

Catalytic Importance of the Substrate Binding Order for the FMNH₂-Dependent Alkanesulfonate Monooxygenase Enzyme[†]

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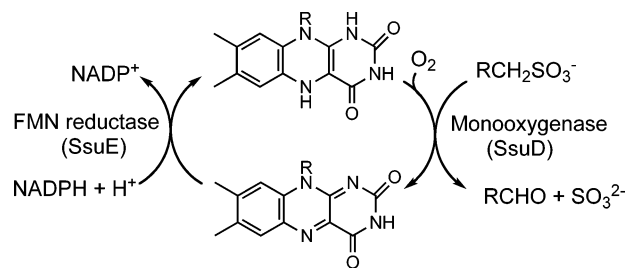
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ABSTRACT: The two-component alkanesulfonate monooxygenase system from *Escherichia coli* includes an FMN reductase (SsuE) and an FMNH₂-dependent alkanesulfonate monooxygenase (SsuD) involved in the acquisition of sulfur from alkanesulfonates during sulfur starvation. The SsuD enzyme directly catalyzes the oxidation of alkanesulfonate to aldehyde and sulfite in the presence of O₂ and FMNH₂. The goal of these studies was to investigate the kinetic mechanism of SsuD through rapid reaction kinetics and substrate binding studies. The SsuD enzyme shows a clear preference for FMNH₂ (K_d , $0.32 \pm 0.15 \mu\text{M}$) compared to FMN (K_d , $10.2 \pm 0.4 \mu\text{M}$) with a 1:1 binding stoichiometry for each form of the flavin. The kinetic trace of premixed SsuD and FMNH₂ mixed with oxygenated buffer was best fit to a double exponential with no observed formation of the C4a-(hydro)peroxyflavin. However, when FMNH₂ was mixed with SsuD and oxygenated buffer an initial fast phase (k_{obs} , 12.9 s^{-1}) was observed, suggesting that the mixing order is critical for the accumulation of the C4a-(hydro)peroxyflavin. Results from fluorimetric titrations with octanesulfonate imply that reduced flavin must bind first to promote octanesulfonate binding. When octanesulfonate was included in the kinetic studies the C4a-(hydro)peroxyflavin was observed at 370 nm when FMNH₂ was not premixed with SsuD, which correlated with an increase in octanal product. There was a clear hyperbolic dependence on octanesulfonate binding, indicating that octanesulfonate binds in rapid equilibrium, and further results indicated there was a second isomerization step following binding. These results suggest that an ordered substrate binding mechanism is important in the desulfonation reaction by SsuD with reduced flavin binding first followed by either O₂ or octanesulfonate.

In *Escherichia coli*, sulfur is typically acquired from inorganic sulfate and assimilated through the cysteine biosynthetic pathway (1–3). Alternative sulfur sources including organic sulfonate and sulfate esters are utilized when inorganic sulfate is limiting in the environment. Sulfate limitation induces specific proteins that enable the organism to acquire and metabolize these alternative sulfur sources. Proteins produced from the *ssu* operon are involved in the uptake and desulfonation of alkanesulfonates in the environment. In addition to an ABC-type transporter, a two-component protein system is required for sulfur acquisition (4). The NAD(P)H¹-dependent FMN reductase (SsuE) catalyzes the reduction of FMN by NAD(P)H followed by the transfer of reduced flavin to the monooxygenase (SsuD), which catalyzes the oxygenolytic cleavage of alkanesulfonate to aldehyde and sulfite (Scheme 1) (4). The released sulfite is reduced and assimilated into sulfur-containing compounds.

Several bacterial flavin reductases associated with two-component systems have been kinetically evaluated (5–10). Although SsuE shares little sequence identity with other

Scheme 1: Reaction Mechanism of the Alkanesulfonate Monooxygenase System



flavin reductases, the mechanism of flavin reduction is similar. In steady-state kinetic assays, the results have shown that SsuE follows an ordered sequential mechanism, with NADPH as the first substrate to bind and NADP⁺ as the last product to dissociate. Reduction of the flavin by NADPH leads to immediate transfer from SsuE to SsuD. While the steady-state kinetic parameters of the system were not significantly altered in the presence of SsuD and octanesulfonate, the kinetic mechanism of SsuE is altered to a rapid equilibrium ordered mechanism. These results suggested that protein–protein interactions are relevant to flavin transfer (5). Results from rapid reaction kinetic studies of flavin reduction by SsuE identified the formation of charge-transfer intermediates between the pyridine nucleotide and flavin substrate (11). Therefore, the mechanism of flavin reduction involving formation of charge-transfer complexes is similar

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¹ Abbreviations: DTNB, 5,5-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediamine tetraacetic acid; FMN, flavin mononucleotide; FMNH₂, reduced flavin mononucleotide; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); SsuE, FMN reductase; SsuD, alkanesulfonate monooxygenase.

to standard flavoproteins. Additional studies have been performed to better understand the mechanism of reduced flavin transfer between SsuE and SsuD (11). Stable protein–protein interactions were identified between the alkane-sulfonate monooxygenase proteins through affinity chromatography and spectroscopic analyses. These studies further support a model involving direct flavin transfer from the reductase to the monooxygenase enzyme.

While the flavin reductase belonging to the alkanesulfonate monooxygenase system has been well characterized, much less is known about the catalytic mechanism of the monooxygenase component. The reaction mechanism of SsuD involves the oxygenolytic cleavage of the carbon–sulfur bond of alkanesulfonates. The α -ketoglutarate-dependent taurine dioxygenase from *E. coli* is another enzyme that utilizes primary aliphatic sulfonates as sulfur sources (12). The mechanism involved for sulfur acquisition by taurine dioxygenase is notably different from the alkanesulfonate monooxygenase system. Taurine dioxygenase utilizes α -ketoglutarate and an iron center to activate O_2 for cleavage of the carbon–sulfur bond, while the alkanesulfonate monooxygenase relies on a reduced flavin for dioxygen activation (4). The SsuD enzyme was previously shown to catalyze the desulfonation of a wide range of alkanesulfonate substrates, and this catalysis was dependent on reduced flavin and dioxygen (4). Comparison of the amino acid sequence identity of SsuD with other two-component monooxygenase enzymes is low; however, SsuD is structurally related to bacterial luciferase (13–15). Specifically, there are several conserved and spatially similar active site residues common to each protein. The catalytic role for several of these residues has been determined in bacterial luciferase; however, their corresponding role in the desulfonation reaction by SsuD remains to be elucidated (16–21).

While the initial characterization of SsuD has been performed, the detailed mechanism of sulfur acquisition by this enzyme has not been fully explored (4). A C4a-(hydro)peroxyflavin intermediate is proposed to be the reactive O_2 species for SsuD and other monooxygenase components belonging to these systems (22–28). Several groups have identified the formation of a flavin adduct when the monooxygenase enzyme is mixed with reduced flavin in the presence of oxygen. Detailed presteady state kinetic analysis of aromatic hydroxylation by *p*-hydroxyphenylacetate 3-hydroxylase has provided valuable insight into the mechanism of hydroxylation (25, 26). The desulfonation of alkane-sulfonates by SsuD likely involves the formation of a C4a-(hydro)peroxyflavin intermediate similar to *p*-hydroxyphenylacetate 3-hydroxylase; however, the kinetic mechanism may be more closely related to bacterial luciferase given their structural and catalytic similarities. Presteady state evaluation of the SsuD reaction has been performed, and a mechanism for sulfur acquisition and substrate binding is described in these studies.

