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Mechanism for sulfur acquisition by the alkanesulfonate monooxygenase system

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ABSTRACT

The bacterial alkanesulfonate monooxygenase system is involved in the acquisition of sulfur from organosulfonated compounds during limiting sulfur conditions. The reaction relies on an FMN reductase to supply reduced flavin to the monooxygenase enzyme. The reaction catalyzed by the alkanesulfonate monooxygenase enzyme involves the carbon–sulfur bond cleavage of a wide range of organosulfonated compounds. A C4a-(hydro)peroxyflavin is the oxygenating intermediate in the mechanism of desulfonation by the alkanesulfonate monooxygenase. This review discusses the physiological importance of this system, and the individual kinetic parameters and mechanistic properties of this enzyme system.

1. Introduction

The flavin-dependent monooxygenase enzymes catalyze diverse reactions critical to biological processes. These enzymes couple the reactivity of the reduced flavin with the activation of molecular oxygen to generate the C4a-(hydro)peroxyflavin oxygenating intermediate. The catalytic versatility of the C4a-(hydro)peroxyflavin is dependent on the active site environment stabilizing the reactive form of the flavin [1–3]. Depending on the reaction catalyzed, the oxygenating flavin intermediate can act as a nucleophile or electrophile in catalysis [4–8]. For nucleophilic reactions the oxidizing flavin intermediate is the C4a-peroxyflavin (Fl-OO^-), while electrophilic reactions are catalyzed by a C4a-hydroperoxyflavin (Fl-OOH) intermediate.

While many flavin monooxygenase enzymes have a flavin prosthetic group, a group of flavin-dependent monooxygenases has been identified that utilize flavin as a substrate rather than a bound prosthetic group. For these enzymes the flavin is reduced by a flavin reductase, and the reduced flavin is subsequently transferred to the monooxygenase enzyme. Details on the elegant mechanistic strategies these enzymes utilize to stabilize the C4a-hydroperoxyflavin and catalyze the oxidation of substrates have been determined for many of these two-component monooxygenase systems. An FMN-dependent two-component monooxygenase system that catalyzes the desulfonation of a broad range of sulfonate substrates generating free sulfite and the corresponding aldehyde has been identified in a number of bacteria [9–13]. The alkanesulfonate monooxygenase (SsuD) is dependent on an FMN reductase (SsuE) to supply reduced flavin for the desulfonation of sulfonated compounds (Fig. 1). The cleavage of a carbon–sulfur bond is a mechanistic challenge for flavoproteins; therefore, the desulfonation reaction catalyzed by the alkanesulfonate monooxygenase system represents a novel catalytic feat for the acquisition of sulfur in bacterial organisms.

2. Sulfur limitation in bacterial systems

Sulfur is essential for all organisms as a component of amino acids and enzyme cofactors. In many bacterial organisms sulfur is acquired through the sulfate assimilation pathway leading to the production of sulfide that is then incorporated into sulfur-containing organic molecules [14,15]. Bacteria grown in the laboratory typically are supplied with inorganic sulfate in the growth media, and sulfur limitation is not observed. Inorganic sulfate is not prevalent in nature; therefore, bacteria must have some way to acquire sulfur from the environment under sulfur-limiting conditions. Sulfonates and sulfonate esters are found as naturally occurring products or xenobiotic compounds [14]. When certain bacteria are under sulfur-limiting conditions, a set of proteins is expressed to allow these bacteria to utilize organosulfonates or sulfonate esters as sulfur sources [9,13,16,17]. The sulfate starvation-induced proteins identified play a role in sulfonate uptake, sulfur acquisition from organic compounds, and protection against reactive oxygen species. The limitation of sulfur may lead to a change in the overall redox balance in the cell, as many of the enzymes that maintain the redox properties of the cellular

Abbreviations: FMN, flavin mononucleotide; $FMNH_2$, reduced flavin mononucleotide; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); SsuE, alkanesulfonate flavin reductase; SsuD, alkanesulfonate monooxygenase; TauD, taurine dioxygenase; LadA, long-chain alkane monooxygenase.

