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# Catalytic role of a conserved cysteine residue in the desulfonation reaction by the alkanesulfonate monooxygenase enzyme ☆

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ABSTRACT

Detailed kinetic studies were performed in order to determine the role of the single cysteine residue in the desulfonation reaction catalyzed by SsuD. Mutation of the conserved cysteine at position 54 in SsuD to either serine or alanine had little effect on $FMNH_2$ binding. The $k_{cat}/K_m$ value for the C54S SsuD variant increased 3-fold, whereas the $k_{cat}/K_m$ value for C54A SsuD decreased 6-fold relative to wild-type SsuD. An initial fast phase was observed in kinetic traces obtained for the oxidation of flavin at 370 nm when $FMNH_2$ was mixed against C54S SsuD ($k_{obs}$, 111 $s^{-1}$) in oxygenated buffer that was 10-fold faster than wild-type SsuD ($k_{obs}$, 12.9 $s^{-1}$). However, there was no initial fast phase observed in similar kinetic traces obtained for C54A SsuD. This initial fast phase was previously assigned to the formation of the C4a-(hydro)peroxyflavin in studies with wild-type SsuD. There was no evidence for the formation of the C4a-(hydro)peroxyflavin with either SsuD variant when octanesulfonate was included in rapid reaction kinetic studies, even at low octanesulfonate concentrations. The absence of any C4a-(hydro)peroxyflavin accumulation correlates with the increased catalytic activity of C54S SsuD. These results suggest that the conservative serine substitution is able to effectively take the place of cysteine in catalysis. Conversely, decreased accumulation of the C4a-(hydro)peroxyflavin intermediate with the C54A SsuD variant may be due to decreased activity. The data described suggest that Cys54 in SsuD may be either directly or indirectly involved in stabilizing the C4a-(hydro)peroxyflavin intermediate formed during catalysis through hydrogen bonding interactions.


## 1. Introduction

Sulfur is a critical element for the growth of bacterial organisms. Many bacteria have inorganic sulfur requirements for the production of sulfur-containing compounds. Inorganic sulfur is poorly represented in aerobic soil, therefore bacteria in these environments must have an alternative process for obtaining this crucial element [1,2]. To meet its sulfur requirements under sulfate-starvation conditions, *Escherichia coli* synthesizes taurine dioxygenase and alkanesulfonate monooxygenase proteins for the acquisition of sulfur from alternative sources [3,4]. The expression of these proteins allows the organism to utilize a broad range of alkanesulfonates during growth when inorganic sulfur is limiting.

The two-component alkanesulfonate monooxygenase system is composed of both an NAD(P)H[1]-dependent flavin reductase (SsuE) and a monooxygenase (SsuD). SsuD belongs to a family of reduced flavin-dependent monooxygenases that use flavin as a substrate instead of a bound prosthetic group [4]. It catalyzes the oxygenolytic cleavage of 1-substituted alkanesulfonates in the presence of $FMNH_2$ and dioxygen, producing the corresponding aldehyde and sulfite. In the proposed mechanism, the C4a-peroxyflavin (II in Scheme 1) makes a nucleophilic attack on the sulfonate functional group to form an initial alkanesulfonate peroxyflavin intermediate (III). A Baeyer-Villiger rearrangement of the flavin adduct (III) followed by proton abstraction by an active site base (IV) generates the aldehyde and sulfite products (V). The reaction catalyzed by SsuD has been partially evaluated through steady-state and rapid reaction kinetic analyses [5]. The octanesulfonate substrate is unable to bind to SsuD unless reduced flavin is present, suggesting that reduced flavin binds first to induce a protein conformational change that allows the alkanesulfonate to bind. The reactions catalyzed by many two-component flavin-dependent monooxygenases have been shown to involve a C4a-(hydro)peroxyflavin intermediate. The formation of the C4a-(hydro)peroxyflavin intermediate in bacterial luciferase in the presence and absence of the aldehyde substrate has been identified through kinetic analyses [6–9]. This flavin intermediate was isolated at low temperatures in the absence of the aldehyde substrate demonstrating the stability of this intermediate in bacterial luciferase [10]. Rapid reaction

*Abbreviations:* FMN, flavin mononucleotide; $FMNH_2$, reduced flavin mononucleotide; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); SsuE, alkanesulfonate flavin reductase; SsuD, alkanesulfonate monooxygenase

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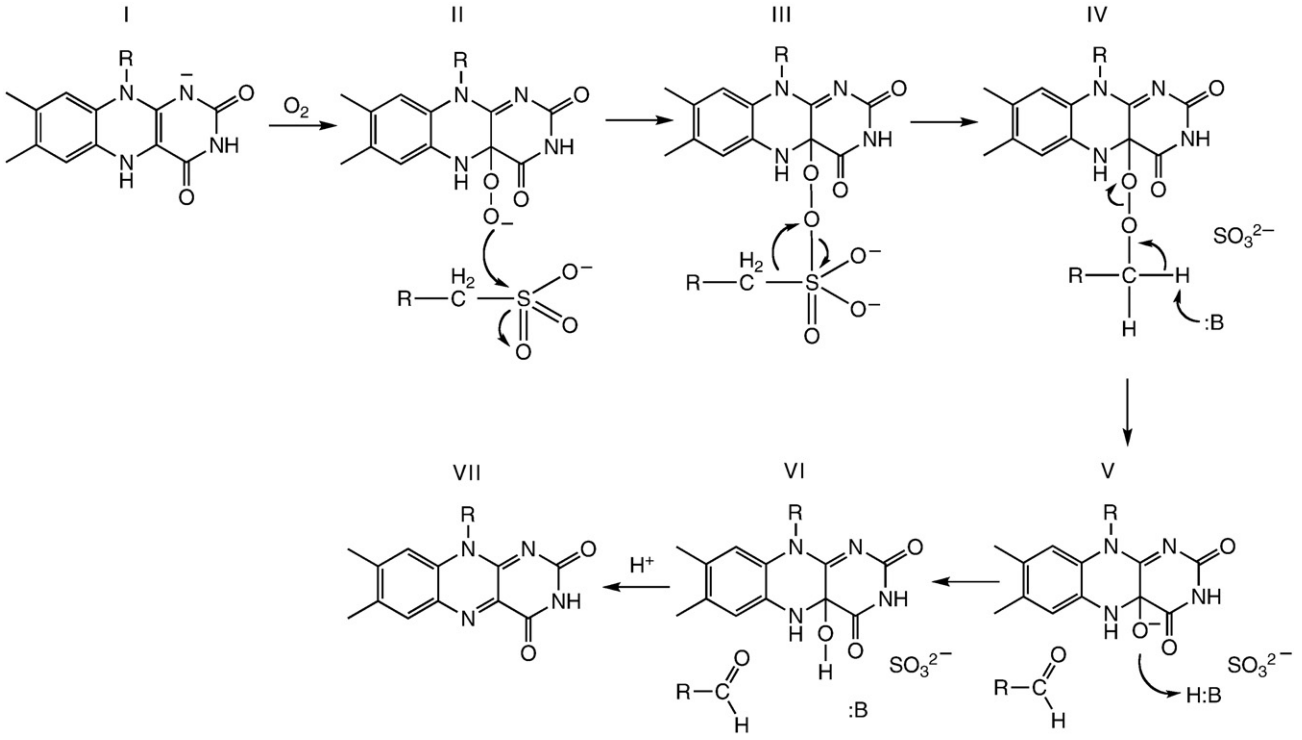


**Scheme 1.**

kinetic evaluation of SsuD also suggests that the C4a-(hydro) peroxyflavin intermediate is formed as proposed in the mechanism, but this flavin intermediate is not as stable as the intermediate generated in the bacterial luciferase reaction [5].

