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Structure and ligand binding properties of the epoxidase component of styrene monooxygenase,^{†,‡}

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Abstract

Styrene monooxygenase (SMO) is a two-component flavoprotein monooxygenase that transforms styrene to styrene oxide in the first step of the styrene catabolic and detoxification pathway of *Pseudomonas putida* S12. The crystal structure of the N-terminally histidine-tagged epoxidase component of this system, NSMOA, determined to 2.3 Å resolution, indicates the enzyme exists as a homodimer in which each monomer forms two distinct domains. The overall architecture is most similar to that of *p*-hydroxybenzoate hydroxylase (PHBH), although there are some significant differences in secondary structure. Structural comparisons suggest that a large cavity open to the surface forms the FAD binding site. At the base of this pocket is another cavity that likely represents the styrene-binding site. Flavin binding and redox equilibria are tightly coupled such that reduced FAD binds apo NSMOA ~8,000-times more tightly than the oxidized coenzyme. Equilibrium fluorescence and isothermal titration calorimetry data using benzene as substrate analog indicate that the oxidized flavin and substrate analog binding equilibria of NSMOA are linked such that the binding affinity of each is increased by 60-fold when the enzyme is saturated with the other. A much weaker ~2-fold positive cooperative interaction is observed for the linked binding equilibria of benzene and reduced FAD. The low affinity of the substrate analog for the reduced-FAD complex of NSMOA is consistent with a preferred reaction order in which flavin reduction and reaction with oxygen precede the binding of styrene, identifying the apo enzyme structure as the key catalytic resting state of NSMOA poised to bind reduced FAD and initiate the oxygen reaction.

Flavins are key cofactors in the reductive activation and transfer of oxygen atoms to organic substrates in the biosynthesis of alcohols, aldehydes, and acids (1,2). Through structural studies, dynamic motions of the flavin isalloxazine ring system have been tracked and linked to discrete catalytic steps associated with substrate binding, oxygenation, and product release (3-8). Flavin-dependent epoxidations, which are less commonly encountered, have important roles in the biosynthesis of cholesterol and plant pigments and in the styrene catabolic and detoxification pathway of *Pseudomonas* bacteria (9-11). The soluble flavoenzyme styrene

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monooxygenase (SMO), which catalyzes the transformation of styrene to *S*-styrene oxide, is readily purified and has proven to be an excellent target for mechanistic studies of flavin-catalyzed epoxidation (12-15). Studies of SMO have provided some insight into the mechanisms of the functionally-related FAD-dependent epoxidases, squalene monooxygenase and zeaxanthin epoxidase, for which practical challenges associated with obtaining reagent quantities of enzyme have limited the extent of previous investigations (9,10). Structural and mechanistic studies of the enantioselective epoxidation reaction catalyzed by SMO may impact the development of new biological methods for producing chemical building blocks (16,17) and for removing styrene and styrene oxide from contaminated regions in the environment (12).

SMO is composed of two components: a 46 kDa FAD-specific styrene epoxidase (SMOA) and a 20 kDa NADH-specific flavin reductase (SMOB). In catalysis, reduced flavin produced by the reductase must be transferred to the epoxidase active site. The flavin thus takes on roles of substrate and coenzyme in a reaction that is different from the single component flavin monooxygenases such as *p*-hydroxybenzoate hydroxylase (PHBH), in which the flavin reduction and oxygen activation reactions occur within the same polypeptide (1,2). In the mechanism of SMO, reduced flavin generated by the reductase migrates to the active site of styrene monooxygenase where it is used in the reductive activation of molecular oxygen in the epoxidation reaction (Scheme 1). The efficient transfer of reduced flavin from the reductase to the epoxidase poses a problem since reduced flavin reacts readily with oxidized flavin and molecular oxygen dissolved in solution (3).

Various mechanistic strategies have evolved to address this problem. Protein-protein interactions between the reductase and monooxygenase components of bacterial luciferase and alkane sulfonate monooxygenase are thought to minimize side reactions of freely diffusing flavin with dissolved oxygen and contribute to the efficient transfer of reduced flavin from the reductase to the monooxygenase in catalysis (18,19), although recent data may not be consistent with a luciferase-oxidoreductase complex (20). The reductase of the two-component 4-hydroxyphenylacetate-3-monooxygenase system of *Acinetobacter baumannii* includes an allosteric domain that binds 4-hydroxyphenylacetate and activates the flavin-reduction reaction in response to increasing availability of the hydroxylase substrate. It has been clearly demonstrated that the combination of allosteric regulation with the high affinity of the monooxygenase component for reduced FAD enables the *A. baumannii* system to function efficiently without the need for the formation of a flavin-transfer complex in catalysis (21).

