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Identification of Critical Steps Governing the Two-Component Alkanesulfonate Monooxygenase Catalytic Mechanism

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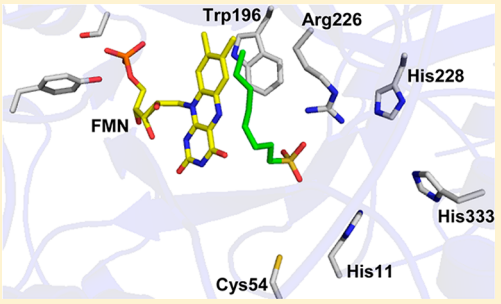
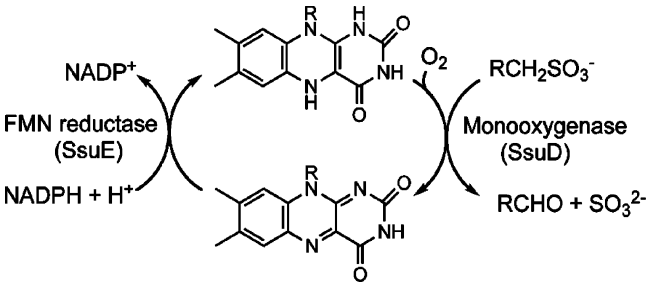
Supporting Information

ABSTRACT: The alkanesulfonate monooxygenase enzyme (SsuD) catalyzes the oxygenolytic cleavage of a carbon−sulfur bond from sulfonated substrates. A mechanism involving acid−base catalysis has been proposed for the desulfonation mechanism by SsuD. In the proposed mechanism, base catalysis is involved in abstracting a proton from the alkane peroxyflavin intermediate, while acid catalysis is needed for the protonation of the $FMNO^-$ intermediate. The pH profiles of k_{cat} indicate that catalysis by SsuD requires a group with a pK_a of 6.6 ± 0.2 to be deprotonated and a second group with a pK_a of 9.5 ± 0.1 to be protonated. The upper pK_a value was not present in the pH profiles of k_{cat}/K_m. Several conserved amino acid residues (His228, His11, His333, Cys54, and Arg226) have been identified as having potential catalytic importance due to the similar spatial arrangements with close structural and functional relatives of SsuD. Substitutions to these amino acid residues were generated, and the pH dependencies were evaluated and compared to wild-type SsuD. Although a histidine residue was previously proposed to be the active site base, the His variants possessed similar steady-state kinetic parameters as wild-type SsuD. Interestingly, R226A and R226K SsuD variants possessed undetectable activity, and there was no detectable formation of the C4a-(hydro)peroxyflavin intermediate for the Arg226 SsuD variants. Guanidinium rescue with the R226A SsuD variant resulted in the recovery of 1.5% of the wild-type SsuD k_{cat} value. These results implicate Arg226 playing a critical role in catalysis and provide essential insights into the mechanistic steps that guide the SsuD desulfonation process.



In *Escherichia coli*, sulfur limitation induces the synthesis of a set of proteins involved in acquiring sulfur from alternative sources.[1,2] The two-component alkanesulfonate monooxygenase system, comprised of a flavin reductase (SsuE) and an alkanesulfonate monooxygenase (SsuD), enables the organism to utilize a broad range of alkanesulfonates as alternative sulfur sources.[2] The SsuE enzyme catalyzes the reduction of FMN by NAD(P)H, and the reduced flavin is transferred to SsuD (Scheme 1). In the proposed mechanism for desulfonation, $FMNH_2$-bound SsuD activates dioxygen to form a C4a-(hydro)peroxyflavin intermediate that is thought to cleave the carbon−sulfur bond of the alkanesulfonate substrate, resulting in the release of sulfite and the corresponding aldehyde (Scheme 2).[3]

Scheme 1



Efforts to elucidate the catalytic mechanisms of flavin-dependent monooxygenase systems continue to be an area of active research. Elucidating catalytic mechanisms of two-component monooxgenases has proven to be particularly challenging in that the reduced flavin cofactor acts as a substrate and not as a prosthetic group. The binding of the reduced flavin substrate provides an additional step that must be accounted for in the kinetic mechanism, as substrate binding and release steps in both SsuD and bacterial luciferase have been linked to one or more conformational changes during catalysis.[1−17] In our previous studies, the mechanism of SsuD was probed by a combination of biochemical and kinetic approaches.[6−8] The octanesulfonate substrate was unable to bind to SsuD until $FMNH_2$ was first bound, which implied an ordered substrate binding mechanism in the SsuD reaction. In addition, evidence for the formation of a C4a-(hydro)peroxyflavin intermediate was obtained by stopped-flow kinetic analyses in the absence or at low concentrations of octanesulfonate.[6,8] In the proposed mechanism, the C4a-peroxyflavin performs a nucleophilic attack on the sulfur group of the alkanesulfonate substrate followed by a Baeyer−Villiger rearrangement leading to the cleavage of the carbon−sulfur bond to generate the alkane

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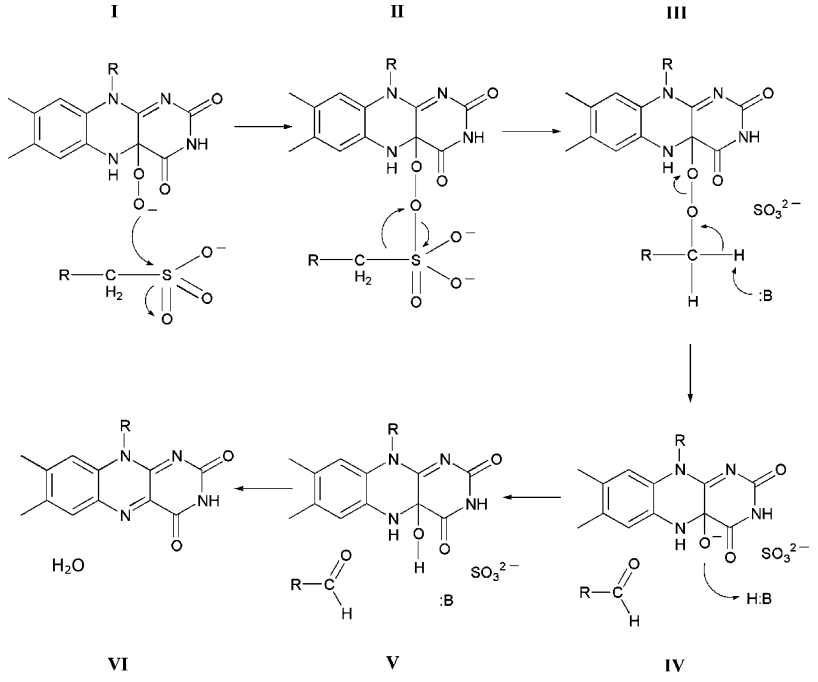
Scheme 2. Proposed Chemical Mechanism of SsuD after All Substrates Are Bound and Dioxygen Has Been Activated



peroxyflavin intermediate (Scheme 2, **II**). A catalytic base has been proposed to abstract a proton from the alkane peroxyflavin intermediate, leading to the release of the aldehyde product from the enzyme following rearrangement (Scheme 2, **III**). Finally, a general acid is proposed to donate a proton to the $FMNO^-$ intermediate triggering a conformational change that opens the enzyme to solvation and promotes product release (Scheme 2, **IV**). Unfortunately, there is little information regarding the exact structures of proposed SsuD isomers. Therefore, identification of the catalytic base and acid would provide valuable information to support the proposed desulfonation mechanism by SsuD.

