Mechanistic Strategies of Two-component Flavin-dependent Monooxygenases Involved in Sulfur Acquisition

by

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Major Department: Biochemistry and Molecular Biology

Abstract

All organisms require sulfur for diverse metabolic and physiological processes. Unlike mammals, bacteria are often deprived of sulfur in their natural habitat and express various proteins that enable these microorganisms to utilize alternative sulfur sources during sulfur starvation. Some of the proteins expressed by bacteria are part of two-component flavin-dependent enzyme systems and include the alkanesulfonate monooxygenase (SsuE/SsuD), methanesulfinate monooxygenase (MsuE/MsuC), and methanesulfonate monooxygenase (MsuE/MsuD) systems. These two-component flavin-dependent systems consist of a flavin reductase (SsuE and MsuE) that supplies reduced flavin to the monooxygenase (SsuD, MsuC, MsuD) for the coordinated desulfonation of alkanesulfonates to obtain sulfite which is ultimately used in the synthesis of sulfur-containing biomolecules.

Of specific interest in this dissertation are the TIM-barrel enzymes, SsuD and MsuD, that share 65% amino acid sequence identity. A combination of kinetic and

computational analyses was performed to evaluate why these structurally and functionally similar monooxygenases had different substrate specificities. Even though SsuD was unable to utilize methanesulfonate and ethanesulfonate as a sulfur substrate in desulfonation assays, both SsuD and MsuD had similar binding affinities for reduced flavin and methanesulfonate. Structural analysis using accelerated molecular dynamic simulations revealed that methanesulfonate is not stabilized within the active site of SsuD due to the absence of a longer alkyl chain that provides an appropriate active site arrangement for catalysis. Under physiological conditions, SsuD would require molecular oxygen to successfully desulfonate alkanesulfonates and yield sulfite. While results from fluorometric titration experiments revealed that SsuD was able to bind methanesulfonate, this might not represent a catalytically active complex given that the experiments were performed anaerobically.

The two-component alkanesulfonate monooxygenase system (SsuE/SsuD) is commonly found in diverse bacteria highlighting its importance in adequately providing bacteria with alternative sulfur sources. The mechanism of this enzyme system is of extreme importance in bacterial sulfur acquisition; however, mechanistic details remain elusive. While SsuD has previously been proposed to employ a C4a-(hydro)peroxy flavin as an oxygenating flavin intermediate, a flavin-N5-oxide was identified as an oxygenating intermediate or formed as the final product during catalytic turnover by several bacterial flavin-dependent enzymes. Structural similarity with some of these enzymes led to the hypothesis that SsuD could also employ a flavin-N5-oxide as an oxygenating intermediate or as the final product. The results obtained from these studies revealed that SsuD does not form the flavin-N5-oxide as a final product, thereby suggesting that this flavin adduct

could be utilized as one of the oxygenating flavin intermediates in the reaction catalyzed by SsuD. Moreover, the studies presented in this dissertation focused on elucidating the roles conserved residues and structural features play in oxygen activation to perform the desulfonation reaction catalyzed by SsuD.

Even though the alkanesulfonate sulfonate monooxygenase system is commonly found in bacteria, *Pseudomonas* species utilize a more complex enzyme system to acquire sulfur during sulfur starvation. This includes the methanesulfinate (MsuE/MsuC) and the methanesulfonate (MsuE/MsuD) monooxygenase systems. MsuC is structurally distinct from SsuD and MsuD and relies on MsuE to supply reduced flavin for the oxidation of methanesulfinate to yield methanesulfonate. While flavoenzymes have been reported to undergo changes in the oligomeric state in the presence of substrates and products to efficiently transfer reduced flavin, the presence of substrates and products did not alter the structural and thermal stability of MsuC. Interestingly, rapid reaction kinetic experiments evaluating the reduced flavin transfer between MsuE and MsuC revealed that reduced flavin was protected by MsuC in the absence of methanesulfinate. This enzymatic protection prevents the autooxidation of reduced flavin which could subsequently result in the generation of reactive oxygen species.

Taken together, the findings presented in this dissertation provide structural and mechanistic insight into the roles diverse flavin-dependent monooxygenases fulfill during sulfur starvation. Given the fact that these enzymes are also found in a wide range of pathogenic bacteria such as the multi-drug resistant *Pseudomonas aeruginosa*, the structural and mechanistic information obtained from these studies could represent a notable target for rational drug design.

Mechanistic Strategies of Two-component Flavin-dependent Monooxygenases Involved in Sulfur Acquisition

A Dissertation

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Dedication

To my parents and sister:

Krish Somai, Shantie Sheombar,

and

Smriti Somai

I am very grateful for your unconditional love, wisdom, and encouragement throughout my life.

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ΔH_{vH}	van't Hoff's enthalpy	145
ABC	ATP-binding cassette	21
aMD	Accelerated molecular dynamics	97
APS	adenosine-5'-phosphosulfate	8
ArPP	5-amino-6-ribityl-amino-2,4(1H,3H)-pyrimidinedione 5'-phosphate	27
ATP	Adenosine triphosphate	2
B_{max}	maximum binding at equilibrium	94
CBS	cystathionine-beta-synthase	4
CD	circular dichroism	120
CF	cystic fibrosis	84
CG	conjugate gradient	97
ChrR	Quinone reductase	63
CSD	Cysteine desulfurases	13
CSE	Cystathionine-gamma-lyase	2
DARPP	2,5-diamino-6-ribosyl-amino-4(3H)-pyrimidinedione 5'-phosphate	27
DBT	dibenzothiophene	47
DBTO	dibenzothiophene sulfoxide	47
$DBTO_2$	dibenzothiophene sulfone	47
DHBP	3,4-dihydroxy-2-butanone-4-phosphate	27
DHPA	3,4-dihydroxylphenyl acetate	67
DMS	dimethyl sulfide	19

SSI Sulfate starvation-induced 15

ssu *Sulfate starvation utilization* 18

sulfo-SBED sulfo-N-hydroxysuccinimydyl-2-(6-[biotinamido]-2-(p-azidobenzamido)-hexanoamido) ethyl-1,3'-dithiopropionate 68

TIM Triosephosphate isomerase 72

T_m melting temperature 145

UPEC uropathogenic *E. coli* 23

UV-Vis Ultraviolet-visible 123

CHAPTER ONE

Literature Review

1.1 Introduction to Sulfur Metabolism

1.1.1 The Physiological Importance of Sulfur

Sulfur is one of the most common elements found on Earth and occurs in different forms ranging from sulfide or sulfate minerals to native sulfur, which are commonly found around volcanic regions.[1,2] Additionally, sulfur is present in the ocean as dimethyl sulfide and as an atmospheric trace gas. Because sulfur exists in multiple valence states (S^{2-} to S^{6+}), it can participate in different chemical processes.[1,3]

All organisms require sulfur for diverse metabolic and physiological processes.[4] Sulfur is an abundant mineral found in the cell and plays a critical role in growth, metabolism, and maintaining an appropriate redox balance within the cellular environment.[2,5-8] Out of the four-sulfur containing amino acids (methionine, cysteine, homocysteine, and taurine), only methionine and cysteine are involved in protein synthesis. Since methionine cannot be synthesized *de novo* in mammals, it is considered an essential amino acid and must be obtained through the diet. Cysteine, on the other hand, is considered non-essential since it can be obtained from methionine metabolism. Unlike mammals, bacteria synthesize both cysteine and methionine *de novo* which subsequently serve as a precursor for other sulfur-containing biomolecules.[4,9,10] Cysteine and methionine play many diverse roles in all organisms.[5,6,8,11]

Methionine is one of the most hydrophobic amino acids and is mostly found in buried locations within the hydrophobic core of globular proteins. Additionally, methionine is

responsible for initiating protein synthesis in the form of *N*-formyl methionine, in both eukaryotes and prokaryotes.[8] Beside its roles as a proteogenic amino acid, methionine is also involved in different metabolic processes as a key intermediate in transmethylation reactions, a precursor for polyamines, nucleotides, and phospholipids, and an intermediate in the choline metabolic pathway. Finally, methionine also serves as an outlet of the folate cycle involved in one-carbon metabolism and as a precursor for other sulfur-containing molecules including but not limited to cysteine, taurine, and glutathione (Figure **1.1**).[6-8,12-15]

The metabolism of methionine in mammals starts with the activation of *S*-adenosylmethionine (SAM) in the presence of ATP by methionine adenosyltransferase (MAT) (Figure **1.1**). Subsequent methyl transfer from SAM to a methyl acceptor leads to the formation of *S*-adenosylhomocysteine (SAH). Hydrolysis of SAH by SAH-hydrolase yields homocysteine (HCY) and adenosine. The aforementioned conversion of methionine to homocysteine and adenosine is referred to as transmethylation. Homocysteine can either be methylated back to methionine or can be further catabolized to cysteine. Remethylation of homocysteine is primarily catalyzed by B_{12}-dependent methionine synthase, however in the liver or kidney methylation can also occur by betaine-homocysteine methyltransferase which involves donation of a methyl group from betaine. The catabolism of homocysteine to cysteine is referred to as a transsulfuration which occurs in the liver, kidney, intestine, and pancreas. In the transsulfuration pathway, homocysteine is irreversibly converted to cysteine by the B_6-dependent enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE).[6-8,16] Similar to mammalian sulfur metabolism, bacterial sulfur metabolism shares some commonalities

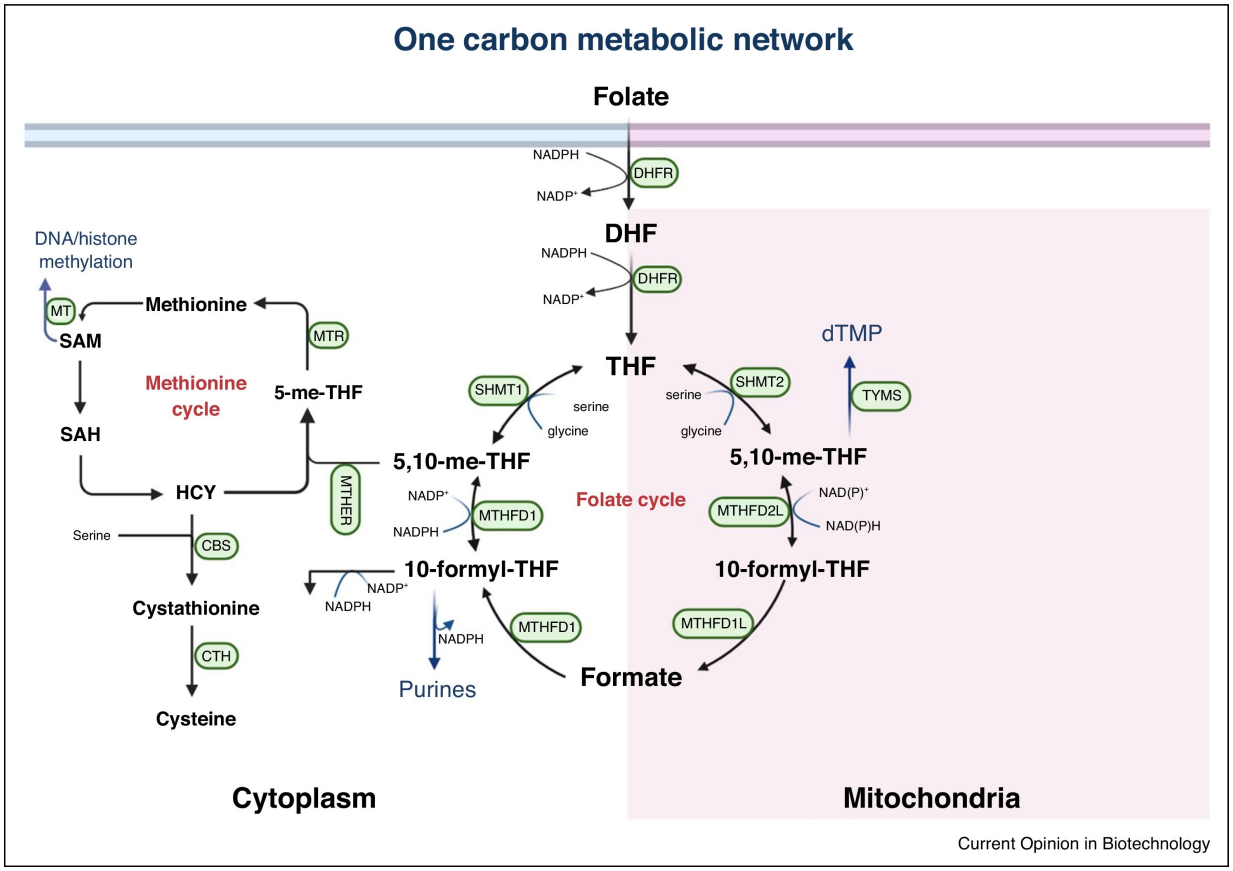

Figure 1.1: Methionine metabolism in mammals and its association with the folate cycle. (Adapted with permission from [318]). Copyright 2021 Elsevier Inc. All rights reserved.

which will be discussed further later in this chapter. Taken together, homocysteine is at the junction of the transmethylation and the transsulfuration pathway and is used as a marker for several diseases. High levels of homocysteine (hyperhomocysteinemia) have been associated with cardiovascular and neurodegenerative diseases such as dementia, Parkinson's disease, and Alzheimer's disease. Hyperhomocysteinemia that further results in several neurodegenerative diseases, has been identified as a result of deficiencies in folate, vitamin B_6, and vitamin B_{12}.[6,7] Hyperhomocysteinemia has also been reported to predominantly arise due to genetic defects of enzymes involved in

homocysteine metabolism which include methionine synthase, cystathionine β-synthase (CBS), and 5, 10-methylene tetrahydrofolate reductase.[17]

Accumulation of homocysteine can be prevented by catabolism that yields the amino acid cysteine. Cysteine plays a crucial role in the synthesis and secondary structure of different proteins due to its ability to form inter- and intrachain disulfide bonds with other cysteine residues. Cysteine is also involved in the production of other key cellular agents, including glutathione (GSH), hydrogen sulfide (H_2S), taurine and sulfate. Glutathione (GSH) is the most common thiol in cells and has different functions including detoxification, protecting macromolecules from reactive oxygen species (ROS), and regulation of protein structure and function.[2,5-8] Lastly, the desulfurization of cysteine results in the formation of hydrogen sulfide which is involved in the regulation of inflammation and apoptosis, preventing oxidative stress, and modulation of neurotransmission.[18-21] Hydrogen sulfide is mainly produced from the conversion of homocysteine to cysteine in the reverse transsulfuration pathway in all organisms. Homocysteine is converted to cysteine through the formation of cystathionine. This conversion is catalyzed by cystathionine-β-synthase and cystathionine-γ-lyase (CSE) in the cytosol. Hydrogen sulfide can also be produced in the reaction of mercaptopyruvate sulfur transferase (MST) which occur in both eukaryotes and prokaryotes. The substrate of mercaptopyruvate sulfur transferase, 3-mercaptopyruvate, is obtained through the deamination of cysteine (Figure **1.2A**). The reaction catalyzed by mercaptopyruvate sulfur transferase involves the donation of a sulfur atom by the 3-mercaptopyruvate substrate to an active site cysteine of the enzyme. This results in the formation of a persulfide intermediate which is subsequently transferred to an acceptor (R-SH) forming another

persulfide (R-S-SH) intermediate. The R-S-SH intermediate can react with another R-SH acceptor leading to the formation of a disulfide (RSSR) and hydrogen sulfide. Cystathionine-β-synthase, cystathionine-γ-lyase, and 3-mercaptopyruvate sulfur transferase are present in the brain, since hydrogen sulfide is involved in different physiological processes that occur in the nervous system.[18-20] Similar to homocysteine,

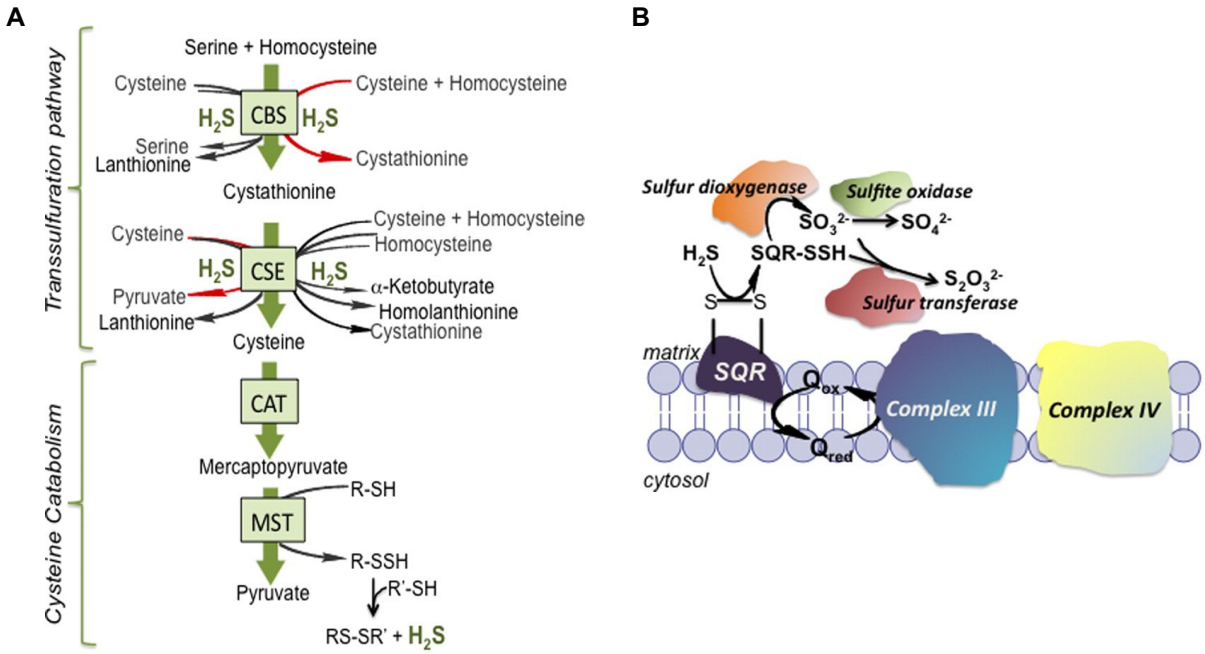

Figure 1.2: Biogenesis and catabolism of endogenously produced hydrogen sulfide. **A.** Hydrogen sulfide producing enzyme involved in the transsulfuration and cysteine catabolism pathways. **B.** The mitochondrial sulfide oxidation pathway coupled to the electron transfer chain. (Adapted with permission from [319]). Copyright. (2010). The Journal of Biological Chemistry.

dysregulation of hydrogen sulfide in humans is consequently linked to various neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, and Huntington's disease.[18] Hydrogen sulfide has been reported to bind and inhibit cytochrome c oxidase; an enzyme involved in the electron transport and subsequently in

ATP synthesis. Inhibition of cytochrome c oxidase (complex Ⅳ) results in overall ATP depletion, therefore leading to an overall decrease in cellular energy. Since the brain requires high amounts of energy for proper function, an overall decrease in ATP interferes with neuron functions such as neurotransmission resulting in various neurodegenerative diseases.[18,22,23]