The SsuD enzyme exists as a homotetramer containing four active sites, with each subunit composed of a triose phosphate isomerase (TIM)-barrel fold. The location of the SsuD active site has been proposed to be located at the C-terminal end of the β-barrel, where the active sites of most TIM-barrel enzymes are found [11]. The identification of the active site is supported by earlier biochemical studies, and the location of conserved amino acid residues in SsuD. Although the amino acid sequence identity is low, SsuD is strikingly similar in overall structure with bacterial luciferase and long-chain alkane monooxygenase (LadA) [12–17]. While luciferase uses a long-chain aliphatic aldehyde as a substrate to catalyze the production of visible light and a corresponding carboxylic acid, LadA oxidizes long-chain alkanes to primary alcohols. These enzymes catalyze distinctly different biochemical reactions; however, they all are members of the FMN-dependent monooxygenase family.

Several amino acids located in the putative active site of SsuD (His228, Tyr331, Arg297, and Cys54) are in a similar spatial arrangement as proposed catalytically relevant amino acid residues from bacterial luciferase (His44, Tyr110, Arg291, and Cys106) and LadA (His311, Tyr63, and Cys14) (Fig. 1) [12–17]. The His44 residue in the α-subunit of bacterial luciferase has been reported to be the catalytic base in the bioluminescence reaction. Mutation of His44 residue to alanine showed marked differences in the intensity of light emitted [18]. The bioluminescence activity could be reconstituted by chemical rescue with the addition of imidazole to the reaction [19]. Substitution of the corresponding residue in LadA was shown to inactivate the enzyme [17]. The conserved Tyr residue has been evaluated in LadA where variants of this residue were shown to completely abolish activity [17]. In the structure of bacterial luciferase with bound FMN, Tyr110 is proposed to be involved in hydrogen bonding interactions with flavin binding residue Asp113 [16,20–22]. A conserved arginine residue in SsuD is also located in a similar position to Arg291 in bacterial luciferase. In both enzymes, this residue is located in an insertion region containing an unstructured loop that is thought to cover the active site once the substrates are bound. Although direct substitution of Arg291 has not been performed, deletion of this unstructured loop region in bacterial luciferase resulted in a decrease in bioluminescence [23]. The catalytic role of Cys106 in bacterial luciferase has been studied in extensive detail. Initial studies demonstrated that chemical modification of the reactive thiol of Cys106 resulted in the inactivation of the enzyme, leading to the assumption that this residue was directly involved in catalysis [24–26]. Substitution of Cys106 was shown to reduce

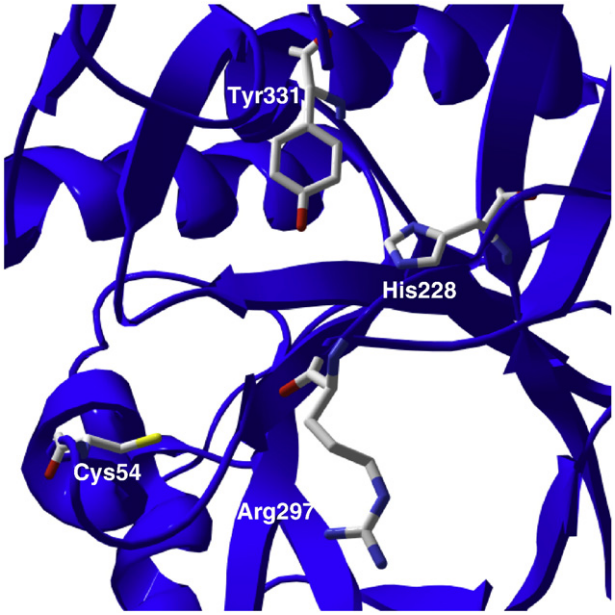


**Fig. 1.** Putative active site of SsuD. The highlighted amino acids are spatially conserved in bacterial luciferase (His44, Tyr110, Arg291, and Cys106) and LadA (His311 and Tyr63). The active site was generated with Swiss PDBViewer (PDB ID:1M41) and rendered with the MegaPov program (7).

bioluminescence, and was suggested to be involved in the stabilization of the C4a-(hydro)peroxyflavin intermediate in bacterial luciferase [27,28]. The Cys106 residue is not directly involved in catalysis, but appears to play an indirect role in maintaining the proper active site conformation for optimal stabilization of the C4a-(hydro) peroxyflavin. Variants of the corresponding Cys14 residue in LadA were unable to catalyze the oxidation of the alkane substrate [17]. Earlier studies demonstrated that cysteine-labeling of SsuD with methylmercury leads to inactivation of the enzyme suggesting a possible role of Cys54 in catalysis [12]; however, the role of the spatially conserved cysteine residue in the desulfonation reaction catalyzed by SsuD has not been evaluated.

The three-dimensional structures of flavin-dependent enzymes that utilize a proposed C4a-peroxyflavin in catalysis are increasingly being determined [12–17,29,30]. A striking feature observed in these structures is the importance of active site residues in maintaining the proper architecture and environment for optimal catalysis. Therefore, understanding the catalytic roles of these active site residues is highly significant in order to fully understand the mechanistic features of these enzymes. Although SsuD is grouped with bacterial luciferase and LadA because of structural similarities, the mechanism of SsuD is quite distinct from these enzymes. Due to the expression of this protein under times of sulfur limitation, Cys54 is the only cysteine residue located in SsuD. The inactivation of cysteine-modified SsuD combined with the spatial similarity of this residue to the functionally significant cysteine residues in bacterial luciferase and LadA suggests that Cys54 may be important in catalysis. In order to determine the function of Cys54 in the desulfonation reaction, variants of the SsuD enzyme were constructed in which the cysteinyl residue was replaced by either serine or alanine. The experiments described in this work define the functional role of the conserved cysteine residue in the catalytic mechanism of SsuD.