Although the amino acid sequence identity is low, SsuD is similar in overall structure to the flavin-dependent monooxygenases bacterial luciferase and long-chain alkane monooxygenase (LadA) as each of these FMN-dependent monooxygenases display similar TIM barrel architectures.[9−15] For bacterial luciferase and SsuD, the binding of reduced flavin induces an apparent conformational change leading to an altered active site environment from the apoenzyme structure.[7,15,16] While three-dimensional structures with oxidized flavin bound within the active site have been obtained for bacterial luciferase and LadA, structures with the reduced flavin bound within the active site have remained elusive for all two-component systems.[9,16] Although there is no three-dimensional structure available for SsuD with substrates bound, the location of the active site of the enzyme has been postulated based on chemical labeling, computer simulations, and structural similarities to bacterial luciferase and LadA (Figure 1). Since the K_d value for FMN and SsuD is higher than for $FMNH_2$, it is reasonable to conclude that the active site environment of SsuD would be different between the oxidized and reduced flavin bound enzyme.[3−7,16] Therefore, even modeling FMN and 1-octanesulfonate into the active site of SsuD based on its close structural homology to bacterial luciferase and LadA would provide only a limited understanding of the active site environment during catalysis. As a result, site-directed mutagenesis, steady-state kinetic assays, pH dependence, and single-turnover rapid-reaction kinetic studies were employed in order to provide a more complete picture of the active site environment of alkanesulfonate monooxygenase as the desulfonation reaction occurs.

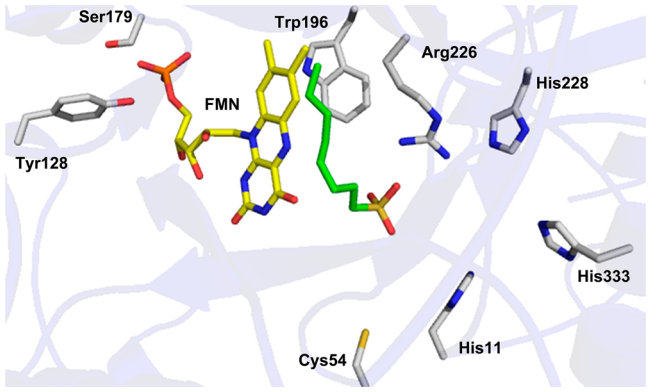


Figure 1. Putative active site of SsuD. The Arg228 guanidinium nitrogen is 7.6 Å from the N1 position of the flavin (yellow) and 4.5 Å from the C1 position of the 1-octanesulfonate substrate (green). The His228 Nε2 is 3.9 Å from the guanidinium moiety of Arg228. The Cys54 thiol is 6.6 Å away from the C1 position of the 1-octanesulfonate substrate and 10.2 Å from the guanidinium moiety of Arg228. The active site was rendered with PyMOL (PDB ID: 1M41), FMN was modeled using its coordinates in LadA (PDB ID: 3B9O), and AutoDock was used to model octanesulfonate.[9,11]

Several amino acids (His228, His11, His333, Cys54, and Arg226) located in the active site of SsuD are in a similar arrangement as catalytically relevant amino acids from bacterial luciferase and LadA (Figure 1).[9−13,16] In our proposed model, an active site base would be involved in proton abstraction from the alkanesulfonate peroxyflavin adduct, leading to the formation of the aldehyde product (Scheme 2, **III**). In bacterial luciferase, His44 in the α-subunit was previously identified as the catalytic base in the bioluminescence reaction as mutation

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of this histidine residue to an alanine showed a decrease in bioluminescence activity.[18,19] The enzymatic activity could be rescued with the addition of imidazole to the reaction at increasing pH.[19] Substitution of His311 in LadA to a phenylalanine was also shown to reduce activity of the LadA enzyme.[9] As a result, His228 of SsuD was suggested as a potential active site base in the desulfonation reaction based on its structural similarity to His44 and His311 in bacterial luciferase and LadA, respectively.[9−13,16,18,19] Additionally, the proposed catalytic mechanism for SsuD includes an active-site acid that would be involved in protonation of the $FMNO^-$ intermediate prior to product release. Cys54 of SsuD was identified as a residue of potential catalytic importance due to its structural similarity to conserved Cys106 of bacterial luciferase and Cys14 of LadA.[9,11,18−23] Although chemical labeling of Cys54 SsuD with methyl mercury led to inactivation of the enzyme, the Cys54 residue was not shown to play a direct role in catalysis.[8] As a result, Arg226 of SsuD was identified as the possible active-site acid due to its location within the active site and the conserved nature of this residue in bacterial SsuD homologues. Further evaluation of these amino acids in the form of chemical rescue, substrate binding, and single-turnover rapid-reaction kinetic studies illuminated crucial steps governing the catalytic mechanism of the SsuD desulfonation reaction.

EXPERIMENTAL PROCEDURES

Materials. *E. coli* strains (XL-1 and BL21(DE3)) and QuickChange site-directed mutagenesis kit were purchased from Stratagene (La Jolla, CA). Plasmid vectors and pET21a were obtained from Novagen (Madison, WI). DNA primers were synthesized by Invitrogen (Carlsbad, CA). Flavin mononucleotide phosphate (FMN), reduced nicotinamide adenine dinucleotide phosphate (NADPH), ethylenediaminetetraacetic acid (EDTA), potassium phosphate (monobasic anhydrous and dibasic anhydrous), streptomycin sulfate, Trizma base, Bis-Tris, glycine, ammonium sulfate, ampicillin, dimethy sulfoxide (DMSO), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), glucose, glucose oxidase, lysozyme, guanidine, and urea were from Sigma (St. Louis, MO). Isopropyl-β-D-thiogalactoside (IPTG), sodium chloride, and glycerol were obtained from Fisher Biotech (Pittsburgh, PA). 1-Octanesulfonate and dithiothreitol (DTT) were purchased from Fluka (Milwaukee, WI). The SsuD and SsuE enzymes were expressed and purified as previously described.[4] The concentration of SsuD and SsuE proteins was determined from A_{280} measurements using a molar extinction coefficient of 47.9 and 20.3 mM^{-1} cm^{-1}, respectively.[4]