Hydrogen sulfide at lower concentrations has been reported to have several advantages including regulation of inflammation and apoptosis.[20,24] However, at higher concentrations this sulfur compound becomes extremely toxic causing eye irritation, shortness of breath, chest tightness, and dizziness. At extremely high concentrations, hydrogen sulfide can cause severe damage to the central nervous system and can even lead to death. Moreover, high concentrations of hydrogen sulfide have been reported to inhibit cytochrome c oxidase and cellular respiration.[18,21,22,25] In order to prevent hydrogen sulfide toxicity, the bioavailability of this sulfur compound must be tightly regulated.[26,27] In mammals, hydrogen sulfide can be broken down by oxidation within the mitochondria. The breakdown of hydrogen sulfide involves the enzymes sulfide quinone oxidoreductase, persulfide dioxygenase, thiosulfate sulfur transferase (also known as rhodanese), and sulfite oxidase (Figure **1.2B**).[28-31] The main products of the oxidation of hydrogen sulfide are thiosulfate, sulfate, and sulfite. The oxidation of hydrogen sulfide within the mitochondria is linked with ATP synthesis.[28,30,32] Electrons obtained from hydrogen sulfide oxidation are transferred to the respiratory chain at the stage of coenzyme Q, thereby enhancing oxygen consumption which ultimately results in an energized inner mitochondrial membrane. Altogether, hydrogen sulfide exhibits both a stimulating and inhibiting role in mitochondrial respiration.[30,32]

Another important role of hydrogen sulfide is its involvement in bacterial antibiotic resistance. Antibiotic resistance of pathogenic bacteria has become a major health concern worldwide.[33-36] Pathogenic bacteria including but not limited to *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus)*, *Acinetobacter baumannii* are known to cause serious infections in patients. Treatment of these infections has become extremely challenging due to resistance of these bacteria to several antibiotics.[37,38] Therefore, this situation requires the development of alternative therapeutic strategies to combat these disease-causing pathogens. The mechanism by which antibiotics elicit their therapeutic effects involves promoting oxidative stress leading to damage of cellular macromolecules, and eventual cell death. The implication of reactive oxygen species (ROS) due to antibiotic usage has subsequently expanded the search of therapeutic strategies.[33,39,40] It has been previously discussed that bacteria produce hydrogen sulfide which has been reported to reduce oxidative stress. The ability to produce hydrogen sulfide has been reported to protect bacteria against oxidative stress induced which is induced upon treatment with antibiotics.[33,41,42] The enzymes involved in hydrogen sulfide production in bacteria have therefore become a promising therapeutic target.[33] Results from gene knockouts experiments with cystathionine-β-synthase, cystathionine-γ-lyase, and 3-mercaptopyruvate sulfurtransferase in *P. aeruginosa* and *S. aureus* revealed an increased sensitivity of these pathogens to various antibiotics. Infections caused by these pathogens typically involves the formation of persisters and biofilms which have a high tolerance to antibiotics.[43] Mutation of the hydrogen sulfide generating enzymes in *P. aeruginosa* and *S. aureus* have also resulted in a significant decrease in the formation of persisters. Persisters have been reported to generate high

amounts of hydrogen sulfide, leading to a decrease in ATP synthesis in addition to a controlled amount of self-poising.[33] This further results in slower rate of metabolism and a high tolerance to antibiotics. Altogether, the inhibition of hydrogen sulfide generating enzymes provide new approaches for drug design which includes the development of hydrogen sulfide inhibitors that could be utilized to combat these disease-causing pathogens.[44]

1.1.2 Sulfur Acquisition by Bacteria

Sulfur is a key component of life due to its involvement in different metabolic, geochemical, and chemical reactions.[1,8,16] Different organisms require sulfur for the synthesis of cysteine and methionine which are further used for diverse metabolic needs.[8,16] Bacteria predominantly rely on sulfate compounds as their main sulfur source. Sulfate can be further assimilated to yield the sulfur containing amino acids cysteine and methionine which are subsequently used for different metabolic purposes (Figure **1.3**).[4] The sulfate compounds are first transported inside the cell using an ATP-binding cassette consisting of ABC-transport proteins.[45] Since the reduction of sulfate to sulfide requires a large amount of energy, sulfate needs to be activated through a reaction with ATP (Figure **1.3**, step 1). This ATP-dependent reaction is catalyzed by ATP sulfurylase and produces adenosine 5'-phosphosulfate (APS). APS is subsequently phosphorylated by APS kinase to 3'-phosphoadenosine 5'-phosphosulfate (PAPS) in a second ATP-dependent reaction (Figure **1.3**, step 2). PAPS is further reduced to sulfite followed by sulfide by the enzymes PAPS reductase and sulfite reductase, respectively (Figure **1.3**, step 3 and 5).[46] Bacterial APS and PAPS reductases have been reported to share a high sequence similarity,

enabling different bacteria to bypass the formation of PAPS (Figure **1.3**, step 4). A similar mechanism has also been observed in plants, algae, and phototrophic bacteria where

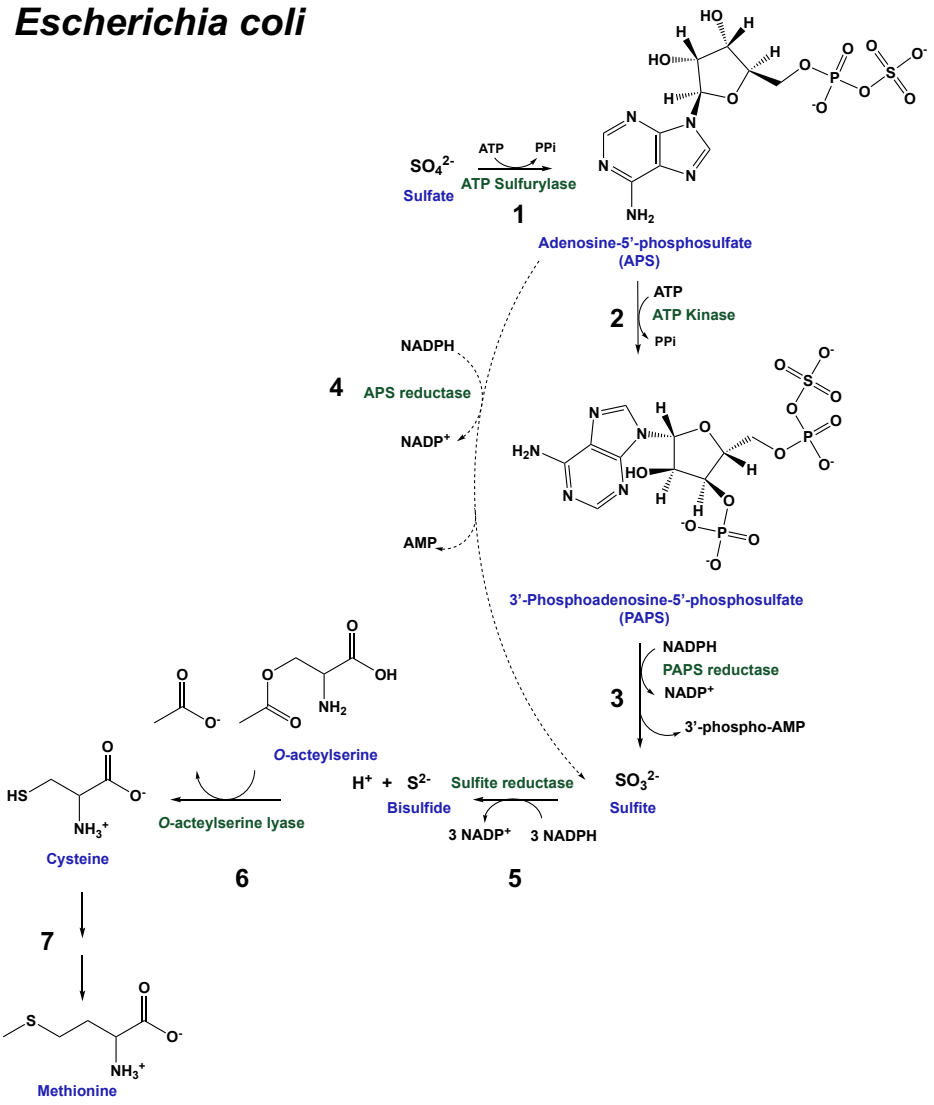

Figure 1.3: Proposed sulfur assimilation pathway and cysteine biosynthesis in *E. coli*. Certain microorganisms and plants can bypass the formation of PAPS. (Adapted from [4,50,312]).

APS is directly reduced to sulfite.[47] The sulfide produced from the reduction of sulfite, reacts with an activated amino acid acceptor which includes *O*-acetylserine, *O*-acetylhomoserine, or *O*-succinylhomoserine to synthesize cysteine which is further

incorporated in other sulfur-containing compounds.[4,47,48] Enteric bacteria such as *Escherichia coli* (*E. coli)* have been reported to synthesize cysteine from the reaction of sulfide and *O*-acetylserine, catalyzed by the enzyme *O*-acetyl-L-serine sulfydrylase (Figure **1.3**, step 5). In the following step, cysteine displaces the succinyl group of *O*-succinylhomoserine to produce cystathionine, a reaction which is catalyzed by cystathionine γ-synthase. β-Elimination of cystathionine catalyzed by cystathionine β-lyase yields homocysteine, ammonia, and pyruvate. In the final step, methylation of homocysteine results in the formation of methionine (Figure **1.3**, step 6). Unlike enteric bacteria; *P. aeruginosa*, *Bacillus*, *Rhizobium*, *Corynebacterium*, *Arthrobacter*, and yeast can obtain methionine through direct sulfhydrylation. The sulfide that is formed in these organisms is immediately transferred to *O*-acetylserine to form homocysteine; a reaction catalyzed by homocysteine synthase (Figure **1.4**, step 6). These organisms typically use homocysteine for the direct synthesis of methionine and are also able to convert cysteine through the reverse transsulfuration pathway (Figure **1.4**, step 7, 8, and 9).[4,48]

Similar to other organisms, sulfur-containing compounds fulfill many key roles in bacteria.[4] In addition to being involved in growth and metabolism, sulfur is also critical for bacterial pathogenicity. Since sulfate is considered the main sulfur source for bacteria, the enzymes involved in the sulfur assimilation pathway represent a notable target for drug development.[49] Inhibition of enzymes involved in this pathway could result in the disruption of cellular redox homeostasis which can be detrimental for pathogenic bacteria. This strategy has been employed in different studies with the human pathogen *Mycobacterium tuberculosis*.[50] Mutation of the genes within the sulfur assimilation pathway has been reported to decrease virulence in macrophage and mouse tuberculosis

infection models. In a different study with *Mycobacterium tuberculosis*, inhibitors were designed to target APS reductase which is the enzyme that catalyzes the first committed

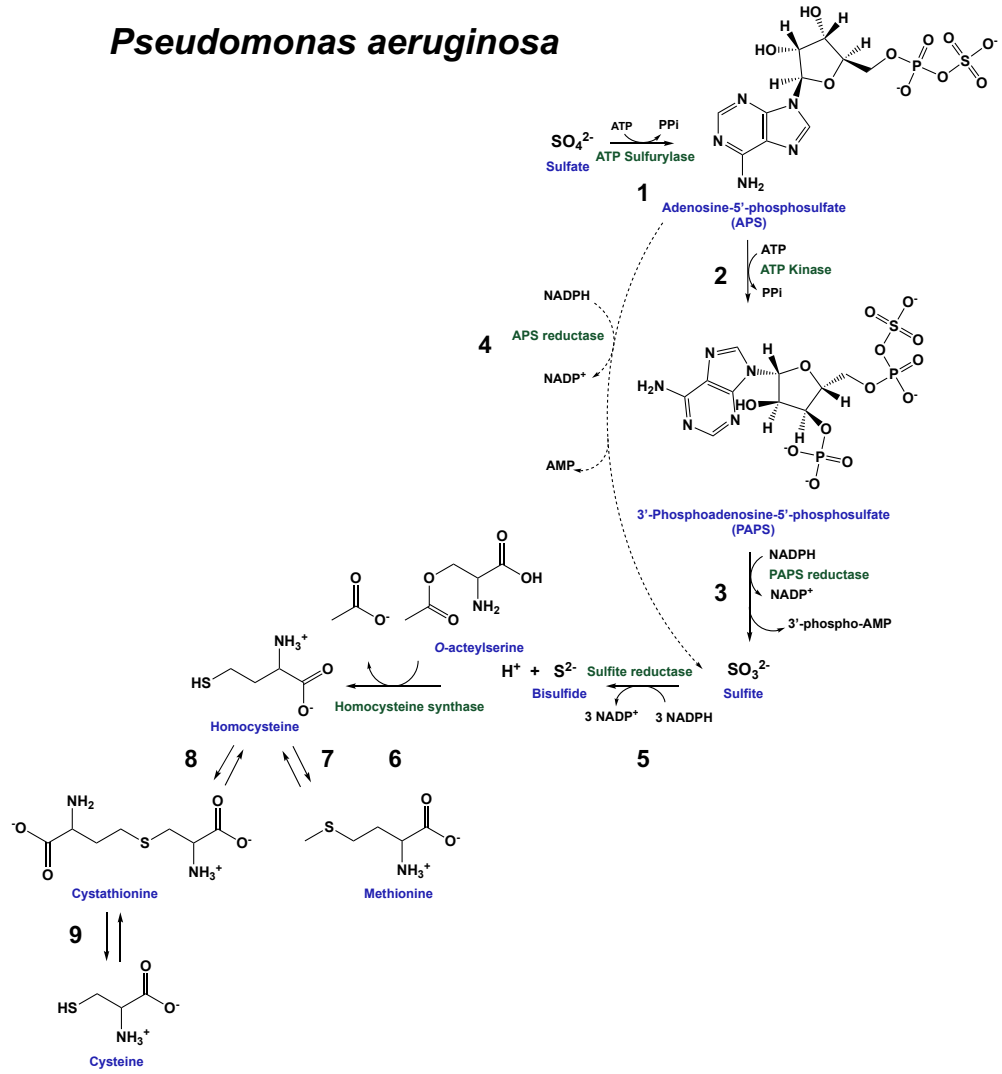

Figure 1.4: Proposed sulfur assimilation pathway and cysteine biosynthesis in *P. aeruginosa*. Certain microorganisms and plants can bypass the formation of PAPS. (Adapted from [4,50,312]).

step in the sulfur assimilation pathway. The inhibitors were reported to display potent bactericidal activity in both wild-type and certain clinical isolates of the pathogen. APS reductase is found in numerous pathogenic bacteria including *P. aeruginosa* and

Salmonella typhimurium; however, this enzyme is not present in mammals.[49] Therefore, inhibition of this enzyme could be a promising strategy for the development of broad-spectrum antibiotics.[50]

1.1.3 The Incorporation of Sulfur in Sulfur-containing Biomolecules

Besides being involved in the synthesis of amino acids, sulfur is also required for the synthesis of cofactors, vitamins, iron-sulfur clusters, and nucleosides that are involved in a variety of reactions (Figure **1.5**). In order to be incorporated in these biomolecules,

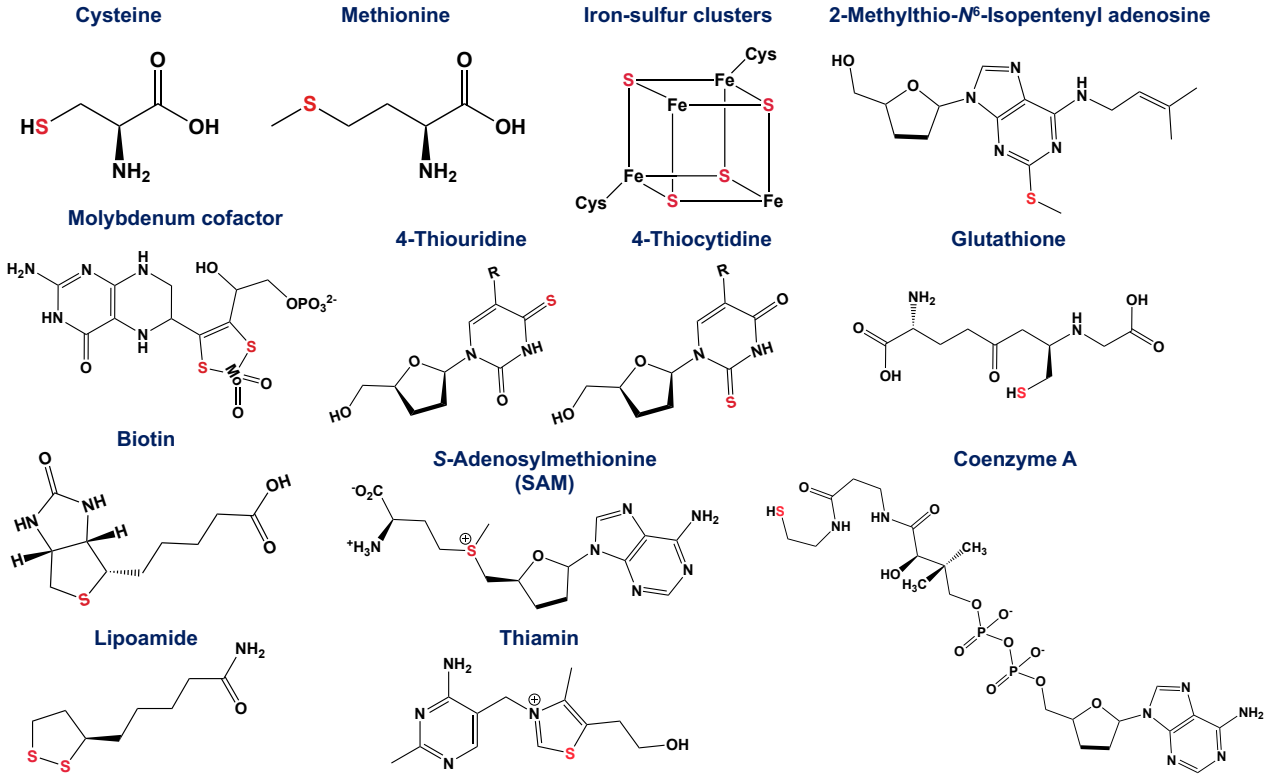

Figure 1.5: Sulfur-containing biomolecules. (Adapted from [52]).

sulfur needs to be reduced and activated.[51] Sulfate and polysulfides are commonly found in nature and serve as substrates for different reductases that further reduce these compounds to sulfide. The synthesis of sulfur-containing biomolecules is proposed to involve the formation of a persulfide intermediate (R-S-SH) which ensures safe trafficking

and delivery of the sulfide.[52] Once sulfide is obtained, it reacts with an active site cysteine residue to form an enzyme persulfide intermediate. The enzyme persulfide intermediate serves as a sulfur donor to an acceptor molecule. Different mechanisms for sulfur donation to acceptor molecules have been proposed. In the first proposed mechanism, the sulfur acceptor performs a nucleophilic attack onto the enzyme persulfide intermediate. In the second mechanism, the sulfur acceptor functions as an electrophile while the enzyme persulfide intermediate performs the nucleophilic attack on a thiol group of the acceptor molecule. In the third proposed mechanism, the enzyme itself participates in sulfur transfer to an iron-sulfur (Fe-S) cluster onto the acceptor.[53] The decomposition of persulfides results in various products including thiols, hydrogen sulfide, disulfides, polysulfides and elemental sulfur (S_8). Persulfides are known to be catalytically versatile, enabling these compounds to participate in several metabolic pathways.[54]