SMOB is not allosterically regulated in this way, and the reduction and epoxidation reactions of the SMO system are efficiently coupled only when free flavin concentrations are low and the monooxygenase is present in >100-fold molar excess over the reductase (15). These features together with sequence homology closely align SMO with 4-hydroxyphenylacetate-3-monooxygenase from *E. coli* W and related proteins (22). Numerical simulations modeling steady-state catalysis of SMO suggest a ternary flavin-transfer complex may be needed to account for the experimentally observed coupling of the NADH oxidation and styrene epoxidation reactions evaluated over a broad range of FAD concentrations and reductase-to-epoxidase ratios (15). However, no direct experimental evidence for protein-protein interactions has been obtained for either styrene monooxygenase or the 4-hydroxyphenylacetate-3-monooxygenase from *E. coli* W (15,23). A common mechanistic theme for styrene monooxygenase and the 4-hydroxyphenylacetate-3-monooxygenases of *E. coli* W and *Acinetobacter baumannii* is a thermodynamic linkage of the flavin-binding and reduction equilibria that favors the binding of reduced flavin over oxidized flavin with great selectivity (15,21,23). This allows the monooxygenase component of each system to effectively scavenge reduced flavin from solution for use in catalysis.

In this work we report the crystal structure of the apo form of NSMOA and identify potential substrate and flavin binding pockets. A detailed analysis of the linked redox and ligand-binding equilibria presented herein demonstrates a strongly cooperative interaction between substrate-analog (benzene) and oxidized-flavin binding equilibria and identifies significant loss of cooperativity in the reduced-FAD complex of NSMOA. The corresponding loss of substrate-analog binding affinity may open the active site to allow oxygen access to react with reduced flavin, and supports a preferred reaction order in which the binding and reaction of reduced-FAD with oxygen precedes the binding of styrene. The equilibrium analysis suggests that in addition to the apo resting state of NSMOA presented here, complexes of apo NSMOA with reduced FAD bound or of NSMOA with oxidized FAD and substrate bound are likely to be the most significant species present in solution prior to reaction of the enzyme with oxygen.

Materials and Methods

Protein Purification

N-terminally histidine-tagged styrene monooxygenase (NSMOA) and styrene monooxygenase reductase were purified by Ni affinity chromatography as described previously (15). NSMOA was then concentrated to approximately 20 mg/mL by using Centricon-30 ultrafiltration devices (Millipore) and stored in 50% glycerol at -20°C.

Fluorescence Spectroscopy

Studies were conducted with a Molecular Devices M5 plate reader. N-terminally histidine-tagged SMOA was exchanged into 20 mM MOPSO buffer adjusted to pH 7 by passage through a Biogel P6 desalting column (Bio-Rad). Following buffer exchange, the protein was concentrated by ultrafiltration and combined with buffer containing FAD and/or benzene in a 96-well black-masked Corning-Costar fluorescence microplate. Equilibria were studied by varying the concentration of NSMOA at defined concentrations of FAD and benzene. Samples were excited at 450 nm and emission was monitored at 520 nm at 25°C with excitation and emission slit widths of 1 mm.

Oxidation-Reduction Potential Measurements

Equilibrium redox potential measurements were conducted in 20 mM MOPSO (pH 7) over range of protein concentrations at 25°C. Spectra of representative redox equilibria of FAD in the presence of NSMOA and reference indicators were recorded with an Ocean Optics USB-2000 spectrophotometer. The solution potential at each point in the titration and the equilibrium midpoint potential of bound FAD in the presence or absence of benzene was computed by using the Nernst equation as described previously (15).

Stopped-Flow Fluorescence Spectroscopy

The kinetics of oxidized FAD binding to NSMOA were measured at 25°C by using an Applied Photophysics SX17 stopped-flow drive unit fitted with PEEK (polyetheretherketone) valves and tubing. Flavin fluorescence was excited with monochromatic 450 nm light along the 0.1 cm light path of the flow cell. Fluorescence emission was detected along the 1 cm light path of the flow cell with an Edmund Optics VG-6 band-pass filter placed in front of the emission photomultiplier tube. The photomultiplier tube was energized with a Pacific Instruments high voltage power supply and the signal digitized with a Pico ADC-216 oscilloscope controlled by PicoScope software.

Isothermal Titration Calorimetry (ITC)

ITC measurements were conducted at 25°C in 20 mM MOPSO buffer (pH 7) by using a MicroCal VP-ITC instrument. In these studies, apo NSMOA was placed in the sample cell of

the calorimeter and titrated with benzene dispensed from the autotitrator syringe set to stir at 307 rpm. To establish the heat of dilution of benzene, a series of injections of benzene were made under identical conditions into MOPSO buffer that contained no enzyme. Enthalpy changes corresponding to the NSMOA-benzene association equilibrium were then computed by numerical integration of the raw ITC data and fit with a single-site model by using Origin 7 software.

Crystallization

NSMOA samples were dialyzed overnight into 20 mM MOPS (pH 7.0) and 5% glycerol prior to crystallization. Samples were pooled and concentrated using an Amicon 10 kDa centrifugal device (Millipore) to concentrations as high as 80 mg/mL. The histidine tag was not removed. Ligand-free crystals of NSMOA appeared within two days by mixing equal volumes of protein at a concentration of 22.9 mg/mL and a reservoir solution consisting of 0.2 M MgCl₂, 0.1 M Tris-HCl (pH 8.5), and 30% (w/v) PEG 4000. All NSMOA crystals were transferred to 10 μ L drops that included the precipitant and 15% (v/v) PEG 400 as a cryoprotectant and immediately flash cooled in liquid nitrogen.

