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Review article

Current understanding of enzyme structure and function in bacterial two-component flavin-dependent desulfonases: Cleaving C–S bonds of organosulfur compounds

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ABSTRACT

The inherent structural properties of enzymes are critical in defining catalytic function. Often, studies to evaluate the relationship between structure and function are limited to only one defined structural element. The two-component flavin-dependent desulfonase family of enzymes involved in bacterial sulfur acquisition utilize a comprehensive range of structural features to carry out the desulfonation of organosulfur compounds. These metabolically essential two-component FMN-dependent desulfonase systems have been proposed to utilize oligomeric changes, protein-protein interactions for flavin transfer, and common mechanistic steps for carbon-sulfur bond cleavage. This review is focused on our current functional and structural understanding of two-component FMN-dependent desulfonase systems from multiple bacterial sources. Mechanistic and structural comparisons from recent independent studies provide fresh insights into the overall functional properties of these systems and note areas in need of further investigation. The review acknowledges current studies focused on evaluating the structural properties of these enzymes in relationship to their distinct catalytic function. The role of these enzymes in maintaining adequate sulfur levels, coupled with the conserved nature of these enzymes in diverse bacteria, underscore the importance in understanding the functional and structural nuances of these systems.

1. Introduction

Sulfur metabolism in bacterial systems is tightly regulated to ensure that this critical element is available when standard sulfur sources are limiting. 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 [1]. Many bacteria typically rely on inorganic sulfate or cysteine as their primary sulfur source but possess alternative pathways for sulfur acquisition when sulfate is limiting [1,2]. Genes expressed during sulfur limitation play a role in organosulfur uptake, sulfur acquisition from organosulfur compounds, and protection against reactive oxygen species [3,4]. Interestingly, several enzymes involved in bacterial sulfur acquisition are two-component (TC) FMN-dependent systems that rely on an FMN reductase and monooxygenase to catalyze the desulfonation of diverse organic sulfur compounds (Scheme 1) [5–9]. In certain bacteria, these TC systems expressed from different operons work together to catalyze the carbon oxidation of environmental organosulfur compounds [1]. The monooxygenases have to coordinate their complex mechanism with the FMN reductase that provides reduced flavin for the desulfonation reaction. The TC FMN-dependent desulfonase systems play a pivotal role in maintaining adequate sulfur levels in a diverse range of bacterial organisms. Catalysis of carbon-sulfur bond cleavage represents an innovative mechanistic approach for TC FMN-dependent desulfonase enzymes. It is

Abbreviations: MsuC, (methanesulfinate monooxygenase); MsuD, (methanesulfonate monooxygenase); MsuE, (methanesulfinate/methanesulfonate monooxygenase FMN reductase); SfnG, (dimethylsulfone monooxygenase); SsuD, (alkanesulfonate monooxygenase); SsuE, (alkanesulfonate monooxygenase FMN-dependent reductase).

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interesting that bacteria invest significant metabolic energy to acquire sulfur, and that two-component FMN-dependent enzymes are specifically involved in these reactions. The TC FMN-dependent desulfonase enzymes contain low sulfur content to preserve sulfur levels in the cell. In addition, the TC FMN-dependent desulfonase systems share similar catalytic and structural properties in both their reductase and desulfonase enzymes. This review will focus on TC FMN-dependent desulfonase enzymes involved in bacterial sulfur assimilation. Although these systems have been identified in diverse bacteria, this review will focus on enzymes biochemically and biophysically characterized from *Escherichia coli*, *Pseudomonas* sp. (*P. fluorescens* Pfl01, *P. aeruginosa* PAO1, *P. putida*), and *Acinetobacter baumanni.* There have been numerous findings on the structure and function of the TC FMN-dependent desulfonase systems, and this review creates a cohesive analysis and assessment of these findings.

2. Genome organization

The expression of genes that encode proteins in the sulfur acquisition and assimilation pathways in bacteria are often under coordinated control. There have been numerous investigations focused on the expression of genes upregulated during sulfur limitation and the subsequent analysis of these individual enzymes [4,6–8]. It is clear operons involved in sulfur acquisition display variations in their expression and gene organization in different bacteria. These differences provide bacteria with diverse pathways for sulfur acquisition.

2.1. Alkanesulfonate gene arrangement (ssu for sulfonate-sulfur utilization)

The *ssuEADCB* gene cluster in *E. coli* was first reported in 1999 and the related operon *ssuEADCBF* in *P. putida* reported soon after [4,7]. Three of the five genes (*ssu*A, *ssu*C, and *ssu*B) produce putative membrane bound transporter proteins while two genes produce SsuE and SsuD protein components required for cleavage of the C–S bond in alkanesulfonates (Fig. 1A). The sixth gene identified in the genome of some *Pseudomonas* sp. (*ssuF*) is classified as a putative molybdate transport protein, but the function of SsuF has not been determined (Fig. 1A).

2.2. Methanesulfonate gene arrangement (msu for methanesulfonate-sulfur utilization)

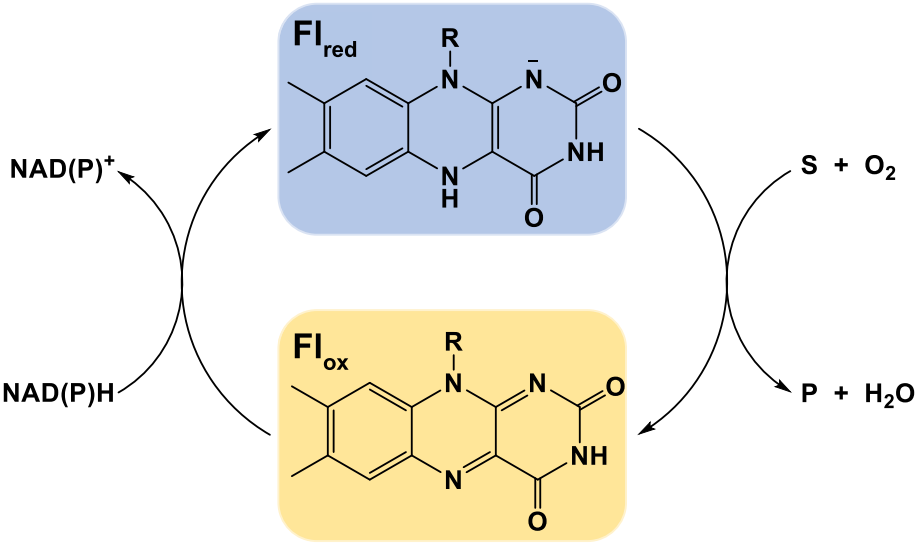
The *msuEDC* gene cluster from *P. aeruginosa* PAO1 was first reported in 1999 [8]. The *msuC* gene product belongs to the acyl-CoA dehydrogenase family and was characterized as a TC FMN-dependent monooxygenase enzyme [8]. The two genes *msu*D and *msu*E are analogous to *ssu*D and *ssu*E. The genes produce the two protein components required for cleavage of the C–S bond in methanesulfonate, the C_1 analog of the alkanesulfonate substrates of SsuD/SsuE. In some *Pseudomonas* sp., the gene encoding the transcriptional regulator enhancer-binding protein SfnR1 is expressed on the same operon *msuEDC* (Fig. 1B) [10]. SfnR1 has been proposed to be involved in the expression of *msuEDC-sfnR1*, *sfnG*, and other target genes involved in dimethylsulfide (DMS)-related metabolism in *P. aeruginosa* PAO1. The *msuEDC-sfnR1* gene cluster is also present in *P. fluorescens* Pfl01 but is not present in the genome of some *P. putida* species.

2.3. Sulfone gene arrangement (sfn for sulfone-sulfur utilization)

The function of the first isolated and purified *sfnG* gene product from the genetically tractable microorganism *P. fluorescens* Pfl-01 was recently reported [9]. The protein SfnG, together with a flavin reductase from the same microorganism (MsuE), converted dimethylsulfone to methanesulfinate, the sulfur-containing substrate for MsuC. In many *Pseudomonas* sp., there appears to be no dedicated FMN reductase directly upstream or downstream of the dimethylsulfone monooxygenase (Fig. 1C). The regulation of the *sfnG* gene remains to be elucidated; however, it has been demonstrated that the production of SfnG is linked to the expression of the msu*EDCsfnR1* gene cluster in *P. aeruginosa* PAO1 under sulfur limiting conditions [10]. An SfnG enzyme and partner FMN reductase was recently characterized from *A. baumanni* (*Ab*SfnG) [11].