The biosynthesis of sulfur-containing biomolecules in eukaryotes and prokaryotes also involves the amino acid cysteine. Sulfur mobilization in these organisms is performed by enzymes referred to as cysteine desulfurases. Cysteine desulfurases utilize a pyridoxal-5'-phosphate (PLP) cofactor for the abstraction of a sulfur atom from cysteine followed by the subsequent transfer to an acceptor molecule (Figure **1.6A**). The abstraction of a sulfur atom from L-cysteine leads to the formation of L-alanine and an enzyme persulfide intermediate.[55] This enzyme persulfide intermediate is required for the mobilization and delivery of sulfur to various acceptor molecules.[52] Eukaryotes and prokaryotes utilize different cysteine desulfurases such as NifS, CsdB, IscS, CsdA (CSD), and SufS.[56-58] NifS and CsdB are homologs that are involved in the biosynthesis of the nitrogenase iron-sulfur (Fe-S) cluster.[56] Additionally, IscS is involved in the biosynthesis

of Fe-S clusters, thionucleosides, and thiamin. IscS also incorporates selenium in selenoproteins as well as selenouridine residues in tRNA. Moreover, the SufS system is functionally similar to the IscS system; however, SufS is used during extreme conditions

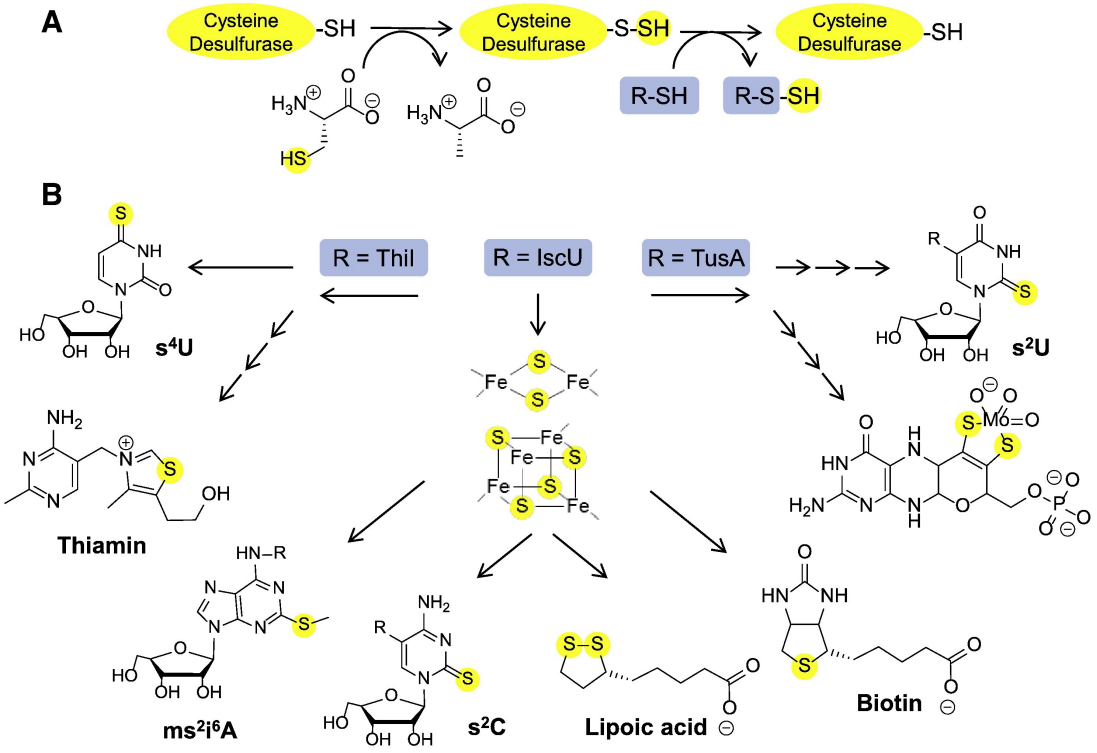

Figure 1.6: Biosynthesis of sulfur-containing biomolecules in *E. coli*. **A.** The transfer of a sulfur atom from L-cysteine to form a persulfide intermediate catalyzed by cysteine desulfurase. **B.** Biosynthetic pathways for the formation of sulfur-containing biomolecules. (Adapted from [53]).

such as oxidative and heavy metal stress as well as iron starvation.[51-53,59,60] Lastly, the CSD (cysteine sulfinate desulfinase) system consisting of CsdA and CsdE, are homologs of SufS and SufE, respectively. CsdA and CsdE are both involved in Fe-S cluster biosynthesis and have been reported to supply sulfur to the Suf machinery.[61] Due to the functional overlap, most organisms require a single cysteine desulfurase serving as a

core for sulfur mobilization in the biosynthesis of thionucleosides, thiamin, iron-sulfur cluster, molybdenum cofactor, biotin, lipoic acid, *S*-adenosyl methionine (SAM), and coenzyme A (Figure **1.6B**). However, additional cysteine desulfurases might be needed depending on the physiological and environmental conditions.[53]

1.2 Sulfur Starvation in Bacteria

1.2.1 Sulfate Starvation Induced Proteins

Inorganic sulfate and cysteine are the main sulfur sources bacteria rely on to meet their sulfur needs. In a laboratory setting, bacteria are typically grown with LB media or minimal media which contain high amounts of inorganic sulfur and cysteine. Inorganic sulfate present in soil typically varies between 1-5% of the total available sulfur content. The remaining 95% of sulfur present in soil exist as organosulfur compounds such as sulfonates, sulfate esters, and sulfamates.[4] Due to the limited availability of inorganic sulfate, bacteria have evolved to utilize specific survival strategies which includes obtaining sulfur from alternative sulfur sources.[4] One of the main alternative sulfur sources bacteria rely on are organosulfonates. Organosulfonates can be obtained from different sources ranging from industrial activities that produce aromatic sulfonates to photo-oxidation of dimethyl sulfide that yields methanesulfonate. Other sources also include animal and algal metabolic by-products that yield taurine, sulfonolipids from plants, or oxidized cysteine.[4,62,63]

Previous studies using two-dimensional gel electrophoresis identified different proteins that were expressed in the absence of sulfate, cysteine, or thiosulfate. These proteins are referred to as the sulfate starvation-induced proteins (SSI proteins) and are found in various Gram-positive and Gram-negative bacteria.[64-66] The SSI proteins found

in *E. coli* have been studied in detail and are encoded by two separate operons, namely the *tauABCD* and the *ssuEABCD* operon (Figure **1.7**). The proteins encoded by *tauABCD* are involved in the uptake and desulfonation of taurine (2-aminoethanesulfonic acid),

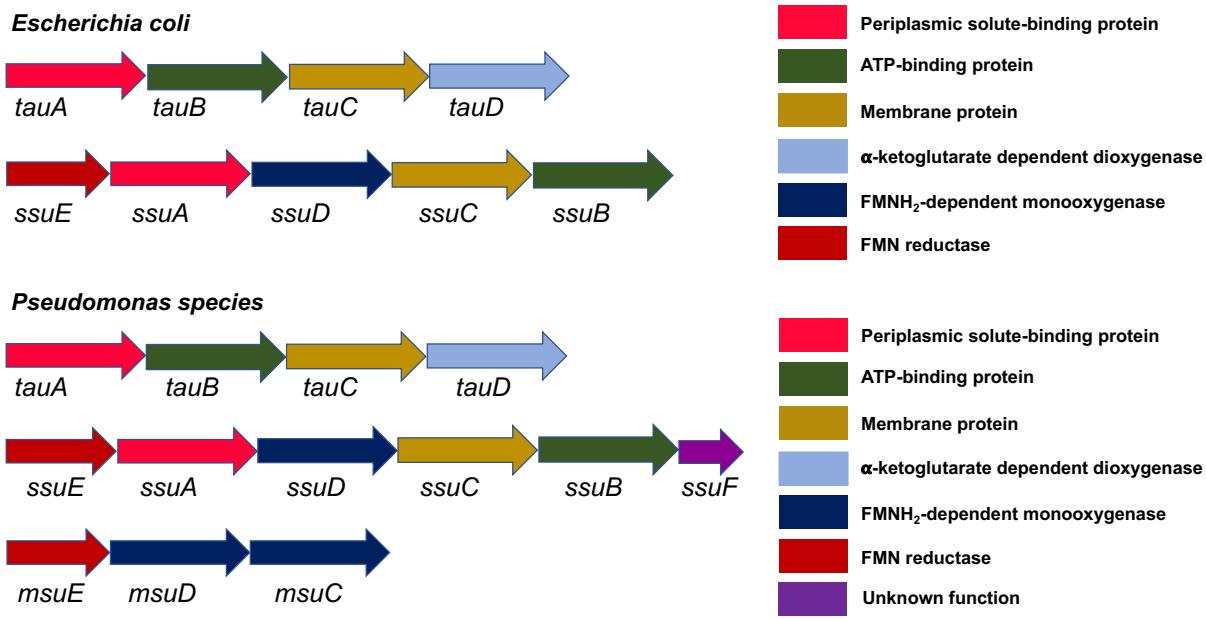

Figure 1.7: Genetic organization of the transporters and enzymes responsible for the desulfonation of taurine and alkanesulfonates in *E. coli* and *Pseudomonas* species. (Adapted from [4,67]).

whereas the *ssuEABCD* genes are required for the uptake and desulfonation of various alkanesulfonates. The process of desulfonation involves the enzymatic cleavage of the carbon-sulfur bond from organosulfonates to yield sulfite.[65-67] The sulfite that is produced can be further used in the synthesis of various sulfur-containing biomolecules. Previous studies with the *tau* and the *ssu* operon revealed that the genes are repressed in the presence of sulfate, cysteine, and thiosulfate. Additionally, it was also revealed that deletion of each cluster resulted in the inability of *E. coli* to utilize various alkanesulfonates as an alternative sulfur source.[65-68]

In the *tau* operon, *tauD* encodes for an iron-dependent α-ketoglutarate-dependent dioxygenase which is involved in the desulfonation of taurine to produce sulfite and aminoacetaldehyde (Figure **1.8**).[4,67] The reaction of TauD involves the initial binding of α-

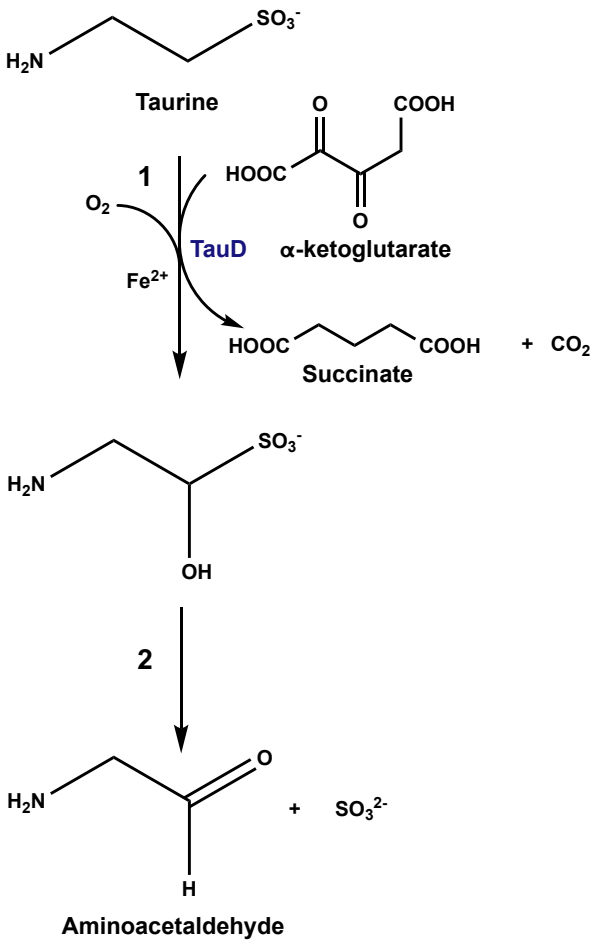

Figure 1.8: Desulfonation of taurine by TauD generating aminoacetaldehyde. (Adapted from [65]).

ketoglutarate in a six-coordinate Fe(Ⅱ) complex followed by the binding of taurine in a five-coordinated Fe(Ⅱ) complex. Molecular oxygen is the third substrate to bind after which succinate and carbon dioxide are released and the Fe(Ⅳ)=O intermediate is formed (Figure **1.8**, step 1). This intermediate is required for the incorporation of oxygen into the substrate to yield aminoacetaldehyde and sulfite (Figure **1.8**, step 2). In addition

to taurine, sulfonates including butanesulfonate, pentanesulfonate and MOPS (3-(*N*-morpholino) propanesulfonic acid) could also serve as a potential substrate for TauD.[65,67,69]

Besides *tauD*, the *ssuE* and *ssuD* genes in the *ssu* (sulfate starvation utilization) operon encode for an NAD(P)H:FMN reductase and an alkanesulfonate monooxygenase, respectively. SsuE and SsuD are part of a two-component system, where SsuE supplies reduced flavin to SsuD (Figure **1.9**).SsuD utilizes reduced flavin obtained from SsuE for

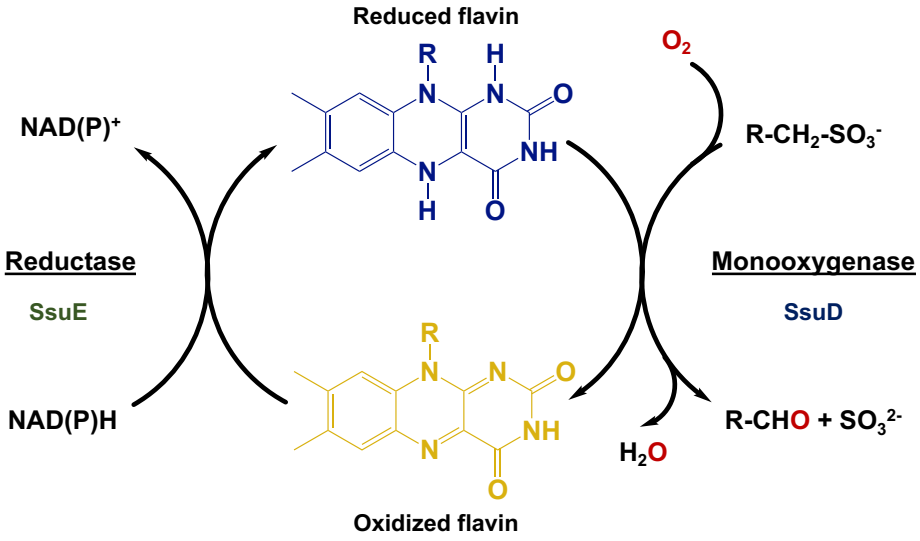

Figure 1.9: Desulfonation of alkanesulfonates generating sulfite catalyzed by the two-component enzymes SsuE and SsuD. (Adapted from [99]).

the activation of molecular oxygen followed by the oxidation of various alkanesulfonates to yield sulfite and form the corresponding aldehyde, water and regenerate FMN.[66] Unlike TauD where both oxygen atoms are incorporated in the product, SsuD incorporates a single oxygen atom in the corresponding aldehyde, whereas the other oxygen atom is reduced and released as water. The SsuE/D system has been reported to desulfonate alkanesulfonates ranging from C2-C10 in carbon chain length. Additionally, this system

has also been reported to utilize 1,3-dioxo-2-isoindolineethanesulfonate, 2-(4-pyridyl)ethanesulfonate, substituted ethanesulfonic acids, *N*-phenyltaurine, 4-phenyl-1-butanesulfonic acid, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), PIPES (piperazine-N,N′-bis(2-ethanesulfonic acid), and MOPS as a potential substrate.[66,70] Homologs of SsuE and SsuD are found in a wide range of bacteria and generally contain both the *ssuE* and *ssuD* gene on the same operon.[71] However, some exceptions include *Bacillus subtilis*, *Burkholderia cenocepacia*, and *Corynebacterium glutamicum* where the *ssu* operon does not contain an *ssuE* gene. For *Bacillus subtilis*, previous studies have reported the presence of a flavin reductase encoded in a separate operon.[72-76] Moreover, it has been proposed that organisms lacking the *ssuE* gene could utilize alternative unrelated flavin reductases for the reduction and subsequent transfer of reduced flavin to SsuD.[77,78] Our lab has identified a gene encoding for an oxidoreductase in the *ssu* operon within the genome of *Bacillus subtilis*. There could be a possibility that the *ssuE* gene from *Bacillus subtilis* has been misannotated in previous studies.

In addition to the *tau* and *ssu* operons, different *Pseudomonas* species have also been reported to utilize arylsulfonates as well as oxidation products of dimethyl sulfide (DMS) as alternative sulfur sources (Figure **1.7**). The genes required for the coordinated desulfonation of dimethyl sulfone, methanesulfinate, and methanesulfonate are encoded by the *sfnFG* and the *msuEDC* operon.[4,71] The *sfnF* gene encodes for an NAD(P)H:FMN reductase that supplies reduced flavin to the dimethyl sulfone monooxygenase which is encoded by the *sfnG* gene (Figure **1.10**). SfnG uses reduced flavin to catalyze the

oxidation of dimethyl sulfone to yield methanesulfinate. The *msuE* gene in the *msuEDC* operon encodes an NAD(P)H:FMN reductase similar to SsuE, whereas the *msuD* and

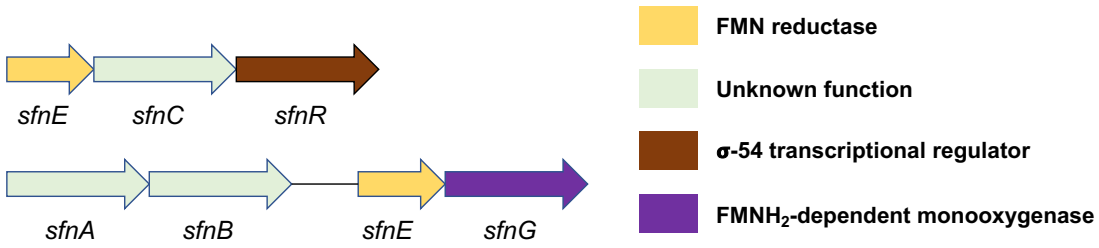

Figure 1.10: Genetic organization of the *sfn* genes involved in the utilization of organosulfonates by *Pseudomonas* species. (Adapted from [96]).

msuC genes encode for a reduced flavin-dependent methanesulfonate monooxygenase and methanesulfinate monooxygenase, respectively. Initial studies with *P. aeruginosa* identified two sets of *ssu* operons, namely *ssuEADCB* and *ssuEDC*.[4,75,79] The latter was revised to the *msuEDC* annotation, thereby identifying the MsuE/C and MsuE/D two-component enzyme systems.[75,79] The *ssu* operon in different *Pseudomonas* species was also reported to contain an extra *ssuF* gene, which was hypothesized to either be a transporter protein or to be involved in the desulfonation of arylsulfonates.[71] In addition to the *ssu*, *sfn*, and *msu* operons, *Pseudomonas* species also expresses the *asf* and *ats* operon which is involved in the uptake, transport, and utilization of aryl sulfonates (and aromatic sulfate esters) as an alternative sulfur source during sulfur limitation. The presence of the *ssu*, *sfn*, *msu, asf*, and *ats* gene clusters in *Pseudomonas* species equips these organisms to utilize a broader range of alternative sulfur sources during sulfur starvation.[4,80]