Substrate-Analog Soaks and Cocrystallization

Several soaking and cocrystallization protocols were developed in an attempt to obtain structures of the flavin and substrate-analog bound forms of NSMOA. Efforts to obtain a reduced FADH₂-bound structure included soaking crystals for 5 to 25 min in solutions containing 0.1-1.0 μ M SMOB in the presence of 0.5-50 mM FAD and 2-100 mM NADH. In addition, crystals were soaked in solutions containing 10-50 mM FAD and 10 mM sodium dithionite. Crystallization of NSMOA in the presence of varying concentrations of SMOB, FAD, NADH and dithionite was attempted both aerobically and in a Coy anaerobic chamber. Finally, solutions (1 μ L) containing 10 mM FAD and 100 μ M-10 mM benzene were added to the crystallization drops for 15 min to 24 h in an effort to obtain crystals of oxidized, substrate-analog bound NSMOA.

Data Collection

Diffraction data were collected at 100 K using Mar225 and Mar300 CCD detectors at LS-CAT (sector 21) beamlines ID-D and ID-F and at GM/CA-CAT (sector 23) beamline ID-D at the Advanced Photon Source (APS, Argonne National Laboratory, Argonne, IL). The data sets were indexed, integrated and scaled with HKL2000 (24). There are two molecules in the asymmetric unit, and the crystals belong to space group *P*6₃ with unit cell dimensions of *a* = *b* = 114.302 Å and *c* = 140.803 Å. Diffraction data statistics are shown in Table 1.

Structure Determination

The structure was solved by multiple isomorphous replacement with anomalous scattering (MIRAS) phasing with autoSHARP (25). Three heavy atom derivatives were obtained by soaking crystals in 10 mM and 28.9 mM K₂PtCl₄ (Pt1 and Pt2 in Table 1) and 1 mM KAu(CN)₂ (Au in Table 1) for 24 h. The heavy atom solutions (1 μ L) were added directly to each drop. SHELXD in autoSHARP located 20 Pt and 10 Au sites, and an initial experimental map was calculated to 2.75 Å resolution using the data obtained for the 28.9 mM K₂PtCl₄ soak as the native data set (Pt2 in Table 1) (25-27). The phasing powers for the derivatives were 1.124 for Pt1, 1.246 for Pt2, and 0.87 for Au. The map was improved by solvent flattening using SOLOMON in autoSHARP with an estimated solvent content of 49.1% (28). The initial figure of merit was 0.50 and the figure of merit after solvent flattening was 0.90.

Model Building and Refinement

An initial model was generated using Buccaneer (29) and was further adjusted manually in Coot (30). This model was subjected to iterative rounds of refinement and rebuilding in Refmac5 (31) and Coot, respectively, using dataset Pt2. A higher resolution native data set was then obtained using a crystal that had been soaked in a solution containing 10 mM FAD and 100 μ M benzene for 24 h (native2 in Table 1). The 2.75 Å resolution model ($R_{\text{work}} = 0.21$ and $R_{\text{free}} = 0.26$) was used as a molecular replacement model in PHASER (32) and further refinement to 2.3 Å resolution was conducted with Refmac5 (Table 1). The final model consists of residues 3-410 in chain A, 4-410 in chain B, and 578 water molecules. A Ramachandran plot calculated with PROCHECK (33) shows that 96.3 % of the residues have the most favorable geometry with the rest in additionally allowed regions. Figures were generated with PyMol (34). Coordinate superpositions were performed using the LSQKAB program (35) or the SSM server (<http://www.ebi.ac.uk/msd-srv/ssm>) (36). Protein-protein interfaces were analyzed with PROTORP (<http://www.bioinformatics.sussex.ac.uk/protorp>). Cavity calculations were performed with CASTp (37) and sequence alignments were prepared with ALINE (38).

Results

Estimation of Benzene-Binding Affinity by Isothermal Titration Calorimetry

It was previously demonstrated that benzene binds to SMOA during catalytic turnover as a competitive inhibitor (15), and in the present work, we use benzene as a non-reactive substrate analog of styrene in the evaluation of the ligand-binding and redox equilibria of NSMOA under aerobic and anaerobic conditions. Substitution of benzene for styrene in these studies prevented problems associated with the oxidation and polymerization of styrene, and protein modification through alkylation. These effects are amplified in equilibrium studies that require long-term exposure of NSMOA to high concentrations of styrene. In addition to providing new insight into the role of ligand binding in the reaction mechanism of NSMOA, the equilibrium dissociation constants of benzene and oxidized and reduced forms of FAD helped us establish the concentration ranges to explore in crystallographic studies.