EXPERIMENTAL PROCEDURES

Materials. FMN, NADPH, EDTA, potassium phosphate (monobasic anhydrous and dibasic anhydrous), sodium chloride, sodium hydrosulfite (dithionite), and 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), glucose, glucose oxidase, and urea were from Sigma (St. Louis, MO). Glycerol was

purchased from Fisher (Pittsburgh, PA). Standard buffer contains 25 mM potassium phosphate, pH 7.5, and 10% glycerol unless otherwise noted. SsuD and SsuE enzymes were expressed and purified as previously described (5). The concentrations of SsuD and SsuE proteins were determined from A_{280} measurements using a molar extinction coefficient of $47.9 \text{ mM}^{-1} \text{ cm}^{-1}$ and $20.3 \text{ mM}^{-1} \text{ cm}^{-1}$, respectively.

Reduced Flavin Binding. Reduced flavin binding to SsuD and SsuE was measured by spectrofluorimetric titration using a similar method described for oxidized flavin (3). Spectra were recorded on a Perkin-Elmer LS 55 luminescence spectrometer (Palo Alto, CA) with an excitation wavelength at 280 nm and an emission wavelength of 344 nm. Anaerobic SsuD or SsuE enzyme solutions were prepared in a glass titration cuvette by at least 15 cycles of evacuation followed by equilibration with ultrahigh purity argon gas. Glucose (20 mM) and glucose oxidase (10 units) were added to remove trace amounts of dioxygen. Flavin prepared in standard buffer was added to an airtight titrating syringe with an oxygen-scrubbing system of glucose (20 mM) and glucose oxidase (10 units). The syringe was incubated in an anaerobic glovebox for 20 min to remove O_2 . The anaerobic FMN solution was photoreduced by irradiation for 30 min in the presence of EDTA (10 mM), and the titration apparatus was assembled in the anaerobic glovebox.

For the titration of SsuD with reduced flavin, a 1 mL solution of SsuD ($0.5 \mu\text{M}$) in standard buffer was titrated with reduced flavin (0.16 – $7.8 \mu\text{M}$) and the fluorescence spectra recorded following a 2 min incubation after each addition. The titration of SsuE ($0.5 \mu\text{M}$) with reduced flavin was performed as described for SsuD; however, the reduced flavin concentration used for the titration ranged from 0.5 to $97.0 \mu\text{M}$. Bound FMNH₂ was determined using the following equation (29):

$$[S]_{\text{bound}} = [E] \frac{(I_0 - I_c)}{(I_0 - I_f)} \quad (1)$$

where $[S]_{\text{bound}}$ represents the concentration of enzyme-bound substrate. $[E]$ represents the initial concentration of enzyme, I_0 is the initial fluorescence intensity of enzyme prior to the addition of substrate, I_c is the fluorescence intensity of enzyme following each addition, and I_f is the final fluorescence intensity. The concentration of FMNH₂ bound was plotted against the free substrate to obtain the dissociation constant (K_d) according to eq 2.

$$Y = \frac{B_{\text{max}} X}{K_d + X} \quad (2)$$

Y and X represent the concentration of bound and free substrate, respectively, following each addition. B_{max} is the maximum binding at equilibrium with the maximum concentration of substrate. K_d is the dissociation constant for the substrate.

Octanesulfonate Binding. Octanesulfonate binding to SsuD or the SsuD–FMNH₂ complex was performed by similar spectrofluorimetric methods used in the flavin binding experiments. A 1 mL solution of SsuD ($1 \mu\text{M}$), either alone or with reduced flavin ($2 \mu\text{M}$), was made anaerobic in a glass titration cuvette as described. Aliquots of octanesulfonate (2.7 – $54 \mu\text{M}$ or 2.7 – $108 \mu\text{M}$) in an airtight titrating syringe

were added to SsuD or the SsuD–FMNH₂ complex. The fluorescence spectra were recorded with an excitation wavelength at 280 nm and emission intensity measurements at 344 nm following a 2 min incubation. The concentration of bound octanesulfonate was determined by eq 1 and plotted against the concentration of free octanesulfonate to determine the dissociation constant (K_d) according to eq 2.

Rapid Reaction Kinetic Analyses. Stopped-flow kinetic analyses were carried out on a thermostatted Applied Photophysics SX. 18 MV stopped-flow spectrophotometer. The stopped-flow instrument was made anaerobic by filling with an O₂ scavenging system containing 25 mM phosphate buffer, pH 7.5, 10% glycerol, 100 mM NaCl with 20 mM glucose, and 10 units of glucose oxidase in the inner system and drive syringe. The oxygen-scrubbing solutions were allowed to stand in the system overnight and were thoroughly rinsed with anaerobic standard buffer prior to performing the experiments. All reactions were carried out in standard buffer at 4 °C. Alternate mixing of the enzyme and substrates were performed in these studies; however, all experiments contained 45 μ M SsuD and 15 μ M FMNH₂. The FMNH₂ solutions were made anaerobic in a glass tonometer and then photoreduced by irradiation for 30 min in the presence of EDTA (10 mM). The O₂ concentration remained constant at 210 μ M (air-saturated buffer), or when evaluating the O₂ dependence was varied from 0.1 to 1.0 mM. The O₂ concentrations were determined on a Hansatech oxygen electrode (Norfolk, UK). The O₂-saturated buffer in experiments monitoring dioxygen dependence was prepared by bubbling 100% O₂ in standard buffer for 30 min in an airtight syringe. Solutions containing different concentrations of O₂ were prepared by mixing different volumes of O₂-saturated buffer with anaerobic buffer solutions. When included in the reaction, the octanesulfonate concentration was constant (100 or 200 μ M), or when monitoring the octanesulfonate dependence varied from 0.5 to 1.0 mM. All experiments were carried out in single-mixing mode by mixing equal volumes of the various solutions and monitored by single wavelength analyses at 370 and 450 nm.

Activity Assay. SsuD activity was assayed based on a previously described method with some modifications (4). Reactions were initiated by the addition of NADPH (500 μ M) into a reaction mixture containing SsuD (0.2 μ M), SsuE (0.6 μ M), FMN (1 μ M), and a range of octanesulfonate concentrations (5–1000 μ M) in standard buffer with 100 mM NaCl at 25 °C. After a 3 min incubation, the reaction was stopped by the addition of urea (2.0 M), and DTNB (1 mM) was added to a 200 μ L aliquot of the reaction solution. The colorimetric reaction was allowed to develop at room temperature for 2 min, and the absorbance measured at 412 nm using a molar extinction coefficient for the TNB anion of 14.1 mM⁻¹cm⁻¹.