1.2.2 Transport of Alkanesulfonates in the Bacterial Cell

The genes expressed by the *tau* and *ssu* operons enable bacteria to utilize alternative sulfur sources in the form of taurine and organosulfonates during sulfur starvation.[4,66,69] The *tau* and *ssu* operons both consist of ABC transporter proteins which are required for the uptake of taurine and organosulfonates (Figure **1.11**).[65,81] The ATP-

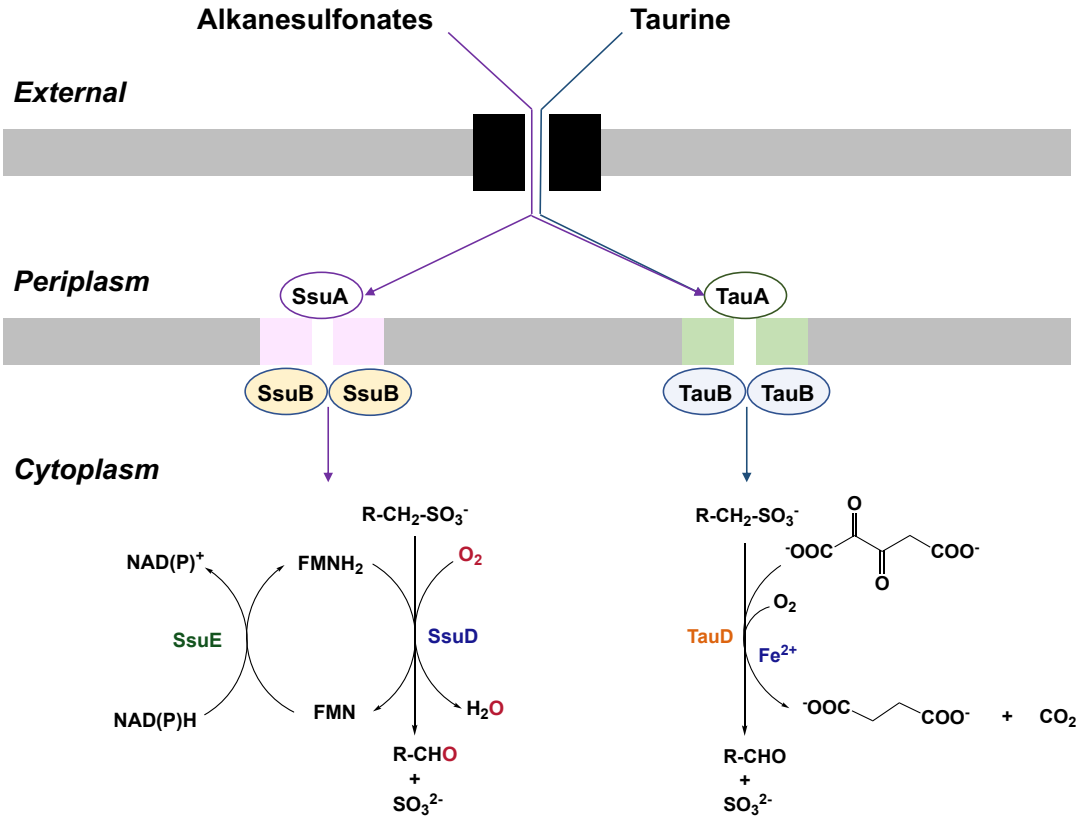

Figure 1.11: The uptake of alkanesulfonates and taurine by *E. coli*. (Adapted from [67]).

binding cassette (ABC) transporter superfamily belongs to one of the largest classes of membrane proteins. These proteins utilize energy from the hydrolysis of ATP for the translocation of different molecules across membranes. ABC transporters are found in both eukaryotes and prokaryotes where they function as either importers or exporters.[82-84] ABC transporters that function as importers are responsible for providing the cell with

nutrients, whereas exporters remove toxins and unwanted molecules from the cell.[84] These types of transporters generally consist of two transmembrane domains that transverse the membrane bilayer, and two nucleotide binding domains which are found in the cytoplasm. The nucleotide binding domains of ABC transporters are reported to be conserved in sequence and structure. ABC transporters from prokaryotes functioning as importers also contain an additional high-affinity binding protein within the periplasm that associates with specific compounds for the subsequent delivery to the proper transporter.[84,85]

The *ssuABC* genes needed for the uptake of organosulfonates encode for a periplasmic solute-binding protein (SsuA), an ATP-binding protein (SsuB), and a membrane protein (SsuC). The *tauABC* genes required for the uptake of taurine, encode for similar proteins.[81] Mutations in the *ssuABC* and *tauABC* genes have been reported to result in the inability of bacteria to utilize the alternative sulfur sources, organosulfonates and taurine, respectively.[4,68] Additionally, deletion of either SsuA, SsuB or SsuC prevented *Corynebacterium glutamicum* and *Bacillus subtilis* from growing on shorter alkanesulfonates; however, this deletion did not affect the ability of these bacteria to grow on long chain alkanesulfonates. It has been proposed that *Corynebacterium glutamicum* could utilize an alternative system, supposedly a fatty acid transporter for the uptake and transport of long chain alkanesulfonates.[74] In bacteria that contain both gene clusters, such as *E. coli*, the *tauABC* genes were found to be responsible for the uptake of shorter alkanesulfonates including taurine, whereas the *ssuABC* genes were essential for the uptake of most alkanesulfonates. Deletion of either one of the transporters did not have an influence on the other.[67,68,74] Besides expression during sulfur limitation, the *tau* and

ssu gene clusters have also been reported to be upregulated in uropathogenic *E. coli* (UPEC) involved in different diseases such as human urinary tract infections. The presence of the *tau* and *ssu* genes in different diseases could be an interesting drug target against several pathogenic bacteria.[86]

1.2.3 Regulation of the *ssi* Operons

The expression of the *ssu* and *tau* operons in *E. coli* are under control of the CysB protein, a LysR-transcriptional regulator.[81,87] LysR-transcriptional regulators are DNA-binding proteins that are involved in the regulation of specific genes.[88] In addition to the *ssu* and *tau* operons, CysB also regulates genes (*cysKJIHPTWAM*) involved in cysteine biosynthesis and sulfate metabolism in enteric bacteria (Figure **1.12,** step B).[89,90] Two sets of compounds referred to as inducers and anti-inducers enable CysB to switch

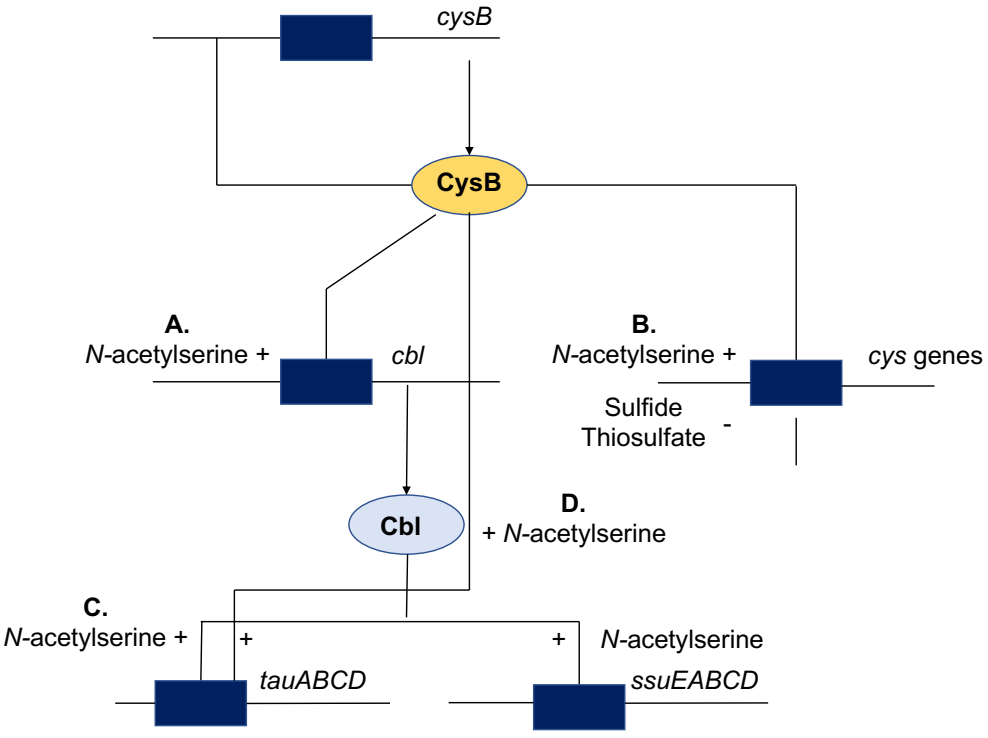

Figure 1.12: Regulation of sulfur assimilation in *E. coli*. (Adapted from [4]).

between the active and repressive states. While sulfide and thiosulfate are known as the anti-inducers of CysB, *O*-acetyl-serine and *N*-acetyl serine are considered the inducers of CysB. The intracellular levels of the inducers and anti-inducers subsequently influence the functional state of CysB. During sulfur limitation decreased sulfide levels are observed, whereas *O*-acetyl-serine and *N*-acetyl serine accumulate resulting in the activation of CysB.[88-90] The activation of CysB leads to expression of sulfate transporters and enzymes of sulfate metabolism (*ssuEABCD* and *tauABCD*) which ultimately results in increased levels of sulfide followed by deactivation of CysB.[81,87]

The expression of the *ssu* and *tau* operons that enable bacteria to utilize alternative sulfur sources, requires CysB in addition to another LysR-transcriptional activator, Cbl (Figure **1.12**, step A). CysB and Cbl share ~41% amino acid sequence identity and are both required for the expression of the *ssi* operon.[81,87,91] Mutations of *cbl* led to the inability of bacteria to utilize taurine and a range of aliphatic sulfonates as an alternative sulfur source during sulfur limitation. It was also revealed that both CysB and CbI are necessary for the expression of the *tau* operon (Figure **1.12**, step C).[87] The roles of CysB and CbI were further investigated with the *ssu* operon, and it was reported that CysB served as an activator for the expression of the *cbl* gene and is not directly involved in the expression of the *ssu* operon (Figure **1.12**, step D).[81,87] For the expression of the *ssu* operon, Cbl binds upstream of the -35 region. Conversely, for the *tau* operon, Cbl binding occurs at -112 to -68 relative to the transcription start. The *tau* operon requires the activation of both CysB and Cbl for expression. Under sulfate-rich conditions the expression of the *ssu* and *tau* operons are repressed due to the presence of adenosine-5'-phosphosulfate which functions as a corepressor of Cbl.[81,87,92]

Similar to enteric bacteria, *Pseudomonas* species also contain CysB as the transcriptional regulator for organosulfur assimilation. However, unlike enteric bacteria *Pseudomonas* species might utilize an alternative mechanism due to the absence of the *cbl* gene.[4,48] In addition to the *ssu* and *tau* operons, *Pseudomonas* species utilize a more complex mechanism for sulfur acquisition that involves the *sfn* and *msu* operons.[4,48,93] The products of the *sfn* and *msu* operons enable *Pseudomonas* species to use oxidation products of dimethyl sulfide as alternative sulfur sources during sulfur limitation.[4,94] Studies with *Pseudomonas putida* DS1 identified the transcriptional regulator, SfnR, that was shown to be required for the expression of the *sfnFG* operon. Moreover, SfnR from *P. putida* allowed this bacterium to utilize dimethyl sulfoniopropionate (DMSP), methionine sulfone, dimethyl sulfide, dimethyl sulfoxide, and dimethyl sulfone as alternative sulfur sources.[95-97] *P. aeruginosa* PAO1 can metabolize DMSP to methanethiol via the demethylation pathway, and the methanethiol can be further converted to generate hydrogen sulfide or dimethyl sulfide. Even though SfnR has been identified as a transcriptional regulator in *Pseudomonas* species, organosulfur utilization during sulfur limitation has not been fully investigated.[95,97]

Many of the SSI enzymes are part of two-component flavin-dependent systems.[4] Besides sulfur acquisition, two-component enzymes are involved in a broad range of reactions. While some of the two-component enzymes utilize flavin as a prosthetic group, the two-component enzymes utilize flavin as a co-substrate in the overall reaction. The alkanesulfonate monooxygenase system, the methanesulfinate monooxygenase system, and the methanesulfonate monooxygenase system all utilize flavin as a co-substrate rather than a bound prosthetic group.[98] This further means that the enzymes belonging

to these systems are purified in the absence of flavin. The absence of flavin as a prosthetic group brings additional challenges with mechanistic evaluation of this enzymes which will be discussed later in this chapter.[99]

1.3 Flavin Enzymology

1.3.1 History of Flavin

Riboflavin was first discovered and isolated from milk in 1879 by the chemist Winter Blyth.[100,101] The isolated compound had a bright yellow color and was initially called lactochrome ("*lacto*" is Latin for milk). After years of extensive research, it was found that this yellow compound was a component of the vitamin B complex and was named riboflavin. The term flavin is used for compounds that contain the 7,8-dimethylisoalloxazine ring which is considered the active portion of these molecules. The name riboflavin does not only indicate the color ("*flavus*" is Latin for yellow) of this vitamin, but also refers to the ribityl sugar side chain at the N10 position of the isoalloxazine ring (Figure **1.13**).[102,103] Riboflavin (vitamin B_2) is a heat-stable, water-soluble vitamin mainly found in milk, dairy, and meat products.[101,102] Even though riboflavin is not directly involved in metabolism, it serves as the precursor for the flavin coenzymes, flavin

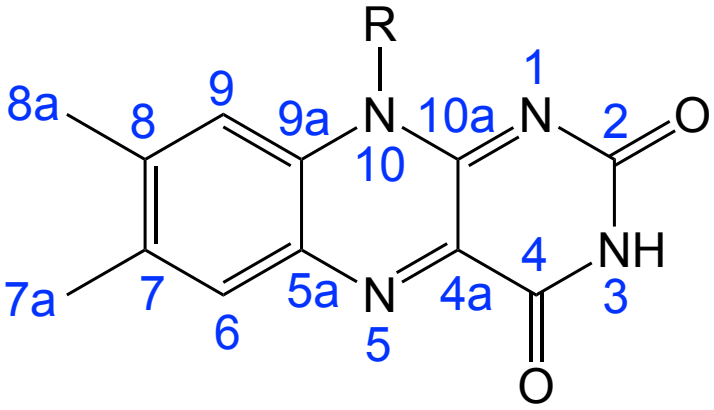

Figure 1.13: The numbering system for the isoalloxazine ring of flavins.

mononucleotide (FMN) and flavin adenine dinucleotide (FAD).[104,105] In addition to the isoalloxazine ring and ribityl sugar, FMN has a phosphate group, and FAD has an adenine dinucleotide attached at the N10 position. Both FMN and FAD play critical roles as cofactors in energy metabolism, maintenance of redox balance, and are required for the proper function of different enzymes involved in a variety of oxidation and reduction reactions that occur in all organisms.[106-109]

Riboflavin can be synthesized by all plants and most microorganisms from guanosine-5'-triphosphate (GTP) and ribulose-5-phosphate which are derived from the purine and pentose phosphate biosynthetic pathways (Figure **1.14** and Table **1.1**).[102,110] The biosynthesis of riboflavin is extremely complex and certain steps remain to be elucidated. In the initial steps of this pathway, GTP is converted into 2,5-diamino-6-ribosyl-amino-4(3*H*)-pyrimidinedione 5'-phosphate (DARPP) by GTP cyclohydrolase Ⅱ (Figure **1.14**, step 1). Conversion of DARPP through sequential deamination, reduction, and phosphorylation reactions results in the formation of 5-amino-6-ribityl-amino-2,4(1*H*,3*H*)-pyrimidinedione 5'-phosphate (ArPP) (Figure **1.14**, steps 2-5). In fungi and Archaea, reduction of DARPP occurs before the deamination step.[102,110,111] However, in eubacteria and plants side chain reduction occurs prior to the deamination step. In the following step, ArPP is seen to be dephosphorylated to ArP, however the mechanism and enzyme involved are currently unknown (Figure **1.14**, step 6).[110] In an alternative pathway, ribulose-5-phosphate is converted into 3,4-dihydroxy-2-butanone-4-phosphate (DHBP) by DHBP synthase (Figure **1.14**, step 7). Both pathways merge into one leading to the condensation of DHBP with ArP to produce 6,7-dimethyl-8-ribityllumazine (DRL). This condensation reaction involves a complex rearrangement and elimination sequence

which is catalyzed by DRL synthase (lumazine synthase) (Figure **1.14**, step 8). In the final step, riboflavin synthase catalyzes the dismutation of DRL to yield riboflavin (Figure **1.14**,

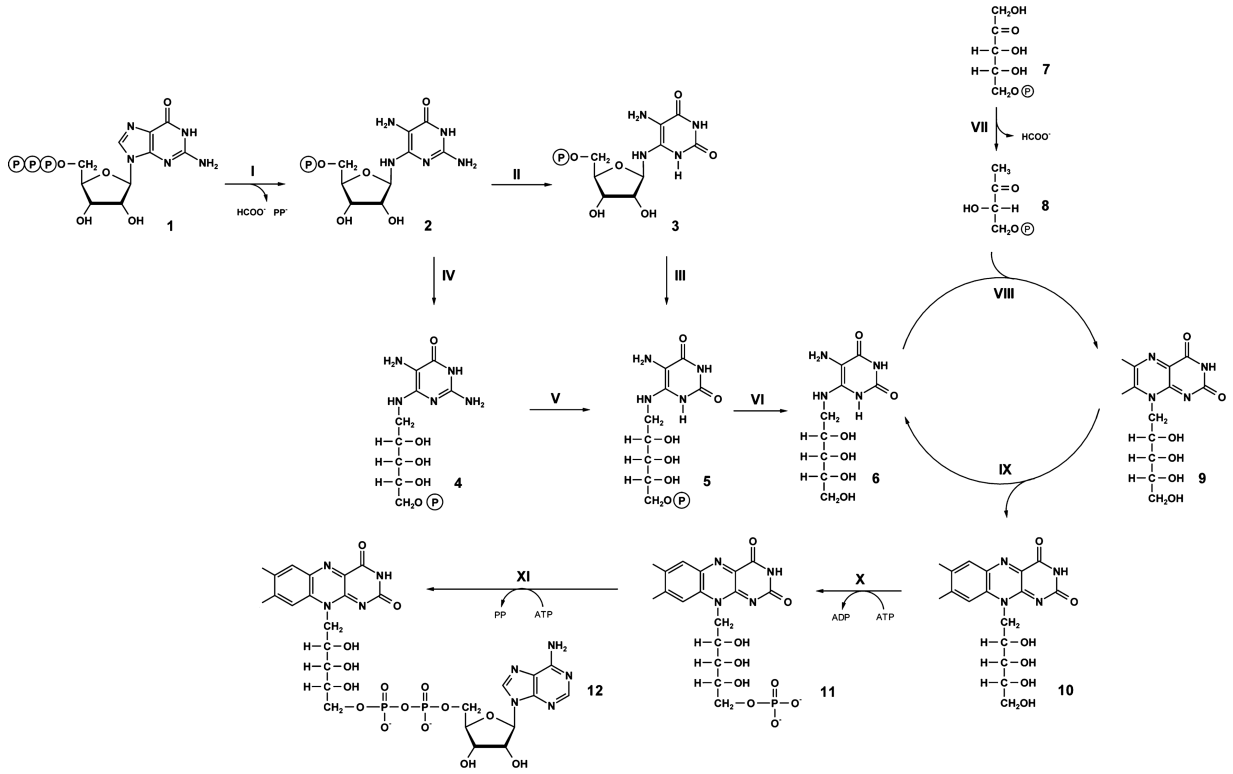

Figure 1.14: Biosynthesis of riboflavin. (Adapted with permission from [111]). Copyright © 2008 Elsevier Inc. All rights reserved.