## 2. Materials and methods

### 2.1. Materials

QuikChange site-directed mutagenesis kit and super-competent cells *E. coli* BL21(DE3) were from Stratagene (La Jolla, CA). Oligonucleotide primers were from Invitrogen (Carlsbad, CA). Potassium phosphate (monobasic and dibasic), flavin mononucleotide phosphate (FMN), nicotinamide adenine dinucleotide phosphate (NADPH), Trizma base, Bis–Tris, glycine, and NaCl were purchased from Sigma (St. Louis, MO). Glycerol was obtained from Fisher (Pittsburgh, PA). Octanesulfonate was from Fluka (Milwaukee, WI). The standard phosphate buffer contained 25 mM potassium phosphate, pH 7.5, and 10% glycerol unless otherwise noted.

### 2.2. Site-directed mutagenesis, expression, and purification

Mutagenesis of the pET21a plasmid containing the *ssuD* gene was performed with the Stratagene QuikChange Site-Directed Mutagenesis Kit. The TGC codon for Cys54 was replaced with GCT and GCG for Ser and Ala, respectively. The *ssuD* Cys54 variants were identified by sequence analysis at Davis Sequencing (Davis, CA). Plasmids harboring the appropriate *ssuD* Cys54 variants were transformed into *E. coli* BL21(DE3) super-competent cells for protein expression and stored at −80 °C. The mutants generated are referred to as C54S (Cys54 to Ser) and C54A (Cys54 to Ala) SsuD. The expression and purification of the variant and wild-type SsuD proteins were performed as previously described [31].

### 2.3. Far-UV circular dichroism

The spectra of the variant and wild-type SsuD enzymes were obtained with an enzyme concentration of 1.2 μM in 25 mM potassium phosphate buffer, pH 7.5, at 25 °C. Buffer exchange was performed to remove all NaCl and glycerol using an Amicon Ultra Centrifugal Filter (Millipore, Billerica, MA) with a MWCO of 10,000 kDa. Far-UV circular dichroism spectra were recorded on a JASCO J-810 spectropolarimeter (Easton, MD). Measurements were taken in 0.2 nm increments in continuous scanning mode from 270 to 180 nm in a 0.1 cm path length cuvette with a bandwidth of 1 nm and a scanning speed of 20 nm/min. Each spectrum is the average of 8 accumulated scans.

### 2.4. $pK_a$ determination of Cys54

The p$K_a$ of Cys54 was determined by measuring the absorbance at 240 and 280 nm over a pH range of 6.2–10.2 in the appropriate buffer (50 mM Bis–Tris, pH 6.2–7.2; 50 mM Tris–HCl, pH 7.2–9.0; 50 mM glycine, pH 9.0–10.2, and 100 mM NaCl). The SsuD enzyme (10 μM final concentration) was equilibrated in each buffer for 10 min prior to measuring the absorbance values. The final protein concentration in solution was determined using the absorption coefficient of SsuD at 280 nm ($\varepsilon_{280} = 4.79 \times 10^4$ $M^{-1}$ $cm^{-1}$). The amount of SsuD in solution and $A_{240}$ values were used to calculate the $\varepsilon_{240}$ at each pH value. The $\varepsilon_{240}$ value was plotted against the pH and the p$K_a$ was determined by a fit to Eq. (1).

$$y = \frac{\left[\left(A \times 10^{-\mathrm{pH}}\right) + \left(B \times 10^{-\mathrm{p}K_a}\right)\right]}{\left(10^{-\mathrm{p}K_a} + 10^{-\mathrm{pH}}\right)} \quad (1)$$

where $y$ is the $\varepsilon_{240}$, $A$ is the lower plateau at high pH, and $B$ is the upper plateau at low pH.

### 2.5. Steady-state kinetic studies

For the variant and wild-type SsuD enzymes the reaction was initiated with the addition of NADPH (500 μM) into a reaction mixture containing SsuD (0.2 μM), SsuE (0.6 μM), FMN (2 μM), NADPH (500 μM), and varying concentrations of octanesulfonate (10–500 μM) in standard buffer with 100 mM NaCl at 25°C. Because of decreased enzymatic activity, a final concentration of 1.0 μM was used in assays with C54A SsuD protein. Following quenching of the reaction, the amount of sulfite product was determined as previously described [5].

### 2.6. Substrate binding studies

Flavin binding to variant and wild-type SsuD enzymes was monitored by spectrofluorimetric titration with either oxidized or reduced flavin. Spectra were obtained on a Perkin Elmer LS 55 luminescence spectrometer (Palo Alto, CA) with an excitation wavelength of 280 nm and emission wavelength of 344 nm. For the titration of SsuD with FMN, a 1.0 mL solution of variant or wild-type SsuD enzyme (1 μM) in standard buffer containing 100 mM NaCl was titrated with a solution of FMN (1.5–65.0 μM for C54S and 4.4–90.8 μM for C54A), and the fluorescence spectra recorded after each addition. Similar experiments were performed with $FMNH_2$, however care had to be taken to keep the solution anaerobic as previously described [5]. Anaerobic solutions of the SsuD variants (0.5 μM) in a glass titration cuvette were titrated with $FMNH_2$ (0.21–6.0 μM for C54S and 0.21–9.4 μM for C54A) in an air-tight titrating syringe. The bound FMN or $FMNH_2$ was determined by the following equation:

$$[\mathrm{FMN}]_{\mathrm{bound}} = [\mathrm{SsuD}]\frac{I_0 - I_c}{I_0 - I_f} \quad (2)$$

where [SsuD] is the initial concentration of enzyme, $I_0$ is the initial fluorescence intensity of SsuD prior to titration, $I_c$ is the fluorescence intensity of SsuD following each addition of FMN or $FMNH_2$, and $I_f$ is the final fluorescence intensity. The concentration of bound FMN or

$FMNH_2$ was plotted against the total FMN or $FMNH_2$ to obtain the dissociation constant ($K_d$) according to Eq. (3):

$$Y = \frac{B_{max}X}{(K_d + X)} \quad (3)$$

where $B_{max}$ is the maximum binding at equilibrium with the maximum concentration of substrate, and $K_d$ is the dissociation constant for the substrate [5].

For the binding of octanesulfonate to variant or wild-type SsuD, a 1.0 mL solution of SsuD (1 μM) with reduced flavin (2 μM) was made anaerobic in a glass titration cuvette as described previously [5]. Aliquots of an anaerobic solution of octanesulfonate (1.3–57.5 μM for C54S and 1.3–73.2 μM for C54A) in an air-tight titrating syringe were added to the SsuD–$FMNH_2$ complex. The fluorescence spectra were recorded with an excitation wavelength at 280 nm and emission intensity measurements at 344 nm. 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. (3).