3. Overall reactions

Monooxygenase desulfonases that belong to the TC FMN-dependent systems involved in sulfur acquisition share similar overall structural properties but have distinct substrate specificities. These systems may have diverged to recognize specific substrates, while still retaining similar catalytic steps. A ubiquitous means for acquiring sulfur during sulfur limiting conditions in diverse bacteria is the alkanesulfonate desulfonase system (*Ec*SsuE/*Ec*SsuD) that catalyzes the conversion of



Scheme 1. Overall reaction of TC FMN-dependent desulfonase enzymes.

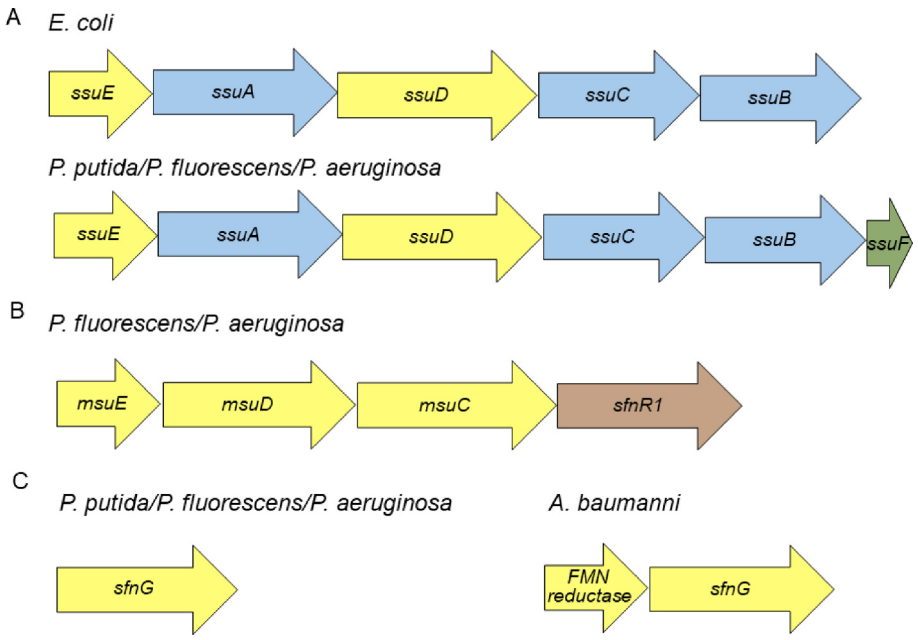


Fig. 1. Genome organization of enzymes involved in desulfonation during sulfur limitation. (A) The arrangement of genes in the *ssuEADCB* operon in *E. coli* K12 (NC_000913.3) and *ssuEADCBF* operon in *P. putida* (NC_021505.1), *P. fluorescens* Pfl01 (NC_007492.2), and *P. aeruginosa* PAO1 (NC_002516.2). The genes producing the FMN-dependent reductase and monooxygenase are yellow and the putative transporter proteins are blue. The gene identified as a putative molybdate transport protein SsuF in *Pseudomonas* sp. is denoted as green. (B) The arrangement of genes in the *msuEDCsfnR1* cluster in *P. aeruginosa* PAO1 (NC_002516.2) and *P. fluorescens* (NZ_LT907842.1). The gene identified as a transcriptional regulator protein *sfnR1* is denoted as brown. (C) The genetic arrangement of the *sfnG* gene in *P. fluorescens*, *P. putida*, and *P. aeruginosa. FMN reductase* and *sfnG* in *A. baumanni* (NZ_CP043953.1).

alkanesulfonates to sulfites and aldehydes [5]. In the overall reaction scheme, the FMN reductase (*Ec*SsuE) catalyzes the reduction of FMN by NAD(P)H followed by the transfer of reduced flavin to the monooxygenase enzyme (*Ec*SsuD). SsuD catalyzes the oxygenolytic cleavage of alkanesulfonate to aldehyde and sulfite using reduced flavin to activate molecular oxygen (Fig. 2A) [4]. The alkanesulfonate transporter is responsible for the uptake of alkanesulfonates from the environment. The presence of the alkanesulfonate system in a broad range of bacteria suggests its role is essential in maintaining cellular sulfur concentrations through the oxidation of alkanesulfonates.

Pseudomonas sp. have a more diverse mechanism for sulfur acquisition when sulfur in the environment is limiting [12]. Some *Pseudomonas* sp. contain multiple two-component FMN-dependent systems for the acquisition of sulfur from organic sulfur sources. $DMSO_2$ is derived through the oxidation of DMS, a secondary metabolite in some marine algae, and is the most abundant biological sulfur compound emitted to the atmosphere [13,14]. In addition, methanethiol is formed during methionine catabolism in some bacteria that can be oxidized further to

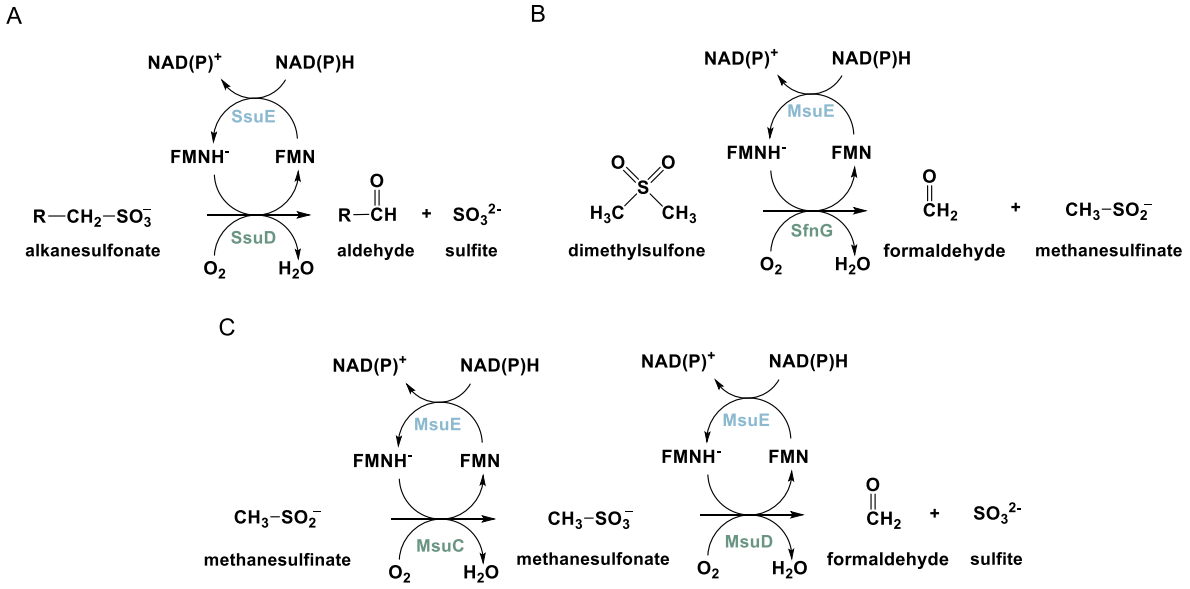


Fig. 2. TC FMN-dependent desulfonase reactions for the acquisition of sulfite from organosulfur compounds. (A) Alkanesulfonate monooxygenase (SsuE/SsuD). The SsuD enzyme can utilize a broad range of alkanesulfonate substrates. (B) Dimethylsulfone monooxygenase (MsuE/SfnG). The SfnG enzyme catalyzes the conversion of $DMSO_2$ to formaldehyde and methanesulfinate. (C) Methanesulfinate/methanesulfonate monooxygenases (MsuE/MsuC and MsuE/MsuD). MsuC and MsuD catalyze the sequential conversion of methanesulfinate to formaldehyde and sulfite.