An assay was also developed to compare the formation of aldehyde product using alternate mixing of reaction components, analogous to reactions carried out in the stopped-flow instrument. The contents of syringe A and B are shown in Table 2, and all reactions were performed in 25 mM potassium phosphate buffer, pH 7.5, 25 °C. Solutions in syringe A always contained the flavin substrate either in the presence or absence of SsuD and the octanesulfonate substrate. Reduced flavin was prepared in an airtight syringe with an O₂-scrubbing system containing glucose (20 mM)

and glucose oxidase (10 units). Syringe A was incubated in an anaerobic glovebox for 20 min to remove O₂, and the flavin was photoreduced in the presence of EDTA (10 mM). The solution in syringe A was then mixed with the solution in syringe B to give a total volume of 1 mL. The reaction mixture was incubated at 25 °C for 5 min, and 1 mL methylene chloride was added to extract the octanal product from the reaction solution. A standard curve was generated with a range of octanal concentrations (1–50 μ M) in 1 mL of methylene chloride, and 50 μ M pentadecane was added as the internal standard (retention time: 16.3 min). The methylene chloride extract solution was analyzed on a Shimadzu gas chromatograph GC-14A equipped with a flame ionization detector. A 1 μ L sample was chromatographed on a 30 m \times 0.25 cm HP-1 (cross-linked methyl silicone gum) column with nitrogen at 180 °C, and the detector temperature was at 250 °C.

Data Analysis. Initial analyses of the single wavelength stopped-flow traces at 370 and 450 nm were performed with the PROKIN software (Applied Photophysics, Ltd.) installed on the stopped-flow spectrophotometer. Global analysis was applied to discern the steps involved in flavin oxidation. A two- or three-step sequential reversible model was adopted during the fitting, and the kinetic traces were resolved into two or three distinct phases depending on the wavelength evaluated. All single wavelength traces at 370 and 450 nm were imported and fitted with Kaleidagraph software (Abelbeck Software, Reading, PA). The single-wavelength traces were fitted to a double or triple exponential using the following equations:

$$A = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + C \quad (3)$$

$$A = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + A_3 \exp(-k_3 t) + C \quad (4)$$

where k_1 , k_2 , and k_3 are the apparent rate constants, A is the absorbance at time t , A_1 , A_2 , A_3 are amplitudes of each phase, and C is the absorbance at the end of the reaction.

The data for the concentration dependence of octanesulfonate on k_1 were fitted with a simplified hyperbolic equation:

$$k_{\text{obs}} = k_{\text{lim}}[S]/(K_d + [S]) \quad (5)$$

where k_{obs} is the observed rate constant, k_{lim} is the limiting rate constant for flavin reduction, K_d is the dissociation constant for the enzyme–substrate complex, and $[S]$ is the substrate concentration.

RESULTS

Reduced Flavin Binding. Previous studies have shown that each protein in the two-component monooxygenase family has a specific affinity for the oxidized or reduced form of the flavin (5). The dissociation constants for the binding of reduced flavin to SsuE and SsuD were determined by fluorimetric titrations. The decrease in the intrinsic protein fluorescence emission intensity due to the binding of FMNH₂ was monitored at 344 nm for each protein. The concentration of bound and free FMNH₂ was calculated according to eq 1, and the concentration of flavin bound to each enzyme was plotted against the concentration of free FMNH₂ added with each aliquot (Figure 1). The dissociation constants for FMN

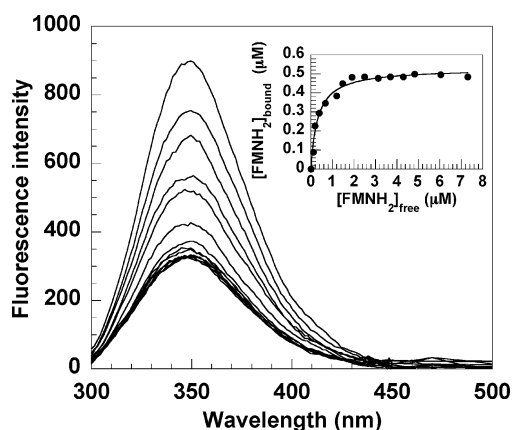


FIGURE 1: Fluorimetric titration of the alkanesulfonate monooxygenase with FMNH₂. The SsuD enzyme (0.5 μM) was titrated with FMNH₂ (0.16–7.8 μM). Emission intensity measurements at 344 nm were measured using an excitation wavelength at 280 nm. The change in fluorescence of the SsuD enzyme following the addition of FMNH₂ was converted to the estimated concentration of bound FMNH₂ (eq 1) and plotted against the concentration of free FMNH₂. Inset: Change in the emission intensity at 344 nm. The solid line represents the fit of the titration curve to eq 2. Each titration was performed in triplicate.

Table 1: Dissociation Constants for FMN and FMNH₂ Binding

	SsuE (μM)	SsuD (μM)
FMN ^a	0.015 ± 0.004	10.2 ± 0.4
FMNH ₂ ^b	15.5 ± 1.3	0.32 ± 0.15

^a Previously reported (5). ^b Determined under anaerobic conditions as described in Experimental Procedures.

and FMNH₂ binding to each alkanesulfonate monooxygenase enzyme are summarized in Table 1. The average K_d value for FMNH₂ binding to SsuD was 0.32 ± 0.15 μM with a binding stoichiometry of one reduced flavin bound per SsuD monomer. A similar binding ratio was also obtained by equilibrium gel filtration. The average K_d value for FMNH₂ binding to SsuE was 15.5 ± 1.3 μM. As a substrate of SsuE and product of SsuD, the K_d for oxidized flavin binding to each alkanesulfonate monooxygenase enzyme was previously determined by similar fluorimetric titration methods described for reduced flavin. The dissociation constants for FMN binding to SsuE and SsuD were 0.015 ± 0.004 μM and 10.2 ± 0.4 μM, respectively (5). These results show that SsuE has a higher affinity for oxidized FMN, while SsuD has a higher affinity for reduced flavin. These contrasting affinities ensure that once SsuE reduces the flavin, it is immediately released and transferred to SsuD.

Kinetic Studies of Flavin Oxidation by SsuD. The short half-life of reduced flavin in the presence of dioxygen often makes it difficult to assay the activity of the monooxygenase in the absence of the flavin reductase for the two-component monooxygenase enzymes. However, stopped-flow kinetic analyses have been used successfully to probe the kinetic mechanism of these enzymes (25–28, 30–32). Stopped-flow kinetic studies in the absence of alkanesulfonate were performed to monitor the oxidation of FMNH₂-bound SsuD at 370 and 450 nm. The formation of the C4a-(hydro)peroxyflavin by SsuD should be observed in a step prior to conversion of the flavin to the oxidized form in the kinetic traces obtained at 370 nm. Single-wavelength kinetic traces were also obtained for the reaction of free FMNH₂ with air-