### 2.7. Rapid reaction kinetic experiments

Rapid reaction kinetic analyses were carried out using an Applied Photophysics SX.18 MV stopped-flow spectrophotometer. Anaerobiosis was established as previously described [5]. All experiments were performed by mixing 15 μM $FMNH_2$ in one drive syringe against SsuD (45 μM) in air-saturated buffer in the other drive syringe. When included in the reaction, varied concentrations of octanesulfonate (0.05–2.0 mM) were mixed with the protein solutions in air-saturated buffer. All experiments were carried out in single-mixing mode by mixing equal volumes of the solutions, and monitoring the reactions by single wavelength analyses at 370 and 450 nm. The single-wavelength traces of each cysteine variant at 370 and 450 nm were fitted to the following equations using the KaleidaGraph software (Abelbeck Software, Reading, PA):

$$A = A_1\exp(-k_1t) + A_2\exp(-k_2t) + C \quad (4)$$

$$A = A_1\exp(-k_1t) + A_2\exp(-k_2t) + A_3\exp(-k_3t) + C \quad (5)$$

where $k_1$, $k_2$, and $k_3$ are the apparent rate constants for the different phases, $A$ is the absorbance at time $t$, $A_1$, $A_2$, and $A_3$ 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 the first phase were fitted with the following equation:

$$k_{obs} = k_{lim}[S] / (K_d + [S]) \quad (6)$$

where $k_{obs}$ is the observed rate constant, $k_{lim}$ is the limiting rate constant for flavin oxidation at saturating octanesulfonate concentrations, $K_d$ is the dissociation constant for the enzyme-substrate complex, and $S$ is the substrate concentration.

## 3. Results

### 3.1. $pK_a$ determination of Cys54

The $pK_a$ value of Cys54 was evaluated to establish the protonation state of the single active site cysteine by monitoring the absorbance of the cysteine thiolate at 240 nm [32–36]. Because SsuD contains only one cysteine residue, determining the $pK_a$ of Cys54 by this method is not complicated by the contribution of additional cysteine thiolates. Inhibition assays with iodoacetamide are less reliable due to the utilization of DTNB in the standard steady-state kinetic SsuD assays. The $\varepsilon_{240}$ values representing the absorbance of the cysteine thiolate were determined at varying pH values between 6.2 and 10.2 (Fig. 2).

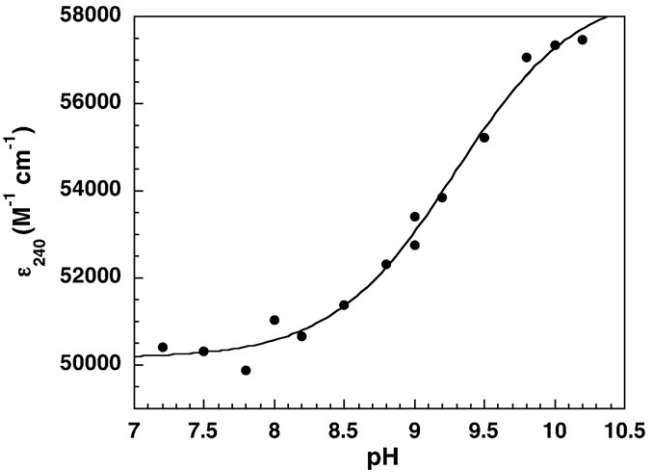


**Fig. 2.** Monitoring cysteine thiolate absorption at varying pH values to determine the $pK_a$ of Cys54. The SsuD enzyme (5 μM) was measured at 240 and 280 nm over a range of pH values (6.2–10.5). The $\varepsilon_{240}$ values at each pH were calculated using the concentration of SsuD in solution and the absorbance obtained at $A_{240}$. The $\varepsilon_{240}$ was plotted against the pH and fit to Eq. (1).

There was a clear dependence on pH with a single apparent $pK_a$ value of $9.3 \pm 0.1$. The difference in the protonated and deprotonated states of Cys54 was 6,000 $M^{-1}$ $cm^{-1}$, which agrees with previously published molar extinction coefficients for cysteine thiolates (4,000–6,000 $M^{-1}$ $cm^{-1}$) [32]. The results indicate that the protonated form of Cys54 likely predominates in SsuD at the pH utilized in the steady-state and rapid reaction kinetic assays.

### 3.2. Steady-state kinetic parameters for octanesulfonate

Steady-state kinetic analyses were performed with each cysteine variant to determine if the substitution of Cys54 led to an alteration in the kinetic parameters previously determined for wild-type SsuD. Results from circular dichroism spectroscopy experiments revealed that there were no obvious perturbations in the overall secondary structure between the Cys54 variants and wild-type SsuD, suggesting that substitution of Cys54 did not cause any major consequences to the protein structure. Because SsuD requires reduced flavin for activity, a coupled assay that includes SsuE in the reaction must be employed. This assay included an excess of SsuE so $FMNH_2$ would not be limiting in the reaction. The steady-state kinetic parameters for the Cys54 variants and wild-type SsuD were obtained by measuring the amount of TNB anion formed at 412 nm from the reaction of DTNB with the sulfite product. The steady-state kinetic parameters were determined by a fit of the data to the hyperbolic dependence of each enzyme on octanesulfonate concentration. The C54S SsuD enzyme showed an approximate 6-fold decrease in the $K_m$ value and a 2-fold decrease in the $k_{cat}$ value resulting in an overall increase in the $k_{cat}/K_m$ value compared to wild-type SsuD (Table 1). The less conservative C54A SsuD variant showed a 2-fold decrease in the $K_m$ value and a 9-fold decrease in the $k_{cat}$ value compared to wild-type, leading to a decrease in the $k_{cat}/K_m$ value. These results suggest that serine was effectively able to substitute for cysteine

**Table 1**
Dissociation constants and steady-state kinetics for C54S, C54A, and wild-type SsuD.

| | $k_{cat}$ ($min^{-1}$) | $K_m$, octanesulfonate (μM) | $k_{cat}/K_m$ ($\mu M^{-1}$ $min^{-1}$) | $K_d$, FMN (μM) | $K_d$, $FMNH_2$ (μM) |
|---|---|---|---|---|---|
| WT SsuD[a] | 51.7 ± 2.1 | 44.0 ± 8.3 | 1.2 ± 0.2 | 10.2 ± 0.4 | 0.32 ± 0.15 |
| C54S SsuD | 28.1 ± 0.4 | 7.5 ± 0.9 | 3.8 ± 0.7 | 11.3 ± 0.6 | 0.47 ± 0.09 |
| C54A SsuD | 5.6 ± 0.1 | 26.4 ± 1.7 | 0.2 ± 0.1 | 13.7 ± 0.4 | 0.53 ± 0.09 |

[a] Previously reported (5).

under steady-state conditions, while the alanine substitution led to a decrease in the steady-state kinetic parameters for SsuD.