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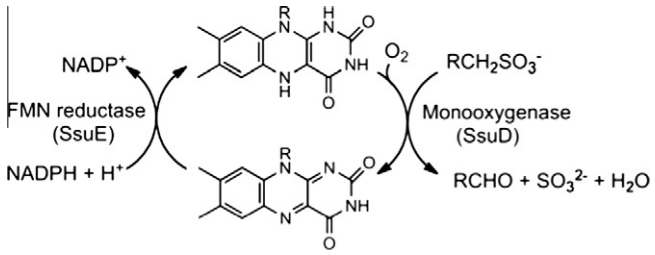

Fig. 1. The mechanism of desulfonation by the alkanesulfonate monooxygenase system. The FMN reductase (SsuE) provides reduced flavin to the monooxygenase enzyme (SsuD). The monooxygenase enzyme catalyzes the carbon–sulfur bond cleavage of a broad range of organosulfonated compounds.

environment utilize redox-active cysteines. Two operons, *tau* and *ssu*, are expressed in specific bacteria during sulfur limitation that allows the organism to utilize aliphatic sulfonates as a sulfur source [10,16]. The *tau* operon encodes for taurine dioxygenase (TauD) that catalyzes the oxygenation of taurine to a hydroxytaurine intermediate, and an ABC-type transporter involved in the cellular uptake of taurine [18]. The hydroxytaurine is unstable and breaks down to yield the aminoacetaldehyde and sulfite product. The *ssu* operon encodes for SsuE and SsuD in addition to an ABC-type transporter for organic sulfonate transport [10]. The TauD enzyme prefers taurine as a substrate, but can catalyze the desulfonation of some alkanesulfonate substrates [18]. Conversely, the alkanesulfonate monooxygenase enzymes can utilize a broad range of organosulfonate substrates, but there was no catalytic activity observed with taurine [18]. The diverse substrate range catalyzed by the alkanesulfonate monooxygenase system includes C-2 to C-10 alkanesulfonates, sulfonated buffers, 1,3-dioxo-2-isoindolineethanesulfonate, and 2-(4-pyridyl)ethanesulfonate [11]. These TauD and SsuE/D enzymes work in parallel to ensure that the bacterial cell has ample sulfur for biosynthetic processes. While the mechanism of TauD has been extensively characterized, the mechanistic and structural properties of SsuE and SsuD have only recently been evaluated [19–26].

3. The FMN reductase of alkanesulfonate monooxygenase

The reductase enzymes associated with two-component systems all catalyze the reduction of flavin with reducing equivalents provided by a pyridine nucleotide. The reductase enzymes typically have a specificity for either FMN or FAD, and that specificity is also observed in the monooxygenase enzyme. The reductases that have been characterized either have a flavin tightly bound with a signature spectrum for oxidized flavin, or the flavin is a substrate during catalysis. The SsuE enzyme was shown to utilize flavin as a substrate, and there was no flavin bound to the enzyme when purified [11,23]. Gel filtration and analytical ultracentrifugation experiments indicated SsuE exists as a dimer in solution. The steady-state kinetic parameters for SsuE with varying flavin concentrations gave a higher k_{cat}/K_m value with FMN than FAD, indicating a preference for FMN [11]. Interestingly, SsuE also has a 1000-fold higher affinity for the oxidized FMN substrate over the reduced product [23,25]. These results indicate that SsuE has a clear preference not only for FMN, but also for the oxidized form of the flavin.

The flavin reductases show different kinetic mechanisms for the binding and subsequent release of the flavin and pyridine nucleotide. Several flavin reductases that have a tightly bound flavin, show a ping pong mechanism for flavin reduction (Fig. 2A) [27,28]. For these enzymes the bound flavin cofactor is first reduced by a pyridine nucleotide. Following reduction, the pyridine nucleotide is released and the second flavin binds and is reduced by the bound flavin. For flavin reductases without a bound flavin, the kinetic mechanism follows an ordered sequential mechanism with either the pyridine nucleotide or flavin binding first to the enzyme (Fig. 2B) [11,28,29]. A ternary complex must be formed before any chemistry can occur with an ordered sequential mechanism. Following flavin reduction either the reduced flavin or oxidized $NADP^+$ is released first followed by the other product. In single-enzyme kinetic assays, SsuE followed an ordered sequential mechanism, with NADPH as the first substrate to bind and $NADP^+$ as the last product to dissociate [23].

There are several reports of observed changes in the kinetic parameters and mechanism for two-component monooxygenase enzymes [28,30–32]. The steady-state kinetic parameters of SsuE were not altered with varying ratios of SsuD included in the reaction [23]. However, the SsuE kinetic mechanism was changed to an equilibrium ordered mechanism in the presence of SsuD and saturating levels of octanesulfonate suggesting the NADPH substrate and $NADP^+$ product are in equilibrium with free enzyme. Therefore, the rate of the reaction for flavin reduction by SsuE was independent of NADPH, and the equilibrium was displaced toward the ternary complex. In addition, there was a 10-fold increase in the K_m value (0.13 ± 0.01 μM) for FMN with SsuD and octanesulfonate included in the reaction [23]. These observed changes were associated with a decrease in the binding affinity of the oxidized flavin. The K_m value (0.016 ± 0.002 μM) for flavin binding to SsuE in the single-enzyme assays was nearly equal to the K_d value (0.015 ± .004 μM), suggesting the K_m value for FMN by SsuE essentially represents the dissociation constant. The increase in the K_m value for SsuE in the presence of SsuD and the octanesulfonate substrate may be attributed to a decrease in the binding affinity for oxidized flavin. Because flavin transfer between proteins is an essential step in catalysis, a lower affinity for oxidized or reduced FMN by SsuE would likely increase the rate of reduced flavin transfer to SsuD. In addition, a change to a rapid equilibrium ordered mechanism displaces the reaction towards the ternary complex and subsequent flavin transfer. Thus, the change in mechanism and observed increase in the K_m value for the oxidized flavin by SsuE in the presence of SsuD and the alkanesulfonate substrate ensures that the critical flavin transfer step in the pathway is preserved.