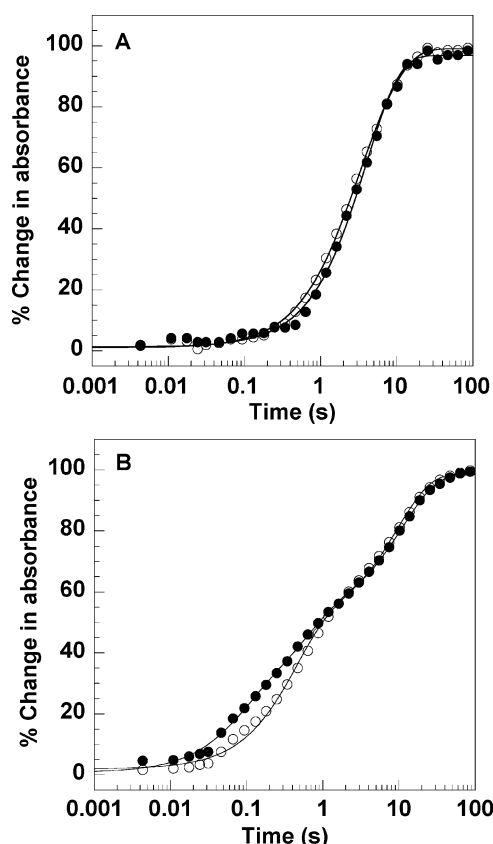


FIGURE 2: The kinetics of flavin oxidation by SsuD with alternate mixing of FMNH₂ and O₂ in the absence of octanesulfonate. Experiments were performed by stopped-flow kinetic analyses at 4 °C. A: Premixed SsuD–FMNH₂ (SsuD: 45 μM, FMNH₂: 15 μM) was mixed with air-saturated buffer. Kinetic traces of flavin oxidation were monitored at 370 (●) and 450 nm (○). B: Free FMNH₂ solution was mixed with SsuD (45 μM) in air-saturated buffer. Kinetic traces of flavin oxidation were monitored at 370 (●) and 450 nm (○). The kinetic traces shown are the average of three separate experiments. The solid lines are the fits of the kinetic traces to eq 3 or 4.

saturated buffer in the absence of SsuD (data not shown). The oxidation of free FMNH₂ was best fit to a double-exponential equation with rates of 2.20 s^{−1} (k_1) and 1.19 s^{−1} (k_2) at 370 nm and 2.04 s^{−1} (k_1) and 1.43 s^{−1} (k_2) at 450 nm, respectively. For the oxidation of FMNH₂-bound SsuD, a higher concentration of SsuD (45 μM) was used relative to FMNH₂ (15 μM) to ensure that the enzyme-catalyzed oxidation of reduced flavin was monitored. Under these conditions, the kinetic traces at both wavelengths increased at almost identical rates in two distinct phases at 370 and 450 nm. These kinetic traces were best fit to a double-exponential equation with rates of 0.43 s^{−1} (k_1) and 0.15 s^{−1} (k_2) at 370 nm and 0.50 s^{−1} (k_1) and 0.16 s^{−1} (k_2) at 450 nm (Figure 2A). The rate of oxidation for FMNH₂-bound SsuD at both wavelengths was significantly slower than the oxidation of free FMNH₂. There was no direct evidence for the formation of a stabilized C4a-(hydro)peroxyflavin intermediate generated during flavin oxidation by SsuD under these conditions. The two phases observed in the kinetic traces can be attributed to flavin oxidation.

Additional stopped-flow kinetic experiments were conducted to obtain information on the binding and oxidation of FMNH₂ by SsuD. When FMNH₂ was mixed with SsuD in air-saturated buffer, the kinetic traces were significantly

altered from the oxidation of premixed SsuD and FMNH₂ (Figure 2B). An initial fast phase was now observed in the reaction at 370 nm that was best fit to a triple-exponential equation with rates of 12.9 s⁻¹ (k_1), 0.95 s⁻¹ (k_2), and 0.08 s⁻¹ (k_3), while the kinetic trace at 450 nm was best fit to a double-exponential equation with rates of 1.3 s⁻¹ (k_1) and 0.09 s⁻¹ (k_2). The increase at 370 nm should be correlated with the C4a-(hydro)peroxyflavin and implies that the flavin adduct is only observed when reduced flavin and SsuD are not premixed. To determine if the fast phase is dependent on dioxygen, free FMNH₂ was mixed with SsuD at varying O₂ concentrations at 370 nm. Interestingly, the rate constant for the first phase was not dependent on increasing O₂ concentrations. The lack of an effect on the fast phase with varied O₂ concentrations may indicate that a slow binding or conformational step masks this dependence, or that the phase represents a step unrelated to formation of the C4a-(hydro)peroxyflavin.

Kinetic Parameters for Octanesulfonate. The steady-state kinetic parameters for SsuD were determined by measuring the amount of TNB anion formed at 412 nm from the reaction of the sulfite product with DTNB. The SsuE enzyme was included in the reaction to provide reduced flavin to the monooxygenase enzyme and was in excess relative to SsuD so reduced flavin would not be limiting in the reaction. There was no measurable amount of TNB anion produced due to side reactions with protein thiols. The K_m and k_{cat} values for octanesulfonate were $44.0 \pm 8.3 \mu\text{M}$ and $51.7 \pm 2.1 \text{ min}^{-1}$, respectively. The value for the catalytic efficiency (k_{cat}/K_m) of the enzyme with octanesulfonate was $1.17 \pm 0.22 \mu\text{M}^{-1} \text{ min}^{-1}$.

Octanesulfonate was also used to determine the binding affinity of the alkanesulfonate substrate to SsuD. Aliquots of an octanesulfonate solution were added to SsuD under anaerobic conditions; however, there was no observable change in the intrinsic protein fluorescence at an emission wavelength of 344 nm (Figure 3A). These results suggest that octanesulfonate is not able to bind directly to SsuD. Ordered binding of the alkanesulfonate substrate to SsuD may be contingent upon the binding of other substrates required for catalysis. The binding of octanesulfonate to the SsuD-FMNH₂ complex was evaluated under anaerobic conditions to determine if flavin-bound SsuD promotes the binding of octanesulfonate. To ensure that all SsuD was bound to FMNH₂, a 2:1 ratio of FMNH₂ to SsuD was used in these studies. The addition of octanesulfonate showed a decrease in the intrinsic fluorescence of the SsuD-FMNH₂ complex at an emission wavelength of 344 nm (Figure 3B). The concentration of bound octanesulfonate and free octanesulfonate was calculated according to eq 1. The concentration of octanesulfonate bound to the SsuD-FMNH₂ complex plotted against the concentration of free octanesulfonate after each addition is shown in the inset of Figure 3B. The K_d value for octanesulfonate binding to the SsuD-FMNH₂ complex was $17.5 \pm 0.9 \mu\text{M}$ with a 1:1 stoichiometric ratio of octanesulfonate to SsuD. These results indicate that the octanesulfonate substrate can bind to SsuD only if FMNH₂ is initially bound and suggests that octanesulfonate binding may be dependent on a conformational change induced by FMNH₂.