### 3.3. Substrate binding affinity to the Cys54 SsuD variants

Spectrofluorimetric titrations were performed to determine whether Cys54 substitutions affect the binding of FMN or $FMNH_2$. The dissociation constants for the binding of oxidized and reduced flavin to the SsuD variants were determined by titrating either FMN or $FMNH_2$ into a sample of C54S or C54A SsuD. The decrease in the intrinsic protein fluorescence emission due to the binding of FMN or $FMNH_2$ was monitored at 344 nm for each variant. The concentration of bound and free flavin was calculated using Eq. (1), and the concentration of flavin bound to each variant was plotted against the concentration of free flavin. The dissociation constants ($K_d$) for FMN and $FMNH_2$ are summarized in Table 1. The average $K_d$ value for $FMNH_2$ binding to each SsuD variant was $0.47 \pm 0.09$ μM and $0.53 \pm 0.09$ μM for C54S or C54A SsuD, respectively. The dissociation constants for FMN binding to C54S and C54A SsuD were determined to be $11.3 \pm 0.6$ μM and $13.7 \pm 0.4$ μM, respectively (data not shown). These results indicate that mutation of the putative active site cysteine residue to either serine or alanine had little effect on FMN or $FMNH_2$ binding when compared to the previously reported $K_d$ value of $0.32 \pm 0.15$ μM ($FMNH_2$) and $10.2 \pm 0.4$ μM (FMN) for wild-type SsuD [5].

It was previously shown that ordered substrate binding occurs in the SsuD reaction, with reduced flavin cofactor binding first before the octanesulfonate substrate can bind [5]. The binding of octanesulfonate to the C54S or C54A SsuD/$FMNH_2$ complex was performed to determine whether the binding of the substrate was affected by the cysteine substitutions. Dissociation constants for the binding of octanesulfonate to the Cys54 SsuD variants were obtained under anaerobic conditions similar to those previously described for titration of each SsuD variant with $FMNH_2$ [5]. The concentration of octanesulfonate substrate bound to each SsuD variant/$FMNH_2$ complex was calculated according to Eq. (1) and plotted against the concentration of free octanesulfonate after each addition (Fig. 3, titration of C54S SsuD). The $K_d$ value for the binding of octanesulfonate to each Cys54 SsuD variant/$FMNH_2$ complex was $2.68 \pm 0.60$ μM and $4.97 \pm 0.58$ μM for C54S and C54A SsuD, respectively. The $K_d$ values obtained for the SsuD variants show a distinct decrease from the values obtained for wild-type SsuD ($17.5 \pm 0.9$ μM) by similar methods [5]. These results suggest that each variant has an increased affinity for octanesulfonate and indicate that the binding of octanesulfonate is affected by the substitution of Cys54 to either serine or alanine.

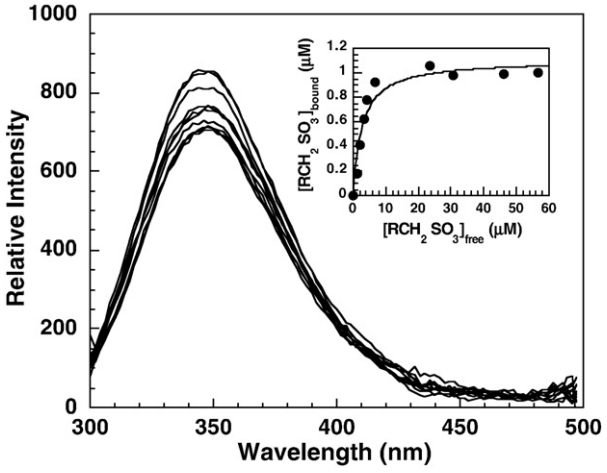


**Fig. 3.** Fluorimetric titration of the C54S SsuD/$FMNH_2$ complex with octanesulfonate. C54S SsuD (1.0 μM) was titrated with octanesulfonate (1.3–57.5 μM). Emission intensity measurements at 344 nm were obtained with an excitation wavelength of 280 nm. The change in the intrinsic fluorescence intensity for the SsuD/$FMNH_2$ complex following the addition of octanesulfonate was converted to concentration of bound octanesulfonate (Eq. (2)) and plotted against the concentration of free octanesulfonate. Inset: Change in fluorescence emission intensity at 344 nm. The solid line represents a fit of the titration curve to Eq. (3).

### 3.4. Kinetic studies of flavin oxidation by the SsuD variants

In order to further define the function of Cys54 in the desulfonation reaction by SsuD, rapid reaction kinetic studies were performed with each of the cysteine variants. Stopped-flow kinetic studies were initially performed by mixing $FMNH_2$ against C54S and C54A SsuD in air-saturated buffer in the absence of the alkanesulfonate substrate, and the oxidation of the flavin monitored at 370 and 450 nm. The Cys54 SsuD variants were maintained at a higher concentration (45 μM) relative to $FMNH_2$ (15 μM) to ensure single-turnover conditions. The rate constants obtained with each variant are summarized in Table 2. The kinetic trace was best fit to a triple exponential equation with rate constants of $111 \pm 15$ $s^{-1}$ ($k_1$), $24.1 \pm 0.8$ $s^{-1}$ ($k_2$), and $0.33 \pm 0.01$ $s^{-1}$ ($k_3$) for SsuD C54S (○, Fig. 4A). An initial fast phase ($k_1$) was observed in the reaction at 370 nm for the C54S SsuD variant that represents the generation of the C4a-(hydro)peroxyflavin intermediate [5]. The rate constants obtained for the last two phases have been assigned to the decay of the flavin intermediate back to the oxidized form. The C54S SsuD variant was able to generate the C4a-(hydro)peroxyflavin, but the rate of formation was increased 10-fold relative to wild-type. The rate constants for the subsequent breakdown of the C4a-(hydro) peroxyflavin were 13-, and 4-fold over wild-type for $k_2$, and $k_3$, respectively. The kinetic trace at 450 nm was best fit to a double exponential equation with rate constants of $9.76 \pm 0.19$ $s^{-1}$ ($k_1$) and $0.33 \pm 0.01$ $s^{-1}$ ($k_2$) (●, Fig. 4A). The C54A SsuD kinetic trace at 370 nm did not exhibit an initial fast phase and was best fit to a double exponential equation with rate constants of $4.70 \pm 0.10$ ($k_1$) and $0.35 \pm 0.01$ ($k_2$) at 370 nm (○, Fig. 4B). Rate constants of $1.99 \pm 0.04$ ($k_1$) and $0.32 \pm 0.01$ ($k_2$) for C54A SsuD were obtained from the fit of the kinetic traces at 450 nm (●, Fig. 4B). These results suggest that the C4a-(hydro)peroxyflavin is only formed with the more conservative cysteine to serine substitution, while substitution of Cys to Ala led to decreased accumulation of the flavin intermediate.