The reduction of flavins by pyridine nucleotides often leads to the formation of a charge-transfer complex between the flavin

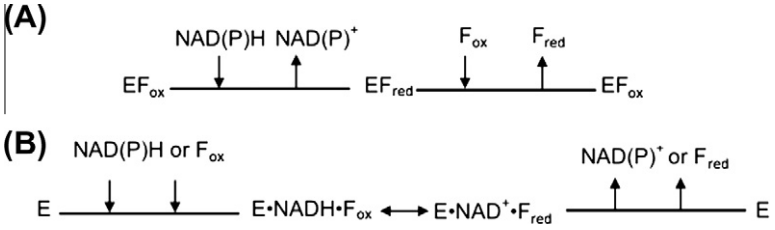

Fig. 2. Mechanisms of flavin reduction by the flavin dependent oxidoreductases. A. Mechanism for flavin reduction with flavin-bound reductases. B. Mechanism for flavin reduction by reductases without a flavin bound. F represents the flavin substrate.

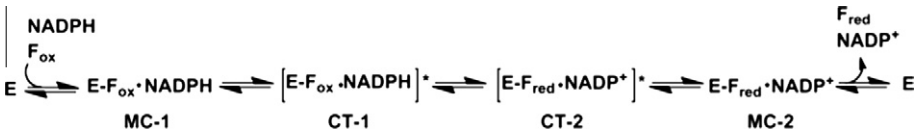

Fig. 3. Kinetic mechanism for flavin reduction by SsuE. The formation of the ternary complex (MC-1) leads to the generation of two charge-transfer complexes (CT-1 and CT-2) between the different redox forms of the pyridine nucleotide and the flavin. The CT-2 complex is converted to the ternary complex with products bound (MC-2). F represents the flavin substrate [24].

and pyridine nucleotide identified as a broad absorbance band between 550 and 800 nm [33–36]. This long-wavelength absorption increase was also observed in enzymes that utilize flavin as a substrate [37]. There were three distinct phases identified in rapid reaction kinetic analyses monitoring flavin reduction at 450 and 550 nm by SsuE that were associated with specific steps in flavin reduction [24]. The NADPH and FMN substrates bind to SsuE to form the ternary complex (MC-1). Following substrate binding, the first phase (k_1, 241 s^{-1}) corresponds to the formation of the initial charge-transfer complex between NADPH with FMN (CT-1)(Fig. 3). The second phase (k_2, 11 s^{-1}) is the step leading to the second charge-transfer complex between reduced $FMNH_2$ with $NADP^+$ (CT-2). The conversion of CT-1 to CT-2 represents the hydride transfer step from the pyridine nucleotide to FMN. The decay of CT-2 to the Michaelis complex of SsuE with bound products (MC-2) represents the final step in flavin reduction (k_3, 19 s^{-1}). Results from isotope studies with [4(R)-^{2}H]NADPH revealed that hydride transfer from NADPH to FMN was the rate-limiting step in flavin reduction [24]. The hydride transfer step was also inhibited at high flavin concentrations, further implicating this step as rate-limiting. Although a charge-transfer complex was observed during flavin reduction by SsuE, the complex generated was relatively weak compared to flavin reductases with a tightly bound flavin. This may be caused by loose flavin binding to the active site of flavin reductases. Given the role of these enzymes in flavin reduction and transfer, a mechanism involving a tightly bound flavin would not be as effective in promoting flavin transfer.