Kinetic Studies of Flavin Oxidation by SsuD in the Presence of Octanesulfonate. Flavin oxidation by SsuD in

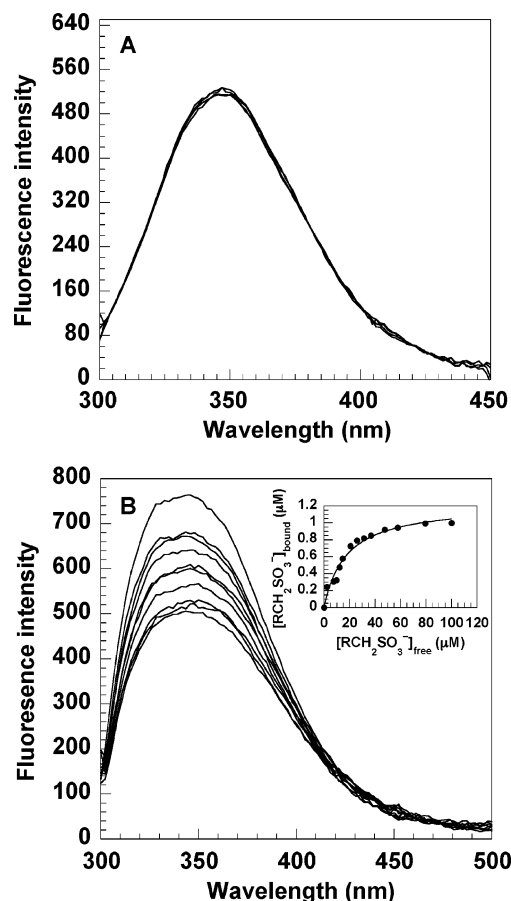


FIGURE 3: Fluorimetric titration of the SsuD enzyme and SsuD-FMNH₂ complex with octanesulfonate. Emission intensity measurements at 344 nm were measured using an excitation wavelength at 280 nm. A: Titration of SsuD with octanesulfonate in the absence of FMNH₂. SsuD enzyme (1 μM) was titrated with octanesulfonate (2.7–54 μM). B: Titration of the SsuD-FMNH₂ complex with octanesulfonate. SsuD (1 μM) was premixed with FMNH₂ (2 μM), and the complex was titrated with octanesulfonate solution (2.7–108 μM). Inset: Change in the emission intensity at 344 nm. The solid line represents the fit of the titration curve to eq 2. Each titration was performed in triplicate.

the presence of octanesulfonate was investigated through stopped-flow kinetic analyses at 370 and 450 nm mixing free FMNH₂ with SsuD and octanesulfonate in air-saturated buffer. At low octanesulfonate concentrations ($\leq 100 \mu\text{M}$), the kinetic traces at 370 nm were best fit to a triple exponential equation (Figure 4). However, as the concentration of octanesulfonate increased the kinetic traces were best fit to a double exponential (data not shown). For the reactions monitored at 370 nm, the k_{obs} for the first (when observed at low octanesulfonate concentrations) and third phase did not vary with the octanesulfonate concentration (data not shown), while the second phase showed a hyperbolic dependence on the octanesulfonate concentration that was best fit to eq 4 to give a K_d of 93 μM and a limiting rate constant of 2.4 s⁻¹ (Figure 4, Inset A). These results are consistent with octanesulfonate binding in two steps. A rapid equilibrium step is involved in the initial binding of octanesulfonate to the enzyme followed by either an irreversible isomerization or chemical step. The chemical step could involve the formation of the C4a-(hydro)peroxyflavin intermediate or carbon-sulfur bond cleavage if O₂ reacts with reduced flavin prior to octanesulfonate binding. Kinetic traces recorded at 450 nm under similar experimental conditions

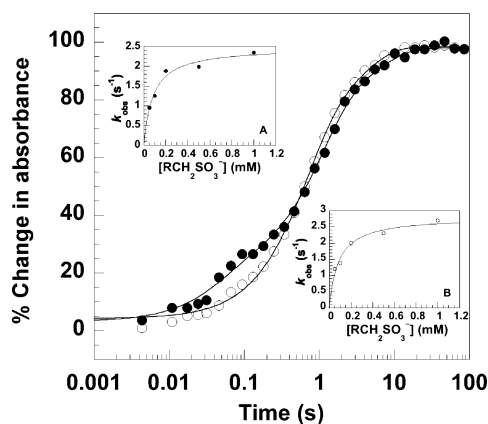


FIGURE 4: Kinetics of flavin oxidation by SsuD in the presence of octanesulfonate. Experiments were performed by stopped-flow kinetic analysis at 4 °C. Flavin oxidation of free FMNH₂ (15 μ M) mixed with SsuD (45 μ M) and octanesulfonate (50 μ M) in air-saturated buffer monitored at 370 (●) and 450 nm (○). Inset A: Octanesulfonate concentration dependence on the k_{obs} for the second phase at 370 nm. Inset B: Octanesulfonate concentration dependence on the k_{obs} for the first phase at 450 nm. The kinetic traces shown are the average of three separate experiments. The solid lines are the fits of the kinetic traces to eq 3 or 4.

Table 2: Determination of Octanal Product from Single Turnover Reactions of SsuD^a

type of reaction: syringe A/syringe B	octanal (μ M)
[FMNH ₂]/ [SsuD, octanesulfonate, O ₂]	5.8
[SsuD, FMNH ₂]/ [octanesulfonate, O ₂]	1.5

^a All reactions were carried out in 25 mM potassium phosphate buffer, pH 7.5, 25 °C. The solutions of SsuD (80 μ M), FMNH₂ (80 μ M), octanesulfonate (200 μ M), and oxygenated buffer (0.21 mM) were mixed in two airtight syringes according to the table. The octanal product was extracted as described in Experimental Procedures and analyzed by gas chromatography. Under the experimental conditions, the retention time of octanal and pentadecane (internal standard) was 8.1 and 16.3 min, respectively.

were best fit to a double-exponential equation. The k_{obs} for the first phase again displayed a hyperbolic dependence on the octanesulfonate concentration with a K_d of 85 μ M and a limiting rate constant of 2.8 s⁻¹ (Figure 4, Inset B), similar to the kinetic parameters obtained at 370 nm.

A fast initial phase was observed when reduced flavin was mixed with the SsuD at low octanesulfonate concentrations. The observed formation and accumulation of the putative C4a-(hydro)peroxyflavin intermediate may be directly related to increased product formation. To determine if the initial fast phase could be correlated with C4a-(hydro)peroxyflavin formation, a single turnover reaction assay for SsuD, analogous to reactions carried out in the stopped-flow instrument, was developed to directly measure the aldehyde product. Single-turnover reaction conditions were established to ensure that the oxidation of reduced flavin was directly coupled to product formation. The SsuD enzyme and substrate were mixed in different orders according to Table 2. The results from these assays showed that 5.8 μ M octanal was produced when FMNH₂ was reacted with premixed SsuD and octanesulfonate in air-saturated buffer, a 4-fold increase compared to the reaction of SsuD–FMNH₂ mixed with octanesulfonate and air-saturated buffer (1.5 μ M octa-