The affinity of benzene binding to the apo enzyme was measured by ITC by titrating apo NSMOA with a concentrated stock solution of benzene. After correcting for the heat of dilution, data were fit according to a non-interacting site equilibrium model, which assumes one benzene-binding site per NSMOA monomer (Figure 1). The best fit through these data indicates that the equilibrium dissociation constant of apo NSMOA for benzene is quite weak with $K_{d1} = 4.20 \pm 0.37$ mM.

Measurement of Linked Benzene and FAD-Binding Equilibria by Fluorescence Titration

The interaction of oxidized FAD and NSMOA was investigated at equilibrium by monitoring the increase in steady-state fluorescence associated with the binding of the flavin. Since oxidized FAD binds weakly to NSMOA, it was necessary to include relatively high concentrations of FAD and NSMOA in the titration. The Molecular devices M5 fluorimeter was found to have a linear fluorescence response up a concentration of 50 μ M FAD. Above this concentration, the signal became complicated by the inner filter effect so FAD concentration was not further increased. Benzene was used in these studies in place of styrene due to the favorable stability and solubility characteristics of this substrate analog. Upon binding to NSMOA, the fluorescence of FAD increases, and equation 1 was derived and used to compute the apparent binding constant of FAD in the presence of various benzene concentrations, where ϵ_1 and ϵ_2 are the molar extinction coefficients for the fluorescence of free and bound forms of FAD. Fits passing through the titration data are shown in the inset of Figure 2.

$$F = \varepsilon_1 [FAD]_{total} + \left(\frac{K_d^{app} + [FAD]_{total} + [SMOA]_{total} - \sqrt{(K_d^{app} + [FAD]_{total} + [SMOA]_{total})^2 - 4[FAD]_{total}[SMOA]_{total}}}{2} \right) (\varepsilon_2 - \varepsilon_1) \quad (1)$$

Use of higher concentrations of NSMOA proved to be experimentally problematic and for this reason it was not possible to record data further into the saturation region of the binding isotherm. This limitation prevented calculation of the endpoint of the titration in the equilibrium reaction of apo NSMOA with FAD. However, the linkage of the benzene and FAD binding equilibria causes the apparent K_d of FAD to decrease hyperbolically such that data from titrations that included benzene could be fit to yield good estimates of ε_2 and the apparent K_d . The data plotted in Figure 2 were fit with Equation 2, which describes the dependence of the apparent K_d of oxidized FAD on the benzene concentration and the equilibrium dissociation constants of apo NSMOA interacting with benzene (K_{d1}) and FAD (K_{d3}) and the oxidized FAD-NSMOA complex interacting with benzene (K_{d4}).

$$K_{dapp} = \frac{K_{d3} \left(1 + \frac{[B]}{K_{d1}} \right)}{\left(1 + \frac{[B]}{K_{d4}} \right)} \quad (2)$$

The best hyperbolic fit through these data imposing the value of K_{d1} determined in the ITC experiment gives limiting values of 1.57 ± 0.79 mM for K_{d3} and 26 ± 20 μ M for K_{d4} .

Redox Measurements

Apparent equilibrium midpoint potentials were computed after spectral deconvolution by using the Nernst equation as described previously (15). Since FAD binds reversibly to NSMOA, both FAD-bound and FAD-free populations were considered in the analysis of redox equilibria. This was accomplished by conducting redox titrations over a range of NSMOA concentrations. These studies were completed in the presence or absence of benzene to further establish the linkage of the flavin reduction and benzene-binding equilibria. The apparent midpoint potentials plotted in Figure 3 were fit with Equations 3 and 4, which give the dependence of the apparent midpoint potential on the concentration of NSMOA for titrations, which excluded or included benzene, respectively.

$$E_m^{app} = E_m^{freeFAD} + \frac{RT}{nF} \ln \left(\frac{\left(1 + \frac{[NSMOA]}{K_{d6}} \right)}{\left(1 + \frac{[NSMOA]}{K_{d3}} \right)} \right) \quad (3)$$

$$E_m^{app} = E_m^{freeFAD} + \frac{RT}{nF} \ln \left(\frac{\left(1 + \frac{[NSMOA]}{K_{d5}} \right)}{\left(1 + \frac{[NSMOA]}{K_{d2}} \right)} \right) \quad (4)$$

Known values for the solution potential of free FAD ($E_m^{freeFAD}$) and the equilibrium dissociation constants for oxidized FAD binding to the benzene-bound (K_{d2}) and apo (K_{d3}) forms of NSMOA, which were determined by fluorescence titration (Figure 2), were included as fitting constants. In this way, it was possible to compute estimates for the remaining dissociation constants ($K_{d5} = 112 \pm 30$ nM and $K_{d6} = 206 \pm 27$ nM) describing the binding equilibria of reduced FAD interacting with the benzene-free and benzene-bound forms of NSMOA, respectively. The linkages of the redox and ligand-binding equilibria of NSMOA are summarized in Figure 4 together with numerical estimates of the corresponding equilibrium midpoint potentials and ligand dissociation constants.