step 9).[110] Riboflavin can be used as a precursor of the cofactors, FMN and FAD (Figure **1.14**, step 10 and 11). The phosphorylation of riboflavin to yield FMN is catalyzed by an ATP-dependent riboflavin kinase, whereas an ATP-dependent FMN adenyltransferase catalyzes the formation of FAD. In eukaryotes, the phosphorylation of riboflavin and subsequent adenylation are catalyzed by two different enzymes.[102,112,113] However, in most prokaryotes a single bifunctional flavokinase/FAD-synthase phosphorylates riboflavin to FMN followed by a subsequent adenylation to yield FAD. The production of FMN and FAD from riboflavin is ATP-dependent.[102,110,111,114,115]

Table 1.1: Intermediates and enzymes involved in the biosynthesis of flavin

Intermediates involved	Enzymes involved
1. Guanosine-5'-triphoshate (GTP)	Ⅰ. GTP-cyclohydrolase Ⅱ
2. 2,5-diamino-6-ribosylamino-4(3*H*)-pyrimidinedione 5'-phosphate (DARP)	Ⅱ. 2,5-diamino-6-ribosyl-amino-4(3*H*)-pyrimidinedione 5'-phosphate deaminase
3. 5-amino-6-ribosyl-amino-2,4(1*H*,3*H*)-pyrimidinedione 5'-phosphate	Ⅲ. 5-amino-6-ribosyl-amino-2,4(1*H*,3*H*)-pyrimidinedione 5'-phosphate reductase
4. 2,5-diamino-6-ribityl-amino-2,4(1*H*,3*H*)-pyrimidinedione 5'-phosphate	Ⅳ. 2,5-diamino-6-ribityl-amino-2,4(1*H*,3*H*)-pyrimidinedione 5'-phosphate reductase
5. 5-amino-6-ribityl-amino-2,4(1*H*,3*H*)-pyrimidinedione 5'-phosphate (ArPP)	Ⅴ. 2,5-diamino-6-ribityl-amino-2,4(1*H*,3*H*)-pyrimidinedione 5'-phosphate deaminase
6. 5-amino-6-ribityl-amino-2,4(1*H*,3*H*)-pyrimidinedione (ArP)	Ⅵ. Unknown phosphatase
7. ribulose 5'-phosphate	Ⅶ. 3,4-dihydroxy-2-butanone 4-phosphate synthase (DHBP synthase)
8. 3,4-dihydroxy-2-butanone 4-phosphate (DHBP)	Ⅷ. 6,7-dimethyl-8-ribityllumazine synthase (lumazine synthase)
9. 6,7-dimethyl-8-ribityllumazine (DRL)	Ⅸ. riboflavin synthase
10. riboflavin	Ⅹ. riboflavin kinase
11. FMN	Ⅺ. FAD synthetase
12. FAD	

Unlike plants and microorganisms, mammals and some prokaryotic and eukaryotic microorganisms (e.g., *Corynebacterium pyogenes, Streptococcus pyogenes, Listeria monocytogenes*, spirochetes, protists) are unable to synthesize riboflavin *de novo* and must acquire this vitamin through their diet.[102] Ariboflavinosis or riboflavin deficiency in mammals has been linked to numerous diseases since riboflavin itself is needed to

synthesize the cofactors FMN and FAD.[101] A deficiency of riboflavin has been reported to result in elevated concentrations of plasma homocysteine which has been linked to an increased risk of cardiovascular disease. Riboflavin deficiency has also been reported to influence iron absorption causing anemia and fatigue. Lastly, a deficiency of riboflavin could also cause various eye disorders such as cataracts, night blindness, and glaucoma.[101,103,116]

Besides riboflavin, FMN, and FAD another group of flavins are naturally produced. This specific group of flavin derivates is known as the 5-deazaflavins in which the nitrogen at the number 5 position of the isoalloxazine moiety is replaced by a carbon (Figure **1.15**).[102,103] Within this group of flavin derivates, the ribitylated 7,8-didemethyl-8-hydroxy-5-deazaflavin is known as coenzyme F_0, whereas the oligoglutamylated derivate is known as coenzyme F_{420}. Both coenzyme F_0 and coenzyme F_{420} are critical in hydride transfer reactions needed for the conversion of carbon dioxide and acetate to methane in methanogenic archaea. In certain streptomycetes, coenzyme F_{420} functions as a cofactor in the biosynthesis of the antibiotics tetracycline and lincomycin. Additionally, certain bacteria (*Mycobacterium* and *Nocardia* spp.) also utilize coenzyme F_{420} as a cofactor of glucose-6-phosphate dehydrogenase.[117-119] Despite its 5-deazaflavin moiety, the chemistry of coenzyme F_{420} resembles that of NAD(P)H rather than FMN or FAD.[103,120] Another naturally occurring flavin produced by *Streptomyces davawensis* is the antibiotic roseoflavin, which has been reported to be active against Gram-positive bacteria.[121] Additionally, alkylated analogs of roseoflavin have also been reported to possess strong antibacterial activity.[122]

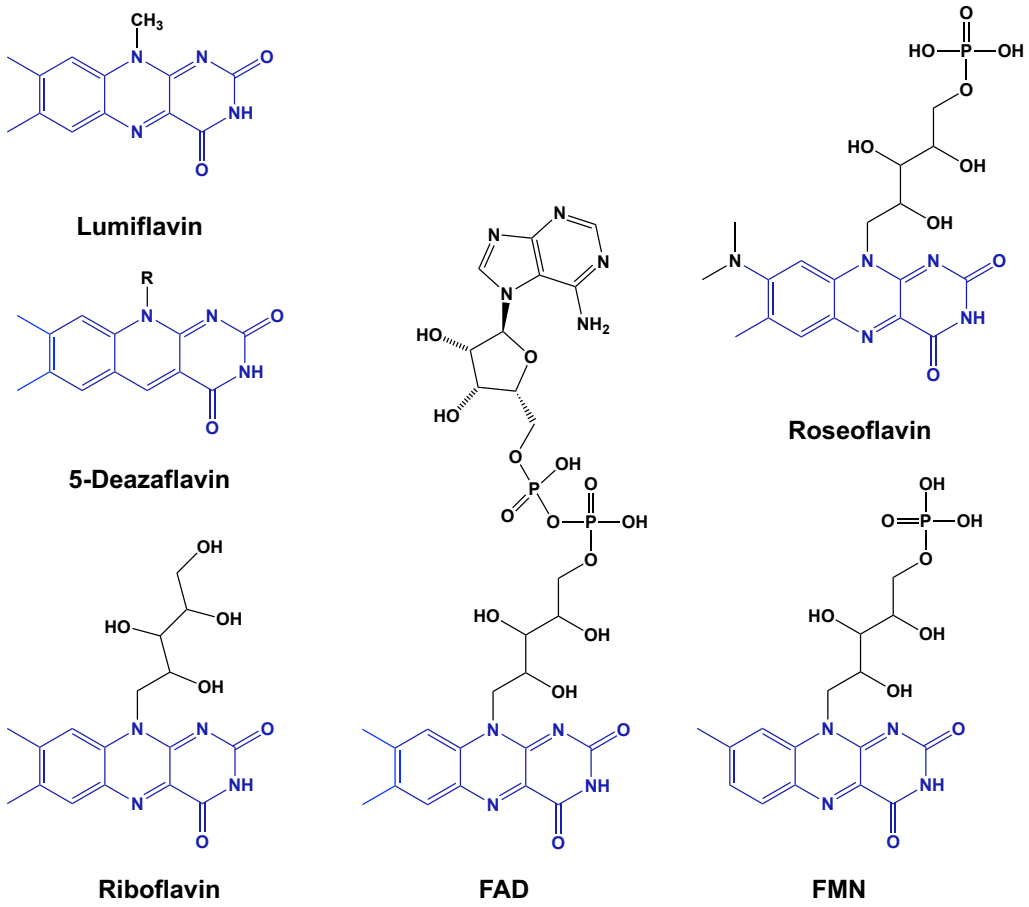

Figure 1.15: Structures of naturally occurring flavins.

1.3.2 Spectral Properties of Flavin

Flavins are involved in numerous redox reactions ranging from electron transport to DNA repair and phototropism in plants.[123] The catalytic versatility of flavins is attributed to its heterocyclic isoalloxazine moiety which can exist in different oxidation states; namely, (1) the oxidized or quinone form, (2) the one-electron reduced or semiquinone form and (3) the two-electron reduced or hydroquinone form. Since flavins are

amphoteric, they can exist as a cation, an anion, or in its neutral form in all three oxidation states depending on the pH (Figure **1.16**).[106,124]

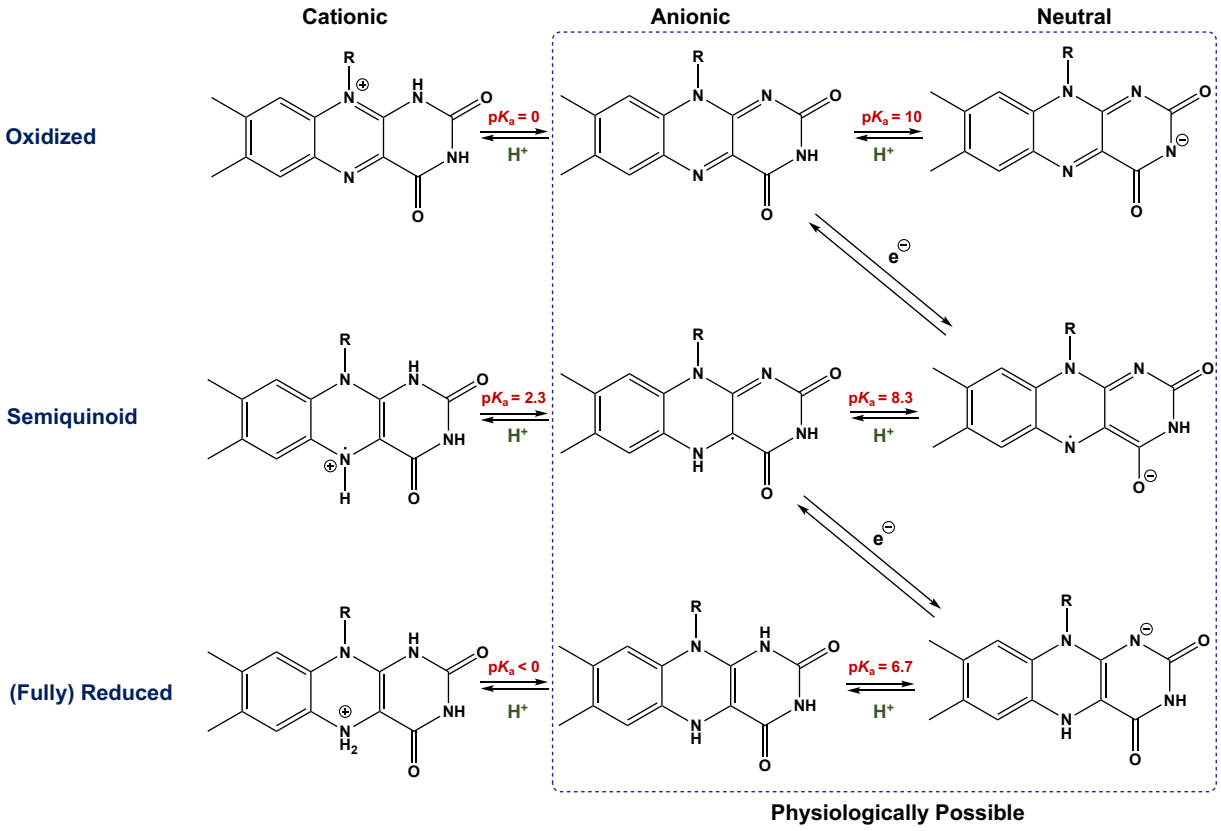

Figure 1.16: The different ionic and redox states of flavins. The anionic and neutral forms of the flavins are typically considered physiologically relevant. The cationic form of the flavins can only be obtained at a very low pH. (Adapted from [132]).

Based on the oxidation state of the isoalloxazine moiety, flavins have different spectral properties.[125] In its oxidized form, flavins exhibit an absorption spectrum with an absorbance maximum at ~450 nm (Figure **1.16**) (Figure **1.17**). In addition to the absorbance properties, oxidized flavin has fluorescence properties with a maximum emission at ~520 nm.[126] One-electron reduction of the oxidized form yields the semiquinone form, which has a weak absorbance at ~450 nm but strongly absorbs at

~370 nm (Figure **1.17**).[127,128] The semiquinone has been reported to exist in the neutral (red in color) or anionic form (blue in color) which are considered physiologically relevant (Figure **1.16**). The neutral semiquinone shows a broad absorbance between 500-650 nm with an absorbance maximum of 580 nm (Figure **1.17**). Conversely, the anionic semiquinone displays a weak absorbance at 450 nm but strongly absorbs at 370 nm

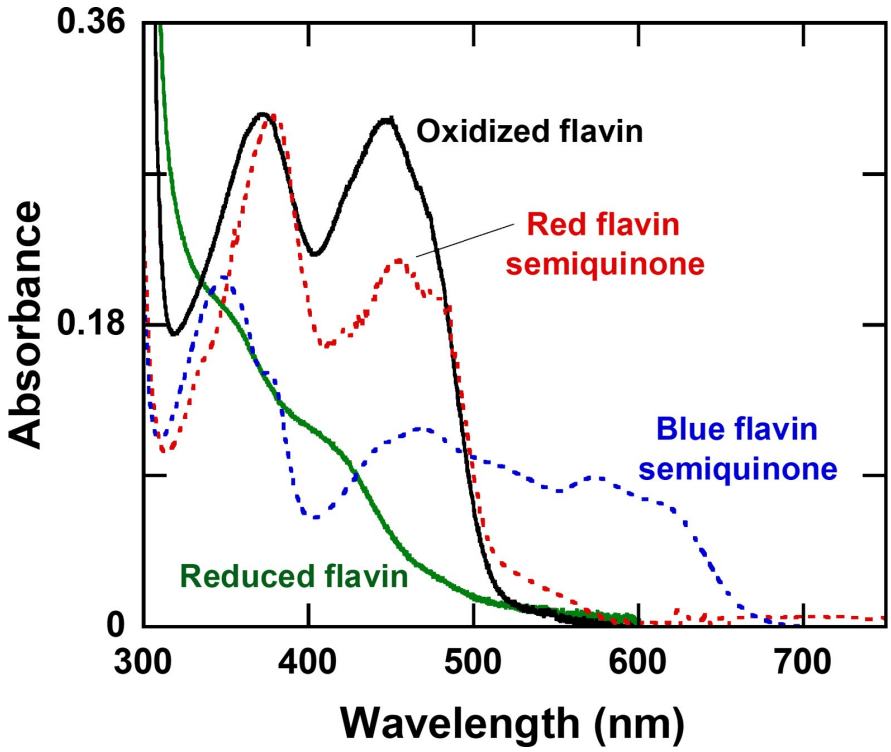

Figure 1.17: Different spectral properties exhibited by the different oxidation states of flavins. (Adapted with permission from [320]). Copyright © 2020 Elsevier Inc. All rights reserved.

(Figure **1.17**).[127,128] Free flavin in its semiquinone form is nonfluorescent, however this flavin form does exhibit fluorescent properties when bound to enzymes due to the influence of the surrounding protein environment.[126] The semiquinone form can also be obtained when a solution of equimolar free oxidized and reduced flavin are rapidly mixed. In the absence of any enzyme, only 5% of the neutral semiquinone is stabilized at a pH

7. However, upon binding with a specific enzyme, the neutral semiquinone is stabilized over a pH range that is influenced by the stability of the enzyme itself. The neutral semiquinone is typically found to be enzymatically stabilized around a pK_a of 8.5, whereas the anionic semiquinone is stabilized at lower pK_a values (Figure **1.16**). The stabilization of the neutral and anionic semiquinone is highly dependent on the active site environment of the enzyme.[106] The fully reduced or hydroquinone form is colorless and does not absorb at ~450 nm (Figure **1.17**). Even though it has been assumed that the hydroquinone form is nonfluorescent, different studies have provided evidence for some fluorescence with flavoenzymes at certain excitation wavelengths. It has also been reported that the fluorescence properties of reduced flavin could be affected by the viscosity and pH of the buffers, the active site environment of the enzyme, and the substitutions of the isoalloxazine ring.[106,127]

An interesting feature of flavins is their ability to form different adducts with their substrates. The characterization of these flavin adducts is a major aspect in flavoenzymology.[129] While certain enzymes stabilize flavin adducts for longer time periods, other enzymes do not stably form these flavin adducts.[130] Conversely, synthesis of these flavin adducts are generally extremely challenging due to their low stability in the absence of the enzyme.[131] The unique spectral features of the different flavin forms enables one to monitor various oxidation states during catalysis, and thereby providing valuable mechanistic information when studying flavoproteins.[125] The ability to provide spectral evidence for the formation of different flavin adducts during various stages of catalytic turnover provides mechanistic details that could be applicable for pharmaceutical and industrial purposes.