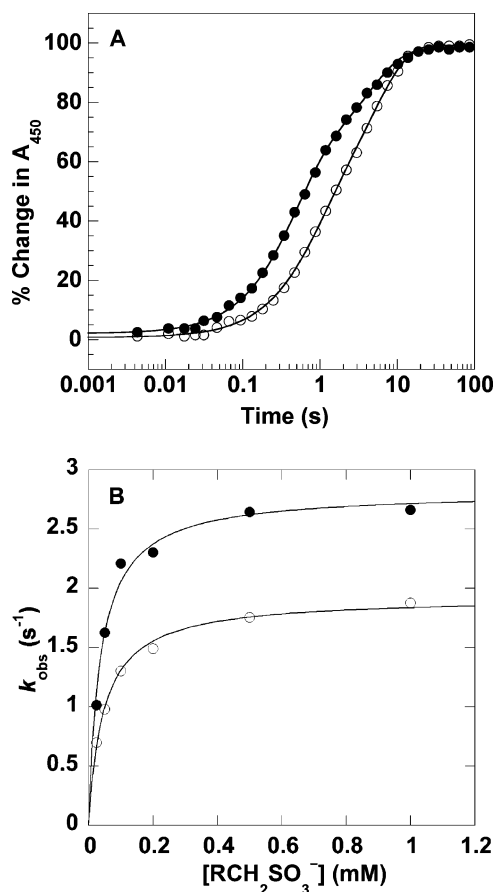
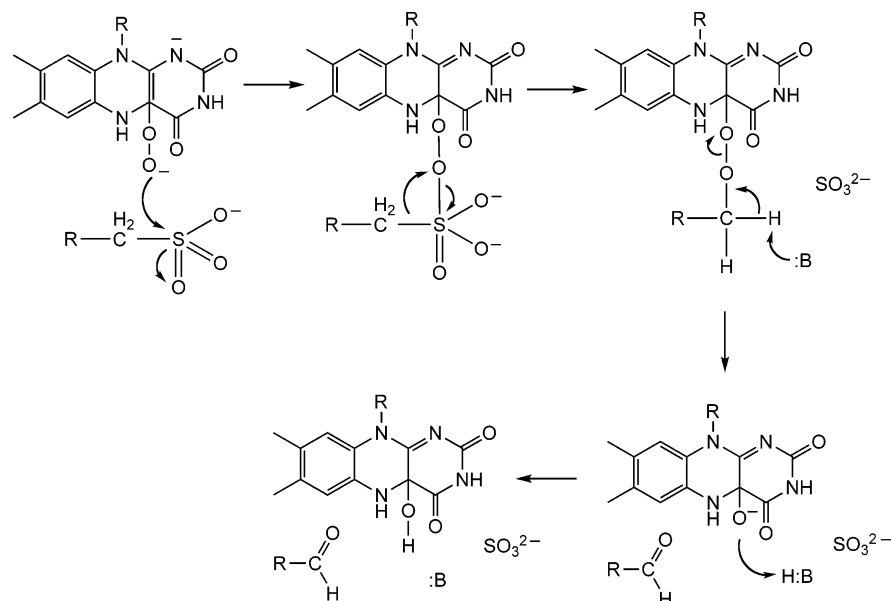


FIGURE 5: Kinetics of flavin oxidation by SsuD with alternate mixing in the presence of octanesulfonate. Experiments were performed by stopped-flow kinetic analyses at 4 °C. A: Kinetic traces of flavin oxidation at 450 nm. (○) Kinetic trace of SsuD–FMNH₂ (SsuD: 45 μ M, FMNH₂: 15 μ M) mixed with octanesulfonate (200 μ M) in air-saturated buffer; (●) Kinetic trace of SsuD–FMNH₂–octanesulfonate (SsuD: 45 μ M, FMNH₂: 15 μ M, octanesulfonate: 200 μ M) mixed with air-saturated buffer. The kinetic traces shown are the average of three separate experiments. The solid lines are the fits of the kinetic traces to eq 3. B: Octanesulfonate concentration dependence on the k_{obs} for the first phase at 450 nm. (○) Plot of k_{obs} for the first phase when SsuD–FMNH₂ is mixed with varied octanesulfonate solutions. (●) Plot of k_{obs} for the first phase when SsuD–FMNH₂–octanesulfonate is mixed with air-saturated buffer. The solid line represents the fit of the curve to eq 5.

nal). The amount of the octanal produced in each reaction should be directly correlated with the amount of C4a-(hydro)peroxyflavin intermediate observed in each reaction. Therefore, increased formation of the C4a-(hydro)peroxyflavin intermediate occurs when reduced flavin is reacted with premixed SsuD and octanesulfonate in air-saturated buffer.

Kinetic Studies of Flavin Oxidation by SsuD with Alternate Octanesulfonate Mixing. The results suggest that the binding of FMNH₂ to SsuD induces an isomerization promoting octanesulfonate binding. However, it was not clear if octanesulfonate binding induces a second isomerization step. If there is an isomerization step following binding of the octanesulfonate substrate, then the oxidation of premixed SsuD–FMNH₂–octanesulfonate should be faster than that of the SsuD–FMNH₂ complex with octanesulfonate and O₂. Figure 5A shows the kinetic traces at 450 nm for two alternate mixing conditions. Two phases were also observed in the reaction of the SsuD–FMNH₂–octanesulfonate complex mixed with air-saturated buffer, a fast phase (k_1)

Scheme 2: Proposed Mechanism of the SsuD Desulfonation Reaction



followed by a slower phase (k_2). At a 200 μM octanesulfonate concentration, the data was best fit to a double-exponential equation with rates of 2.31 s^{-1} (k_1) and 0.18 s^{-1} (k_2). The rate of the fast phase (k_1) for the SsuD-FMNH₂-octanesulfonate complex reaction with O₂ was approximately 2-fold faster than that of SsuD-FMNH₂ complex reaction with octanesulfonate in air-saturated buffer (1.30 s^{-1}). The rate dependence on octanesulfonate concentration was performed to determine if any of the individual steps were dependent on the alkanesulfonate concentration. Kinetic analyses at 450 nm for the oxidation of the SsuD-FMNH₂-octanesulfonate complex at varying octanesulfonate concentrations mixed with air-saturated buffer showed a clear hyperbolic dependence on k_1 (Figure 5B). A fit of the data gave a K_d value of 37.4 μM and a limiting rate constant of 2.8 s^{-1} . The octanesulfonate-dependent experiments were repeated by mixing the SsuD-FMNH₂ complex with increasing amounts of octanesulfonate in air-saturated buffer. The k_{obs} for the first phase also showed a hyperbolic dependence on the octanesulfonate concentration with a K_d value of 100.8 μM and a limiting rate constant of 1.9 s^{-1} (Figure 5B). The lower K_d value and higher rate observed with the reaction of the FMNH₂-bound SsuD and octanesulfonate complex with air-saturated buffer supports the existence of an isomerization step following octanesulfonate binding.

DISCUSSION

While the kinetic mechanism of the flavin reductase has been extensively studied in the two-component alkane-sulfonate monooxygenase system, the mechanism of the monooxygenase enzyme in this system is still not well understood (5, 6). The detailed mechanism of SsuD should prove interesting given the unique role of this enzyme in carbon-sulfur bond cleavage. Several groups have shown that rapid reaction kinetic analyses are a valuable tool for probing the reaction mechanism of the two-component monooxygenase enzymes (25–28, 30–32). The results from these substrate binding studies and rapid reaction kinetics analyses provide insight into the substrate binding specificity of SsuD, and apparent protein isomerizations relevant to

catalysis. Understanding the catalytic mechanism of SsuD is important to further probe the role of this enzyme system in bacterial sulfur acquisition.