Construction of Variant Proteins. A recombinant pET21a plasmid containing the *ssuD* gene was used to construct variants of the SsuD enzyme. Primers for each variant were designed as 29 base oligonucleotides containing the desired mutation. The CAT codon for His228, His11, and His333 was replaced with GCG (H228A, H11A, and H333A). The CGT codon for Arg226 was replaced with CGC (R226A), AAA (R226K), and CAT (R226H). The variant constructs were confirmed by DNA sequencing analysis at Davis Sequencing (University of California, Davis). Confirmed variants were transformed into *E. coli* BL21(DE3) competent cells for protein expression and stored at −80 °C. Each SsuD variant protein was purified as previously reported.[4]

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra of wild-type SsuD and variants were obtained by mixing 1.2 μM of enzyme in 10 mM potassium phosphate buffer, pH 7.5, and 100 mM NaCl at 25 °C. Spectra were recorded on a Jasco J-810 spectropolarimeter (Easton, MD). Measurements were taken in 0.1 nm increments from 300 to 185 nm in a 0.1 cm path length cuvette with a bandwidth of 1 nm and a scanning speed of 50 nm/min. Each spectrum is the average of eight scans. Background correction was performed using the default parameters within the Jasco J-720 software.

Dependence of Kinetic Parameters on pH. The activities of the H228A, H11A, H333A, C54A, and wild-type SsuD enzymes were routinely assayed at 25 °C as previously described with the following modifications: reactions employed a range of 1-octanesulfonate concentrations (10−5000 μM) in either 50 mM Bis-Tris (pH range of 5.8−7.2), 50 mM Tris-HCl (pH range of 7.2−9.0), or 50 mM glycine (pH range of 9.0−10.0).[6,8,10] Higher enzyme concentrations were utilized for the R226A, R226K, and R226H SsuD enzymes due to decreased activity. For those variants, reactions were initiated by the addition of NADPH (500 μM) into a reaction mixture containing a SsuD variant (3 μM; R226A, R226K, or R226H SsuD), SsuE (9 μM), FMN (5 μM), and a range of 1-octanesulfonate concentrations (10−5000 μM) in 50 mM Tris-HCl (pH 7.5) at 25 °C. Each buffered solution was supplemented with 100 mM sodium chloride to maintain the ionic strength, and overlapping assays were performed in Bis-Tris and Tris-HCl at pH 7.2 and in Tris-HCl and glycine at pH 9.0 in order to ensure activity was independent of the buffer used. All assays were performed in triplicate, and steady-state kinetic parameters were determined by fitting the data to the Michaelis−Menten equation. The pH dependence of k_{cat} and k_{cat}/K_m were best fit to either a single ionization model (eq 1), double ionization model (eq 2), or a single ionization model with a sticky proton (eq 3).

$$\log y = \log\left[C/\left(1 + \frac{H}{K_1}\right)\right] \quad (1)$$

$$\log y = \log\left[C/\left(1 + \frac{H}{K_1} + \frac{K_2}{H}\right)\right] \quad (2)$$

$$\log y = \log\left[C\left(1 + \frac{H}{K_1}\right)/\left(\left(1 + \frac{H}{K_2}\right)\left(1 + \frac{H}{K_3}\right)\right)\right] \quad (3)$$

In eqs 1−3, H is $[H^+]$, y is k_{cat} or k_{cat}/K_m, C is the pH-independent value of y, and K_1, K_2, and K_3 represent the dissociation constant for groups on the SsuD−$FMNH_2$ complex. The pH stability of SsuD was determined by preincubating 20 μM enzyme in the appropriate buffer (pH 5.8−10.0) at 25 °C for 30 min. The preincubated SsuD (0.2 μM) was then assayed as previously described in 50 mM Tris-HCl, at pH 7.5, and 100 mM NaCl. The activity of SsuE was assayed as previously described monitoring NADPH oxidation at A_{340} in a reaction mixture containing 0.6 μM FMN and 0.04 μM SsuE.[4] The effect of pH on the kinetic parameters of wild-type SsuE was determined by performing a series of activity assays with varied NADPH concentrations (10−200 μM) in 50 mM Bis-Tris (pH range of 5.8−7.2), 50 mM Tris-HCl (pH range of 7.2−9.0), or 50 mM glycine (pH range of 9.0−10.0).

Substrate Binding Affinity. Binding of reduced flavin to the variant SsuD enzymes was monitored by spectrofluorimetric titration as previously described.[6] A solution of R226A or R226K SsuD (0.5 μM) in 25 mM potassium phosphate, pH

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7.5, 10% glycerol, and 100 mM NaCl (1.0 mL total volume) was titrated with a solution of $FMNH_2$ (0.26−8.26 μM) under anaerobic conditions. After excitation at 280 nm, the fluorescence emission intensity at 344 nm was recorded following each addition of $FMNH_2$. Bound $FMNH_2$ was determined using the equation

$$[S]_{bound} = [E]\frac{I_0 - I_c}{I_0 - I_f} \quad (4)$$

where $[S]_{bound}$ 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 $FMNH_2$ bound was plotted against the free substrate to obtain the dissociation constant (K_d) according to eq 5.

$$Y = \frac{B_{max}X}{K_d + X} \quad (5)$$

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.

Octanesulfonate binding to the SsuD variants was investigated as previously described by similar fluorimetric titration methods employed for flavin binding.[6] Aliquots of an anaerobic solution of octanesulfonate (2.5−108 μM) in an airtight titrating syringe were added to an anaerobic solution of either R297A or R297K SsuD (1 μM), with $FMNH_2$ (2 μM) in 25 mM potassium phosphate, pH 7.5, 10% glycerol, and 100 mM NaCl (1.0 mL total volume). The fluorescence spectrum for each titration was recorded with an excitation wavelength at 280 nm and emission intensity measurements at 344 nm. The concentration of 1-octanesulfonate bound was determined by eq 4 and was plotted against the free substrate to obtain the dissociation constant (K_d) according to eq 5.

Rapid Reaction Kinetic Analyses. Stopped-flow kinetic analyses were carried out as previously described using an Applied Photophysics SX.18 MV stopped-flow spectrophotometer.[6] All experiments were performed by mixing $FMNH_2$ (25 μM) in one drive syringe against SsuD (35 μM) in air-saturated buffer (25 mM Tris-HCl, pH 8.5, 100 mM NaCl) in the other drive syringe. When included in the reaction, varied concentrations of 1-octanesulfonate (20−1000 μM) were added to the SsuD solution in air-saturated buffer. All experiments were carried out in single-mixing mode by mixing equal volumes of the solutions, and the reactions monitored by the change in absorbance at 370 and 450 nm over 100 s. Kinetic traces were fit to the following equation with KaleidaGraph software (Abelbeck Software, Reading, PA).

