styrene monooxygenase epoxidase component
FAD	flavin adenine dinucleotide
NADH	nicotinamide adenine dinucleotide
NSMOA(FAD_{red})	NSMOA with reduced FAD bound
NSMOA(FAD_{ox})	NSMOA with oxidized FAD bound
NSMOA(FAD_{OOH})	NSMOA with bound FAD C(4a)-hydroperoxide
NSMOA(FAD_{OH})	NSMOA with bound FAD C(4a)-hydroxide
SMOB	styrene monooxygenase reductase component

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FIGURE 1.

Reaction of NSMOA with Styrene and Oxygen Monitored by Absorbance and Fluorescence. Data recording the reaction of 25 μM NSMOA (FADred) with 134 μM oxygen and 125 μM styrene at 25°C monitored by absorbance at (Δ) 375 nm, ($\circ$) 390 nm, and ($\square$) 450 nm, and by fluorescence emission at 520 nm ($\diamond$). Data representative of the reaction kinetics at pH 6.4 and pH 8.8 are shown in plots A and B, respectively. Solid lines represent the best exponential fits through the data. Relative fluorescence intensities are scaled to allow comparison with absorbance data.

FIGURE 2.

pH- and Temperature-Dependence of the Observed Rate Constants from the Reaction of NSMOA with Oxygen and Styrene. Rate constants ($\circ$) k_1 , ($\square$) k_2 , (Δ) k_3 , and ($\diamond$) k_4 estimated by exponential fitting of time-resolved absorbance and fluorescence data. Solid curves passing through the data represent the best fits of the observed rate constants as a function of pH at 15, 20, and 25°C in plots A–C, respectively.

FIGURE 3.

Energetic Analysis of Observed Rate Constants and Kinetically Resolved K_a Values in the Reaction Mechanism of NSMOA. Eyring plots of limiting values of rate constants (Δ) k_1 and ($\diamond$) k_2 at low pH (panel A) and high pH (panel C.) Limiting values of rate constants ($\circ$) k_3 and ($\square$) k_4 at low pH (panel B) and high pH (panel D). Van't Hoff plots of the macroscopic K_a values associated with each observed rate constant are shown in panels E–H.

FIGURE 4.

Time-Resolved Spectra Representative of Flavin Peroxide and Hydroxide Intermediates Detected in the Epoxidation Reaction of NSMOA. Upper pair of spectra are representative of the peroxide intermediate recorded 100 ms after mixing NSMOA bound with reduced FAD with oxygen at ($\diamond$) pH 6.4 and (Δ) pH 8.8. Lower pair of spectra are representative of the hydroxide intermediate recorded 100 ms after mixing reduced FAD bound NSMOA with oxygen and styrene at ($\square$) pH 6.4 and ($\circ$) pH 8.8. Black-lines are smooth fits through the data provided for clarity.

FIGURE 5.

Identification of the Reaction Phase Coupled to the Synthesis of Styrene Oxide. Computed concentration profiles showing the transformation (■) reduced enzyme to yield first (□) FAD-peroxide, and then (◇) the fluorescent intermediate observed the reaction of NSMOA with oxygen and styrene. Dashed lines indicate the time dependence of styrene oxide production if the fluorescent intermediate is (○) a product of or (Δ) responsible for the epoxidation reaction. A plot of the experimentally observed reaction fluorescence (●) is included for direct comparison to the concentration profile. Rapid-quench time points (○) recovered 0.1 and 1 second after mixing and assayed by GC-MS.

FIGURE 6.

Stabilization of Flavin Peroxide Intermediate by Benzene. Plot A shows the absorbance changes at 450 nm (0.1cm pathlength) corresponding to the reaction of 86 μM NSMOA(FADred) at 25°C with air-saturated, 0.1 M MOPSO pH 7 containing ($\circ$) 0 mM, ($\square$) 0.25 mM, 0.5 ($\diamond$), and (Δ) 5 mM benzene. Inset plot shows kinetics of the same reaction monitored at 375 nm in the presence (Δ) or absence ($\circ$) of 5 mM benzene. In plot B, the absorbance recorded at 450 nm 100 seconds after mixing is fit as a function of benzene concentration as described in the text.

FIGURE 7.

Ligand Binding Equilibria in the NSMOA(FAD_{OOH}) decomposition reaction. At sub-millimolar benzene concentrations, the enzyme partitions primarily into benzene-bound forms of NSMOA(FAD_{OOH}) and NSMOA(FAD_{ox}). The benzene-binding reactions presented occur rapidly on the time scale of the NSMOA(FAD_{OOH}) decomposition reaction. K_{d1} , K_{d3} , and K_{d7} are all greater than 1 mM. K_{d2} , K_{d4} and K_{d8} are each in the tens of micromolar range.

FIGURE 8.