1.3.3 Flavoproteins

The term flavoproteins is utilized for proteins that rely on a flavin cofactor to perform their biological functions. Flavoproteins can be further categorized depending on the type of reactions they catalyze, the nature of substrates they utilize, the number of electrons transferred per catalytic cycle, and their physicochemical and structural properties.[132] Flavin cofactors are involved in numerous enzymatic reactions and can participate in both one- and two-electron transfer reactions.[106,109,124] The chemical versatility of flavins is influenced by the flavin environment which typically is the active site of the flavoprotein of interest.[106] Flavoproteins can also be categorized as canonical flavoproteins and flavin-free flavoproteins. Canonical flavoproteins are proteins in which the flavin is a prosthetic group, whereas flavin-free proteins do not have a covalently bound flavin and are supplied with free flavin.[124,133] The covalent attachment of flavins to the protein occurs either through the C6 atom or the C8α-methyl group of the isoalloxazine ring (Figure **1.13**). In canonical flavoproteins, this covalent bond is formed with cysteine, tyrosine or histidine residues.[109,134] The redox potential for the two-electron reduction of flavin is usually around -200 mV, however this value can vary from -400 mV to +60 mV depending on the protein-milieu.[109] The presence of a positive charge is proposed to increase the redox potential, whereas a negative charge or hydrophobic environment are thought to lower this value.[109,135,136] Additionally, different studies have reported that the covalent interaction in canonical flavoproteins could increase the oxidative power of the flavin, indicating the importance of the protein environment in refining the chemical properties of the flavin.[137]

Depending on their ability to react with molecular oxygen, flavoproteins can be further categorized into flavin dehydrogenases, reductases, oxidases and monooxygenases.[129] The flavin dehydrogenases typically do not react or react poorly with O_2 and are involved in two-electron and one-proton transfer reactions from the substrate to the flavin cofactor to produce the dehydrogenated product.[138] Poor reactivity of flavin dehydrogenases with O_2 mainly leads to the formation of hydrogen peroxide and superoxide anion as the reaction products.[129] Some examples of flavin dehydrogenases include succinate, alcohol, and acyl-CoA dehydrogenase.[138-140] Flavin reductases on the other hand catalyze the reduction of flavins by utilizing electrons from NADPH or NADH.[141] Examples of this particular group of enzymes include glutathione reductase and sulfite reductase.[142,143] Conversely, flavin oxidases such as glucose oxidase and D-amino acid oxidase utilize O_2 as an electron acceptor to oxidize the substrate and generate H_2O_2.[106,129] In these enzymes, the substrate transfers two electrons to the flavin to first generate the hydroquinone form followed by the anionic semiquinone flavin intermediate. Afterwards, a single electron is transferred from the anionic semiquinone flavin intermediate to O_2 followed by the formation of H_2O_2.[129] Finally, flavin monooxygenases such as *p*-hydroxyphenylacetate hydroxylase (HPAH) and cyclohexanone monooxygenase use reduced flavin to activate O_2. Following activation, one oxygen atom is incorporated into the organic substrate and the other oxygen atom is reduced and releases as water.[144,145]

Flavoproteins have been reported to react differently with O_2.[129] While some flavoproteins are involved in electron transport and can contribute to oxidative stress, other flavoproteins such as glutathione reductase prevent the formation of reactive

oxygen species.[106,146] The flavin cofactor, specifically the isoalloxazine moiety of FAD and FMN are typically found in a buried location within the core of flavoproteins.[129] It is currently unclear why certain flavoproteins (dehydrogenases and reductases) do not react (or react very slowly) with O_2 while other flavoproteins (oxidases and monooxygenases) utilize O_2 as a substrate in the overall reaction. For flavin oxidases and monooxygenases that utilize the hydrophobic O_2 as a substrate in their reactions, hydrophobic routes have been proposed to facilitate the passive diffusion of O_2 to the active site.[129,147,148] Conversely, flavin dehydrogenases and reductases that do not react with O_2 have been hypothesized to utilize distinct protein dynamics and structural features that would prevent the diffusion of O_2 to the active site. Therefore, the difference in protein structure with the different flavoproteins has been proposed to modulate the different reactivities with O_2.[129,149]

1.3.3.1 Flavin Reductases

Flavin reductases catalyze the reduction of FMN, FAD or riboflavin using the reduced pyridine nucleotides, NADH and NADPH as an electron donor.[141] The reduced flavin obtained from this reaction is proposed to function as an electron mediator in different reactions. Flavin reductases also play an important role in different biological reactions including: (1) iron metabolism, (2) ribonucleotide reductase activation, and (3) methemoglobin reduction.[141,150-152] The flavin reductases can be further categorized in two different classes. The class I flavin reductases do not contain a covalently bound flavin cofactor, whereas the class II flavin reductases do have a flavin prosthetic group.[141,152,153] In terms of the mechanism of flavin reduction, class I flavin reductases utilize a sequential mechanism where either the flavin (FMN or FAD) or pyridine

nucleotide (NADH or NADH) binds first to the enzyme (Figure **1.18A**). During the sequential reaction a ternary complex is formed prior to flavin reduction. After the flavin is reduced, either reduced flavin or the oxidized pyridine substrate (NAD^+ or $NADP^+$) dissociates followed by the other reaction products. Conversely, class II flavin reductases that have a flavin cofactor bound follow a ping-pong mechanism in which the bound flavin is reduced by the pyridine substrate (NADH or NADPH) followed by the dissociation of the oxidized pyridine nucleotide (NAD^+ or $NADP^+$) (Figure **1.18B**). After the dissociation

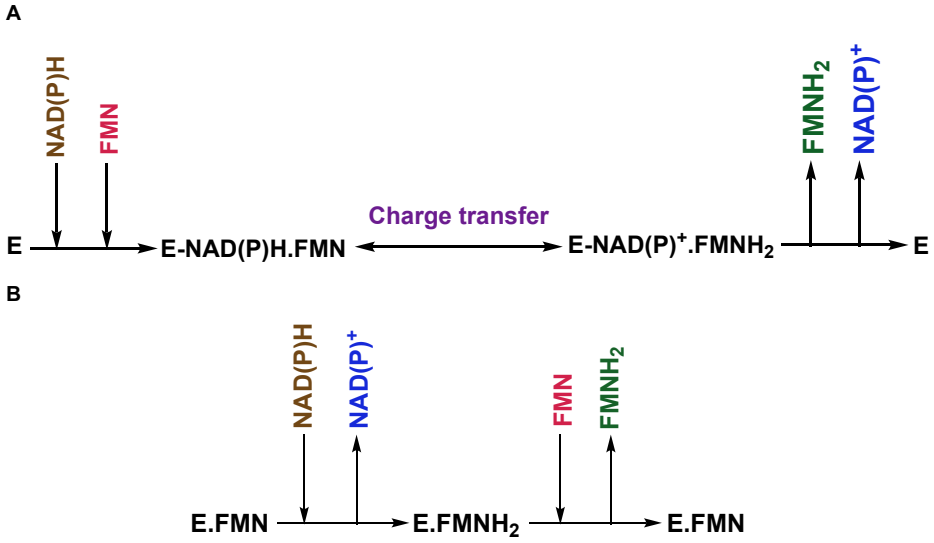

Figure 1.18: Order of substrate binding in flavin reductases. **A.** Flavin reductases that do not contain a tightly bound flavin follow an ordered-sequential mechanism. **B.** Flavin reductases that do contain a tightly bound flavin follow a ping-pong mechanism. (Adapted from [98]).

of the oxidized pyridine nucleotide, a second flavin binds to the enzyme and is reduced by the initially bound flavin cofactor.[141,143,153] Flavin reductases typically show a preference for FMN or FAD and NADH/NADPH.[154,155] Flavin reductases are also stereospecific for the hydrogen atom on the nicotinamide ring of the pyridine substrate.

Depending on whether the *pro-R* or *pro-S* hydrogen atom is transferred, flavin reductases are categorized as A- or B-side enzymes, respectively.[141,156,157]

1.3.3.2 Flavin Monooxygenases

Flavin-dependent monooxygenases are involved in various oxygenation reactions and are highly regio- and enantioselective.[129,155,158] As mentioned previously, flavin-dependent monooxygenases activate O_2 and incorporate a single oxygen atom to produce the oxidized product while reducing the second oxygen atom as water.[129] Although the reaction between O_2 and organic molecules is spin-forbidden, different enzymes including flavin-dependent monooxygenases have found a way to utilize O_2 for the oxygenation of an organic substrate. In its triplet ground state O_2 has two unpaired electrons, whereas most organic molecules are in a singlet state with all their electrons paired (Figure **1.19**, step 1).[129,159] In order to react with O_2, flavin must be in the reduced form. Spin conservation in the reaction between O_2 and reduced flavin requires two unpaired electrons in the products, thereby forming a complex of superoxide and the flavin radical (Figure **1.19**, step 2). Organic radicals and superoxide are typically very unstable; however, the stability can be greatly enhanced by the protein environment.[129,159] The complex between superoxide and the flavin radical is commonly referred to as a caged radical pair which diffuses into different oxygenating flavin intermediates (Figure **1.19,** step 3 and 4).[133,144,159]

Flavin-dependent monooxygenases have been reported to commonly utilize a C4a-hydroperoxy flavin or a C4a-peroxy flavin as the oxygenating flavin intermediate. These flavin intermediates form a carbon-oxygen bond between the superoxide radical and the isoalloxazine ring (at the C4a position) of the reduced flavin radical. The C4a-

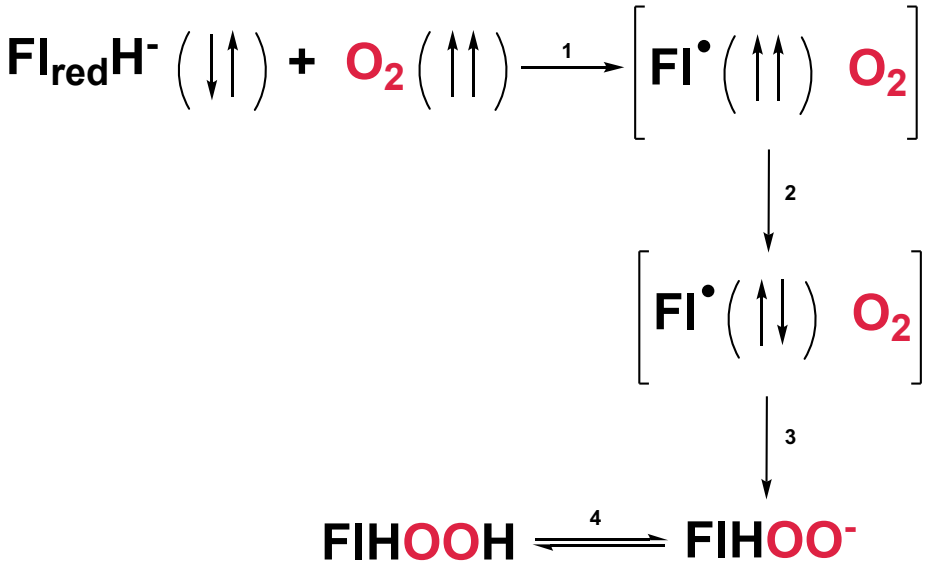

Figure 1.19: The activation of molecular oxygen by reduced flavin. (Adapted from [106]).

(hydro)peroxy flavin intermediates are used for the subsequent transfer of a single oxygen atom in the substrate to give the oxidized product.[108,160-162] Depending on their protonation states, the C4a-(hydro)peroxy flavin intermediates can function as a nucleophile or an electrophile. The C4a-peroxy flavin intermediate is able to act as a nucleophile, while the C4a-hydroperoxyflavin tends to serve as an electrophile in different monooxygenation reactions (Figure **1.20**, steps 2 and 5).[109,159,163] These intermediates are typically formed with second order rate constants between 10^4-10^6 M^{-1} s^{-1}.[164] These intermediates are involved in various chemical reaction such as hydroxylation, halogenation, epoxidation, light emission, sulfoxidation, and Baeyer-Villiger oxidation.[129,158,159] The stability of these oxygenating flavin intermediates strongly varies depending on the protein environment. The C4a-peroxy flavin intermediate in bacterial luciferase has been successfully isolated using low temperatures and this intermediate has been reported to have a lifetime measured in days.[165] Different studies also suggest that active site amino acids as well

as the N5 of the flavin could be involved in stabilizing these oxygenating flavin intermediates through hydrogen bonding.[130]

The C4a-(hydro)peroxy flavin intermediates were considered universal intermediates in different reactions catalyzed by flavin monooxygenases.[131] Recent

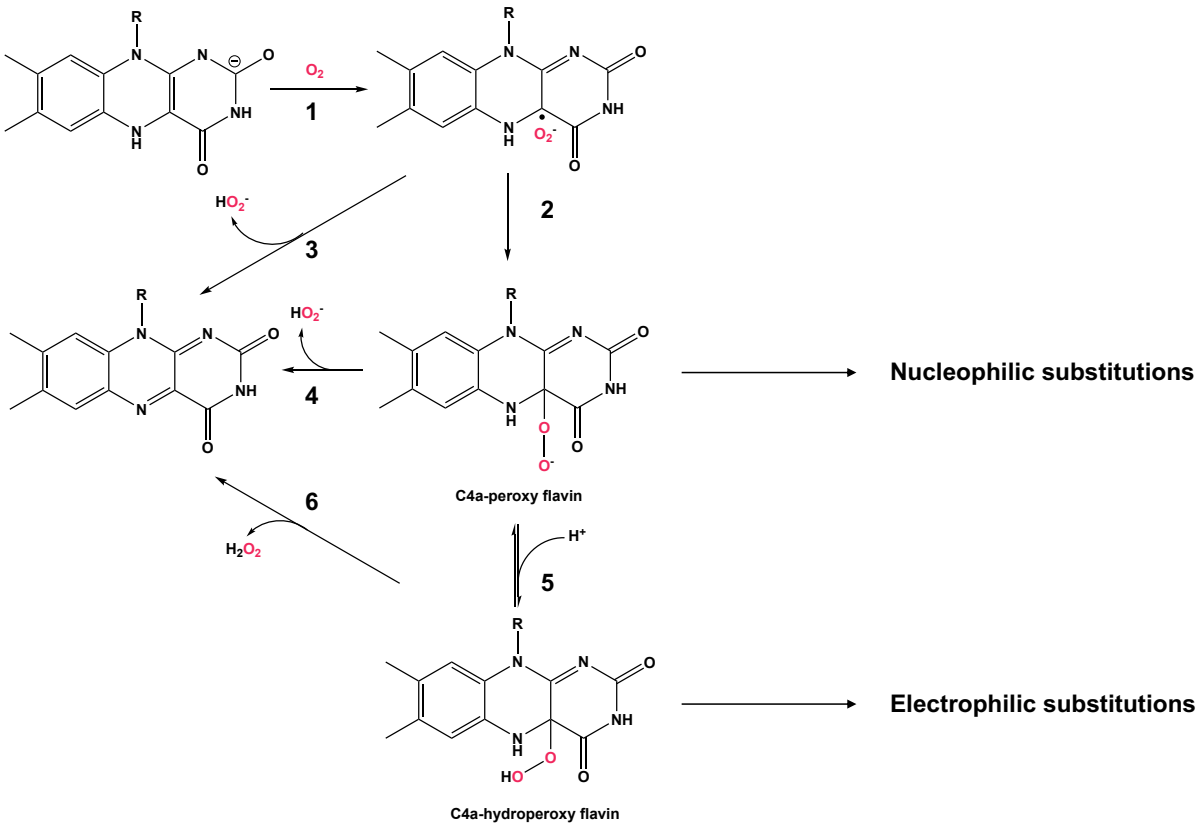

Figure 1.20: Formation of reactive flavin intermediates following the activation of reduced flavin by molecular oxygen. (Adapted from [106]).

studies with different bacterial enzymes provided spectroscopic evidence for the formation of another flavin adduct which is known as the flavin-N5-oxide.[131,166-171] This intermediate was first observed in the FAD-dependent enzyme EncM that is involved in the biosynthesis of bacterial polyketide antibiotic enterocin.[169] In addition to EncM, evidence for the formation of a flavin-N5-oxide as the final product has been provided

with RutA, which is a flavin monooxygenase involved in the catabolism of uracil (Figure **1.21**, step 4).[167,170] A mechanism has been proposed for this enzyme where the initially

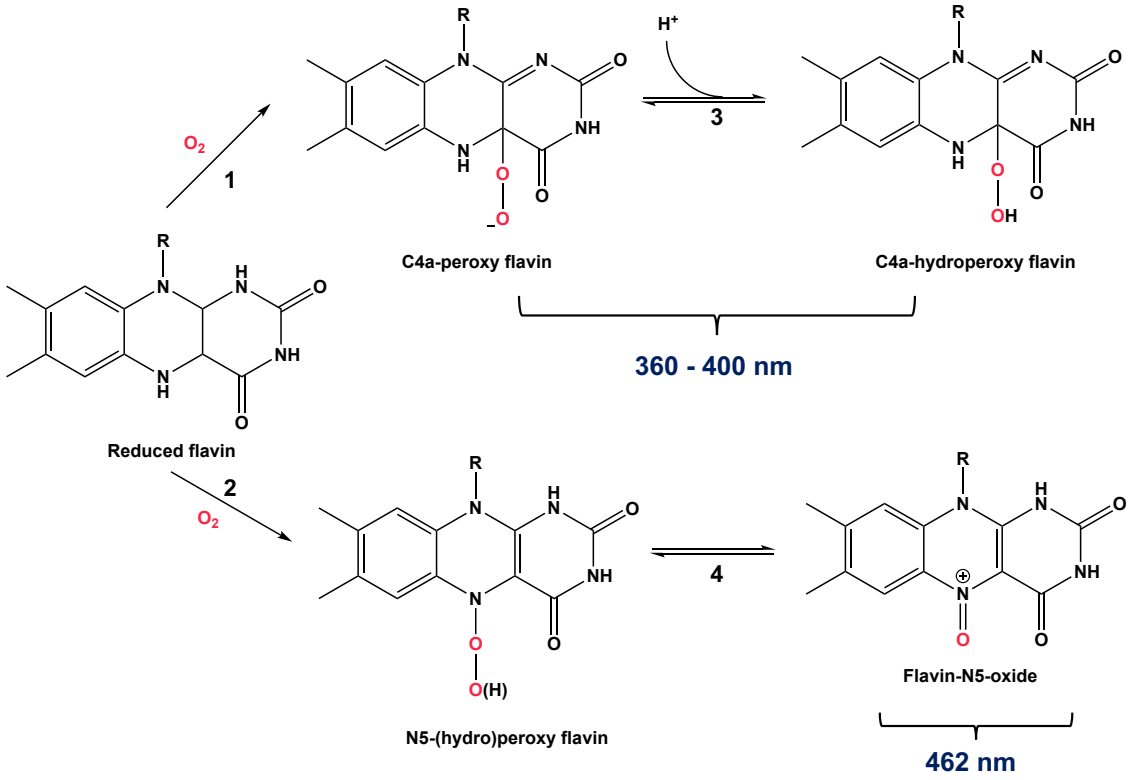

Figure 1.21: Different flavin intermediates that can be formed after the activation of reduced flavin by molecular oxygen.

formed flavin-N5-peroxide promotes substrate cleavage followed by the formation of the flavin-N5-oxide as the final product. The flavin-N5-peroxide is formed in a similar manner as the C4a-peroxy flavin; however, this intermediate is obtained through the formation of a carbon-oxygen bond between the superoxide radical and the isoalloxazine at the N5 position rather than the C4a position of the reduced flavin radical (Figure **1.21**, step 2).[170] Besides EncM and RutA, two other flavin monooxygenases namely DszA and HcbA1 have been reported to employ the flavin-N5-oxide as a final product during catalytic turnover.[166, 168] Despite the spectral evidence for the flavin-N5-oxide in the

aforementioned monooxygenases, there remain many unanswered questions on why this flavin adduct has been overlooked in the past.[53] One of the possible explanations could be the artifactual reduction of this flavin adduct by X-ray radiation, thereby preventing its stabilization and subsequent isolation.[172,173] Another possibility could be the presence of dithiothreitol (DTT) during protein purification and storage which reduces the flavin-N5-oxide.[174] Lastly, the spectrum of the flavin-N5-oxide (462 nm) is similar to that of oxidized flavin (450 nm) and could easily be mistaken for FMN.[175]

Flavin-dependent monooxygenases can be categorized using different criteria, such as the type of reaction catalyzed, the nature of the substrates involved, homology, and topology of three-dimensional structures. Another criterion is the strategy flavin monooxygenases utilize to reduce FMN and FAD.[158] Based on this criteria, three different groups can be identified, namely (1) the one-component monooxygenases, (2) the two-component monooxygenases, and (3) the internal monooxygenases.[129] One-component monooxygenases contain a tightly bound FAD cofactor during the entire catalytic cycle, and utilize the pyrimidine substrates, NADH or NADP as an electron donor for flavin reduction. On the other hand, two-component monooxygenases require a separate NAD(P)H-dependent reductase to deliver reduced FMN or reduced FAD for the activation of O_2 followed by the oxygenation of the substrate. Moreover, the internal monooxygenases utilize a single substrate as an electron donor for flavin reduction and monooxygenation.[129] Lastly, depending on whether the substrate is bound flavin monooxygenases can be further divided into “bold” and “cautious” monooxygenases.[159] Since flavin monooxygenation reactions could produce toxic H_2O_2, “cautious” monooxygenases require the substrate to be bound first prior to the reduction of flavin.