Flavin-Binding Kinetics

The binding of oxidized FAD to NSMOA monitored by stopped-flow fluorescence spectroscopy is shown in Figure 5. The binding reaction studied under pseudo first order reaction conditions results in three kinetic phases characterized by two initial rapid increases followed by a slow decrease in fluorescence emission intensity. Data shown in the plot were subjected to a logarithmic time averaging scheme to improve signal to noise and to optimize the data spacing for exponential fitting.

Crystal Structure

The structure of NSMOA was refined to 2.3 Å resolution (Table 1). An N-terminal histidine tag was not visible in the electron density map. Many of the side chains were visible in the MIRAS experimental map and most had well defined electron density in the final $2F_o - F_c$ map. The NSMOA monomer comprises two globular domains spanned by a long α helix (Figure 6A). Domain A consists of a four-stranded parallel β -sheet ($\beta 1$, $\beta 2$, $\beta 5$, and $\beta 6$) flanked by helices $\alpha 1$, $\alpha 5$, and $\alpha 6$ as well as two 3_{10} helices, $\alpha 2$ and $\alpha 7$. A β hairpin formed by $\beta 12$ and $\beta 13$, helix $\alpha 12$, and the N-terminal part of helix $\alpha 13$ can also be considered part of this domain. Domain B includes a 7-stranded mixed β -sheet ($\beta 3$, $\beta 4$, $\beta 7$, $\beta 8$, $\beta 9$, $\beta 10$, and $\beta 11$) flanked by helices $\alpha 8$, $\alpha 9$, $\alpha 10$, and $\alpha 11$ on one side and the C-terminus of $\alpha 13$ and helices $\alpha 14$, $\alpha 15$, $\alpha 16$, and $\alpha 17$ on the other. The two NSMOA molecules in the asymmetric unit form a homodimer (Figure 6B), consistent with gel filtration analysis (14). The two monomers are very similar and superpose with a rmsd of 0.31 Å for 407 Ca coordinates. The interface region, which buries 1034 Å² per monomer, consists primarily of loop regions along with 3_{10} helices $\alpha 2$ and $\alpha 9$ and helix $\alpha 6$. Despite extensive soaking and cocrystallization experiments involving FAD, FADH₂, and benzene at concentrations demonstrated to bind to NSMOA in the solution studies, no electron density attributable to benzene or FAD was detected in $F_o - F_c$ maps.

Discussion

Structural Comparisons

Searches with the DALI server (39) indicate that NSMOA is most similar to PHBH from *Pseudomonas fluorescens*. Although structures of the hydroxylase components from two two-component systems have been determined recently (40,41), the single component PHBH is a better structural match. Superposition with the PHBH Phe161Ala variant structure (PDB accession code 1CC4, top hit in DALI search) (42) yields an rmsd of 2.47 Å for 309 Ca coordinates. This mutant and the related Arg166Ser variant were prepared to probe the roles of these residues in NADPH binding by PHBH. NSMOA also closely resembles PHBH from *Pseudomonas aeruginosa* (4). Superposition of NSMOA and PHBH shows that the cores of both domains are similar, with some significant differences on the periphery of the molecule and in loop regions (Figure 7). For example, there is an insertion in PHBH domain A that forms a β -hairpin near helix $\alpha 6$ in NSMOA. This β -hairpin is also present in other homologs, including the flavin hydroxylase RebC (43). In addition, the C-terminus of NSMOA is more extended

and includes additional helices not present in PHBH. The regions connecting domains A and B also differ somewhat, with residues 152-166 in NSMOA forming a loop whereas the corresponding region in PHBH is an extended β -strand. Although PHBH is also dimeric in solution, it crystallizes with one monomer in the asymmetric unit. Two interfaces between symmetry-related molecules are observed in its structure (44), of which neither resembles the NSMOA dimer interface.

Flavin Binding Site

Despite extensive efforts, a structure in the presence of FADH₂ or FAD and benzene was not obtained. The FAD binding cavity can be identified by comparison to the PHBH structure, however (Figures 7 and 8). In the location of the FAD in PHBH (45), NSMOA has a large cavity that could accommodate FAD and is open to the surface (Figure 8), which would allow transfer of reduced FADH₂ from SMOB. Similar to PHBH, the isoalloxazine ring is predicted to reside at the interface between the two domains with the ribityl group and adenosine ring extending into domain A. The cavity is occupied by a number of water molecules in the current structure. In PHBH, the flavin ring interacts with residues Gly 46, Val 47, Gly 298, Leu 299, and Asn 300. Residues Val 48, Ala 49, Gly 307, Ala 308, and Asn 309 occupy these positions in NSMOA (Figure 9). The carbonyl oxygen of Ala 308 is oriented nearly identically to that of Leu 299 in PHBH, suggesting it may interact with both ribose oxygen position O3' and the flavin carbonyl oxygen O2. Residue Asn 309 in NSMOA may also be involved in hydrogen bonding with the flavin carbonyl oxygen O2. Residue Asp 295 in NSMOA occupies a similar position to PHBH Asp 286, which interacts with the ribityl chain.