### 3.5. Kinetic studies of flavin oxidation by SsuD variants in the presence of octanesulfonate

Rapid reaction kinetic analyses were also employed to determine if flavin oxidation by the Cys54 SsuD variants was affected by the addition of the octanesulfonate substrate relative to wild-type SsuD. The oxidation of flavin by the SsuD variants in the presence of octanesulfonate substrate was monitored by stopped-flow analyses at 370 and 450 nm by mixing free $FMNH_2$ (15 μM) with either C54S or C54A SsuD (45 μM) and octanesulfonate (0.05–2.0 mM) in air-saturated buffer. Three phases were previously observed in kinetic traces for wild-type SsuD at low octanesulfonate concentrations (≤100 μM) [5]. The initial fast phase at 370 nm was attributed to formation of the C4a-peroxyflavin (II in Scheme 1) or an octanesulfonate peroxyflavin

**Table 2**
Rate constants for the oxidation of reduced flavin by C54S, C54A, and wildtype SsuD in the absence of octanesulfonate.

| | 370 nm | | | 450 nm | |
|---|---|---|---|---|---|
| | $k_1$ ($s^{-1}$) | $k_2$ ($s^{-1}$) | $k_3$ ($s^{-1}$) | $k_1$ ($s^{-1}$) | $k_2$ ($s^{-1}$) |
| WT SsuD[a] | $12.9 \pm 0.3$ | $1.80 \pm 0.03$ | $0.08 \pm 0.01$ | $2.37 \pm 0.01$ | $0.10 \pm 0.01$ |
| C54S SsuD | $111 \pm 15$ | $24.1 \pm 0.8$ | $0.33 \pm 0.01$ | $9.76 \pm 0.19$ | $0.33 \pm 0.01$ |
| C54A SsuD | $4.70 \pm 0.10$ | $0.35 \pm 0.01$ | | $1.99 \pm 0.04$ | $0.32 \pm 0.01$ |

[a] Previously reported (5).

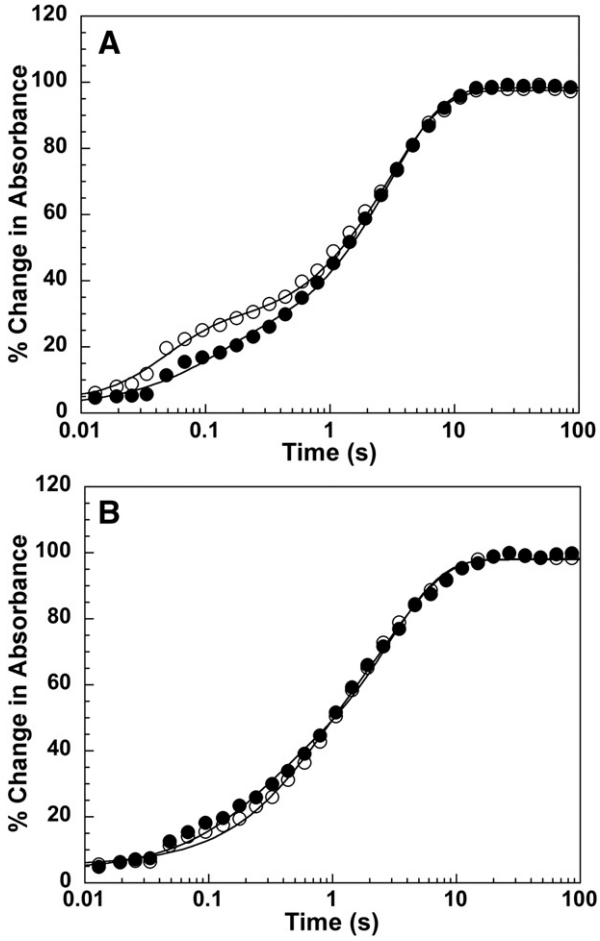


**Fig. 4.** Kinetics of flavin oxidation by C54S and C54A SsuD in the absence of octanesulfonate substrate. Stopped-flow kinetic experiments were performed with the C54S or C54A SsuD variant (45 μM) combined with $FMNH_2$ (15 μM) at 4 °C. A: $FMNH_2$ in a tonometer was mixed against C54S SsuD in air-saturated buffer. The kinetic traces shown are an average of three separate experiments following flavin oxidation at 370 (○) and 450 nm (●). The solid lines are fits of the kinetic traces to Eq. (3) or (4). B: $FMNH_2$ in a tonometer was mixed against C54A SsuD in air-saturated buffer. The kinetic traces shown are an average of three separate experiments following flavin oxidation at 370 (○) and 450 nm (●). The solid lines are the fits of the kinetic traces to Eq. (4) or (5).

intermediates (III or IV) [5]. At higher octanesulfonate concentrations there were only two phases observed that correspond to flavin oxidation, suggesting the C4a-(hydro)peroxyflavin does not accumulate to significant levels with wild-type SsuD. The initial fast phase at 370 nm was not observed for either the C54S or C54A SsuD variants even at low octanesulfonate concentrations, and all kinetic traces were best fit to a double exponential equation (Fig. 5A and B). With both SsuD variants, the $k_{obs}$ values for the first phase at 370 nm showed a hyperbolic dependence on substrate concentration, giving a $K_d$ and limiting rate constant of $160 \pm 20$ μM and $1.58 \pm 0.08$ $s^{-1}$ for C54S SsuD, and $190 \pm 40$ μM and $1.55 \pm 0.13$ $s^{-1}$ for C54A SsuD (Fig. 5A and B, insets). These results from the rapid reaction kinetic studies indicate that there were no substantial changes in the $K_d$ or $k_{obs}$ values compared to the values obtained for wild-type SsuD ($K_d$, 93 μM; $k_{obs}$, 2.4 $s^{-1}$). The absence of an initial fast phase suggests that the C4a-(hydro)peroxyflavin does not accumulate with the Cys54 SsuD variants in the presence of octanesulfonate.

## 4. Discussion

Even though they share low amino acid sequence identity, the alkanesulfonate monooxygenase from *E. coli* is similar in overall structure to bacterial luciferase and LadA [12–17]. The monomeric units of these enzymes adopt a TIM-barrel fold, and there are several conserved amino acid residues in SsuD with spatial counterparts in the active site of bacterial luciferase and LadA. The spatial correlation between conserved amino acid residues in these enzymes suggests that the active site of SsuD is located at the C-terminal end of the TIM-β-barrel [12]. While the function of these conserved residues in LadA have not been fully evaluated, the catalytic role of several of these amino acid residues has been identified for bacterial luciferase. The amino acid Cys106 in bacterial luciferase lies along a wall of a small cavity that projects off a larger pocket, and is proposed to be in close contact with the pyrimidine structure of the isoalloxazine ring of the flavin [13–15]. It was established that chemical modification of this active site cysteine results in the complete loss of enzymatic activity [26]. Substitution of the cysteine residue to alanine, valine, and serine resulted in an active luciferase enzyme with decreased bioluminescence. Although the rate of formation of the C4a-(hydro)peroxyflavin intermediate was not compromised in these variants, the rate of decay for the flavin intermediate was increased relative to wild-type [28]. The luciferase enzyme from *Vibrio fischeri* contains a valine residue at position 106 in the α subunit in a similar position as the cysteine in luciferase from *V. harveyi* based on sequence alignments, suggesting