Dependence of Epoxidation Kinetics on Solution Ionic Strength. (A) Fluorescence data recorded after mixing 50 μ M NSMOA(FADred) with aerobic 0.1 M MOPSO buffer pH 7 containing 250 μ M styrene and ($\circ$) 0 M, ($\square$) 0.1M, (Δ) 1M, and ($\diamond$) 2M NaCl at 25 $^{\circ}$ C. (B) Rate constants from exponential fitting of the fluorescent intermediate ($\bullet$) formation and ($\circ$) decay plotted as a function of NaCl concentration.

FIGURE 9.

Reaction of the FAD C(4a)-peroxide Intermediate of NSMOA with Styrene. 99.6 μM NSMOA(FADred) was first mixed (1:1) with aerobic buffer to generate 49.8 μM FAD-peroxide intermediate. After 100 ms of reaction time, the resulting NSMOA(FAD-peroxide) intermediate was mixed (1:1) with various concentrations of styrene in 0.1M MOPSO buffer pH 7. Time-dependent changes in fluorescence emission from single turnover reactions of NSMOA(FAD-peroxide) with ($\diamond$) 63 μM , ($\square$) 100 μM , (Δ) 138 μM , and ($\circ$) 175 μM styrene. Curves passing through the data represent the best global fit through the data set. The inset plot shows the linear dependence of the observed rate constant of the fluorescence formation phase on styrene concentration.

SCHEME 1.
Intermediates in the Epoxidation Mechanism of NSMOA.

SCHEME 2.

Reaction Coordinate for the Epoxidation of Styrene by NSMOA.

Table 1

Limiting pH-dependent rate constants and pKa values at 25°C

Rate Constant	Low pH limit	High pH limit	Associated pKa
k_1	$129 \pm 4.9 \text{ s}^{-1}$	$182 \pm 3.7 \text{ s}^{-1}$	7.2 ± 0.2
k_2	$107 \pm 0.8 \text{ s}^{-1}$	$65.6 \pm 0.8 \text{ s}^{-1}$	7.7 ± 0.1
k_3	$0.2 \pm 0.08 \text{ s}^{-1}$	$25.2 \pm 0.03 \text{ s}^{-1}$	8.3 ± 0.1
k_4	$0.2 \pm 0.1 \text{ s}^{-1}$	$6.6 \pm 0.1 \text{ s}^{-1}$	7.6 ± 0.1

Table 2
Activation parameters in the styrene epoxidation mechanism of NSMOA at 25°C

Rate Constant	$\Delta G^\ddagger_{(\text{low pH})}$ (kcal. mol. ⁻¹)	$\Delta G^\ddagger_{(\text{high pH})}$ (kcal. mol. ⁻¹)	$\Delta H^\ddagger_{(\text{low pH})}$ (kcal. mol. ⁻¹)	$\Delta H^\ddagger_{(\text{high pH})}$ (kcal. mol. ⁻¹)	$T\Delta S^\ddagger_{(\text{low pH})}$ (kcal. mol. ⁻¹)	$T\Delta S^\ddagger_{(\text{high pH})}$ (kcal. mol. ⁻¹)
k ₁	14.3 ± 0.5	14.3 ± 0.5	9.8 ± 1.4	13.3 ± 1.2	-4.7 ± 0.8	-1.0 ± 0.1
k ₂	14.4 ± 0.1	14.4 ± 0.1	20.3 ± 1.6	51.1 ± 7.5	5.7 ± 0.5	36.1 ± 5.5
k ₃	18.2 ± 9.1	15.3 ± 0.1	-9.2 ± 7.0	1.9 ± 0.8	-27.5 ± 14.6	-13.6 ± 23.1
k ₄	8.2 ± 7.3	16.1 ± 2.0	-1.9 ± 10.4	34.3 ± 2.5	-20.1 ± 35.3	18.1 ± 1.4

$\Delta G^\ddagger = -RT \ln \left(\frac{h}{k_B T} k_{\text{obs}} \right)$ and the rate constants listed in Table-I.

Table 3

Ionization energy parameters of kinetically resolved Ka values at 25°C.

Kinetically Resolved pKa	ΔG (kcal. mol. ⁻¹) ^I	ΔH (kcal. mol. ⁻¹)	$T\Delta S$ (kcal. mol. ⁻¹)
pKa ₁	9.8 ± 0.3	28.2 ± 8.1	18.4 ± 8.1
pKa ₂	10.5 ± 0.1	20.2 ± 19.7	9.6 ± 20.1
pKa ₃	11.3 ± 0.1	8.2 ± 1.0	-3.0 ± 1.1
pKa ₄	10.4 ± 0.1	25.6 ± 4.5	15.2 ± 4.6

^I Calculated using the equation $\Delta G^{O'} = 2.303RT \times pKa$ and pKa values listed in Table-1.