methanesulfinate/methanesulfonate. $DMSO_2$ is converted to methanesulfinate by dimethylsulfone monooxygenase (SfnG), which can utilize different FMN reductases depending on the organism (Fig. 2B) [6,15–18]. Some *Pseudomonas* sp. do not produce a partner FMN reductase to supply reduced flavin to SfnG. Given the coordinated expression of the *msu* operon and *sfnG* gene in some *Pseudomonas* sp., MsuE expressed from the methanesulfonate monooxygenase operon was used as the reduced flavin donor in experimental studies [9]. Although not specifically stated, studies with *Ab*SfnG likely utilized a coexpressed FMN reductase [11]. The methanesulfinate produced is oxidized in some *Pseudomonas* sp. to methanesulfonate by the methanesulfinate monooxygenase system (MsuE/MsuC), and the methanesulfonate is further oxidized to sulfite and formaldehyde by methanesulfonate monooxygenase (MsuE/MsuD) (Fig. 2C) [9]. Most *Pseudomonas* sp. also express SsuE and SsuD when sulfur is limiting to ensure that multiple sources for acquiring sulfur are available [12]. All of these systems are centered on providing sulfur that can be incorporated into different cellular metabolites. The reductase and monooxygenase enzymes that catalyze carbon-sulfur bond cleavage from *E. coli* and *Pseudomonas* sp. share between 30 and 80 % amino acid sequence identity. The high amino acid sequence identity suggests that they have evolved similar structural properties and mechanistic strategies for desulfonation, while still maintaining distinct substrate specificities.

4. Structural properties of the FMN reductases and monooxygenases

4.1. FMN reductases

4.1.1. Overall structural properties of the FMN reductases

Three-dimensional structures for FMN reductases linked to desulfonation systems have only been determined for *Ec*SsuE and *Pp*MsuE [19,20]. These enzymes have the flavodoxin-like fold characteristic of several enzymes belonging to the NAD(P)H:FMN reductase family (Fig. 3A) [19]. Although the TC FMN reductases belong to the NAD(P)H:FMN reductase family, members of the family are also comprised of canonical FMN reductases that have a bound FMN cofactor and directly catalyze reduction reactions. The three-dimensional structure of *Ec*SsuE exists as a dimer of dimers which is essential for substrate-induced structural changes. The three-dimensional structure of SsuE with excess flavin substrate added to preformed crystals had either one or two FMN bound per monomer [19]. A tightly bound FMN was in a protected site (Site 1) stabilized by noncovalent interactions from the peptide backbone and side chains. A loosely bound FMN that did not occupy each monomer was bound to a second site (Site 2). The electron density of this second FMN was weak suggesting that the flavin was partially bound and/or disordered. Reduction of bound FMN eliminated the loosely bound flavin at Site 2. Alternatively, it was speculated that the second flavin could be an artifact of the crystallization conditions. Therefore, it is not clear if there is an actual role for the FMN bound at the second site since there is only one FMN bound in titration experiments [21]. Mechanisms incorporating one or two FMN molecules were proposed based on the second binding site. If FMN is bound to Site 1 only, the FMN would be reduced and directly transferred to SsuD. Including Site 2 in the mechanism, the tightly bound FMN would be reduced, and electrons would be transferred to the loosely bound FMN at the second site prior to transfer to SsuD. It has also been proposed that the secondary site could be used as a temporary binding site for reduced flavin following reduction at Site 1 and prior to transfer to SsuD [21].

The TC FMN-reductase enzymes involved in sulfur acquisition are classified into a subgroup of the NAD(P)H:FMN reductase family based on a distinct secondary structural element that is absent in canonical FMN-bound members of the family [19]. All members of the subclass contain a π-helix located at the tetramer interface. Generally, π-helices are characterized by a single amino acid insertion into an α-helix to provide an evolutionary advantage through enhancement or gain of function [23]. The insertion of an amino acid into an established α-helix generates a change in intrastrand hydrogen bonding that results in wide turns [23–25]. The proposed insertional residue for *Ec*SsuE is a Tyr residue whereas MsuE from different *Pseudomonas* sp. has a His residue (Fig. 3B) [19,20]. In the tetrameric structure, the hydroxyl group of Tyr118 in *Ec*SsuE forms hydrogen bonding interactions with the carbonyl oxygen of Ala78 across the dimer/dimer interface (Fig. 4A) [19]. The amide nitrogen of Ala78 forms a hydrogen bond with the carbonyl oxygen of FMN C4 bound to the same subunit leading to a hydrogen-bonding network between subunits. In addition to the hydrogen-bonding interactions, Tyr118 forms π-stacking interactions at the tetramer interface (Fig. 4B) [26]. Conversion of Tyr118 to an Ala in *Ec*SsuE converted the π-helix to an α-helix and resulted in a dimeric structure that was not able to form a tetramer due to steric hindrance at the dimer/dimer interface [20]. However, deletion of the Tyr118 insertional residue did not convert the FMN-dependent reductase to an α-helix observed in canonical flavin reductases in the NAD(P)H:FMN reductase. The region surrounding the Tyr118 insertion was disordered and resulted in a disruption in the π-helix. Based on these studies, it is likely that the π-helix secondary structure in *Ec*SsuE was not formed by a single amino acid insertion residue, but additional structural properties contributed to the formation of the secondary structure.

4.1.2. Oligomeric changes in the FMN reductases

Oligomeric modulations are important in regulating the catalytic properties of some enzymes and are often utilized to regulate catalysis and complex mechanistic features [27–31]. Some TC FMN reductases associated with desulfonation reactions undergo changes in the

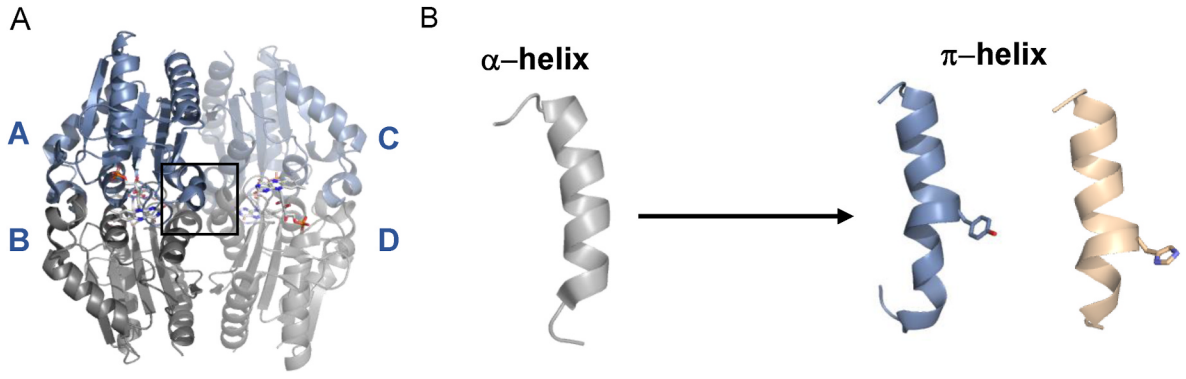


Fig. 3. Structural properties *Ec*SsuE and *Pf*MsuE. (A) Overall tetrameric structure of the TC FMN reductases. The enzymes exist as tetramers or dimers. Shown is the structure of *Ec*SsuE with the π-helices boxed at the tetrameric interface. The blue/gray subunits represent the dimer/dimer pairs (Chains A/B and C/D). Each subunit has a single bound flavin in the active site. (B) A canonical NAD(P)H:FMN reductase containing an α-helix. The π-helix is formed from the insertion of a Tyr or His residue into an α-helix. PDB: 4PTZ, *Ec*SsuE; AlphaFold structure of *Pf*MsuE [19,22].