$$A = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + C \quad (6)$$

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

Guanidinium Rescue Experiments. Two experimental conditions were used in order to evaluate the ability of guanidinium to compensate for the arginine amino acid in R226A SsuD. In the first experiment, the enzymatic activity of R226A SsuD was determined by conducting the previously described activity assay with 1-octanesulfonate (1 mM) and a range of guanidine concentrations (5−200 mM) in 50 mM Bis-Tris (pH range of 5.8−7.2), 50 mM Tris-HCl (pH range of 7.2−9.0), or 50 mM glycine (pH range of 9.0−10.0). The second experiment focused on the effect of guanidinium on the steady-state kinetic parameters of R226A SsuD by measuring the enzymatic activity with varying concentrations of octanesulfonate (10−5000 μM) in the presence of 100 mM guanidine−HCl in 50 mM Bis-Tris (pH 6.2), 50 mM Tris-HCl (pH 7.5), or 50 mM glycine (pH 9.5). For each experimental condition, the reactions were initiated with NADPH (500 μM) into a reaction mixture containing R226A SsuD (3.0 μM), SsuE (9.0 μM), and FMN (5.0 μM). Control experiments were performed where guanidine and/or R226A SsuD were not included in the reaction. The sulfite product was quantified as previously described.[6,10]

RESULTS

Dependence of Wild-Type SsuD Kinetic Parameters on pH. In order to gain insight into catalytically relevant ionizations governing the desulfonation reaction of SsuD, the k_{cat} and k_{cat}/K_m values for wild-type SsuD were measured as a function of pH from pH 5.8−10.0 (Figure 3A,B). SsuD showed optimal catalytic activity between pH 7.2 and 8.5 where the pH-independent value was found to be 93 ± 5 min^{-1} for k_{cat} and 1.9 ± 0.1 μM^{-1} min^{-1} for k_{cat}/K_m (Table 1). SsuD was found to be soluble over the entire experimental pH range, as full activity was restored when SsuD that had been preincubated at specific experimental pH values was introduced into an activity assay at pH 7.5. The pH dependence of k_{cat} for SsuD revealed two titratable residues with pK_a values of 6.6 ± 0.1 and 9.5 ± 0.1 (Table 1). These results indicate that a group with a pK_a value of around 6.6 must be deprotonated, and a group with a pK_a value of around 9.5 must be protonated to support catalysis up through product release. Interestingly, only one titratable amino acid residue with a pK_a value of 6.9 ± 0.1 was extrapolated from the pH dependence of k_{cat}/K_m, suggesting that a functional group on the free enzyme or substrate must be deprotonated in order to commit through the first-irreversible step. Since the SsuD enzyme works in conjunction with the SsuE enzyme as part of a two-component system, pH dependence controls were performed in order to verify that the experimental pK_a values were not attributable to functional groups participating in the SsuE reductase half-reaction (data not shown). The SsuE reductase half-reaction did not demonstrate any dependence on pH over the experimental range, suggesting that experimental pK_a values can be attributed solely to functional groups on the SsuD enzyme and/or its substrates.

Table 1. pH Dependence of Steady-State Kinetic Parameters for Wild-Type SsuD and Variants

	k_{cat}		k_{cat}/K_m
SsuD	pK_1	pK_2	pK
wild-type	6.6 ± 0.1	9.5 ± 0.1	6.9 ± 0.1
H228A	6.6 ± 0.1	9.6 ± 0.1	7.1 ± 0.1
H11A	6.8 ± 0.2	9.5 ± 0.2	7.0 ± 0.1
H333A	6.6 ± 0.1	9.5 ± 0.1	7.0 ± 0.1
C54A	6.3 ± 0.1	—[a]	7.2 ± 0.2

[a]Value could not be determined within the experimental pH range.

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pH Dependence of SsuD Variants. In an effort to identify the groups contributing to the pK_a values governing the pH dependence of kinetic parameters for wild-type SsuD, several SsuD variants were constructed with substitutions at amino acid groups likely to be responsible for these pK_a values. The pK_a values of 6.6 ± 0.1 and 6.9 ± 0.1 seen for the pH dependences of k_{cat} and k_{cat}/K_m, respectively, are similar to those that might be expected for a histidine residue acting as an active site base in an enzyme.[9−13,18−20,24] As a result, pH-dependent studies were conducted on H228A, H11A, and H333A SsuD in order to determine whether the absence of the imidazole functional group at these positions resulted in a shift in or absence of the pK_a in the corresponding pH profiles compared to wild-type SsuD. For H228A SsuD, the pH-independent value was 57 ± 2 min^{-1} for k_{cat} and 0.54 ± 0.07 μM^{-1} min^{-1} for k_{cat}/K_m. For H11A and H333A SsuD, the pH-independent value for k_{cat} was 66 ± 3 and 82 ± 2 min^{-1}, respectively, and 2.1 ± 0.2 and 1.8 ± 0.2 μM^{-1} min^{-1} for k_{cat}/K_m, respectively (Table 2). The pH dependence of k_{cat} and k_{cat}/K_m for each of the SsuD variants revealed titratable groups with pK_a values similar to those determined for the wild-type enzyme (Table 1). The results indicated that neither of these histidine residues was contributing exclusively to the lower pK_a value in the k_{cat} nor k_{cat}/K_m pH dependence profile for wild-type SsuD. Interestingly, the presence of a slight "hollow" in the k_{cat} pH profile for H228A and H11A SsuD and "hump" in the k_{cat}/K_m pH profile for H228A SsuD suggested the presence of a sticky substrate with these variants (Figure S1). The H228A SsuD variant in particular appears to promote the dissociation of this ionizable group proton when octanesulfonate is present.[24,25] Despite this intriguing result, the overall impact on SsuD activity as a result of the alanine substitutions to these positions was negligible. As a result, neither amino acid residue was considered critical to catalysis.

Table 2. pH-Independent Steady-State Kinetic Parameters for Wild-Type SsuD and Variants

	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
WT SsuD[a]	93 ± 5	1.9 ± 0.1
H228A SsuD	57 ± 2	0.54 ± 0.07
H11A SsuD	66 ± 3	2.1 ± 0.2
H333A SsuD	82 ± 2	1.8 ± 0.2
C54A SsuD[a]	16 ± 1	0.43 ± 0.08
R226A SsuD	ND[b]	ND
R226K SsuD	ND	ND
R226A SsuD with 100 mM guanidine	1.26 ± 0.08	0.008 ± 0.002

[a]Values were obtained under current experimental conditions. Previously reported values were in 25 mM phosphate buffer.[25,27] [b]No activity detected.