The substrate bound to the monooxygenase stabilizes the (hydro)peroxy flavin intermediate from wasteful elimination of H_2O_2.[129,159] Conversely, "bold" monooxygenases rapidly reduce the flavin followed by subsequent formation of the (hydro)peroxy flavin intermediate regardless of the presence of substrate. Even without the substrate bound, "bold" monooxygenases effectively protect the (hydro)peroxy flavin intermediate from wasteful elimination of H_2O_2.[159]

Flavin monooxygenases perform regio- and/or enantioselective monooxygenation reactions which are extremely challenging when using nonenzymatic methods.[129,158,159,176] Therefore, this particular group of enzymes represent notable biocatalytic tools.[158] Altogether, the distinct capabilities of flavin monooxygenases combined with further advancement of enzyme redesign methods and process engineering could represent promising biocatalytic tools.[158]

1.4 Two-component Flavin-dependent Enzymes in Sulfur Acquisition

1.4.1 Two-component Flavin-dependent Enzymes in Sulfur Acquisition: A Review

The two-component flavin-dependent enzyme systems require two enzymes, namely the flavin reductase and the monooxygenase to catalyze the overall reaction. The flavin reductase catalyzes the reduction of flavin (FMN or FAD) using reducing equivalents from NADPH or NADH.[98] After reduction of the flavin, reduced flavin is transferred from the reductase to the monooxygenase. The monooxygenase requires reduced flavin for the activation of molecular oxygen followed by the subsequent oxidation of the substrate to yield the oxidized product, water, and oxidized flavin (Figure **1.22**).[98] Flavin reductases commonly have a higher affinity for the oxidized form of the flavin, whereas the monooxygenases prefer the reduced form.[98,177] Generally, the genes

encoding for the flavin reductase, and the monooxygenase are found on the same operon. The two-component enzyme systems catalyze a diverse set of reactions ranging from the oxidation of environmental polycyclic compounds and the biosynthesis of antibiotics to the oxidation of various sulfonated and chelating compounds.[98]

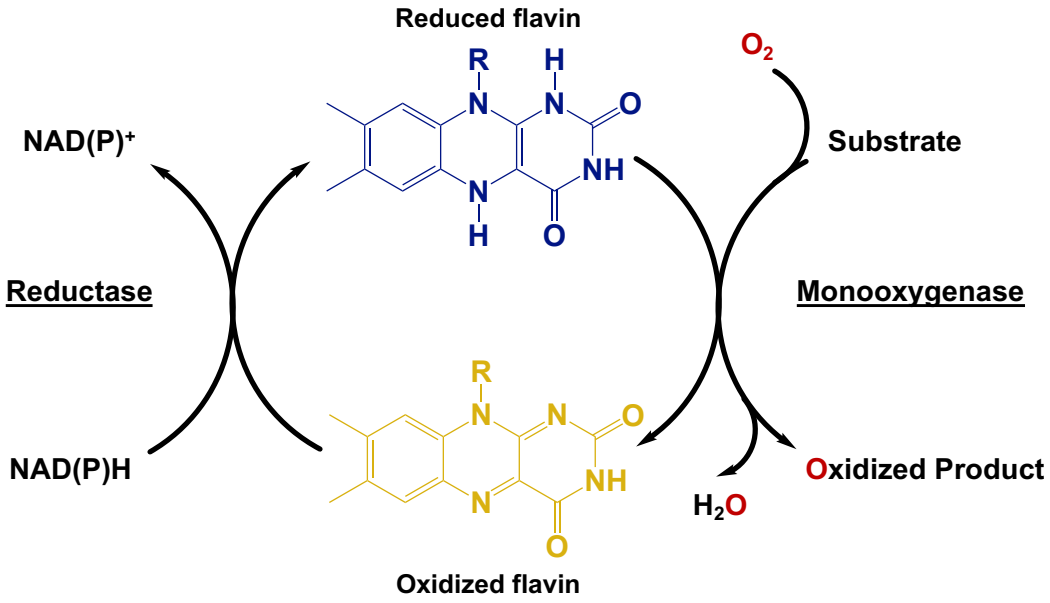

Figure 1.22: Two-component enzymes system contain a separate reductase and monooxygenase to catalyze the overall reaction. The reductase reduces flavin required by the monooxygenase to perform the oxidative half reaction. (Adapted from [99]).

Two-component flavin-dependent systems are involved in various chemical reactions (Figure **1.23**).[98] These enzyme systems are commonly found in bacteria and provide these microorganisms with sources for various elements such as carbon, nitrogen, and sulfur. Bacteria utilize these elements for diverse metabolic and physiological processes.[178] The two-component flavin-dependent enzymes have also been reported to play a critical role in bacterial sulfur acquisition. These two-component flavin-dependent enzymes enable bacteria to utilize alternative sulfur sources during

sulfur starvation.[98,99,179] In addition to bacterial sulfur acquisition, some of these two-component flavin-dependent enzymes also have industrial advantages.[158] The

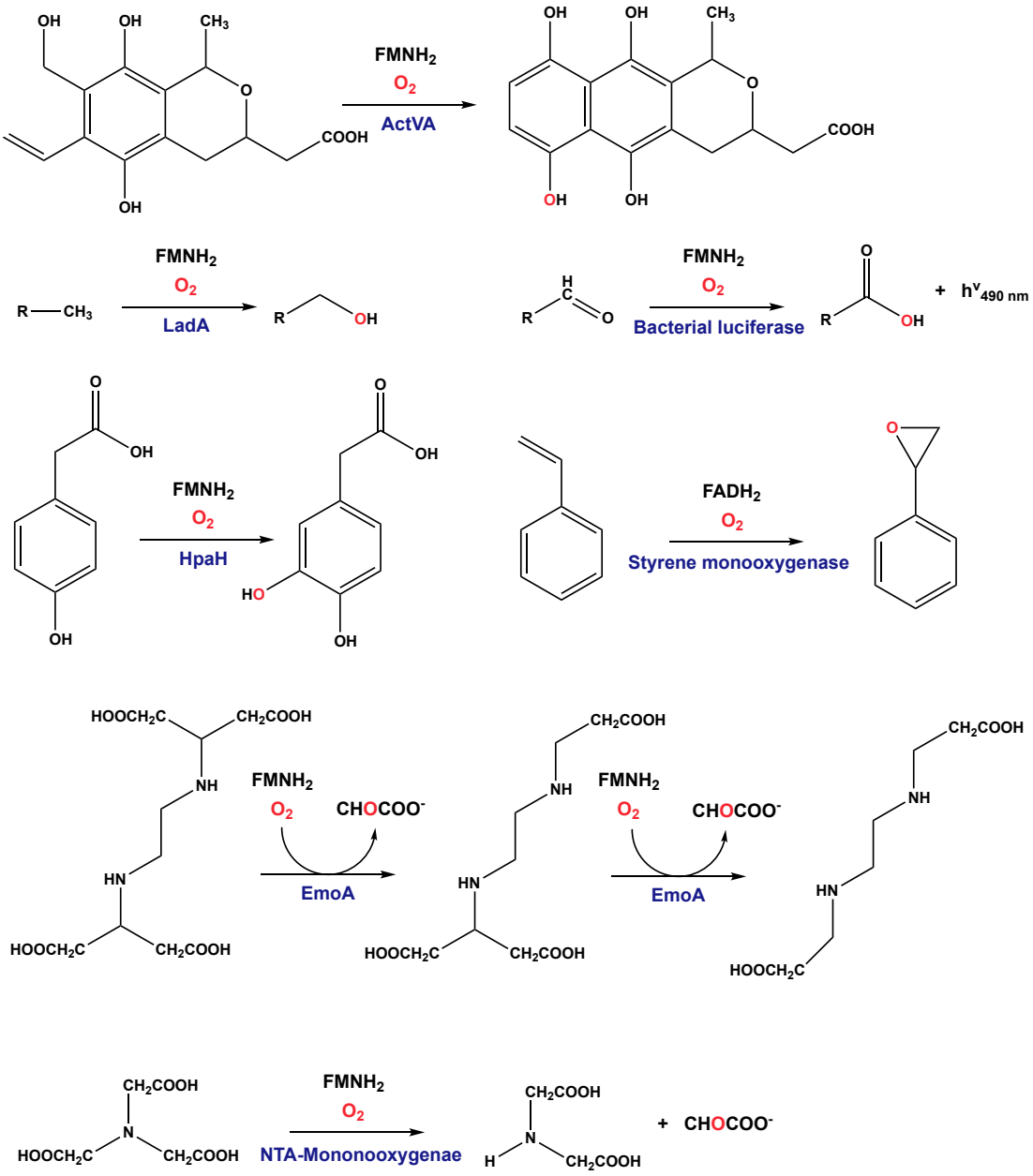

Figure 1.23: Examples of various two-component flavin-dependent enzyme systems. (Adapted from [98]).

dibenzothiophene two-component flavin-dependent enzymes found in certain bacteria catalyzes the stepwise conversion of dibenzothiophenes to sulfite.[166,180,181]

Dibenzothiophenes have been reported to serve an alternative sulfur source for different bacteria.[4] A common pathway bacteria utilize to desulfurize benzothiophene and dibenzothiophenes is known as the "4S" pathway which is named after the four sulfur-containing intermediates. This "4S" pathway has been extensively studied in *Rhodococcus erythropolis* and involves the enzymes DszA, DszB, DszC, and DszD (Figure **1.24**).[180,182,183] The first step in this pathway is catalyzed by DszC, which is an $FMNH_2$-dependent monooxygenase. DszC utilizes $FMNH_2$ supplied by the NADH:FMN

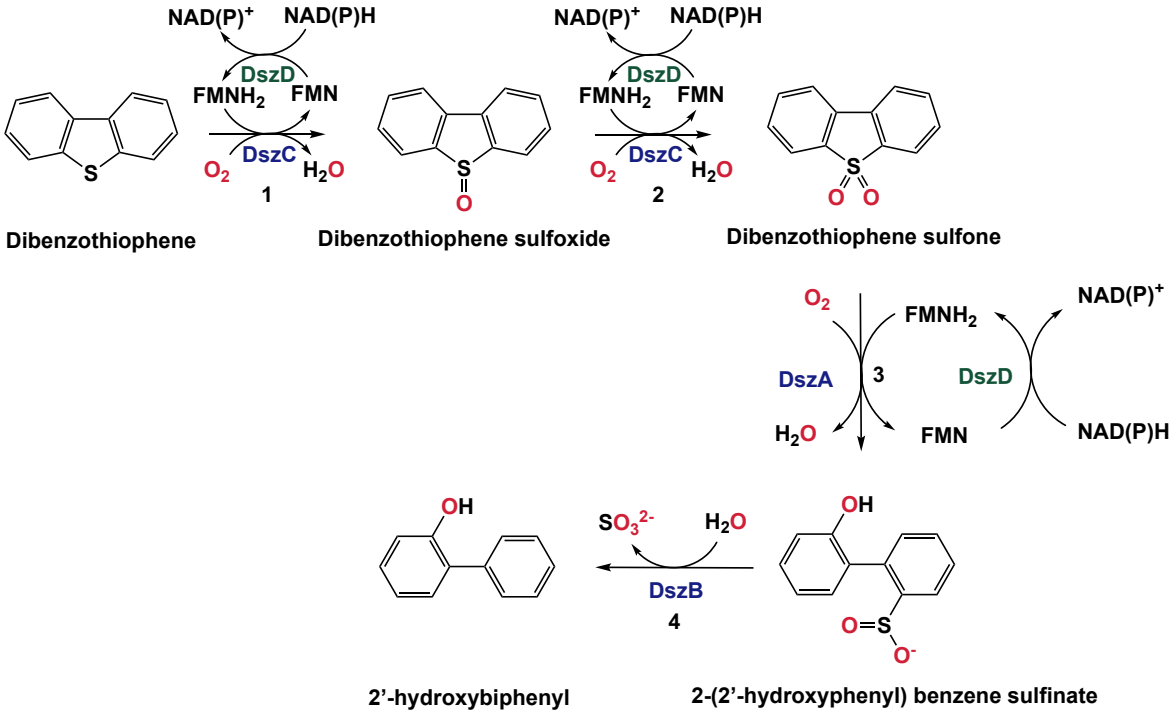

Figure 1.24: The "4S" pathway demonstrating the biodesulfurization of dibenzothiophenes by bacterial enzymes encoded by the *dsz* genes. (Adapted from [321]).

reductase DszD to catalyze two-step conversion of dibenzothiophene (DBT) to dibenzothiophene sulfoxide (DBTO) followed by dibenzothiophene sulfone ($DBTO_2$) (Figure **1.24**, steps 1 and 2). Dibenzothiophene sulfone is further oxidized and cleaved by DszA to form 2-(2'-hydroxyphenyl) benzene sulfinate (HPBS) (Figure **1.24**, step 3).

Similar to DszC, DszA is also a monooxygenase that relies of DszD for the reduction of FMN. In the final step within this pathway, DszB desulfinase cleaves the carbon-sulfur bond of 2-(2'-hydroxyphenyl) benzene sulfinate to yield sulfite and 2-hydroxybiphenyl (Figure **1.24**, step 4).[184-188] In addition to serving as an alternative sulfur source, dibenzothiophenes are also commonly found in fossil fuels and have been a contributing factor to the emission of harmful sulfur dioxides into the atmosphere.[180,189,190] Due to the disadvantages of the conventional hydrodesulfurization methods, scientists are actively considering alternative routes for the desulfurization of fossil fuels which includes biodesulfurization in order to limit the emission of sulfur dioxide. Biodesulfurization takes advantage of bacterial species to remove sulfur from crude oil and, represents a promising target for biocatalysis.[180,191,192]

In addition to the dibenzothiophene two-component flavin-dependent enzymes, the SsuE and SsuD enzymes are also critical for bacterial sulfur acquisition. Both SsuE (flavin reductase) and SsuD (monooxygenase) are part of a two-component flavin-dependent system that allow bacteria to utilize alternative sulfur sources during sulfur limitation.[65,98,179] Homologs of both enzymes are found in diverse bacteria, indicating the importance of this enzyme system.[66,193] Depending on their habitat, certain bacteria including *Pseudomonas* species contain a broader set of two-component flavin-dependent enzymes that they can utilize during sulfur limitation.[4,48,62] Besides SsuE and SsuD, *Pseudomonas* species also express the *sfn* and *msu* operons that allow these species to utilize dimethyl sulfide and its oxidation products as alternative sulfur sources. Dimethyl sulfide can be oxidized to dimethyl sulfoxide followed by dimethyl sulfone (Figure **1.25**, steps 1 and 2).[4] The two-component enzyme flavin-dependent enzymes

SfnF (flavin reductase) and SfnG (monooxygenase) have been reported to catalyze the oxidation of dimethyl sulfone to methanesulfinate (Figure **1.25**, step 3).[194] Methanesulfinate is oxidized to methanesulfonate by the enzymes MsuE and MsuC

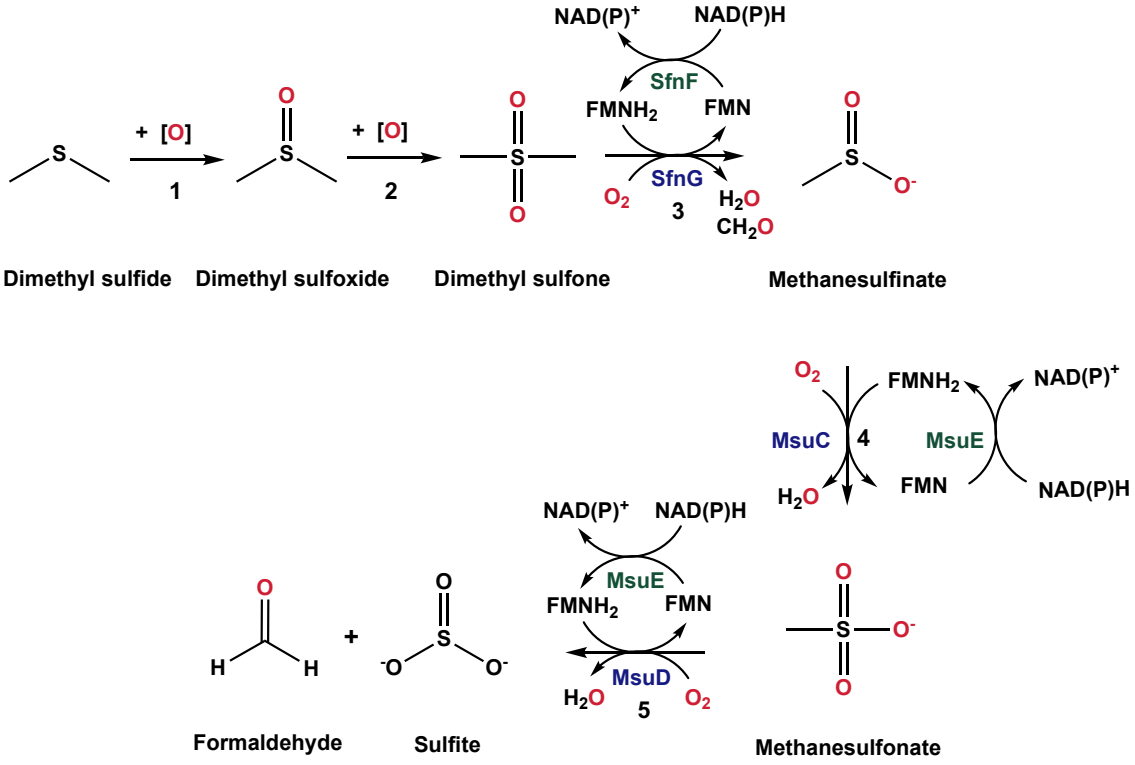

Figure 1.25: Desulfurization of dimethyl sulfide (DMS) by *Pseudomonas* species that utilize the oxidation products of DMS as alternative sulfur sources. (Adapted from [95,194]).