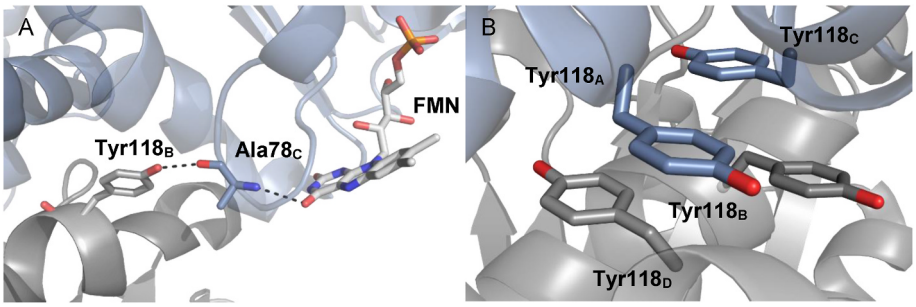


Fig. 4. Noncovalent interactions of the π-helix insertional amino acid residue in *Ec*SsuE. (A) Hydrogen bonding interactions are formed between the Tyr118 insertional residue and the α-carbonyl group of Ala78 across the dimer/dimer interface. The α-amide of Ala78 forms hydrogen bonding interactions with the C4 carbonyl oxygen of FMN bound to Site 1. (B) Tyr118 π-stacking interactions at the tetramer interface of *Ec*SsuE. The blue/gray subunits represent dimer/dimer pairs (A/B and C/D). PDB: 4PTZ, *Ec*SsuE [19].

oligomeric state with the binding of substrates that may assist in regulating flavin reduction and subsequent transfer to the monooxygenase. The initial quaternary structural analysis of SsuE through size-exclusion chromatography demonstrated that the enzyme existed as a dimer in solution [5]. Conversely, FMN-bound SsuE existed as a tetramer in the three-dimensional structure [19,20]. Further investigations demonstrated that SsuE exists as a tetramer in solution but shifts to a tetramer/dimer equilibrium in the presence of the flavin substrate [32]. Conversely, *Pa*MsuE shifted from a dimer to a tetramer in the presence of FMN. The oligomeric changes for both enzymes occurred along the dimer/dimer interface of the tetramer. Similar changes in the oligomeric state were not observed with NADPH for either *Ec*SsuE or *Pa*MsuE. The differences in the quaternary structure of *Ec*SsuE and *Pa*MsuE in the absence and presence of flavin is of particular interest. The FMN binding site is located closer to the dimer/dimer interface in the FMN reductases. Therefore, conversion from a tetramer to a tetramer/dimer equilibrium in the FMN/*Ec*SsuE complex would promote flavin transfer to SsuD. The oligomeric shift of *Pa*MsuE from a dimer to a tetramer would seem to prevent flavin transfer as the active sites would be buried between the dimer/dimer interface. These differences in the oligomeric states between *Ec*SsuE and *Pa*MsuE with FMN bound were attributed to their different functional roles. *Ec*SsuE provides reduced flavin to SsuD only, but *Pa*MsuE provides reduced flavin to both MsuC and MsuD. Therefore, *Pa*MsuE would need to protect reduced flavin until MsuC and MsuD are available for reduced flavin transfer. Although an oligomeric change occurs with the binding of FMN, it is not known how the reduced flavin product may alter these oligomeric states.

4.2. TC FMN monooxygenase desulfonase structures

4.2.1. Overall structure of the TC monooxygenase desulfonases

Despite high amino acid sequence identity between MsuD and SsuD there are primary and tertiary structural differences leading to key distinctions in their function. *Ec*SsuD and *Pf*MsuD are both group C flavin-dependent alkanesulfonate monooxygenases that are structurally represented by a $(\beta/\alpha)_8$ triose isomerase phosphate (TIM)-barrel fold [33,34]. Beyond the core tertiary fold, there are insertion sequences that deviate from the core TIM-barrel structure. The two enzymes share many specific tertiary features that are broadly grouped into the lid region, the dimer interface, and the C-terminal tail.

The insertion region that spans residues D250-L282 in *Pf*MsuD contains the lid region that encloses the active site and is disordered in the absence of substrates [34]. This region parallels a comparable insertion region in *Ec*SsuD that is similarly disordered (Fig. 5A) [33,34]. Interestingly, for *Pf*MsuD, this region is only ordered in the presence of its bound substrates (Fig. 5B). The lid region closes over the active site with both substrates bound to protect the active site from bulk solvent. It is apparent that this lid region is important to catalysis as the lid becomes more ordered and makes important noncovalent interactions when substrates are bound.

The dimer interface in both *Pf*MsuD and *Ec*SsuD consists of a β-hairpin and α-helix separate from the TIM-barrel fold and the outward facing regions of two TIM-barrel α-helices (Fig. 6) [33,34]. These structural elements in both *Pf*MsuD and *Ec*SsuD are positioned similarly, and there are intermolecular bonds along this dimer interface that remain consistent in both enzymes. Another feature that is similar between *Pf*MsuD and *Ec*SsuD is the four-helix bundle that constitutes the tetramer interface (Fig. 6). Although part of the TIM-barrel, two of the core helices in both monooxygenases combine with the same helices in a symmetry related monomer to form the four-helix bundle of the main tetramer interface. This bundle is again identical in position but only partially similar in the intermolecular bonds formed between helices. The elements that make up the quaternary interfaces in the two are consistent in *Pf*MsuD and *Ec*SsuD, making up a dimer of dimers to form the tetramer.

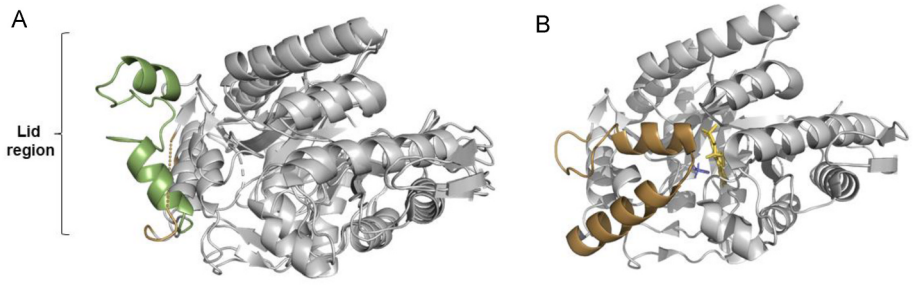


Fig. 5. Mobile lid region of *Pf*MsuD and *Ec*SsuD. (A) The lid region of *Ec*SsuD (green) and *Pf*MsuD (brown) in the absence of substrates. Both structures lacked electron density in the loop region. A model of the loop region of *Ec*SsuD was generated from computational studies. The missing loop region of *Pf*MsuD is designated with a dashed line (brown). (B) *Pf*MsuD with methanesulfonate (blue) and flavin (yellow) bound. The two helices that make up the lid are ordered with the binding of substrates and close over the active site. PDB: 7JV3,7K14, *Pf*MsuD; 1M41, *Ec*SsuD [33,34].