4. The alkanesulfonate monooxygenase enzyme

4.1. Structural properties of alkanesulfonate monooxygenase

The SsuD monooxygenase enzyme catalyzes the desulfonation of a broad range of organosulfonated products to generate the corresponding aldehyde and sulfonate [11]. The desulfonation reaction is catalyzed through the formation of a C4a-(hydro)peroxyflavin intermediate generated by the reaction of dioxygen with reduced flavin provided by SsuE [25]. The location of these enzymes on the same operon during sulfur limitation ensures that a flavin reductase is available to supply reduced flavin for the monooxygenase reaction. Although, the three-dimensional structure of SsuE has not been reported, the three-dimensional structure of SsuD was determined in the absence of substrates [22]. The overall structure of SsuD is similar to the flavin-dependent monooxygenases bacterial luciferase and LadA [22,38–40]. All members of this family form a triosephosphate isomerase (TIM)-barrel fold, with the active site located at the C-terminal end of the β barrel (Fig. 4)[22,38–40]. These proteins vary from canonical TIM-barrel structures by the presence of several insertion regions. One insertion region present in SsuD contains a loop that was largely unresolved in the three-dimensional structure, suggesting conformational mobility in this region (Fig. 4) [22]. This disordered region lies near the putative active site of SsuD, and is highly conserved in all SsuD homologs. It is postulated that the disordered loop in SsuD closes over the active site following the binding of substrate(s), and may be responsible for the conformational changes observed in kinetic studies [25]. In several TIM-barrel proteins, these flexible loops contribute to the structure of the enzyme active site or play a functional role both in the binding of substrates and in enzyme catalysis [41–43]. The closing of the loop over the active site protects the substrate and catalytic intermediates from bulk solvent [44–50]. While a portion of this loop region was unstructured in initial crystal structures of bacterial luciferase in the absence of substrates, a structuring of the loop in the α subunit was observed in a recent three-dimensional structure of bacterial luciferase with bound oxidized FMN [51]. Although the structure of SsuD was determined in the absence of substrates, a similar structuring of the unresolved loop region may also occur with the binding of substrates. This lid-gating mechanism would be catalytically important for the two-component monooxygenase enzymes to sequester reduced flavin, and protect flavin intermediates generated during catalysis.

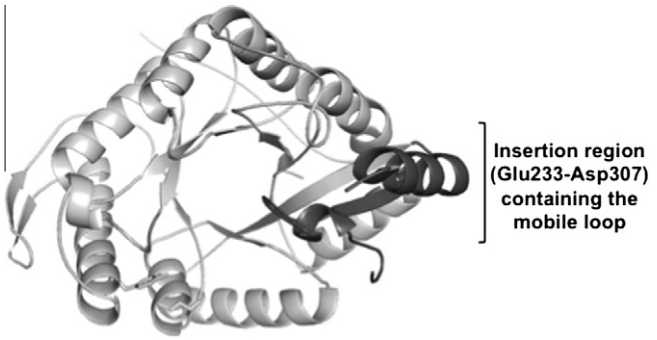

Fig. 4. Structure of the monomeric unit of SsuD. The SsuD enzyme exists as a TIM-barrel structure with four insertion sequences. The insertion sequence highlighted in dark gray represents the proposed lid region. Amino acid residues 250–282 of this insertion region were poorly defined. The structure was generated and rendered with PyMOL (PDB ID: 1M41) [22].

While loop closure may assist in protecting the reduced flavin from bulk solvent, there are other mechanistic properties of the enzyme that ensure the flavin is effectively transferred to SsuD. The preference of the flavin reductase and monooxygenase for different redox forms of the flavin appears to play an essential role in flavin transfer between these two enzymes. The SsuD enzyme had a 40-fold lower K_d value for reduced flavin compared to SsuE, while SsuE showed a 600-fold lower K_d value for oxidized flavin compared to SsuD [25]. This ensures that once SsuE reduces the flavin, the reduced flavin is immediately released and bound by SsuD. This inherent feature of the alkanesulfonate monooxygenase system prevents uncoupling of the desulfonation reaction due to nonenzymatic oxidation of the reduced flavin, and is an inherent mechanistic property in several two-component monooxygenase systems.

4.2. Catalytic features of desulfonation

The flavin monooxygenases utilize a C4a-(hydro)peroxyflavin to catalyze a diverse range of reactions. The monooxygenase enzymes can catalyze the oxidation of substrates through electrophilic or