The upper pK_a value of 9.5 ± 0.1 for the pH dependence of k_{cat} for wild-type SsuD was consistent with the value for a cysteine residue within the active site.[8,24] For C54A SsuD, the pH-independent values for k_{cat} and k_{cat}/K_m correspond with the 5-fold decrease in activity reported previously (Table 2).[8] The decrease in activity for C54A SsuD supports the possibility that Cys54 could be contributing to the upper pK_a value at 9.5 ± 0.1 for k_{cat}. Interestingly, the cysteine to alanine substitution resulted in the elimination of the upper pK_a value from the k_{cat} pH profile. The pH dependence of k_{cat} for C54A SsuD revealed only a single titratable group with an apparent pK_a value of 6.3 ± 0.1 and a single titratable group with an apparent pK_a value consistent to that of wild-type SsuD for the pH dependence of k_{cat}/K_m (Table 1). These pK_a values are apparent because the pH dependence data were fit to eq 1 for comparison with the values obtained for wild-type SsuD. However, "hollows" present in both the k_{cat} and k_{cat}/K_m pH profiles for C54A SsuD indicate the presence of a sticky proton in the enzyme−substrate complex (Figure 3C,D).[24,25] When fitted to eq 3, the k_{cat}/K_m pH profile for C54A SsuD revealed three pH-dependent terms with values of 7.0 ± 0.5, 7.8 ± 0.2, and 6.0 ± 0.5. Similar pK_a values of 7.1 ± 0.7, 7.4 ± 0.6, and 5.8 ± 0.5 were also obtained for the k_{cat} pH profile. These results indicate that the cysteine to alanine substitution causes the proton associated with the ionizable group on the acidic limb of the pH profile to become sticky when octanesulfonate is present.[24,25]

Despite arginine having a pK_a of 12.5 in free solution, the active-site residue, Arg226, was identified as a group potentially contributing to the pK_a value at 9.5 ± 0.1 for the pH dependence of k_{cat}. While current and previous experimental evidence dismissed the possibility of Cys54 serving as the active-site acid in SsuD, nearby Arg226 was found to be in close proximity to the pK_a value of an active-site arginine during catalysis.[8,24−28] Interestingly, catalytic sulfite production was not detected over the pH range for those assays involving R226A or R226K SsuD, even at increased protein concentrations. The absorbance values at 412 nm for 2-nitro-5-benzoic acid production were similar to the absorbance values obtained in control experiments in the absence of enzyme. The absorbance values were below the detectable limit for sulfite of 4 μM. As a result, pH-independent and -dependent k_{cat} and k_{cat}/K_m values could not be determined for R226A or R226K SsuD. The lack of activity seen with these SsuD variants supports Arg226 as the active-site acid contributing to the upper pK_a value at 9.5 ± 0.1 for k_{cat}.

Substrate Binding Affinity. Fluorescent titrations were performed to determine if the lack of detectable activity was due to a disruption in substrate binding. To evaluate flavin binding, $FMNH_2$ was titrated into a sample of R226A or R226K SsuD, and the spectra recorded with an excitation wavelength at 280 nm and emission intensity measurements at 344 nm. Each Arg226 SsuD variant had comparable K_d values for $FMNH_2$ binding as wild-type SsuD (1.49 ± 0.24 and 2.02 ± 0.25 μM for R226A or R226K SsuD, respectively) (Figure S2), suggesting that substitution of the arginine residue to either lysine or alanine did not alter the binding affinity of $FMNH_2$.[6] Titrations were also performed to determine if there was a measurable change in the affinity for 1-octanesulfonate binding to the Arg226 SsuD variants with $FMNH_2$ bound. The K_d value for 1-octanesulfonate binding to each SsuD variant/$FMNH_2$ complex was 10.5 ± 1.1 and 20.1 ± 2.5 μM for R226A or R226K SsuD, respectively (Figure S3). These K_d values are comparable to the values obtained for wild-type SsuD (17.5 ± 0.9 μM) and suggest that substitution of the arginine residue to either lysine or alanine does not alter the binding of 1-octanesulfonate to the SsuD variant/$FMNH_2$ complex in fluorescent titrations.[6] Therefore, the absence of activity in the Arg226 SsuD variants was not due to substantial changes in the binding affinity for $FMNH_2$ or 1-octanesulfonate.

Rapid Reaction Kinetic Analyses. The role of Arg226 in the desulfonation reaction was further evaluated through rapid reaction kinetic analyses to determine if modification of the flavin oxidation steps were responsible for the lack of activity observed with the Arg226 SsuD variants. The oxidation of

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$FMNH_2$ by R226A or R226K SsuD in the absence or presence of 1-octanesulfonate (10−500 μM) was monitored at 450 nm by stopped-flow kinetic analyses. The kinetic traces obtained at 450 nm for each variant represent the oxidation of reduced flavin (Figure 2A,B). The kinetic traces were best fit to a

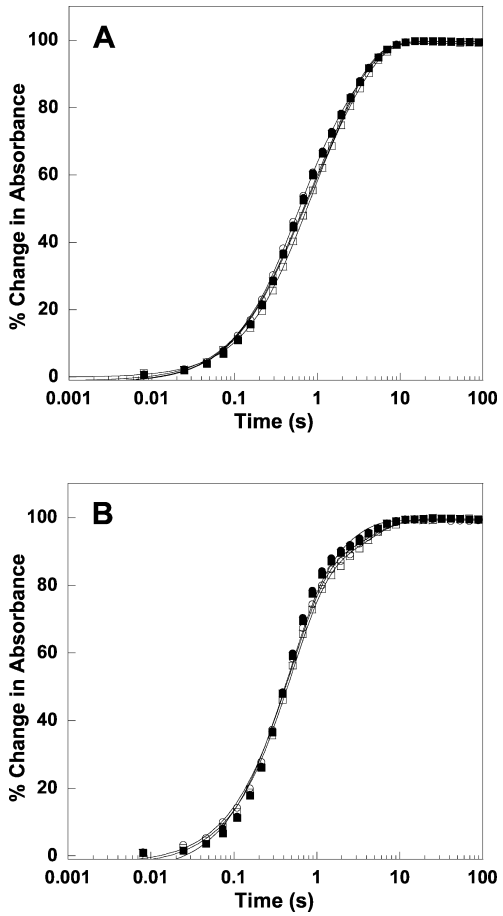

Figure 2. Kinetic traces of flavin oxidation by R226A and R226K SsuD. Single turnover experiments were performed by stopped-flow kinetic analysis at 4 °C in 50 mM Tris-HCl, pH 8.5, and 100 mM sodium chloride. (A) Single turnover kinetic traces were obtained after mixing free $FMNH_2$ (25 μM) with R226A SsuD (35 μM) in air-saturated buffer at 370 nm (●) and 450 nm (■) and after mixing free $FMNH_2$ (25 μM) with R226A SsuD (35 μM) and 1-octanesulfonate (100 μM) in air-saturated buffer at 370 nm (○) and 450 nm (□). (B) The single turnover traces employ the same conditions described for (A), but with R226K SsuD. The kinetic traces shown represent an average of three separate experiments. The solid lines are the fits of the kinetic traces to eq 6.