There are also a number of differences between NSMOA and PHBH in this putative FAD binding site. The NSMOA main chain adopts a different conformation from PHBH spanning residues 32-46. As a result, Arg 43 and Asn 46, which are equivalent to PHBH Arg 42 and Arg 44, of which both are involved in FAD binding, are not properly positioned to interact with FAD. Additionally, differences in secondary structure cause NSMOA residues Lys 145 and Tyr 146 to extend into the pocket where they would interfere with binding of the adenosine ring. However, the orientation of the adenosine ring observed in the structure of *Comamonas testosteroni* 3-hydroxybenzoate hydroxylase (46) can be accommodated by the NSMOA structure. Finally, Ser 13 within the first helix of PHBH, which interacts with the pyrophosphate group is replaced with alanine in NSMOA. Several residues adjacent to the flavin binding site in PHBH, including Phe 161, His 162, Arg 166, and Arg 269, are important for NADPH binding (8,42). These residues are not conserved in NSMOA and the corresponding regions adopt completely different conformations, consistent with NSMOA not interacting directly with pyridine nucleotides.

Substrate Binding Site

At the bottom of the FAD binding site, a likely substrate-binding cavity is present (Figure 8). This cavity is located in the analogous position to the *p*-hydroxybenzoate site in PHBH (42) and is large enough to accommodate styrene. The pocket is adjacent to where the FAD isoalloxazine ring is predicted to sit and abuts the surface of the 7-stranded β -sheet in domain B. The putative substrate-binding site is completely buried within the protein core, with the only access via the FAD binding site. A number of hydrophobic residues, including Val 184, Met 186, Ile 196, Ile 198, Val 211, Gly 305, Phe 382, and Phe 386, line the cavity (Figure 10). Also surrounding the pocket are His 76, Tyr 78, Gln 306, and Asn 309. Previous predictions of the styrene-binding site based on computational modeling identified residues Phe 235, Val 303, Leu 220, Ile 198, and Val 222 as surrounding the styrene (47). Of these residues, only Ile 198 is present in the cavity in the current structure. Val 303 is nearby, but its side chain points away from the pocket, and the remaining residues are quite distant. In PHBH, residue Pro 293 interacts with the 3-OH group of the product and is thought to stabilize the transition state

(48). The analogous residue in NSMOA, Pro 302, occupies the same position, but cannot engage in the same way with the styrene ring in the substrate epoxidation reaction. Other residues involved in binding *p*-hydroxybenzoate include Arg 214, Arg 220, and Tyr 201 and are replaced with Thr 200, Ala 209, and Met 186 in NSMOA, respectively. This increase in hydrophobicity is consistent with binding styrene. The active site residue His 72, which is thought to function in a proton relay from solvent to the aromatic ring hydroxyl of *p*-hydroxybenzoate (49) corresponds to Tyr 73 and Phe 74 in NSMOA. Other differences along the proton relay path identified in the PHBH structure include the substitution of Phe 386 for PHBH Tyr 385 and Val 210 for PHBH Tyr 222. The presence of non-hydrogen bonding side chains in these positions suggests that NSMOA does not employ the same type of proton shuttle system that has been invoked in the PHBH mechanism.

Crystallographic and kinetic data have resolved three conformations of PHBH, which are thought to be relevant to catalysis (1,48). Two of these arrangements, designated “in” (PDB accession code 1PBE) (45) and “out” (PDB accession code 1DOD) (4), differ primarily in the position of the isoalloxazine ring, which has moved to more sequestered or solvent exposed locations, respectively. In addition, *p*-hydroxybenzoate is bound in the flavin “in” structure, whereas the flavin “out” structure contains the reaction product, 3,4-dihydroxybenzoate. In a third structure, termed “open” (PDB accession code 1K0I) (8), a more substantial movement of protein allows substrate and product access to the active site. Given substantial differences in regions bordering the binding sites (e.g. NSMOA residues 123-126, 143-146, and 268-275) (Figure 7), it is difficult to compare NSMOA to these subtle variants. However, it is clear that the FAD is exposed to solvent and that the predicted substrate binding cavity is not. Therefore, the current structure may be comparable to the flavin “out” conformation.

Catalytic Significance of Apo Enzyme and Complexes with Substrate and FAD

The intracellular concentration of oxidized FAD is estimated to be less than 100 μ M (23) and the growth of *P. putida* S12 is inhibited at styrene concentrations greater than 300 μ M (50), indicating that intracellular oxidized flavin and styrene concentrations are likely to be low relative to the millimolar equilibrium constants determined in this study for interaction with apo NSMOA. This suggests that the apo enzyme structure reported here represents a significant fraction of SMOA present in solution under typical cellular conditions.