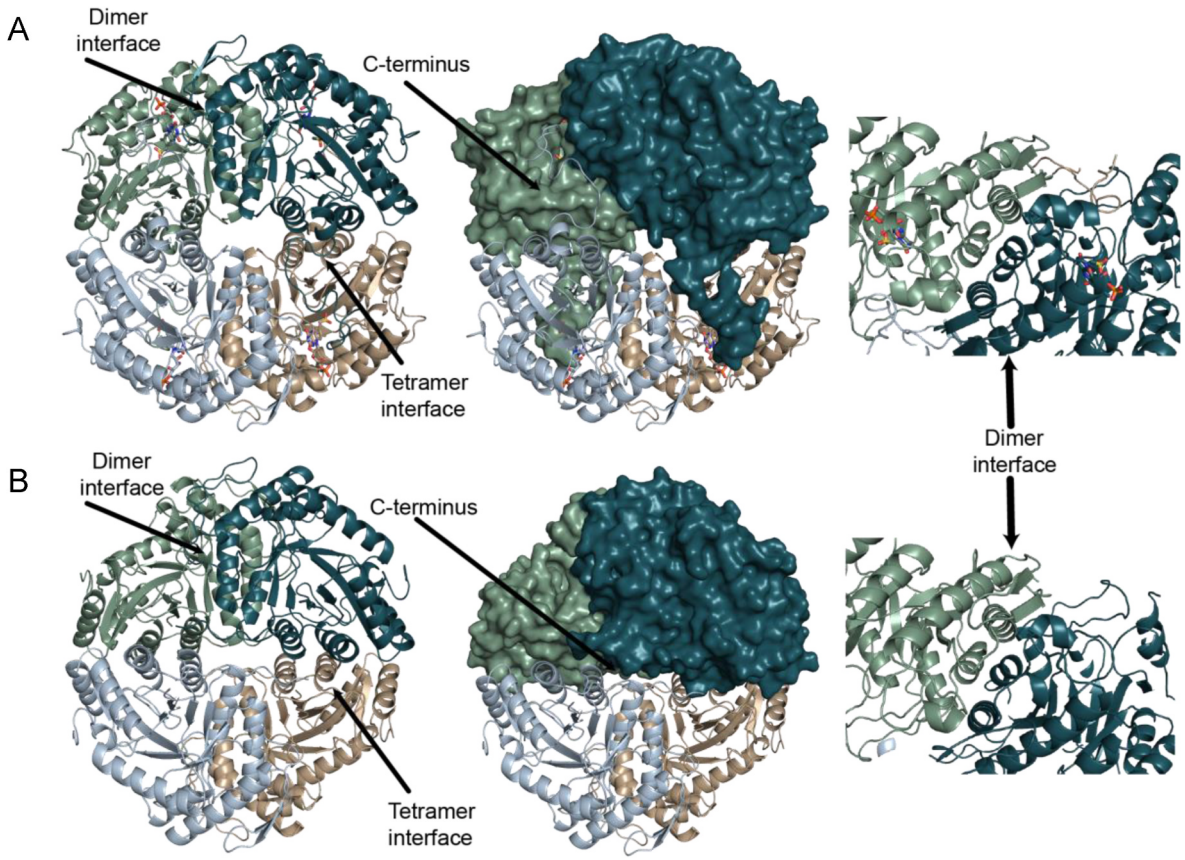


Fig. 6. Comparisons of *Pf*MsuD and *Ec*SsuD tetramers show strong structural similarities at the dimer and tetramer interfaces. Liganded *Pf*MsuD (A, PDB 7JW9) and unliganded *Ec*SsuD (B, PDB 1M41) chains A, B, C and D are colored teal, green, light blue, and tan, respectively. Figures are displayed as follows: left, cartoon representations of the tetramer; middle, cartoon and surface representations of the two dimers; right, the dimer interface looking downward from the top of the tetramer. Surface representations for chains A and B show how the dimer of dimers structure in *Pf*MsuD can be strengthened by the C-termini that reach across the tetramer interface. In *Ec*SsuD, the C-termini are seen to run parallel to the dimer instead. The ligands FMN and methanesulfonate are displayed in sticks with oxygen in red, sulfur in yellow, phosphorous in orange, nitrogen in blue, and carbons matching each monomer's color [33,34].

4.2.2. Conformational changes in the TC monooxygenase desulfonases

In addition to the ligand-dependent ordering of the active site mobile lid region discussed above, the final region of note is the C-terminal tail distinct from the TIM-barrel core (Fig. 6). This broad structural feature differs between *Pf*MsuD and *Ec*SsuD. In both enzymes, as well as other group C flavin-dependent monooxygenases, the C-terminus is long and often disordered. In the ternary complex of *Pf*MsuD the C-terminus extends over into the active site of the opposing monomer and forms several interactions including a secondary shell interaction to the substrate binding site (Fig. 6A) [34]. Five prolines in the C-termini enable the structural contortions needed for this winding path between monomers. In the published structure of *Ec*SsuD the C-terminus is a partially built linear chain that makes no interactions (Fig. 6B) [33]. The C-termini of *Ec*SsuD and many of their homologs contain multiple proline, glycine, and alanine residues that would potentially allow similar sharp bends and turns; however, a comparable tertiary structure of *Ec*SsuD has not been determined. As a primary structural feature that differs between *Pf*MsuD and *Ec*SsuD, it may be that the C-termini along with the active site residues from the mobile lid region are key components that enable the different substrate specificities. SfnG adopts only a moderate level of structural identity with SsuD and MsuD (~30 %) from multiple bacteria; therefore, further structural studies of SfnG will be important to reveal the structural similarities and differences to SsuD and MsuD.

4.3. Protein-protein interactions

Transfer of flavin from the FMN reductase to the monooxygenase must be tightly regulated to avoid nonenzymatic oxidation of the flavin during transfer, and protein-protein interactions would be a viable mechanism to mediate such an event. Protein-protein interactions have been identified between *E. coli* SsuE and SsuD [36,37]. The regions of *Ec*SsuE that interact with *Ec*SsuD are located at the dimer/dimer interface. Therefore, the tetramer of *Ec*SsuE would need to undergo an oligomeric shift to a dimer in order for this region to effectively form protein-protein interactions with *Ec*SsuD. As noted, the binding of both oxidized FMN and *Ec*SsuD have been shown to effectively trigger an oligomeric shift that would promote protein-protein interactions [32]. Because MsuE provides reduced flavin to both MsuC and MsuD in *Pseudomonas* sp., conserved structural features of MsuE may be important in promoting protein-protein interactions with both enzymes [20]. The shift of MsuE from a dimer to a tetramer with the binding of oxidized FMN would potentially hide the interaction sites at the tetramer interface [32]. For MsuE, flavin reduction or the presence of the monooxygenase partner may facilitate an equilibrium shift to a dimer to promote flavin transfer and/or protein-protein interactions. Therefore, the interaction event with the monooxygenase partners may lead to the tetramer/dimer equilibrium shift that promotes flavin transfer. Formation of the tetramer would assist in the regulation of flavin transfer from MsuE to either MsuC or MsuD until the flavin is reduced and the monooxygenase is available for transfer. The SfnG enzyme does not have a dedicated FMN reductase in some *Pseudomonas*

sp. and may rely on MsuE or SsuE to supply reduced flavin. *Pf*SfnG catalyzed the oxidation of $DMSO_2$ to methanesulfinate with $FMNH_2$ provided by *Pf*MsuE [9]. If flavin transfer is dependent on protein-protein interactions, then common structural features may facilitate these interactions.

An area of interest in the two-component FMN-dependent systems is how conserved structural features contribute to flavin transfer. It had been speculated that the π-helix is involved in promoting flavin transfer from the TC FMN reductase to the partner monooxygenase [19,20]. A primary region of amino acid sequence identity within the sulfur-acquiring FMN reductases corresponds to the α-helix (region 78–89) and π/α-helix (region 119–125) of *Ec*SsuE that was protected in HDX-MS experiments in the presence of *Ec*SsuD (Fig. 7A) [37]. The complementary protected sites of SsuD were located on an α-helix (D251–A261) and lid region (Fig. 7B). Protected regions identified on *Ec*SsuD were located near the active site opening and are connected to each other by the mobile loop region. As mentioned, several of the TC flavin-dependent desulfonases that have been characterized are able to utilize reduced flavin supplied by FMN-dependent reductases from other systems [38,39]. This characteristic supported a mechanism for flavin transfer that would not be dependent on protein-protein interactions. As noted, conserved motifs could be involved in promoting flavin transfer from the FMN reductase to the monooxygenase through protein-protein interactions. Sulfur-acquiring TC reductases and enzymes share >30 % amino acid sequence identity and similar structural features that includes the π-helix. To support these structural observations, sulfite production was detected in coupled studies with *Pa*MsuE/*Ec*SsuD and *Ec*SsuE/*Pa*MsuD, and the specific activities were comparable to the established FMN reductase/monooxygenase pairs [20]. Although preliminary results suggest that protein-protein interactions can form between alternative FMN reductase and desulfonase pairs, the regions that promote these protein-protein interactions have not been confirmed. Identifying the putative interaction regions would provide valuable information on how these enzymes have evolved to share common structural features to promote flavin transfer.