double-exponential equation, and the rate constants obtained either with or without octanesulfonate were within error to the values obtained for wild-type SsuD.[6] Additional studies were performed monitoring the oxidation of $FMNH_2$ by R226A or R226K SsuD in the absence or presence of 1-octanesulfonate (10−500 μM) at 370 nm (Figure 2A,B). The kinetic traces at 370 nm for R226A SsuD were best fit to a double-exponential equation with rate constants of 2.24 ± 0.10 s^{-1} (k_1) and 0.41 ± 0.03 s^{-1} (k_2) in the absence of octanesulfonate (Figure 2A, ●) and 1.90 ± 0.02 s^{-1} (k_1) and 0.40 ± 0.01 s^{-1} (k_2) with the addition of octanesulfonate (Figure 2A, ○). Kinetic traces at 370 nm for R297K SsuD were also best fit to a double-exponential equation with similar rate constants within error as R297A SsuD, 2.09 ± 0.03 s^{-1} (k_1) and 0.26 ± 0.01 s^{-1} (k_2) in the absence of octanesulfonate (Figure 2B, ●), and 2.14 ± 0.04 s^{-1} (k_1) and 0.27 ± 0.05 s^{-1} (k_2) with octanesulfonate included in the reaction (Figure 2B, ○). The rate constants were similar to the values obtained from the kinetic traces of the Arg226 SsuD variants monitored at 450 nm, indicating that the C4a-(hydro)peroxyflavin intermediate was never formed. The C4a-(hydro)peroxyflavin intermediate has an absorbance peak observable at 370 nm, but not at 450 nm.[6] In kinetic traces of wild-type SsuD at 370 nm in the absence and at low concentrations (<100 μM) of octanesulfonate an initial phase corresponding to the formation of the C4a-(hydro)peroxyflavin intermediate was previously observed.[6] With the Arg226 SsuD variants, there was no initial phase identified at 370 nm, and the corresponding kinetic trace essentially overlaid with the normalized kinetic trace obtained at 450 nm (Figure 2). If the intermediate had formed, these two traces would not have overlaid because the ratio of the extinction coefficients at 370 and 450 nm for the C4a-(hydro)peroxyflavin intermediate is greater than the comparable ratio for fully oxidized flavin.[29] Therefore, if the C4a-(hydro)peroxyflavin was generated in the first phase, the kinetic traces at 370 nm would appear similar to those observed for wild-type SsuD.[6,30−36] The inability of R226A or R226K SsuD to generate the C4a-(hydro)-peroxyflavin intermediate correlates with the absence of activity in steady-state kinetic experiments and suggests the peroxyflavin does not accumulate to detectable levels during turnover.

Guanidinium Rescue Experiments. From a structural standpoint, the arginine to alanine mutation in the active site of SsuD is equivalent to the removal of a guanido moiety from the side chain of Arg226. The kinetic and biochemical properties that have been affected by such a mutation should be at least partially restored in the presence of exogenous guanidine.[36−38] The effect of chemical rescue on the kinetic parameters of R226A SsuD was determined over the experimental pH range. Approximately 1.5% of the wild-type SsuD k_{cat} value was restored by the addition of 100 mM guanidine to the R226A SsuD assay (Table 2). The rescue of k_{cat} by guanidinium was pH independent which was attributed not only to free guanidinium cation (pK_a = 13.6) remaining protonated throughout the experimental pH range, but the outward displacement of pK_a values relative to the wild-type enzyme beyond the experimental pH range. An outward shift of pK_a values observed only in the k_{cat} pH profile is indicative of a non-pH-dependent, but normally rate-limiting, slow step following the catalytic reaction.[24−26] The pH dependence of k_{cat}/K_m for guandinium rescue of R226A SsuD activity could not be determined due to absorbance values being below the detectable limit for sulfite production (4 μM) at low pH under assay conditions employing low concentrations of 1-octanesulfonate. The lack of activity seen with the lysine variant demonstrates that SsuD activity is not dependent simply on the charge associated with the Arg226 in the active site; the hydrogen-bonding interactions of the guanido moiety of Arg226 are also of particular importance to SsuD catalysis. The combination of low activity and the apparent displacement of pK_a values with the addition of guanidinium suggests Arg226 plays a role in the rate-limiting product release step, potentially through mediation of a conformational change.[24−26]

■ DISCUSSION

Similar active-site environments often promote diverse functionalities despite exhibiting nearly identical structural

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motifs. Although members of the bacterial luciferase superfamily share common structural motifs and some conserved amino acid sequence identities, the catalytic mechanisms employed by each enzyme appear to be quite distinct.[9−13,15−22] In the mechanisms of bacterial luciferase and LadA, a histidine residue has been determined to serve as an active-site base in each of these enzymes.[9−13,16,18,19] As a result, the conserved His228 amino acid residue of SsuD had been proposed to serve in this same capacity based on its similar structural orientation to the histidine active-site base in bacterial luciferase and LadA.[9−13,16] To further probe the role of active site residues in catalysis, the pH dependence on the k_{cat} and k_{cat}/K_m values of SsuD and SsuE were determined in the pH range of 5.8−10.0. Experiments were limited to this pH range because SsuD reacts adversely with many buffers as aminosulfonic acids are substrates of SsuD. Although previous kinetic parameters were established in potassium phosphate at pH 7.5, use of this buffer was abandoned after it was found to inhibit the SsuD reaction.[6−8] As a result, Bis-Tris (pH range of 5.8−7.2), Tris-HCl (pH range of 7.2−9.0), and glycine (pH range of 9.0−10.0) were used to establish the kinetic parameters over these pH ranges.

The k_{cat}/K_m pH profile indicates the optimal protonation state of ionizable groups located on the free-enzyme and/or substrates in order for the reaction to commit to the first irreversible step of catalysis, while the pH dependence of k_{cat} reflects ionizable groups on the enzyme−substrate complex that are required for catalysis.[24,25,40,41] Unlike the pH profile for k_{cat}/K_m, the k_{cat} pH profile reflects the necessary protonation state of groups necessary for catalysis to occur including product release. The k_{cat}/K_m pH profile for the SsuD reaction revealed a single titratable group with a pK_a value of 6.9 ± 0.1. Additionally, the wild-type SsuD pH profile of k_{cat} revealed a similar titratable pK_a value of 6.6 ± 0.1. Despite the differing pK_a values of 6.9 and 6.6 for the k_{cat}/K_m and k_{cat} profiles, respectively, these values likely represent the same group. The altered pK_a value could be the result of a change in the active-site environment upon the formation of the first enzyme−substrate complex where the pK_a value of the group is 6.9 before formation of the enzyme−substrate complex but is perturbed to 6.6 upon binding of one or more of the substrates.[24,42] Previous studies have indicated that the desulfonation reaction by SsuD occurs through an ordered binding mechanism where $FMNH_2$ must bind to the enzyme first, followed by either 1-octanesulfonate or O_2.[6] If the lower pK_a value represents the same group in both the k_{cat}/K_m and k_{cat} profiles, then it can be concluded that the deprotonated form of this group is necessary both for formation of the final enzyme−substrate complex and for catalysis. It is unlikely that this pK_a value can be attributed to the ionization of 1-octanesulfonate because of the relatively low pK_a value of the sulfonate group.[43]

The pH dependence on the kinetic parameters of SsuD obtained from the current study identified a group with a pK_a consistent with a histidine residue to be essential for catalysis. However, the present study failed to identify His228 or any other active-site histidine residue serving as an active-site base in the SsuD enzyme. This pK_a value was present in the pH profiles of all His and Cys variants with only minor perturbations in the value (Table 1). These minor perturbations were attributed to the presence of a sticky substrate.[24,25] However, the presence of this sticky substrate had a negligible effect on the overall k_{cat}/K_m and k_{cat} pH-independent values, suggesting that while these amino acid residues may be involved in maintaining the active-site environment through minor substrate binding interactions, they are not critical to catalysis. Therefore, the pH profiles for H228A, H11A, H333A, and Cys54 SsuD confirm that neither of these amino acid residues is contributing solely to this lower pK_a value and that the group responsible for this ionization has yet to be identified.