Oxidized FAD and benzene-binding equilibria of NSMOA are strongly linked, such that the binding of each increases the affinity of NSMOA for the other by ~60-fold based on the ratios of K_{d1}/K_{d4} or K_{d3}/K_{d2} (Figure 4), so if both substrate and oxidized FAD are present in solution, they will cooperatively bind the enzyme with much higher affinity. Thus, in addition to the apo enzyme, NSMOA with both oxidized FAD and styrene bound is expected to be present in the cell. Complexes of NSMOA with only FAD or styrene bound are expected to represent a much smaller fraction of the total enzyme.

Flavin reduction is linked to an ~8000-fold increase in flavin-binding affinity of the apo enzyme (K_{d3}/K_{d6}), but there is only an ~200-fold increase in flavin-binding affinity for the NSMOA-benzene complex (K_{d2}/K_{d5}). In either case, it can be concluded that reduced FAD not only binds tightly to NSMOA, but also competitively displaces oxidized FAD from the enzyme whether or not substrate analog is bound.

Substrate analog binds the reduced-FAD complex of NSMOA with an affinity that is only about two times greater than its affinity for the oxidized-FAD complex (K_{d1}/K_{d7}). This 30-fold decrease in cooperativity of substrate-analog and reduced-FAD binding equilibria translates into a substrate analog binding affinity for the reduced-FAD complex of NSMOA that is only ~2 times greater than that observed for the oxidized FAD complex (K_{d7} ~2.3 mM vs K_{d7} ~4.2 mM). This interaction is too weak to account for the catalytic activity of NSMOA, which

has an apparent K_m of 5 μM for styrene and K_i for benzene of 170 μM (15). For this reason, the complex of NSMOA with reduced FAD and styrene bound is expected to represent a relatively small fraction of the total forms of enzyme present during catalysis.

Functional Implications

Giving consideration to both the physiologically relevant concentration ranges of substrate and FAD and the observed linkage of ligand-binding and redox equilibria in this system, it can be concluded that the four most catalytically relevant states of the enzyme prior to its reaction with molecular oxygen are those shown in Scheme 1. The complex with substrate and oxidized FAD (A) forms in a strongly cooperative fashion and may represent a means of buffering the intracellular oxidized FAD concentration and maintaining styrene in proximity to the active site while NSMOA is awaiting the arrival of reduced FAD. The cooperative linkage of oxidized-FAD and substrate-analog NSMOA-binding equilibria indicates the apo enzyme structure (B) will be a predominant form of the enzyme under conditions when reduced-FAD is absent. The fraction of the enzyme having only substrate or oxidized FAD bound will be less significant under these conditions. Reduced FAD binds with very high affinity to apo NSMOA and will also easily competitively displace oxidized FAD from the complex of enzyme with substrate and oxidized FAD. The reduced-FAD enzyme complex (C) has very low affinity for substrate, such that in the presence of reduced FAD, only a small fraction of the enzyme will be present as a reduced FAD and substrate-bound complex. Clearance of substrate from the active site may be necessary to allow oxygen access to react with the reduced FAD in the catalytic mechanism. It can be concluded that the crystal structure presented here represents one of the three most catalytically relevant states of the enzyme prior to its reaction with molecular oxygen.

The kinetics of tracking the binding of oxidized FAD to NSMOA by stopped-flow fluorescence spectroscopy strongly support the idea that oxidized flavin binding to NSMOA involves at least three steps corresponding to changes in the flavin electronic environment. Part of the investment in binding energy associated with the formation of the low affinity oxidized-FAD-NSMOA complex may be in the conformational reorganization of the FAD binding pocket of apo NSMOA into a configuration that accommodates the binding of FAD. The details of these interactions will have to await the outcome of future structural studies.

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Abbreviations

FAD	flavin adenine dinucleotide
ITC	isothermal titration calorimetry
NADPH	nicotinamide adenine dinucleotide phosphate
PHBH	<i>p</i> -hydroxybenzoate hydroxylase
rmsd	root mean square deviation
SMO	styrene monooxygenase
SMOA	styrene monooxygenase A

SMOB styrene monooxygenase B

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Figure 1.

Calorimetric titration of apo NSMOA with benzene. Top, raw data corresponding to 54 $5\ \mu\text{L}$ injections of $5\ \text{mM}$ benzene into $18\ \mu\text{M}$ NSMOA monomer in $20\ \text{mM}$ MOPSO buffer (pH 7) at 25°C . Bottom, least squares fit with a single, non-interacting site model. Parameters from this fit are $\Delta G = -3.24 \pm 1.7\ \text{kcal}\cdot\text{mol}^{-1}$, $\Delta H = -26.17 \pm 1.9\ \text{kcal}\cdot\text{mol}^{-1}$, and $\Delta S = +77 \pm 40\ \text{kcal}\cdot\text{mol}^{-1}$.

Figure 2.