nucleophilic substitution reactions. Nucleophilic substitution reactions as observed with cyclohexanone monooxygenase and bacterial luciferase involve a C4a-peroxyflavin (Fl-OO^-) intermediate, while electrophilic substitution reactions as observed with the aromatic hydroxylases utilize a C4a-hydroperoxyflavin (Fl-OOH) [4–8]. The C4a-(hydro)peroxyflavins have a signature absorbance depending on the protonation state of the intermediate that is distinct from other flavin forms [7,52,53]. The type of flavin peroxide utilized in the oxidation mechanism is highly dependent on the local environment stabilizing the protonated or deprotonated intermediate. Evidence for the formation of C4a-(hydro)peroxyflavin by SsuD was provided through rapid reaction kinetic analyses [25]. When SsuD was mixed with reduced flavin in stopped-flow kinetic studies the kinetic trace gave three phases at 370 nm, while only two phases were observed in kinetic traces obtained at 450 nm. The C4a-(hydro)peroxyflavin intermediate was detected at 370 nm at low octanesulfonate concentrations ($\leqslant$100 μM) when $FMNH_2$ was mixed with SsuD and octanesulfonate in oxygenated buffer, and three distinct phases were observed (Fig. 5) [25]. The initial phase (k_1) at 370 nm following mixing of $FMNH_2$ with SsuD and octanesulfonate was assigned to a flavin adduct that forms prior to regeneration of FMN, and was attributed to the C4a-(hydro)peroxyflavin oxygenating intermediate. The rates obtained for the last two phases were assigned to the decay of the C4a-(hydro)peroxyflavin intermediate back to the oxidized form (k_2 and k_3). This oxygenated flavin intermediate was not observed at higher octanesulfonate concentrations. At increasing octanesulfonate concentrations the reaction of octanesulfonate with the C4a-(hydro)peroxyflavin would be rapid and the flavin peroxide would not accumulate to detectable levels. A phase attributed to the decay of the C4a-hydroperoxyflavin (k_2 at 370 nm) showed a hyperbolic dependence on the octanesulfonate concentration, suggesting the binding of octanesulfonate occurs in two steps [25]. The octanesulfonate binds in rapid equilibrium to SsuD followed by an isomerization step or direct carbon-sulfur bond cleavage. The chemical step could involve the formation of the C4a-(hydro)peroxyflavin intermediate or carbon-sulfur bond cleavage if O_2 reacts with reduced flavin prior to octanesulfonate binding.

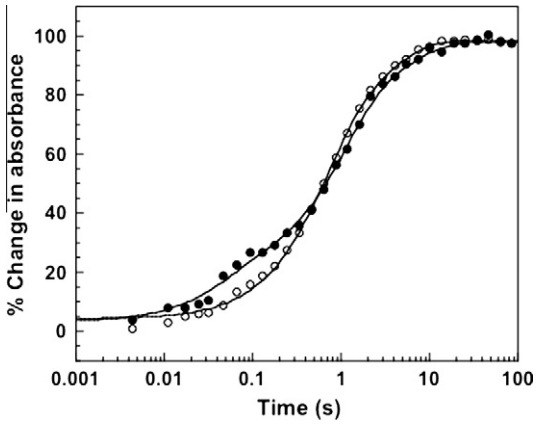

Fig. 5. The kinetics of flavin oxidation by SsuD in the presence of octanesulfonate. Experiments were performed by stopped-flow kinetic analyses at 4 °C. Free $FMNH_2$ solution was mixed with SsuD and octanesulfonate in air-equilibrated buffer. Kinetic traces of flavin oxidation were monitored at 370 (●) and 450 nm (○) [25].

Results from spectrofluorimetric titration experiments demonstrated that the octanesulfonate was unable to bind to SsuD unless reduced flavin was bound first [25]. Therefore, the mechanism of SsuD is dependent on the reduced flavin binding prior to the octanesulfonate. These results suggest an $FMNH_2$-induced conformational change is essential to allow octanesulfonate to bind. The addition of substrates to SsuD likely occurs through a partial ordered process initiated by the binding of reduced flavin. An ordered binding kinetic mechanism has been identified for several two-component monooxygenase enzymes [54–56]. The reduced flavin is the first substrate to bind, and the subsequent addition of substrates varies depending on the enzyme being evaluated. While a mechanism involving loop closure over the active site has been proposed for SsuD, the conformational change induced by reduced flavin binding most likely does not involve complete loop closure over the active site as this would prevent octanesulfonate from binding to SsuD. Therefore, a second conformational change has been proposed to occur with the binding of octanesulfonate triggering loop closure over the active site. Evidence to support this second conformational change was obtained by rapid reaction kinetic analyses with alternate mixing of substrates monitored at 450 nm [25]. There was a hyperbolic dependence on k_{obs} with increasing octanesulfonate concentrations with each alternate mixing condition. The anaerobic SsuD-$FMNH_2$-octanesulfonate ternary complex mixed with oxygenated buffer showed a two-fold faster rate for the first phase of flavin oxidation (k_1) than the reaction with SsuD-$FMNH_2$ mixed with octanesulfonate and oxygenated buffer. As a result, the decay of the reduced flavin occurred at a faster rate if octanesulfonate was premixed with SsuD and $FMNH_2$. In addition, the faster rate for the decay of the flavin intermediate was directly correlated with an increase in product formation. The K_d value for octanesulfonate was 3-fold lower when SsuD, $FMNH_2$, and octanesulfonate were premixed, indicative of an isomerization step following octanesulfonate binding [25]. The octanesulfonate would be in equilibrium in the ternary complex prior to mixing with oxygenated buffer, and an isomerization step would be masked leading to the overall effect of an increase in rate and octanesulfonate binding affinity. Based on results from kinetic studies, a partial model demonstrating substrate binding was determined for SsuD (Fig. 6) [25]. Either octanesulfonate or dioxygen binds following the initial binding of reduced flavin to SsuD to form the SsuD-$FMNH_2$ complex. However, the faster rate and lower