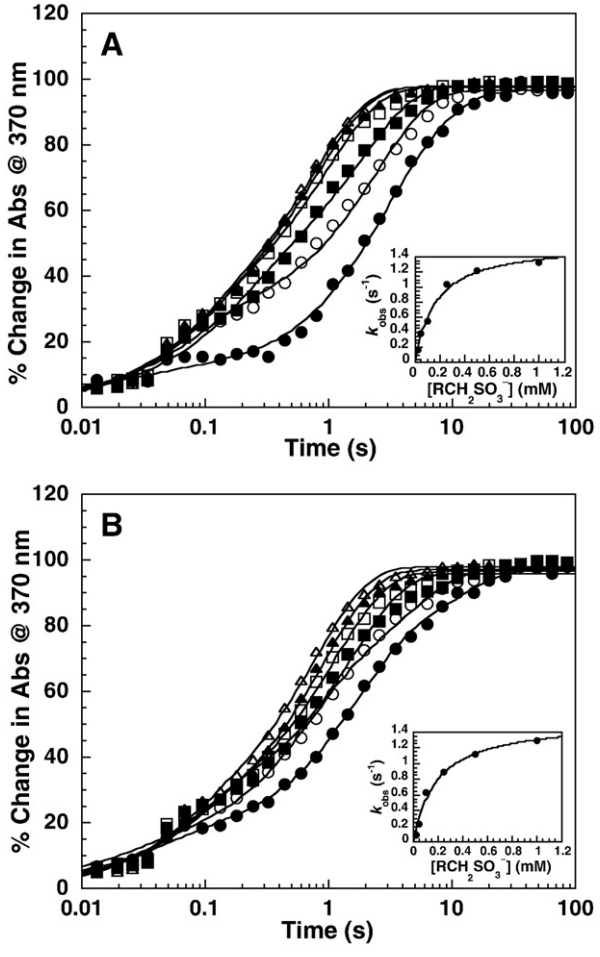


**Fig. 5.** Kinetics of flavin oxidation by C54S and C54A SsuD in the presence of octanesulfonate. Stopped-flow kinetic experiments were performed by mixing $FMNH_2$ (15 μM) in a tonometer against C54S or C54A SsuD (45 μM) in the presence of variable amounts of octanesulfonate (0.025 (●), 0.05 (○), 0.1 (■), 0.25 (□), 0.5 (▲), and 1.0 (△) mM) in oxygenated buffer. The kinetic traces shown represent an average of three separate experiments. A: Anaerobic $FMNH_2$ mixed against C54S SsuD with varying concentrations of octanesulfonate substrate. Inset: Dependence on the $k_{obs}$ at varying octanesulfonate concentration for the second phase at 370 nm. B: Anaerobic $FMNH_2$ mixed against C54A SsuD with varying concentrations of octanesulfonate substrate. Inset: Dependence on the $k_{obs}$ at varying octanesulfonate concentrations for the second phase at 370 nm. The solid lines are the fits of the kinetic traces to Eq. (6).

that a thiol is not directly essential for bioluminescence activity [27,28]. The increase in the decay of the C4a-(hydro)peroxyflavin intermediate suggested that the cysteine is involved in stabilizing this intermediate. While studies suggest the interaction between the thiol and flavin is not direct, it is essential in preserving the correct structural conformation for C4a-(hydro)peroxyflavin stabilization. A recent three-dimensional structure of bacterial luciferase with flavin bound shows Cys106 projecting toward the C-4a position on the isoalloxazine ring of the flavin moiety at a distance of 4.1 Å [16,37]. Substitution of the corresponding cysteine to alanine in LadA eliminated the catalytic activity of the enzyme.

Due to its role in sulfur assimilation, the SsuD enzyme contains a limited number of sulfur-containing amino acids. Therefore, the location of the single cysteine residue within the putative active site of SsuD suggests a possible role for Cys54 in catalysis. In addition, this residue is highly conserved in all putative SsuD enzymes from a diverse group of bacteria. Initial studies have shown that labeling of the conserved Cys54 residue in the active site of SsuD led to inactivation of the enzyme, but the role of this residue in catalysis was not evaluated [12]. Site-directed mutagenesis was used to substitute the Cys54 residue with serine and alanine. In steady-state kinetic assays, the substitution of Cys54 altered the kinetic parameters of SsuD. The SsuD C54S and C54A variants gave $k_{cat}$ values that were 2- and 9-fold lower than wild-type SsuD, respectively. However, the concentration of the C54A SsuD enzyme had to be increased 5-fold over the concentration of C54S and wild-type SsuD in order to detect any appreciable activity. The $K_m$ values with octanesulfonate were decreased 6- and 2-fold for the C54S and C54A SsuD variants, respectively. The changes in the steady-state kinetic parameters led to a 3-fold increase in the $k_{cat}/K_m$ value for C54S SsuD, while the $k_{cat}/K_m$ value for C54A SsuD decreased 6-fold. These results suggest that the less conservative cysteine to alanine substitution had a greater effect on the steady-state kinetic parameters, while the serine was able to adequately substitute for cysteine.

Comparable steady-state kinetic parameters for the C54S SsuD variant as wild-type indicated that Cys54 may be involved in substrate binding through hydrogen bonding and/or maintaining the structural integrity of the active site, as serine would also be able to provide similar interactions. However, the $K_d$ values determined for reduced or oxidized flavin binding to the C54A SsuD variant were similar to the values obtained for wild-type SsuD indicating that Cys54 was not directly involved in flavin binding. This is in contrast to the results from fluorescence titration experiments with bacterial luciferase, where mutation of the cysteine at position 106 to alanine, serine, or valine resulted in an increase in the $K_d$ value for $FMNH_2$ [27]. Previous kinetic evaluation of SsuD demonstrated that two conformational changes likely occur with the binding of reduced flavin and octanesulfonate. Therefore, optimal contacts by the cysteine residue may only be obtained with both substrates bound, and the affinity of the flavin for the Cys54 SsuD variants in the absence of octanesulfonate would likely be similar to wild-type SsuD.

The $K_d$ values for octanesulfonate binding were also determined with each of the SsuD variants by titrating $FMNH_2$-bound C54S or C54A SsuD with octanesulfonate. The $K_d$ value for both variants led to an increase in the binding affinity. It was previously demonstrated through kinetic studies that incubation of wild-type SsuD with reduced flavin in the absence of oxygen results in the conversion of the complex to an inactive form that must undergo an isomerization to the active form of the enzyme before catalysis can occur [5]. Because this conversion may only occur in the presence of dioxygen, octanesulfonate is likely binding to the inactive complex in the static anaerobic titration experiments. Titration of octanesulfonate into the inactive form of the $FMNH_2$-bound SsuD variants may lead to alternative interactions of the substrate and active site amino acid residues in the absence of dioxygen leading to a greater affinity of SsuD for octanesulfonate. However, these interactions may not be favorable for catalysis.