5. Reaction mechanism and kinetics

5.1. TC FMN-dependent reductases

Flavin reduction by the TC FMN reductases relies on reducing equivalents provided by pyridine nucleotides. Unlike FMN reductases with a bound FMN cofactor, TC FMN reductases transfer the reduced flavin product to the monooxygenase partner for oxygen activation. The detailed mechanism of flavin reduction for the TC FMN-dependent reductases has primarily been evaluated for *Ec*SsuE [21,40]. However, recent steady state kinetic analyses with *Pa*MsuE and *Pf*MsuE have provided insights on the substrate preference for the MsuE/MsuD system [9,20,34]. Both *Ec*SsuE and MsuE from *Pseudomonas* sp. form a ternary complex with the flavin and pyridine nucleotide prior to catalysis. *Ec*SsuE has a clear preference for FMN with a 40-fold higher affinity for FMN compared to FAD [5,21]. Although NADPH has been utilized in most of the studies with *Ec*SsuE, the enzyme has a similar affinity for both NADH and NADPH when measuring pyridine nucleotide oxidation [5]. *Pf*MsuE showed no clear preference for FMN or FAD but showed a substrate preference for NADH [9,34]. Not all *Pseudomonas* sp. show a comparable pyridine nucleotide preference. It is interesting that *Pa*MsuE prefers to utilize NADH when measuring pyrimidine nucleotide oxidase activity but shifts to a preference for NADPH when supplying reduced flavin to *Pa*MsuD [20]. This change in preference suggests that MsuD is altering the substrate specificity of MsuE.

Because FMN reductases from TC systems utilize flavin as a substrate, the binding and release of substrates and products must be coordinated for effective reduced flavin transfer. The *Ec*SsuE enzyme showed an ordered sequential mechanism with FMN binding first followed by NADPH [21]. Following flavin reduction, the reduced flavin product is released first so that it can be transferred to the *Ec*SsuD monooxygenase and then $NADP^+$ is released. It was noted that the reaction was altered from an ordered to a rapid equilibrium ordered mechanism in the presence of *Ec*SsuD and the monooxygenase substrate [21]. This ensures that in the presence of the monooxygenase substrate partner, the E/NADPH formed first is converted to the ternary complex with increasing FMN. In the presence of saturating FMN the E/NADPH formed is converted to the ternary complex to provide SsuD with reduced flavin even if NADPH is limiting. Rapid reaction kinetic analysis identified two charge transfer complexes prior to flavin reduction (Fig. 8) [40]. CT-1 represents the charge complex between NADPH and FMN and CT-2 represents charge-transfer between $FMNH_2$ and $NADP^+$. Results from rapid reaction kinetic studies with deuterated NADPH revealed a rate-limiting step on hydride transfer from NADPH to FMN to form CT-2.

As mentioned, *Ec*SsuE diverges from the FMN-bound canonical flavoproteins due to the presence of a π-helix at the tetramer interface. Studies with Tyr118 EcSsuE variants provided valuable insight into the functional properties of the enzyme [26,41]. Substitution of Tyr118 to Ala altered the ability of *Ec*SsuE to release the flavin following reduction or support NADPH oxidation [41]. However, the FMN was reduced by one equivalent of NADPH. Therefore, the tightly bound reduced FMN was protected from molecular oxygen. The *Ec*SsuE variant was now able to protect reduced flavin from oxidation but was not able to release

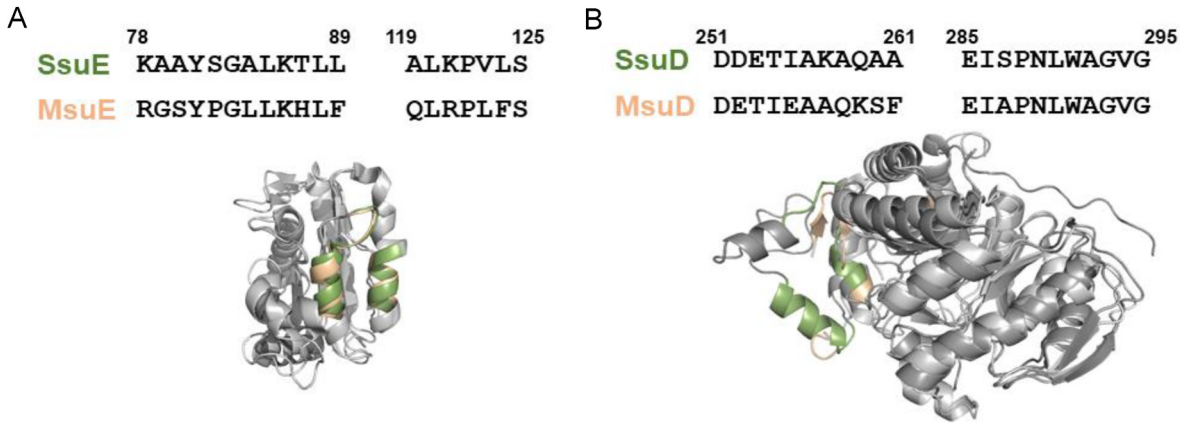


Fig. 7. Conserved putative interaction sites in the TC FMN reductase and monooxygenase enzyme. (A) Alignment of the proposed interaction region with the monooxygenase partner. Overlay of *Ec*SsuE and *Pf*MsuE with the interaction region highlighted. PDB: 4PTZ, *Ec*SsuE; AlphaFold structure of *Pf*MsuE [19,22]. (B) Alignment of the proposed interaction region with the reductase partner. Overlay of *Ec*SsuD and *Pf*MsuD with the interaction region highlighted. PDB: 1M41, *Ec*SsuD; 7JV3, *Pf*MsuD [33,34].

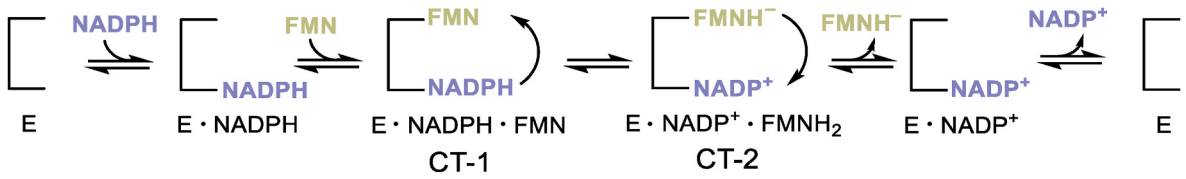


Fig. 8. Reaction scheme for FMN reductases. The binding of NADPH and FMN forms a ternary complex. CT-1 is the charge complex between NADPH and oxidized FMN. CT-2 is the charge complex between reduced flavin and NADPH. Reduced flavin is released first and transferred to the monooxygenase and $NADP^+$ is released last.

$FMNH_2$ to *Ec*SsuD despite still being able to form protein-protein interactions with *Ec*SsuD [26]. The gain of function provided by the π-helix may result in a more dynamic region that promotes the release of reduced flavin. The conversion to an α-helix decreased this flexibility resulting in a more rigid structure as observed for the canonical FMN reductases within the same family. Although the π-helix likely serves the same functional role in both SsuE and MsuE, the insertional residue that is proposed to generate the secondary structure is not interchangeable. *Ec*SsuE demonstrated no FMN reductase activity when the Tyr118 insertional residue was altered to the His residue found in MsuE, but *Pa*MsuE showed a similar catalytic efficiency when the His 126 insertional residue was substituted with Tyr [20]. It was not apparent how switching the insertional residue changed the enzyme structure and if the π-helix was still intact. The functional role of the π-helix in the subclass of the NAD(P)H:FMN reductase family remains unclear. The primary structural difference between the TC FMN-dependent reductases belonging to this family and the canonical FMN reductases with a bound flavin is the π-helix. Therefore, the functional role of this distinct secondary structure could be to promote flavin transfer to the monooxygenase partner.