Studies focused on determining the affinity of both SsuE and SsuD for FMNH₂ were central in establishing the preferred redox form of the flavin for each enzyme. Results from fluorescent titration experiments show that both SsuD and SsuE bind FMNH₂ with a 1:1 ratio, which is consistent with the binding stoichiometry of one oxidized FMN to SsuE and SsuD (5). SsuD had a 40-fold lower K_d value than SsuE for FMNH₂, while SsuE showed a 600-fold lower K_d value compared to SsuD for FMN (Table 1). Based on these results the monooxygenase has a greater affinity for FMNH₂ than the flavin reductase, while the flavin reductase can bind FMN more tightly than the monooxygenase. The preference of the flavin reductase for FMN and the monooxygenase for FMNH₂ is a commonly observed feature in the two-component monooxygenase family (24, 25, 29, 33). The preference of the flavin reductase and monooxygenase for different redox forms of the flavin may play an essential role in flavin transfer between these two enzymes.

A C4a-(hydro)peroxyflavin is widely accepted as a common intermediate for flavin-dependent monooxygenase reactions (23–28). The flavin-dioxygen adduct can either act as an electrophile or nucleophile depending on the reaction catalyzed. A C4a-(hydro)peroxyflavin is also thought to be the reactive flavin intermediate in the desulfonation of alkanesulfonates by SsuD (4). More specifically, the C4a-peroxyflavin (Fl-OO[•]) would seem the plausible intermediate in the reaction making a nucleophilic attack on the sulfonate functional group to form an initial alkanesulfonate peroxyflavin intermediate (Scheme 2). A Baeyer–Villiger rearrangement of the flavin adduct would lead to the generation of the aldehyde and sulfite products. Given the structural similarity of SsuD to bacterial luciferase it was expected that the peroxyflavin would be a highly stable intermediate observable at a range between 300 and 400 nm (34–37). However, results from stopped-flow analyses in the absence of the alkanesulfonate substrate show that the order of addition of FMNH₂ and O₂ determines whether the

formation of the putative C4a-(hydro)peroxyflavin intermediate is easily observed at 370 nm (Figure 2). The overall oxidation of premixed FMNH₂-bound SsuD is significantly slower than that of free FMNH₂. This is likely due to the protected nature of FMNH₂ within the active site of SsuD. The kinetic traces obtained at 370 and 450 nm for the premixed SsuD solution increased at almost identical rates in two distinct phases with a faster initial phase (k_1) and a slower second phase (k_2) (Figure 1). There was no direct evidence for the formation of a stabilized C4a-(hydro)peroxyflavin intermediate, and the kinetic traces observed represent the generation of FMN. However, when free FMNH₂ was mixed with SsuD in oxygenated buffer, the kinetic traces show a different pattern compared to the oxidation of premixed SsuD–FMNH₂ and free FMNH₂ (Figure 2). The kinetic trace now showed three phases, and a new fast phase (k_{obs} at 12 s⁻¹) was observed in the kinetic traces obtained at 370 nm that was absent from the kinetic traces obtained with premixed SsuD and FMNH₂. This fast phase was also absent in the kinetic traces obtained at 450 nm. The rates obtained for the last two phases were similar to the rates obtained with premixed SsuD and FMNH₂ (k_1 and k_2) and are assigned to the decay of the flavin intermediate back to the oxidized form. This initial fast phase following mixing of FMNH₂ with SsuD in oxygenated buffer may represent a flavin adduct that forms prior to regeneration of FMN. In the absence of octanesulfonate, the probable intermediate is the C4a-(hydro)peroxyflavin. These results suggest that a C4a-(hydro)peroxyflavin intermediate is likely generated in the desulfonation reaction catalyzed by SsuD and the identification of this intermediate is dependent on the order of addition of FMNH₂ and O₂ to SsuD. It was expected that the initial fast phase identified in the kinetic trace at 370 nm would be dependent on O₂ concentration, but there was no clear dependence observed. This could be due to a slow binding or conformational step that masks this dependence.

The dependence on the enzyme and substrate mixing order for the observed formation of the C4a-(hydro)peroxyflavin was also reported for the flavin-dependent halogenase RebH (28). A three-form model for binding and complex formation of RebH and FADH₂ has been proposed in this enzymatic reaction. An active ternary complex was initially formed that readily reacted with O₂ to form the C4a-(hydro)peroxyflavin intermediate. Under anaerobic conditions, the ternary complex appears to convert to an inactive form that is not immediately reactive with O₂ but can undergo a moderately slow conversion back to the active ternary complex once O₂ is available. This slow conversion back to the active form would not allow the C4a-(hydro)peroxyflavin to accumulate to detectable levels. Double-mixing experiments also provided evidence for the conversion of the inactive complex to a dead-end complex. For the dead-end complex, FADH₂-bound RebH reacts with O₂ to form oxidized flavin with no significant accumulation of C4a-(hydro)peroxyflavin. Similar protein dynamics could be involved in the SsuD reaction, which would explain the lack of accumulated C4a-(hydro)peroxyflavin when SsuD and FMNH₂ are premixed. While evidence was obtained for the readily reversible inactive form, double-mixing experiments will need to be performed to fully determine if the ternary dead-end complex is also formed in SsuD. Alternatively, the formation of the C4a-

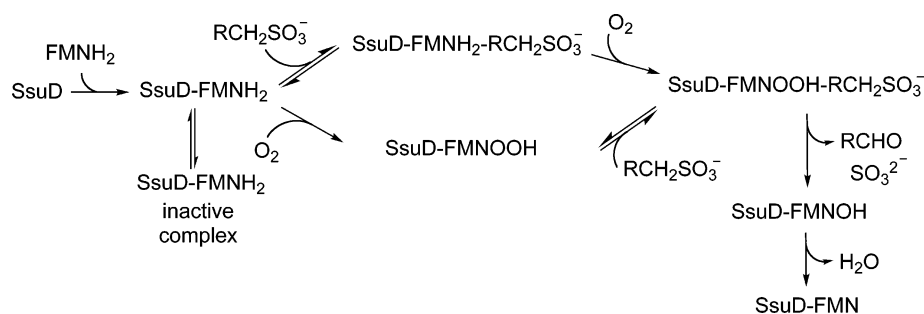
(hydro)peroxyflavin intermediate may form rapidly in SsuD within the dead time of the instrument. When SsuD is mixed with FMNH₂ the observed intermediate may be caused by a slower isomerization step that precedes the formation of the C4a-(hydro)peroxyflavin. This would alter the time scale in which the flavin intermediate is formed allowing the C4a-(hydro)peroxyflavin to accumulate.