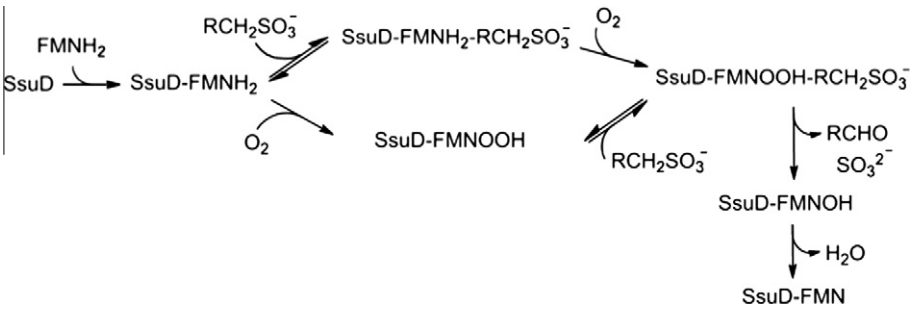

Fig. 6. Sequential substrate binding mechanism for SsuD. Binding of reduced flavin induces a conformation change that allows the octanesulfonate to bind. While either dioxygen or octanesulfonate could bind following flavin binding, kinetic evidence supports the upper pathway with octanesulfonate binding prior to dioxygen [25].

K_d value for octanesulfonate binding observed with premixed SsuD, octanesulfonate, and $FMNH_2$ suggests that octanesulfonate binds prior to oxygen. The binding of oxygen to the preformed SsuD-FMN-octanesulfonate complex would prevent unproductive oxidation of the reduced flavin.

4.3. Proposed mechanisms of desulfonation

The structural similarity of SsuD to bacterial luciferase suggests that the C4a-peroxyflavin (Fl-OO^-) is likely the oxygenating flavin intermediate. However the stability of the flavin intermediate in the SsuD reaction is not comparable to bacterial luciferase. In bacterial luciferase, this intermediate is so stable in the absence of the aldehyde substrate that it was isolated at 4 ℃ [52,53]. Recent structural studies suggest that a hydrophobic cavity lies in front of C4a on the isoalloxazine ring in monooxygenase enzymes, which is essential in stabilizing the C4a-(hydro)peroxyflavin [1,2,40,51,57]. This packed hydrophobic cavity is important in creating a solvent-free environment that prevents rapid breakdown of the unstable flavin intermediate. The inability of SsuD to adequately stabilize the C4a-(hydro)peroxyflavin intermediate may be due to the increased electrostatic environment of the active site. The organosulfonated substrates would require a more electrostatic active site to stabilize the sulfonate functional group. Therefore, with SsuD there is a delicate balance between stability of the sulfonated substrate and stabilization of the C4a-(hydro)peroxyflavin intermediate.

Based on kinetic results of SsuD and studies of other flavin monooxygenases two possible mechanisms can be envisioned for the SsuD enzyme. Because the nature of the flavin peroxide has not been identified, catalysis by SsuD could occur through a C4a-hydroperoxyflavin (Fl-OOH) or C4a-peroxyflavin (Fl-OO^-) intermediate. In the first mechanism, reduced flavin reacts with dioxygen to form a C4a-hydroperoxyflavin intermediate, and an active site base abstracts a proton from the C1 carbon of the alkanesulfonate to generate a carbanion intermediate (Fig. 7A-II and III). The carbanion intermediate makes a nucleophilic attack on the hydroperoxyflavin to generate an unstable 1-hydroxyalkanesulfonate that decomposes to the corresponding aldehyde and sulfite (Fig. 7A-IV and V). The 1-hydroxyalkanesulfonate intermediate would be comparable to the hydroxytaurine generated in the oxidation of taurine by TauD [18]. In the second mechanism, reduced flavin forms a peroxyflavin intermediate (Fig. 7B-II). Attack of the peroxyflavin on the sulfur atom of the organosulfonate would generate a peroxyflavin-organosulfonate adduct (Fig. 7B-II and III). A Baeyer–Villiger rearrangement of the flavin adduct would release the sulfite product and generate a peroxyalkane intermediate. (Fig. 7B-IV) Hydrogen abstraction from C1 of the alkane by an active-site base would lead to heterolytic cleavage of the oxygen-oxygen bond of the alkane-flavin adduct to form the aldehyde product and the C4a-hydroxyflavin (Fig. 7B-IV and V). Each of the proposed mechanisms involves the activation of dioxygen by reduced flavin to form the oxygenating C4a-(hydro)peroxyflavin. The difference is whether the distal oxygen is protonated, which is highly dependent on the active-site environment.