5.2. Mechanism of desulfonation in the two-component FMN-dependent monooxygenase desulfonases

5.2.1. Steady-state kinetic properties

The steady-state kinetic properties of the TC FMN-dependent desulfonases have revealed important information regarding the order of substrate binding and substrate specificity. Kinetic studies of *Ec*SsuD supported a model where $FMNH_2$ binds first followed by alkanesulfonate and molecular oxygen (Fig. 9) [42]. Molecular oxygen binding last would ensure that formation of the oxidizing flavin intermediate would be coupled to carbon-sulfur bond cleavage. Both *Ec*SsuD and *Pf*MsuD are not able to bind the alkanesulfonate substrate unless reduced FMN is bound first [34,42]. Therefore, the binding of $FMNH_2$ may organize the active site for binding the designated substrate by positioning active site residues for substrate recognition and catalysis. However, there are currently no desulfonase structures with reduced flavin bound.

The SsuD enzyme from *E. coli* has a broad substrate range and can acquire sulfur from multiple alkanesulfonate sources. Results from previous studies have demonstrated that *Ec*SsuD catalyzes the desulfonation of linear alkanesulfonates (C4–C12), some substituted ethanesulfonates, and sulfonated buffers [5,43]. Although methanesulfonate could bind to SsuD in anaerobic titration studies, the enzyme is unable to utilize methanesulfonate as a sulfur source. Computational investigations demonstrated the instability of methanesulfonate in the active site of the ternary complex, suggesting a longer alkane is necessary for effective binding of the alkanesulfonate substrate to *Ec*SsuD [43]. For bacteria that have a broader substrate range, the MsuE/D enzymes provide a mechanism for the desulfonation of C1 metabolites. The broader substrate range is often observed in bacteria that are more widely distributed in the environment [1]. For instance, many *Pseudomonas* species are found in aquatic and terrestrial environments, and would have access to methanesulfonate compounds formed from the oxidation of dimethylsulfide [44]. In initial studies, MsuD from *P. aeruginosa* PAO1 was not able to utilize dimethylsulfone and showed reduced activity with C2 and C4 alkanesulfonates in activity assays with the cell lysate [8]. Later studies revealed that *Pa*MsuD was able to catalyze the desulfonation of C8–C10 alkanesulfonates with a similar catalytic efficiency as methanesulfonate in steady-state kinetic assays [43]. The alteration in catalytic efficiency with chain length may be attributed to changes in alkane packing with the closure of the mobile loop region that stabilizes the longer alkanes. The *Pf*MsuD enzyme showed comparable activity with alkanesulfonates of varying carbon length (C1, C5, and C8), similar to what was observed with *Pa*MsuD [34]. The methanesulfonate substrate can be formed from the carbon-sulfur bond cleavage of dimelthylsulfone by SfnG and further oxidation by the TC monooxygenase MsuC, or from methionine catabolism. Together these enzymes cover a broad range of organosulfur compounds to provide bacteria with diverse sulfur sources when sulfur is limiting.

5.2.2. Conformational changes involved in substrate binding

The FMN-dependent monooxygenase desulfonases expressed during sulfur limitation all have a TIM-barrel fold, with the active site located at the C-terminal end of the β-barrel [33,34]. As mentioned, these proteins vary from TIM-barrel structures by the presence of several insertion regions. Similar to other TIM-barrel proteins, there is an insertion sequence that contains a lid region positioned over the active site that

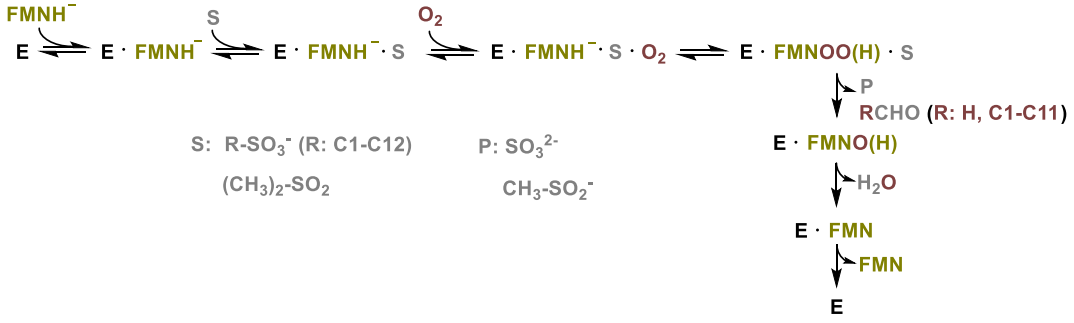


Fig. 9. Reaction scheme for TC FMN-dependent desulfonases. $FMNH_2$ binding leads to a conformational change that facilitates the binding of the organosulfur compounds. S represents the preferred substrates for MsuD (methanesulfonate), SsuD (C4–C12 octanesulfonates), and SfnG (dimethylsulfone). P represents the products for MsuD/SsuD (sulfite) and SfnG (methanesulfinate).

participates in substrate binding in the closed conformation [33–35]. The TC FMN-dependent monooxygenase desulfonases form a complex with the organosulfur substrate, $FMNH_2$, and O_2 prior to catalysis. Dynamic structural changes initiated by the lid region are associated with the binding of each substrate [34,35,45]. *Ec*SsuD could not bind the octanesulfonate substrate unless reduced FMN was bound first, and *Pf*MsuD structural studies also supported a model for the binding of methanesulfonate being dependent on FMN binding [34,42]. Computational and structural studies support a partial closing of the lid with $FMNH_2$ bound that reorganizes the active site [34,35]. A conserved Arg (Arg297) residue on the insertion sequence of SsuD protects flavin intermediates formed during catalytic turnover [45]. It was initially proposed that the Arg residue interacts with the phosphodianion of the FMN phosphate similar to other TIM-barrel enzymes, but there are no structures with substrates bound for SsuD. The loop region of *Pf*MsuD has a comparable conserved Arg (Arg296) residue in a similar position as SsuD [34]. Substitution of Arg296 to Ala resulted in the loss of desulfonation activity. The *Pf*MsuD three-dimensional structure with FMN and methanesulfonate bound showed an interaction of the conserved Arg residue with the sulfonate oxygens. Therefore, the desulfonases differ from canonical TIM-barrel proteins that utilize the conserved Arg residue to form electrostatic interactions with phosphate groups. There was no catalytic activity observed when the mobile loop region of *Ec*SsuD was deleted, and reduced FMN was not protected from unproductive oxidation [46]. A combination of hydrogen-bonding interactions and loop dynamics play a role in protecting the substrates from bulk solvent. The binding of $FMNH_2$ is proposed to promote conformational changes that promote alkanesulfonate binding and the proper orientation of catalytic residues.

5.2.3. *Oxygenating flavin intermediates involved in desulfonation*

While the TC FMN dependent enzyme involved in sulfur assimilation have different substrate specificities, they likely utilize a common overall mechanism for desulfonation based on the reaction catalyzed and their similar structural properties. The utilization of a C4a-(hydro) peroxyflavin as the oxygenating intermediate for flavin monooxygenase desulfonases had been proposed for decades. In some flavoprotein monooxygenases the C4a-(hydro)peroxyflavin is a stable intermediate that can be identified at 340 nm [47–50]. Based on previous studies with other two-component monooxygenase enzymes, two different mechanisms were proposed for *Ec*SsuD that involved a C4a-peroxyflavin or C4a-hydroperoxyflavin oxygenating intermediate [42,51]. Experimental evidence to support different aspects of the mechanism were obtained; however, a stable C4a-(hydro)peroxyflavin intermediate was never identified [42,51,52]. Even though similar stability was not observed for *Ec*SsuD, it was proposed that the reaction proceeded through a C4a-(hydro)peroxyflavin intermediate given the mechanistic information regarding flavin monooxygenases at that time.