Previously, deuterium kinetic isotope studies on bacterial luciferase indicated that the N1 position of $FMNH_2$ (pK_a = 6.2) contributes a comparable lower pK_a value of 6.9.[44] It is possible the N1 position of $FMNH_2$ is contributing to the lower pK_a value in SsuD as well: as is the case with bacterial luciferase, either the protonated species of $FMNH_2$ does not bind or does not form the C4a-(hydro)peroxyflavin at a significant rate.[44] An interesting observation was the identification of a "hollow" from fits of the C54A SsuD k_{cat}/K_m and k_{cat} profiles, indicating the presence of a sticky proton in the enzyme−substrate complex.[24,25] This "hollow" indicates octanesulfonate dissociates faster than the ionizable proton in the C54A SsuD variant.[24,25] Previously, the formation of the C4a-(hydro)-peroxyflavin intermediate has been shown to be stabilized by the presence of Cys54 in SsuD and Cys106 in bacterial luciferase.[8,22,23] In the three-dimensional structure of bacterial luciferase, Cys106 is directly interacting with bound FMN.[16] If the N1 position of $FMNH_2$ is responsible for the lower pK_a value in SsuD, then the observed hollow on the acidic limb of the pH profile indicates Cys54 would be required for the rapid dissociation of the N1 proton from $FMNH_2$ when octanesulfonate is present.[24,25] In this model, rapid dissociation of the N1 proton would be required for the C4a-(hydro)peroxyflavin intermediate to form at a significant rate.[44] Therefore, these combined results indicate Cys54 of SsuD directly interacts with the C4a-(hydro)peroxyflavin intermediate and promotes its formation by facilitating the dissociation of the proton located at the N1 position of $FMNH_2$.

A conserved but previously uncharacterized arginine residue within the active site, Arg226, was determined to serve a crucial role in SsuD catalysis. Alanine and lysine substitutions to the 226 position of SsuD resulted in complete inactivation of the enzyme. Therefore, the pH dependence on the kinetic parameters of R226A and R226K SsuD could not be determined as neither variant could catalyze detectable levels of sulfite production. The similar substrate affinities of R226A and R226K SsuD to wild type ruled out that substrate binding is altered from these substitutions. Although substrate binding was not affected, there was no observable formation of the C4a-(hydro)peroxyflavin intermediate in rapid reaction kinetic studies.[6] Kinetic traces at 370 nm obtained for the oxidation of $FMNH_2$ with R226A and R226K SsuD in the absence of octanesulfonate substrate were fitted to a double-exponential equation (eq 6). There was no phase (k_1) correlating with the formation of the C4a-(hydro)peroxyflavin intermediate as was previously observed with wild-type SsuD in stopped-flow kinetic studies.[6] In addition, there was no dependence on k_2 with increasing octanesulfonate concentrations as previously observed with wild-type SsuD.[6] The inability of the C4a-(hydro)peroxyflavin intermediate to be generated correlates with the lack of activity for the Arg226 SsuD variants.

In considering the proposed catalytic mechanism for SsuD in combination with the results of the present study, it is reasonable to conclude that Arg226 could possess multiple functions. Positively charged groups, such as an arginine, are highly conserved near the active site of Baeyer−Villiger flavin

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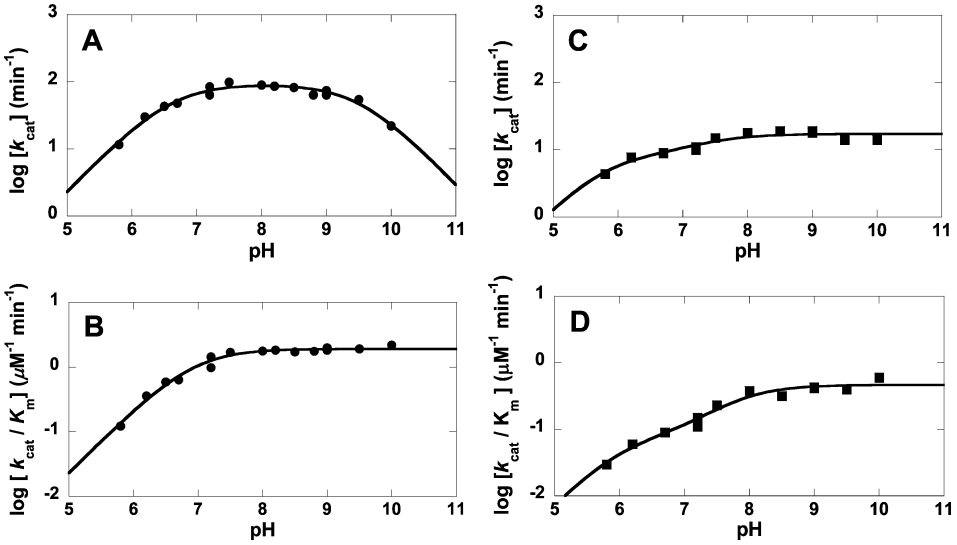

Figure 3. pH dependence of wild-type and C54A SsuD activity. Reactions were initiated by the addition of NADPH (500 μM) into a reaction mixture containing wild-type SsuD (0.2 μM), SsuE (0.6 μM), FMN (2 μM), and a range of 1-octanesulfonic acid concentrations (10−5000 μM) in either 50 mM Bis-Tris (pH range of 5.8−7.2), 50 mM Tris-HCl (pH range of 7.2−9.0), or 50 mM glycine (pH range of 9.0−10.0) and 100 mM sodium chloride at 25 °C. Because of decreased activity, reactions with C54A SsuD employed increased concentrations of C54A SsuD (1.2 μM), SsuE (3.6 μM), and FMN (5 μM). pH dependence of wild-type SsuD (●) A: k_{cat} values; B: k_{cat}/K_m values; pH dependence of C54A SsuD (■); C: k_{cat} values; D: k_{cat}/K_m values. Each point is the average of at least three separate experiments. Solid lines for A, B, C, and D are the fits of data to eq 2, eq 1, eq 3, and eq 3, respectively.