Evidence exists for the formation of the C4a-(hydro)peroxyflavin intermediate in the SsuD catalyzed reaction through rapid reaction kinetic studies [5]. The flavin intermediate was only detectable if SsuD and reduced flavin were not premixed in the tonometer, leading to conversion of the complex to the proposed inactive form. As illustrated in the initial kinetic investigations of SsuD, this species could be more specifically called a C4a-peroxyflavin intermediate ($FMN\text{-}OO^-$) resulting in a nucleophilic attack on the sulfonate group of the alkanesulfonate to form an alkanesulfonate peroxyflavin adduct. Kinetic studies were performed to determine if the C4a-(hydro) peroxyflavin intermediate was observed in stopped-flow reactions when reduced flavin was mixed with the Cys54 SsuD variants. Three phases were identified in the kinetic trace of C54S SsuD mixed against $FMNH_2$ at 370 nm. An initial fast phase ($k_1 = 111\ s^{-1}$) was observed that is attributed to the formation of the C4a-(hydro)peroxyflavin intermediate, and is 10-fold greater than the rate constant obtained with wild-type SsuD. As previously observed for wild-type, this fast phase was not present in the kinetic trace obtained at 450 nm. The rate constants obtained for the last two phases at 370 nm were approximately 13- and 4-fold greater for $k_2$ and $k_3$, respectively. These phases have previously been assigned to the decay of the C4a-(hydro)peroxyflavin. Therefore, although there was increased accumulation of the flavin intermediate, the rate of breakdown for this intermediate also increased. The kinetic traces obtained at 370 and 450 nm for the C54A SsuD variant were similar with no initial fast phase observed at 370 nm, suggesting that there was no accumulation of the proposed C4a-(hydro)peroxyflavin with this variant. It is uncertain whether the C4a-(hydro)peroxyflavin does not form with this C54A SsuD variant in the absence of substrate, or if the flavin intermediate does not accumulate to detectable levels in the absence of substrate. Stopped-flow analyses on essential thiol variants of bacterial luciferase, where the reactive cysteinyl residue at position 106 in the α subunit was replaced with alanine, serine, and valine, were also performed in the absence of the aldehyde substrate [28]. When mixing the luciferase variants and reduced flavin with oxygenated buffer, a C4a-(hydro)peroxyflavin intermediate was observed in all three mutants with rate constants equal to that of wild-type luciferase [28].

Rapid reaction kinetic experiments were also performed for each Cys54 SsuD variant in the presence of octanesulfonate. Similar kinetic experiments with wild-type SsuD showed an initial fast phase attributed to the formation of the C4a-(hydro)peroxyflavin only at low octanesulfonate concentrations ($\leq$100 μM). The reaction was thought to occur rapidly in the presence of increasing concentrations of the octanesulfonate substrate so that no appreciable levels of C4a-(hydro)peroxyflavin intermediate accumulated. With both SsuD variants there was no initial fast phase at 370 nm representing the formation of the C4a-(hydro)peroxyflavin even at low octanesulfonate concentrations, and the kinetic traces were best fit to a double exponential equation. At concentrations of octanesulfonate greater than 100 μM, the first phase representing flavin oxidation at 370 nm displayed a hyperbolic dependence on octanesulfonate concentration [5]. The $k_{obs}$ and $K_d$ values for the Cys54 SsuD variants were similar to the values obtained with wild-type SsuD. There was an alteration in the $K_d$ values obtained using this method compared to static fluorescent titration experiments with octanesulfonate due to inherent differences in the two experimental procedures. In the titration experiments the octanesulfonate substrate is likely binding to the inactive form of the SsuD–$FMNH_2$ complex, and the values obtained may not be relevant to the catalytic conditions. The increase in $K_d$ values for the variants does not seriously impact catalysis, as the $k_{cat}/K_m$ value for C54S SsuD was actually 3-fold higher than wild-type. Although there was no initial fast phase at 370 nm representing formation of the C4a-(hydro)peroxyflavin with either cysteine variant, both variants possessed activity. Therefore, the flavin intermediate must be generated in the reaction. The $k_{cat}/K_m$ value

increased with the C54S SsuD variant, and an increased rate of formation and decay of the C4a-(hydro)peroxyflavin was observed in the absence of octanesulfonate. Conversely, the $k_{cat}/K_m$ value decreased with the C54A SsuD variant, and there was no initial rate representing C4a-(hydro)peroxyflavin formation in the absence of octanesulfonate. These results suggest that the decreased accumulation of the C4a-(hydro)peroxyflavin intermediate at low octanesulfonate concentrations with C54S SsuD may be due to increased reactivity of the flavin intermediate; while the decreased accumulation with C54A SsuD is likely due to destabilization of the active site environment.

The results from rapid reaction kinetic studies with the Cys54 SsuD variants support the importance of hydrogen bonding interactions in the stabilization of the C4a-(hydro)peroxyflavin intermediate either through direct interactions or by preserving the structural integrity of the active site. The cysteine thiol and not the thiolate is likely involved in stabilizing this intermediate, as the serine residue can compensate for cysteine in catalysis. The less conservative C54A SsuD variant would be unable to stabilize the flavin intermediate and would likely lead to alterations in the local environment of the C4a-(hydro) peroxyflavin. The p$K_a$ of Cys54 was $9.3 \pm 0.1$, suggesting that this residue would likely be protonated during catalysis. While the p$K_a$ of Cys54 may vary during catalysis due to localized structural changes that occur in the active site once the substrates are bound, the p$K_a$ value represents a reasonable approximation of the protonation state of Cys54. Putative SsuD enzymes from some bacterial organisms contain a serine or threonine in place of the conserved cysteine residue further supporting the role of this residue in hydrogen bonding interactions.

While the three-dimensional structure of proteins associated with the luciferase family has been determined there are no structures available with reduced flavin bound, making it difficult to ascertain the location of the conserved cysteine residue relative to the flavin. The three-dimensional structures of LadA and bacterial luciferase have been determined with oxidized flavin [16,17]. However, there is currently no structural information available for SsuD with substrates bound. For LadA it was reported that there were no major conformational changes between the flavin bound and unbound structures [17]. Results from kinetic studies of the bacterial luciferase reaction suggest that there is an isomerization step that occurs following reduced flavin binding, which correlates with recent structural observations [8,16]. Experimental evidence suggests that conformational changes may occur with the binding of reduced flavin in bacterial luciferase and SsuD that lead to perturbations in the flavin environment, so flavin modeling and structural determination with oxidized flavin may not accurately define these conformational changes [15,16]. Despite these concerns, modeling of bacterial luciferase with flavin places Cys106 close to the pyridine portion of the isoalloxazine ring [15]. The recent structure of bacterial luciferase with flavin bound also supports these observations [16]. Our analyses suggest that the Cys14 residue from LadA is in a similar orientation as the conserved cysteine residues in bacterial luciferase and SsuD. Using the oxidized flavin coordinates of LadA we were able to determine that Cys54 would also be in close proximity to the pyrimidine of the isoalloxazine ring. These combined results support the role of Cys54 in the stabilization of the C4a-(hydro)peroxyflavin either through direct interactions with the flavin or in helping to maintain the active site environment. Obtaining a three-dimensional structure of SsuD with substrates bound will be essential in evaluating the active site flavin environment.

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