4.4. Catalytically relevant amino acids

Although there is no three-dimensional structure available for SsuD with substrates bound, the putative active site of the enzyme is likely located at the C-terminal end of the β-barrel as is typically

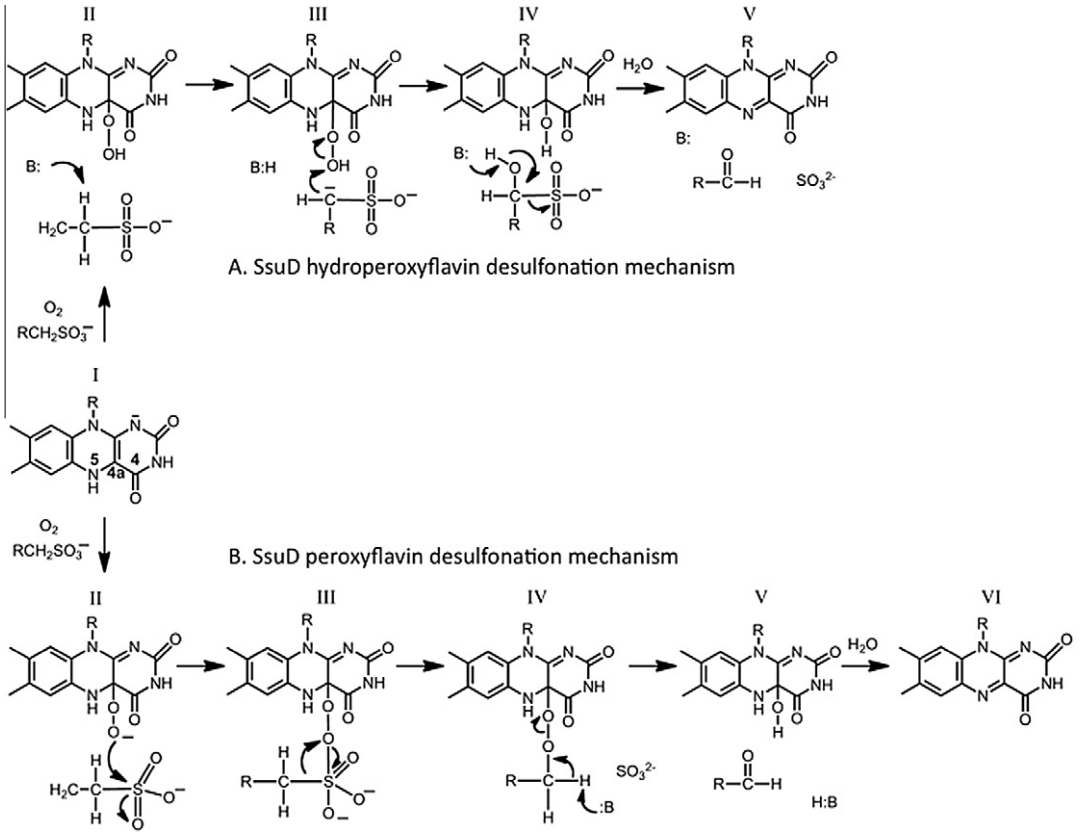

Fig. 7. Proposed mechanisms for desulfonation by SsuD. A. The upper pathway represents the mechanism of desulfonation by SsuD involving a C4a-hydroperoxyflavin intermediate. B. The lower pathway represents the mechanism of desulfonation by SsuD involving a C4a-peroxyflavin. B and BH represent active site amino acids that act as general bases or acids in catalysis.