Estimate of K_d for oxidized FAD binding to NSMOA in the presence of saturating benzene. Solutions containing 50 μM oxidized FAD and 0–1.2 mM benzene were titrated with NSMOA. The inset shows raw data from titrations at 35 ($\bullet$), 100 ($\blacksquare$), and 250 ($\circ$) μM benzene fit with the quadratic equation described in the text to yield apparent K_d values. Apparent K_d values plotted as a function of benzene concentration were then fit to find the best estimates of the K_d value of oxidized FAD for NSMOA in the presence and absence of saturating benzene.

Figure 3.

Estimates of NSMOA-bound FAD equilibrium redox potential in the presence (●) and absence (○) of 1.2 mM benzene. Apparent FAD midpoint potentials are plotted as a function of the NSMOA concentration at which they were determined. The inset shows an example of the solution-potential data used to compute the apparent midpoint potential at 80 μ M NSMOA.

Figure 4.

Ligand binding and redox equilibria of NSMOA. Equilibrium dissociation constants are: $K_{d1} = 4.20 \pm 0.37$ mM for the interaction of benzene with apo NSMOA measured by ITC; $K_{d2} = 26 \pm 20$ μ M and $K_{d3} = 1.57 \pm 0.79$ mM for the binding of oxidized FAD to the benzene-bound and apo forms of NSMOA, respectively, as determined by fluorescence titration; $K_{d5} = 112 \pm 30$ nM and $K_{d6} = 206 \pm 27$ nM for the binding of reduced FAD to the benzene-bound and apo forms of NSMOA, respectively, as determined by electrochemical titration; $K_{d4} = 69.6 \pm 64$ μ M and $K_{d7} = 2.3 \pm 1.9$ mM for the binding benzene to the reduced and oxidized FAD complexes of NSMOA, respectively, computed from linkage relationships ($K_{d4} = K_{d1}K_{d2}/K_{d3}$ and $K_{d7} = K_{d1}K_{d5}/K_{d6}$). The solution potential of free FAD is $E_{m1} = -212$ mV (15). The equilibrium midpoint potentials of FAD bound to the benzene-free and bound forms of NSMOA calculated from the linkage relationships $E_{m2} = E_{m1} - RT/nF \cdot \ln(K_{d2}/K_{d5})$ and $E_{m3} = E_{m1} - RT/nF \cdot \ln(K_{d3}/K_{d6})$ are -97 mV and -142 mV, respectively.

Figure 5.

Kinetics of oxidized FAD binding to NSMOA monitored by stopped-flow fluorescence spectroscopy. A solution of 280 μM NSMOA was rapidly mixed with 50 μM FAD in the stopped-flow cell. A four-exponential fit through the data yields estimated rate constants of $k_1 = 265 \text{ s}^{-1}$, $k_2 = 120 \text{ s}^{-1}$, $k_3 = 21 \text{ s}^{-1}$, and $k_4 = 0.34 \text{ s}^{-1}$.

Figure 6. Overall structure of NSMOA. (A) The NSMOA monomer. Domain A is shown in blue and domain B is shown in green. Secondary structure elements are labeled. (B) The NSMOA dimer colored as in (A).

Figure 7. Stereosuperposition of NSMOA (green) and PHBH (magenta, PDB accession code 1CC4). The FAD and *p*-hydroxybenzoate bound to PHBH are shown as stick representations.

Figure 8.

Surface representation of NSMOA with FAD and *p*-hydroxybenzoate from PHBH structure superposed. Positions of residues predicted to interact with FAD are shown in blue and positions of residues in the predicted substrate-binding pocket are shown in red. The FAD and substrate binding pockets are surface accessible.

Figure 9.
Structure-based sequence alignment of SMOA and PHBH. The secondary structure elements for SMOA are indicated.

Figure 10.
Residues lining proposed styrene binding pocket in NSMOA.

Scheme 1.

Table 1
Data collection and refinement statistics

	native1 ^a	native2	Pt1	Pt2	Au
Data Collection					
wavelength (Å)	1.03	0.98	1.05	1.03	1.03
resolution (Å)	50.0-2.90 (3.00-2.90)	50.0-2.27 (2.31-2.27)	50.0-3.30 (3.42-3.30)	50.0-2.75 (2.80-2.75)	50.0-2.91 (2.96-2.91)
R _{syn}	0.144 (0.405)	0.093 (0.378)	0.107 (0.360)	0.099 (0.386)	0.115 (0.377)
I/σI	20 (10.5)	30.9 (6.5)	23.4 (9.4)	19.0 (5.8)	28 (7.9)
completeness (%)	100 (100)	100 (100)	99.7 (99.4)	98.9 (100)	100 (100)
redundancy	11.4 (11.5)	11.6 (11.6)	10.0 (9.6)	5.7 (5.8)	11.5 (11.6)
Refinement					
Resolution		42.4-2.30			
no. of reflections		43907			
R _{work} /R _{free}		0.21/0.24			
no. of atoms					
Protein		6429			
Water		578			
average B factor (Å ²)		18.9			
root-mean-square deviations					
bond lengths (Å)		0.006			
bond angles		0.891			

^aThe native1 dataset was collected at the GM/CA-CAT beamline; all others were collected at LS-CAT beamlines.