The stability of C4a-(hydro)peroxyflavin intermediates formed in two-component monooxygenase reactions are quite variable depending on the enzyme under investigation. Strong kinetic evidence supports the formation of a C4a-(hydro)peroxyflavin intermediate in numerous two-component monooxygenase systems (23–28). In luciferase, this intermediate is so stable in the absence of the aldehyde substrate that it was isolated at 4 °C (23). Recent structural studies of *p*-hydroxyphenylacetate hydroxylase from *Acinetobacter baumannii* suggest that a hydrophobic cavity lies in front of C4a on the isoalloxazine ring (38). A packed hydrophobic cavity may be required to create a solvent-free environment that prevents rapid breakdown of the unstable flavin intermediate. The C4a-(hydro)peroxyflavin intermediate signal in the SsuD reaction is weak compared to other two-component monooxygenase enzymes, which implies this intermediate may show decreased stability due to lower hydrophobicity within the SsuD active site. While SsuD shares low amino acid sequence identity with bacterial luciferase, the arrangement of conserved residues within the putative active site are highly similar (13, 14). Therefore, one would expect to observe increased stability of the C4a-(hydro)peroxyflavin in SsuD as seen with bacterial luciferase. Obtaining the structure of SsuD with flavin bound will help to determine the nature of the flavin environment within the active site.

Results from fluorimetric titrations imply that octanesulfonate is not able to bind to SsuD unless FMNH₂ is first bound, as there is no observable intrinsic fluorescence intensity change with the addition of octanesulfonate to SsuD. The fluorescence intensity was significantly quenched with the addition of octanesulfonate to the SsuD–FMNH₂ complex giving a K_d value of 17.5 μ M (Figure 3). These results suggest that the alkanesulfonate substrate is not the first substrate that binds to SsuD, and an FMNH₂-induced conformational change is necessary to allow octanesulfonate to bind. The addition of substrates to SsuD likely occurs through a partial ordered process initiated by the binding of reduced flavin. An ordered binding mechanism has been demonstrated through kinetic analyses for several two-component monooxygenase enzymes (25, 28, 31). The reduced flavin is always the first substrate to bind, and the subsequent addition of substrates varies, depending on the enzyme being evaluated. In bacterial luciferase, the aldehyde substrate can bind before or after reaction of the O₂ with reduced flavin (31). For *p*-hydroxyphenylacetate 3-hydroxylase, the reduced flavin also binds first and the addition of either O₂ or hydroxyphenylacetate is random. However, double-mixing stopped-flow experiments support the reaction of O₂ with FMNH₂ to form the C4a-(hydro)peroxyflavin prior to binding of *p*-hydroxyphenylacetate (28).

The C4a-(hydro)peroxyflavin intermediate was also detected at 370 nm at low octanesulfonate concentrations when FMNH₂ was mixed with SsuD and octanesulfonate in air-saturated buffer, and three distinct phases were observed

Scheme 3: Order of Substrate Binding for SsuD



(Figure 4). However, this oxygenated flavin intermediate did not accumulate to detectable levels at higher octanesulfonate concentrations. This indicates at high octanesulfonate concentrations the reaction of octanesulfonate with the C4a-(hydro)peroxyflavin is rapid and does not accumulate to significant levels. The decay of the C4a-(hydro)peroxyflavin showed a hyperbolic dependence on the octanesulfonate concentration, which suggests that the binding of octanesulfonate occurs in two steps. The octanesulfonate binds in rapid equilibrium to SsuD followed by an isomerization step or direct carbon-sulfur bond cleavage. However, there was still no observed O₂ dependence on the initial fast phase at 370 nm seen at lower octanesulfonate concentrations. When premixed SsuD and FMNH₂ were mixed with octanesulfonate in oxygenated buffer there was no initial increase at 370 nm corresponding to the formation of the C4a-(hydro)peroxyflavin. An assay analogous to reactions carried out in the stopped-flow instrument was developed to measure the aldehyde product. Higher product formation should be observed if the initial fast phase at 370 nm attributed to C4a-(hydro)peroxyflavin correlates with product formation. As a single turnover reaction, the amount of product is dependent on the intermediates generated in each reaction. There was a 4-fold increase in the amount of octanal produced when FMNH₂ alone was reacted with premixed SsuD-octanesulfonate-O₂ compared to the reaction of SsuD-FMNH₂ with octanesulfonate and O₂ (Table 2). The octanesulfonate is unable to bind to SsuD unless FMNH₂ is first bound; therefore, even though octanesulfonate is present with SsuD, it is not yet bound to the enzyme. These results further support the presence of an inactive complex between SsuD and FMNH₂. The lower octanal product produced with premixed SsuD and FMNH₂ may be due to the slow conversion of the SsuD-FMNH₂ binary complex back to the active form in the presence of octanesulfonate and O₂. When SsuD is not premixed with the reduced flavin, the inactive complex is unable to form.

The previous results suggest that an isomerization step precedes octanesulfonate binding. Evidence to support this observation was obtained by alternate mixing experiments monitored at 450 nm: SsuD-FMNH₂ mixed with octanesulfonate in oxygenated buffer, and premixed SsuD-FMNH₂-octanesulfonate mixed with oxygenated buffer. The *k*₁ for the decay of the reaction when octanesulfonate was premixed with SsuD and FMNH₂ is 2-fold faster than the reaction that had not been premixed with octanesulfonate (Figure 5A). As a result, the decay of the flavin intermediate occurs at a faster rate if octanesulfonate is premixed with SsuD and FMNH₂. The faster rate for the decay of the flavin intermediate is directly correlated with an increase in product

formation (data not shown). In addition, the *K*_d value is 3-fold lower when SsuD, FMNH₂, and octanesulfonate are premixed (Figure 5B). An isomerization step would be masked in this reaction because octanesulfonate would already be bound to form the ternary complex, and the net effect would be a rate increase and decrease in the octanesulfonate binding affinity.

A minimal model for substrate binding can be defined by the described experiments. After binding of reduced flavin to SsuD to form the SsuD-FMNH₂ complex, the enzyme can carry out the reaction through one of two paths: (1) SsuD-FMNH₂-octanesulfonate is formed prior to reacting with O₂, and (2) the SsuD-FMNH₂-O₂ intermediate is formed prior to reacting with octanesulfonate. A faster rate and lower *K*_d value for octanesulfonate binding was observed with premixed SsuD, octanesulfonate, and FMNH₂ suggesting that octanesulfonate binds prior to O₂. On the basis of these experiments it would appear that O₂ is the last substrate to bind, thereby ensuring that formation of the C4a-(hydro)peroxyflavin is fully coupled to desulfonation; however, both paths are represented in the model.

In summary, this study has elucidated the reaction mechanism of the alkanesulfonate monooxygenase component (Scheme 3). The results suggest an ordered substrate binding mechanism where SsuD binds FMNH₂ first leading to a conformational change that promotes the binding of octanesulfonate. Reaction of FMNH₂ with O₂ to form a C4a-(hydro)peroxyflavin intermediate is directly involved in desulfonating the alkanesulfonate substrate to produce the corresponding aldehyde and sulfite. This substrate binding order plays an essential role in catalysis, and significant protein dynamics during the course of the reaction was shown to be involved in both FMNH₂ and alkanesulfonate binding. Kinetic studies with SsuD variants are now being implemented to investigate the mechanism of desulfonation.

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