monooxygenases in order to stabilize peroxyflavin intermediates.[32,45,46] The absence of this positively charged stabilizing group near the active site can lead to inactivation of the enzyme due to destabilization of the flavin intermediate. The absence of activity even with the lysine substitution of Arg226 is likely due to an altered position of the positive charge, resulting in the inability of this variant to stabilize the C4a-(hydro)peroxyflavin intermediate. Based on the results from the chemical rescue experiments with guanidine, the orientation of and chemical properties associated with the guanido functional group of Arg226 are as crucial to catalysis as its charge. Product release also appears to be affected by the addition of guanidine, given that the recovery of wild-type SsuD k_{cat} is pH independent. This pH independence is likely due to the outward displacement of pK_a values beyond the experimental pH range, suggesting that while the free guanidinium ion rescues catalytic activity in R226A SsuD, product release remains compromised.[24−26] An active site acid has been proposed to play a role in the SsuD mechanism by protonating the FMNO$^-$ intermediate leading to the release of H_2O and the FMN product through a conformational change.[4,6,7,10,11,14,17] In the proposed chemical mechanism for SsuD, protonation of the FMNO$^-$ intermediate by the catalytic acid would be the step prior to product release. If Arg226 is the catalytic acid, and the guanidinium ion is serving the role of this catalytic acid in the R226A SsuD variant, then the product release step could potentially be a limiting factor in guanidinium's ability to rescue k_{cat}. Although exogenous guanidine may be able to compensate for some of the steric and electrostatic interactions normally attributed to Arg226, the guanidinium moiety would be less effective in transferring a proton to the FMNO$^-$ intermediate and signaling the proposed conformational change linked to product release. These combined results support Arg226 playing a dual role in SsuD catalysis by facilitating oxygen activation through stabilization of the C4a-(hydro)peroxyflavin intermediate and by promoting product release through a conformational change associated with protonation of the FMNO$^-$ intermediate.

Experimental results from studies performed on Cys54 SsuD provided support for Arg226 as the active site acid in the SsuD chemical reaction. The protonated form of Cys54 with a pK_a in the free enzyme of 9.3 ± 0.1 was previously shown to be involved in stabilizing the C4a-(hydro)peroxyflavin in SsuD either through direct interactions with the flavin or in helping to maintain the active site environment, but not playing a direct role in SsuD catalysis as the active-site acid (Figure 1).[8] However, the substitution of Cys54 SsuD with Ala resulted in the elimination of the upper pK_a from the k_{cat} pH profile (Figure 3C). Although these combined results indicate Cys54 is contributing to the upper pK_a in the wild-type enzyme, the modest 5-fold drop in the k_{cat} and k_{cat}/K_m values compared to wild-type SsuD does not support Cys54 as the catalytic acid (Table 1). Two possible models would support the apparent disappearance of the upper pK_a: the pK_a value at 9.5 corresponds to Cys54, and there is a second pK_a value in the wild-type enzyme occurring outside the experimental pH range corresponding to the catalytic acid; or the pK_a value at 9.5 corresponds to the catalytic acid, and the cysteine to alanine substitution resulted in a shift of this pK_a value to a value outside of the experimental pH range. Both models suggest that Cys54 participates in a step occurring after the first irreversible step through product release and support Arg226 playing the role of the catalytic acid. Additionally, the cysteine to alanine substitution resulted in the outward displacement of the lower apparent pK_a value in the k_{cat} pH profile when compared to wild-type SsuD but did not affect the apparent pK_a value seen in the k_{cat}/K_m pH profile (Table 1). An outward shift of pK_a values observed only in the k_{cat} pH profile is indicative of a non-pH-dependent, but normally rate limiting, slow step following the catalytic reaction.[24−26] Since the outward pK_a shifts are only present on the pH dependence of k_{cat} for C54A SsuD, the results suggest the rate of product release is slowed by the Cys

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to Ala substitution.[24−26] Previous studies have demonstrated that a combination of electrostatic and hydrogen-bonding interactions can perturb the pK_a values (≥2 units) of catalytic groups in an enzyme active site.[27] Such studies indicate that the pK_a of an arginine group could be perturbed within the active site of an enzyme through a complex hydrogen-bonding network.[24−28] Considering both Cys54 and Arg226 have been linked to stabilization of the C4a-hydroperoxyflavin intermediate, it would be reasonable to conclude a direct or indirect interaction between the two amino acid side chains. Therefore, the data support the possibility that Cys54 is involved in altering the pK_a of Arg226 from a more typical value of 12.5 to 9.5 during catalysis. This result combined with the absence of detectable SsuD catalytic activity in the SsuD arginine variants suggest that Arg226 is contributing to the pK_a value at 9.5 ± 0.1 for wild-type SsuD. Additionally, at least some $FMNO^-$ intermediate must be forming in the reaction with C54A SsuD to account for the catalytic activity of the variant. In this case, the catalytic acid would be required for the protonation of any $FMNO^-$ intermediate that is able to form despite the compromised active site environment (Scheme 2, **IV**). Therefore, it is proposed that Arg226 must be present in order to stabilize the peroxyflavin intermediate and promote product release through a conformational change linked to the protonation of the $FMNO^-$ intermediate.

Similarities between the sequences and three-dimensional structures of related enzymes can offer key insight as to the identity of important groups in catalysis for a particular enzyme. These similarities are evident between the well-characterized bacterial luciferase and the more recently characterized SsuD and LadA two-component monooxygenases. Nevertheless, the present study serves as an example of the different roles conserved groups can play in the mechanisms of such enzymes. The amino acids His44 and His311 were reported to serve as active-site bases in the mechanisms of bacterial luciferase and LadA, respectively.[9−13,15−23] However, results from the characterization of His228 SsuD determined this active-site histidine is not playing this role in SsuD catalysis. Instead, pH dependence studies, steady-state kinetic studies, and single-turnover rapid-reaction kinetic studies highlighted Arg226 to be playing a crucial role as the potential active-site acid in SsuD catalysis. These results are consistent with our proposed mechanism in that conserved arginine residues near the active site are often crucial players in the mechanisms of Baeyer−Villiger flavin monooxygenases.[32,45,46] These results provide an important foundation in the illumination of the SsuD catalytic mechanism. Future studies involving deuterium solvent isotope effects, kinetic isotope effects, and an expanded characterization of nearby, but previously uncharacterized residues will undoubtedly offer a much clearer window into the intricacies of SsuD catalysis.

■ ASSOCIATED CONTENT

Supporting Information

H228A and H11A pH profiles and fluorescent titrations to determine the K_d values for substrate binding; representative data included to correlate with the values provided in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

DTNB, 5,5-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; FMN, flavin mononucleotide; $FMNH_2$, reduced flavin mononucleotide; NADPH, nicotinamide adenine dinucleotide (phosphate); SsuE, alkanesulfonate flavin reductase; SsuD, alkanesulfonate monooxygenase; LadA, long-chain alkane monooxygenase.

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