observed for TIM-barrel structures [22]. Several conserved amino acids located in the active site of SsuD are in a similar arrangement as catalytically relevant amino acids from bacterial luciferase and LadA (Fig. 8) [22,38,39,51]. There is a sole cysteine (Cys54) located in the putative active site of SsuD that is in a similar spatial position to Cys106 in bacterial luciferase [26]. This Cys54 residue was shown to play a role in stabilizing the C4a-(hydro)peroxyflavin intermediate, which is similar to the catalytic role of Cys106 of bacterial luciferase [58]. The Cys106 residue is within hydrogen-bonding distance to the pyrimidine ring of the flavin in the three-dimensional structure of bacterial luciferase [51]. These results suggest that the conserved residues among the flavin-dependent monooxygenases are not only structurally related but also have similar catalytic functions. A histidine residue (His 44) in the α-subunit of bacterial luciferase was shown to be a catalytic base in the bioluminescence reaction [59]. Mutation of His44 to alanine showed a significant decrease in the intensity of light emitted. It was found that the enzymatic activity could be rescued with the addition of imidazole to the reaction at increasing pH [59]. The three-dimensional structure of LadA places His311 in a similar position as His44 in bacterial luciferase. Substitution of His311 with phenylalanine led to a loss in the catalytic activity of LadA, suggesting a similar role for this residue as an active site base [40]. Both putative mechanisms for SsuD involve a catalytic base in the reaction mechanism. Based on the similar spatial arrangement of this residue with bacterial luciferase, His228 of SsuD was proposed to be the putative active site base directly involved in catalysis. Substitutions of His228 to alanine, aspartate, or lysine were generated to evaluate the effects of these variants on catalysis. The H228A and H228 K SsuD variants showed a 50-fold decrease in the k_{cat}/K_m values compared to wild-type, while a 200-fold decrease k_{cat}/K_m values was observed with the H229D SsuD variant. A more substantial decrease in the steady-state kinetic parameters was expected for the substitution of an active site base. The His228 SsuD variants were also able to bind $FMNH_2$ and octanesulfonate with similar affinities as wild-type SsuD. Rapid reaction kinetic analyses of the H228A SsuD variant gave comparable rate constants as wild type SsuD indicating that this amino acid was not involved in a step involving C4a-(hydro)peroxyflavin formation or subsequent chemical steps involving carbon-sulfur bond cleavage. The larger decrease in the k_{cat}/K_m values for the H229D SsuD variant suggests that His228 may be involved in the stabilization and proper orientation of an amino acid residue important in catalysis. There are two conserved histidines (His11 and His333) and an arginine (Arg226) located within the active site of SsuD [22]. The proposed flavin binding site was approximated based on the superposition of the flavin-bound LadA structure with SsuD. This SsuD structural model places the proposed active site amino acid residues in close proximity to the flavin isoalloxazine ring (Fig. 8).

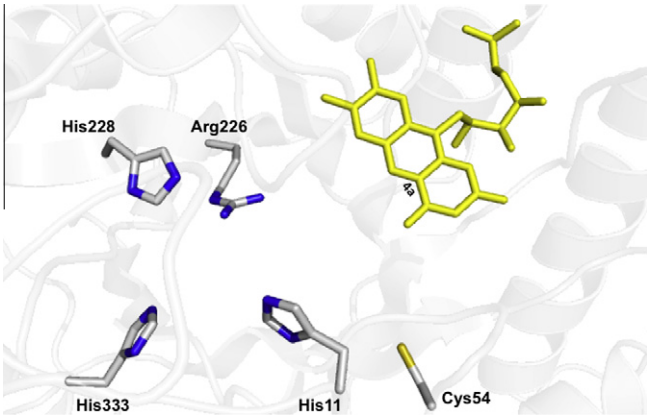

Fig. 8. Putative active site of SsuD. The highlighted amino acids are conserved residues in SsuD. The flavin was modeled into SsuD using the coordinates for LadA. The active site was generated and rendered with PyMOL (PDB ID: 1M41 and 3B90) [22,40].

5. Conclusions

The alkanesulfonate monooxygenase system is found in a large number of bacterial organisms for the acquisition of sulfur during sulfur limitation. In most bacterial organisms, this system relies on two enzymes to catalyze the desulfonation of a diverse group of organosulfonated compounds, in addition to transport proteins for the uptake of these compounds from the environment. The SsuD monooxygenase is dependent on SsuE to supply reduced flavin for the reaction; however, the mechanism of flavin transfer in the alkanesulfonate monooxygenase system has not been determined. The altered kinetic mechanism observed with SsuE in the presence of SsuD and octanesulfonate suggests there is communication between the two enzymes [23]. The desulfonation reaction catalyzed by SsuD is dependent on a C4a-(hydro)peroxyflavin intermediate to catalyze the cleavage of the carbon-sulfur bond. The TIM-barrel structure of SsuD containing an unstructured loop region suggests that a lid gating mechanism is necessary for the protection of the flavin intermediates generated during catalysis, which correlates with the conformational changes identified in kinetic studies [22]. The structural similarity of SsuD to the bacterial luciferase family has provided mechanistic insight into the diverse reactions catalyzed by these enzymes. There are conserved amino acid residues in SsuD that are spatially conserved in the bacterial luciferase family that likely play a similar mechanistic role [22]. While much of the kinetic details of these enzymes are known, there is still a great deal to be evaluated regarding the detailed mechanistic properties of these enzymes.

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