In addition to the C4a-(hydro)peroxyflavin, the activation of molecular oxygen by reduced flavin in some two-component flavin-dependent monooxygenases occurs through a flavin-N5 oxygenating intermediate [53–60]. The anionic flavin semiquinone can form with high spin density at the N5 position of the isoalloxazine ring resulting in N5 adducts. Flavin-N5-oxide has been identified as an oxygenating intermediate and as the final end-product formed in catalysis. Many of these monooxygenase enzymes that use an N5-adduct are involved in C-X heteroatom bond cleavage, which plays a role in catabolism of organic molecules or degradation of xenobiotics [61]. To differentiate between flavin-N5 adduct intermediates it is crucial to investigate whether they can be identified within certain groups or classes of enzymes. Interestingly, a flavin-N5-peroxide has been proposed to be the oxygenating intermediate in several TC flavin monooxygenases. The two-component flavin-dependent enzymes involved in the initial studies share conserved structural properties with the monooxygenases involved in desulfonation (SsuD, MsuD, and SfnG) [53]. Specifically, conserved polar amino acid residues create a pocket for molecular oxygen binding and stabilization of the superoxide anion in enzymes proposed to form a flavin-N5-oxygenating intermediate [53]. In monooxygenases that use a C4a-(hydro)peroxyflavin oxygenating intermediate, the polar amino acids are missing. In addition, it was noted that non-polar residues occupy the oxygen binding site in these monooxygenases and would prevent the reaction of the superoxide anion with flavin at the N5 position [53,61]. The results from these studies suggest the active site architecture of flavin monooxygenases directs the reactivity between molecular oxygen and the flavin at the C4a or N5 position. Interestingly, the two-component flavin-dependent monooxygenase desulfonases contain similar conserved amino acid residues as enzymes that have been proposed to utilize a flavin-N5-peroxide intermediate. Based on recent findings, two different mechanisms have been proposed for the desulfonation enzymes (SsuD and MsuD) that involve a flavin-N5-peroxide intermediate (Fig. 10A) [34,62]. In the first mechanism, nucleophilic attack of the N5-peroxyflavin on the α-carbon of an alkanesulfonate would release the sulfite product and form a flavin-N5-peroxyalkane intermediate (Fig. 10B, upper path) [62]. Proton abstraction by an active site base on the α-carbon of flavin-N5-peroxyalkane would generate formaldehyde and N5-hydroxyflavin. Protonation of the N5-hydroxyflavin would generate water and the oxidized flavin product. A mechanism involving an N5-peroxyflavin has also been proposed for MsuD [34]. The N5-peroxyflavin abstracts a proton from methanesulfonate to form a carbanion that makes a nucleophilic attack on the distal hydroxyl on the N5-hydroperoxyflavin (Fig. 10B, lower path). An additional proton abstraction would lead to a rearrangement and desulfonation of the sulfonated substrate generating N5-hydroxyflavin that would resolve to form the oxidized flavin product. Recent computational investigations supported the mechanism proposed for *Pf*MsuD involving the N5 oxygenating intermediate over oxygen activation by C4a [63]. There have been no flavin-N5-peroxyflavin spectral intermediates observed; therefore, it is unclear which mechanism is more viable. The major difference between the two proposed mechanisms is the timing of sulfite release in the reaction path, which could be exploited in experiments to support a specific mechanism. A primary kinetic isotope effect for C1 deuterated octanesulfonate with *Ec*SsuD supported the deprotonation of C1 as the rate-limiting step, and solvent isotope effect studies support a protonation event after the rate-limiting step [52]. Therefore, both proposed mechanisms would be supported by the isotope studies. Recently the mechanism of SfnG from *A. baumannii* was proposed to occur through oxidation of dimethylsulfone to formaldehyde and methanesulfinate by flavin-generated hydrogen peroxide [11]. In the proposed mechanism hydrogen peroxide is deprotonated by an active site base. The peroxide intermediate then makes a nucleophilic attack on the methyl carbon of $DMSO_2$ generating methane sulfinate and methyl hydroperoxide that undergoes a rearrangement to formaldehyde. The use of substrate analogs and variants with altered catalytic properties could lend support to a particular pathway.

6. Summary

The preferred bacterial sulfur source, inorganic sulfate, is limiting in the terrestrial environment [1]. However, bacteria are adept at utilizing alternative organosulfur sources under these limiting conditions. The TC FMN-dependent desulfonase enzymes provide an alternative means of obtaining sulfur through acquisition from organic compounds [3,64,65]. The prevalence of these enzymes from diverse bacteria demonstrates the importance of maintaining adequate sulfur sources in bacteria. The similar function and overall structure of two-component FMN-dependent desulfonase systems involved in sulfur acquisition suggests the reductases and desulfonases utilize comparable mechanisms to catalyze diverse reactions. Combined structural and functional evaluation of these enzymes have provided valuable insight into the mechanistic advantage of having a separate FMN reductase and monooxygenase enzyme to catalyze a single reaction, and how they work

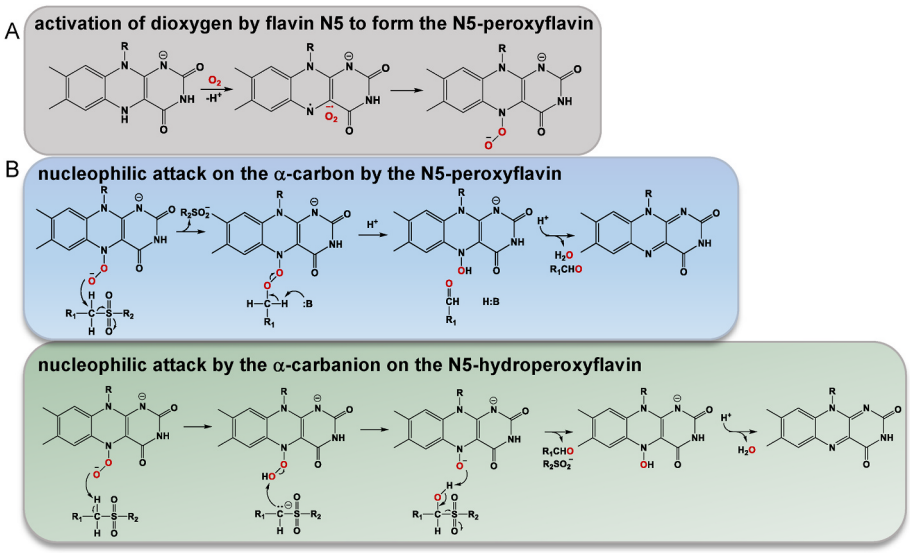


Fig. 10. Proposed mechanisms of the TC-FMO enzymes involved in sulfur assimilation. A. Dioxygen is activated to form the superoxide anion and reacts with the flavin-N5 to form N5-peroxyflavin. B. Proposed mechanisms for the TC-FMO desulfonases. Blue panel: The N5-peroxyflavin makes a nucleophilic attack on the α-carbon with the release of sulfite. The flavin-N5-peroxyalkane is resolved by an active site base releasing the aldehyde product. (B) The N5-peroxyflavin abstracts a proton to form N5-hydroperoxyflavin intermediate, and the α-carbanion makes a nucleophilic attack on the N5-hydroperoxyflavin. Abstraction of the hydroxyl proton by the flavin-N5-oxide forms the aldehyde product. In both mechanisms the N5-hydroxyflavin is resolved by the protonation of the hydroxyl group forming the oxidized flavin and water. R_1:-H for dimethylsulfone and methanesulfonate; C1-C11 alkanes for alkanesulfonates. R_2: CH_3 for dimethylsulfone; O^- for alkanesulfonates.

together to carry out their metabolic functions. A combination of oligomeric and conformational changes facilitates the regulation of flavin transfer and substrate oxidation. Structural and functional evaluation of these enzymes has provided insight into which catalytic steps and structural features are conserved among these enzymes, and how subtle modifications at the active site allow these enzymes to recognize specific substrates. These enzymes likely share similar catalytic steps for desulfonation but utilize distinct substrate recognition due to differences in the active site structure. The distinction in substrate recognition coupled with the novel chemistry involving C–S bond cleavage can be exploited to design new pathways for biocatalysis. Additional studies are needed to fully understand how the TC FMN-dependent reductases and desulfonases utilize similar coordinated mechanisms for desulfonation.

CRediT authorship contribution statement

Jeremy J.M. Liew: Writing – original draft. **Denyce K. Wicht:** Writing – original draft. **Reyaz Gonzalez:** Writing – original draft. **Daniel P. Dowling:** Writing – review & editing, Conceptualization. **Holly R. Ellis:** Writing – review & editing, Writing – original draft, Conceptualization.

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