(Figure **1.25**, step 4).[195] Finally, methanesulfonate is desulfonated by MsuE and MsuD to sulfite and formaldehyde (Figure **1.25**, step 5).[196] SfnG, MsuC, and MsuD are monooxygenases that require $FMNH_2$ from a partner flavin reductase. SfnF and MsuE are both flavin reductases that supply $FMNH_2$ to their partner monooxygenase. The enzyme couples consisting of SfnF/SfnG, MsuE/MsuC, MsuE/MsuD are part of two-component flavin-dependent systems that enable bacteria to utilize a broad range of alternative sulfur sources during times of sulfur scarcicity.[4,194,196,197]

1.4.2 The NAD(P)H-dependent Flavin Reductases: SsuE and MsuE

Flavin reductases that are part of two-component systems, supply reduced flavin to their partner monooxygenase to perform the oxidative half reaction.[98] Since most of the initial characterization has been performed with the monooxygenases SsuD and MsuD, this review will be focused on their partner reductases, SsuE and MsuE, respectively. The flavin reductases SsuE and MsuE are found in different bacteria and are part of the two-component alkanesulfonate and methanesulfinate/methanesulfonate monooxygenase systems, respectively. Both SsuE and MsuE provide their respective partner monooxygenases with reduced flavin for the subsequent oxidation of a broad range of alkanesulfonates. While SsuE supplies reduced flavin to the partner monooxygenase SsuD, MsuE supplies reduced flavin to the monooxygenases MsuC and MsuD.[4,179,196,197]

Based on the SCOP database flavin reductases are grouped into three distinct folds which includes the ferredoxin reductase, the nitroreductase, and the flavodoxin-like superfamilies.[98,198] SsuE and MsuE share 30% amino acid sequence identity and belong to the flavodoxin-like superfamily due to their flavodoxin fold (Figure **1.26**) (Figure **1.27**). Flavin reductases adopting the flavodoxin fold contain five parallel β-strands in the center flanked by α-helices.[198] Moreover, flavin reductases belonging to the flavodoxin-like superfamily can be further categorized based on the Dali structural similarity into the NAD(P)H:FMN reductases, the quinone reductases, the WrbA-like proteins, and the flavodoxin-related proteins (Figure **1.28 A**). Both SsuE and MsuE belong to the NAD(P)H:FMN reductases, and members within this group adopting the flavodoxin fold have been generally characterized as homodimers or homotetramers.[198] Similar to SsuE

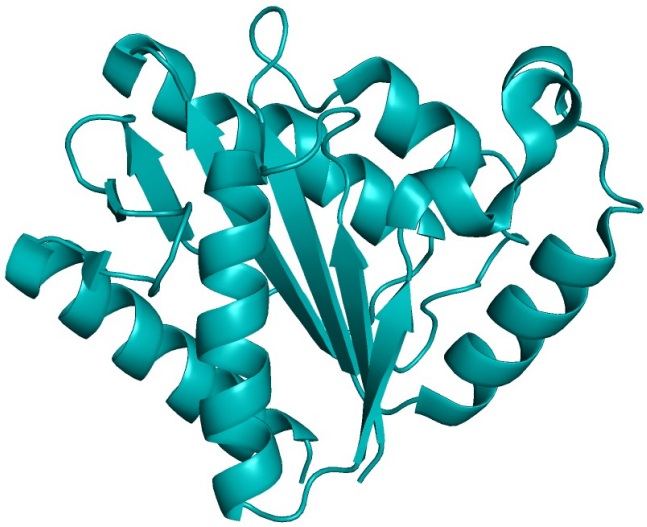

Figure 1.26: The NAD(P)H:FMN reductases belonging to two-component enzyme systems adopt a flavodoxin fold. The crystal structure of SsuE portraying its flavodoxin fold. PDB: 4PTZ

and MsuE, other flavin reductases including EmoB and an oxidoreductase (PDB: 3k1y) are grouped in a distinct category under the NAD(P)H:FMN reductases (Figure **1.28 A**). These flavin reductases are part of two-component systems and contain a conserved π-helix found on the α4 helix (Figure **1.28 B**). The π-helix has been proposed to mediate reduced flavin transfer from the reductase to the partner monooxygenase and participate in protein-protein interactions.[98,198-201] The role of the π-helix found in both SsuE and MsuE will be further discussed in this section. Even though both SsuE and MsuE share similar structural features and are involved in bacterial sulfur acquisition, initial studies have focused on elucidating the structural and mechanistic roles of SsuE.

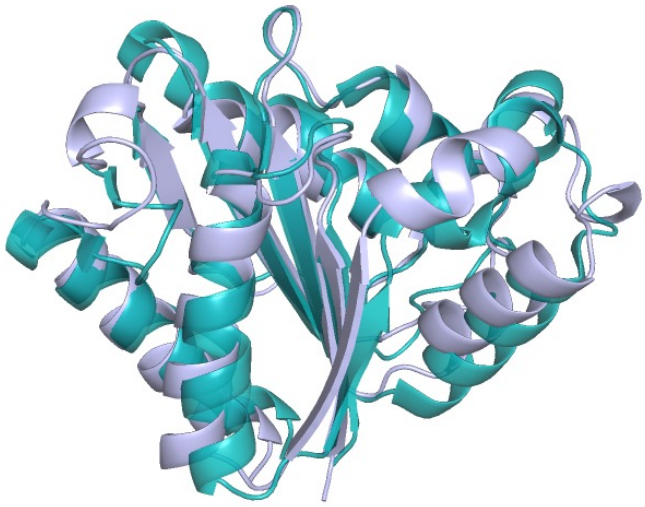

Figure 1.27: Overlay of SsuE (PDB: 4PTZ; displayed in teal) and MsuE (generated with AlphaFold; displayed in purple tint)[322] depicting their flavodoxin fold.

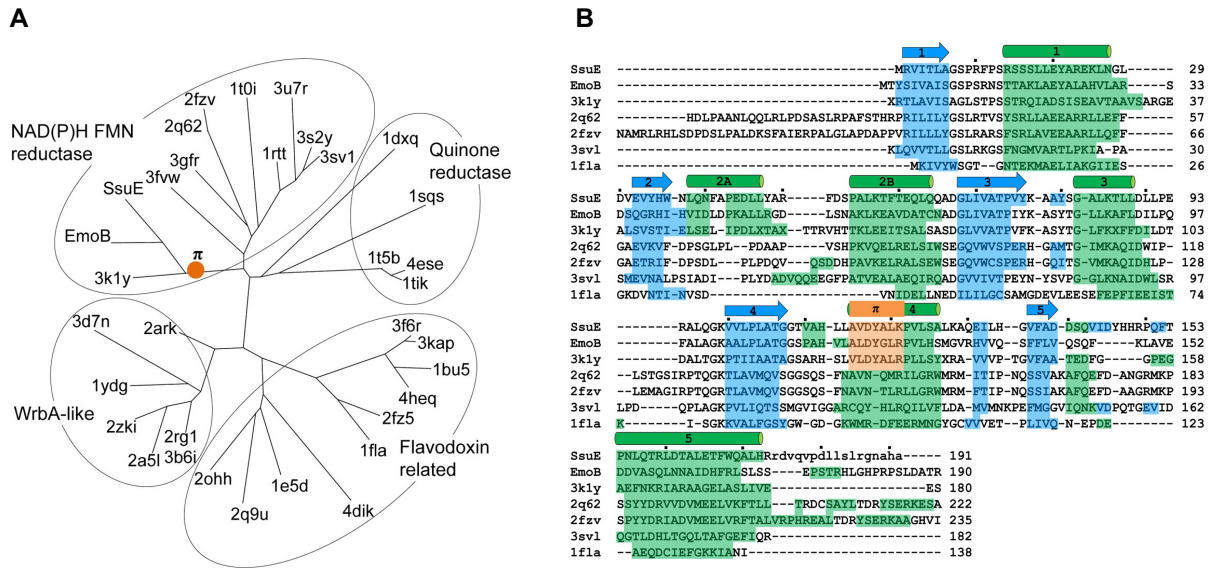

Figure 1.28: Comparison of SsuE with its homologues. **A.** Phylogenetic tree of flavodoxin superfamily including reductases of known structures categorized based on the Dali structural similarity. **B.** Structure-based sequence alignments of EmoB, a reductase from *Corynebacterium diphteria* (PDB: 3K1Y), an NADPH-dependent FMN reductase from *Shigella flexneri* (PDB: 2FZV), the chromate reductase from *E. coli* (PDB: 3SV1), and the G57D mutant flavodoxin from *Clostridium beijerinckii* but with the wild-type sequence (PDB: 1FLA) with SsuE. (Adapted with permission from [198]). Copyright (2014) American Chemical Society.

Unlike canonical flavoproteins, SsuE and MsuE do not contain a covalently bound flavin and instead use flavin as a co-substrate during catalysis.[201] Previous studies with SsuE reported a higher binding affinity (1000-fold) for FMN compared to $FMNH_2$. In addition, SsuE has been reported to have a higher affinity for FMN compared to FAD and follows an ordered-sequential mechanism.[179] The catalytic mechanism of SsuE was evaluated using steady-state and rapid reaction kinetics and involved the initial binding of NADPH followed by FMN. The reduction of flavin by NADPH occurs through the formation

of a ternary complex which has been observed at 550 nm and involved three phases.[202] The initial phase was reported to resemble the formation of a charge-transfer complex between NADPH and FMN. In the second phase, a charge-transfer complex is formed between $NADP^+$ and $FMNH_2$, and this phase was also identified to be the rate limiting step (Figure **1.29**). The identification of hydride transfer as the rate limiting step was also confirmed using [4(R)-^{2}H] NADPH. Finally, the last phase resembled the decay of the charge transfer complex and could also represent the release of $FMNH_2$ followed by $NADP^+$.[202]

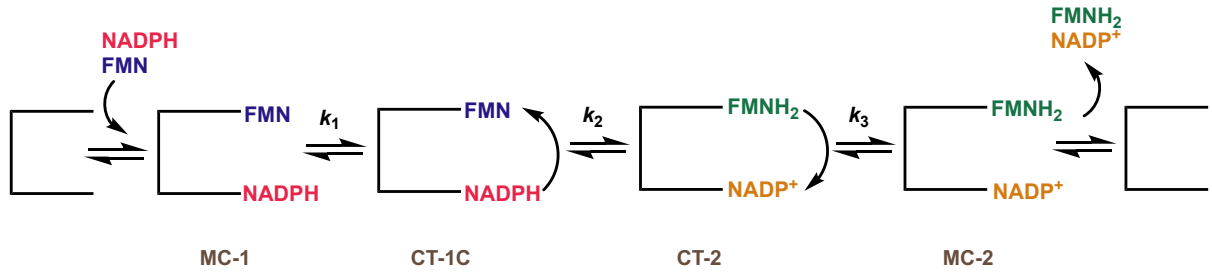

Figure 1.29: Formation of a charge-transfer complex by SsuE. (Adapted from [202]).

The catalytic mechanism of SsuE was further evaluated in the presence of its partner monooxygenase, SsuD. Results from previous studies revealed that the presence of SsuD did not change the steady-state kinetic parameters of SsuE. However, when both SsuD and octanesulfonate were included, the catalytic mechanism of SsuE changed from an ordered-sequential to a rapid-equilibrium mechanism.[179] Therefore, the substrate (NAD(P)H) and product ($NAD(P)^+$) are in equilibrium with free SsuE. From these studies, it was also revealed that the reduction of flavin by SsuE does not depend on the concentration of NADPH. Instead, the equilibrium was shifted towards the formation of the ternary complex. Moreover, in single-enzyme assays with only SsuE present, the K_m was similar to the K_d obtained from fluorometric titrations.[179] However, in the presence of

SsuD and octanesulfonate the K_m was shown to increase by 10-fold. This indicated that the presence of SsuD resulted in a decreased binding affinity of SsuE for flavin, assuming that the K_d is equal to the K_m in the presence of SsuD. This weaker affinity in the presence of SsuD would be essential for the reduced flavin transfer from the reductase to the monooxygenase. The change in the catalytic mechanism from an ordered sequential to a rapid equilibrium mechanism in the presence of SsuD and octanesulfonate, suggests that the presence of SsuD can modulate the activity of SsuE. Moreover, the presence of SsuD and octanesulfonate were reported to be crucial for the reduced flavin transfer.[179] Despite the structural similarities between SsuE and MsuE, the latter supplies reduced flavin to MsuC and MsuD and has therefore been proposed to utilize a different mechanism as compared to SsuE. Mechanistic studies with MsuE are yet to be performed to account for mechanistic similarities and differences as compared to SsuE.

In order to understand how the structure influences the function of SsuE, various studies were performed that evaluated the role of structural features of SsuE in the overall reaction. SsuE was initially characterized as a dimer in the absence of substrates using gel filtration studies; however, in the three-dimensional structure, SsuE formed a tetramer.[66,198] Therefore, the oligomeric state of SsuE was further evaluated using analytical ultracentrifugation studies.[198] The results from these studies revealed that in the absence of FMN, SsuE exists as a tetramer; however, in the presence of FMN a tetramer-dimer equilibrium was observed. SsuE has been reported to undergo changes in the oligomeric state using different experimental approaches. These changes in oligomeric states by SsuE have been proposed to facilitate reduced flavin transfer to the partner monooxygenase, SsuD.[198]

Both SsuE and MsuE contain a conserved π-helix found on the α4 helix.[199-201] The π-helices are a structural feature established by an intrastrand hydrogen bonding network between an amide and carbonyl group that are five amino acid residues apart. In addition, the π-helices are usually characterized by a single amino acid insertion into an α-helix, resulting in an enhancement or gain of protein function.[203-205] Approximately 15% of the known proteins are proposed to contain a π-helix.[205] The π-helices are often overlooked and misannotated as α-helices by algorithms that assign secondary structures when analyzing the three-dimensional structure of proteins.[199,203,205] The amino acids found in π-helices are generally aromatic or large aliphatic amino acids, causing a wider turn at the point of insertion. Structural evaluation of enzymes containing π-helices, led to the hypothesis that these secondary structures could play a role in substrate or cofactor recognition. In metalloproteins and soybean lipoxygenase, the π-helices are critical for substrate recognition and proper placement of coordinating metal ligands, respectively.[206,207] In SsuE, the π-helix is located at the tetramer interface and is formed by the insertion of Tyr118 into the α4 helix.[198-200] Conversely, MsuE contains a histidine insertional residue (His126) at the bulge site of the π-helix (Figure **1.30**).[201] In the three-dimensional structural of SsuE, Tyr118 participates in a hydrogen bonding network between its hydroxyl group and the oxygen atom of the backbone carbonyl of Ala78 located across the tetramer interface. The amide nitrogen of Ala78 also participates in hydrogen bonding with the O4 atom of FMN when bound to the active site (Figure **1.31**).[198,199]

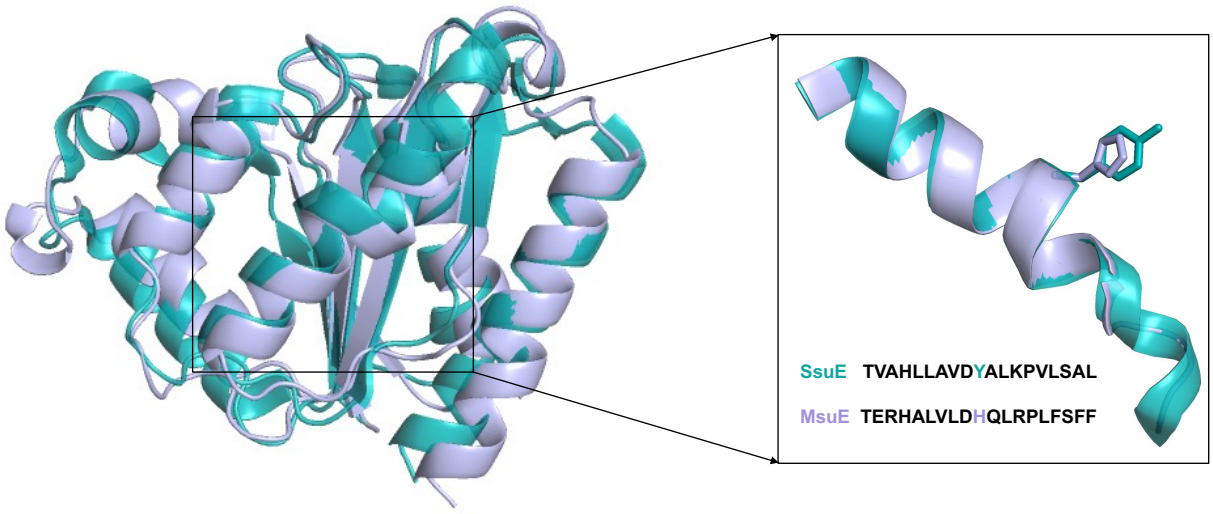

Figure 1.30: Overlay of SsuE (teal) and MsuE (purple tint) showing the region containing the π-helix. While the π-helix in SsuE is formed by the insertion of Tyr118, the one formed in MsuE is due to the insertion of His126. (Adapted from [200]).

To further evaluate the role of Tyr118 in π-helix formation, a Tyr118 to Ala SsuE variant was generated. Substitution of the Tyr118 to an alanine resulted in a flavin-bound enzyme. Unlike the wild-type enzyme, the Y118A SsuE variant did not have any measurable activity in desulfonation assays and had slow reactivity in NADPH oxidase assays. These results suggested that even though the flavin was reduced, it was not effectively released and transferred to SsuD.[199] The disruption of the π-helix in SsuE due to the Tyr118 to Ala substitution could therefore lead to the inability of SsuE to efficiently transfer reduced flavin to the monooxygenase. Substitution of Tyr118 with an alanine could have led to a disruption of this hydrogen bonding network and thereby affected the reduced flavin transfer. The results obtained from this study also revealed that besides maintaining structural integrity, the π-helix in SsuE could be essential for reduced flavin transfer to SsuD. The presence of π-helices in various two-component flavin reductases

including the SsuE/D enzyme system has been hypothesized to be involved in the transfer of reduced flavin from the reductase to the monooxygenase partner.[199]

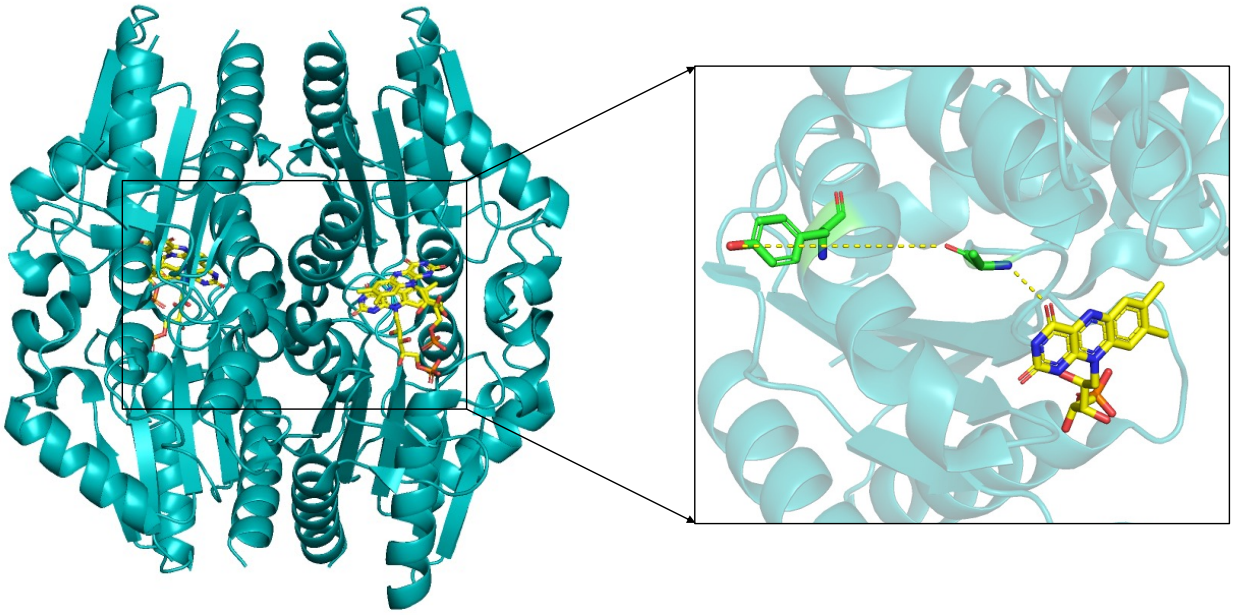

Figure 1.31: Hydrogen bonding interactions between Tyr1188 and Ala78 across the tetramer interface of SsuE. The amide group of Ala78 forms hydrogen bonding interactions with the C4 carbonyl oxygen of the bound flavin within the active site. (PDB 4YTZ). (Adapted from [200]).

The role of the π-helix in SsuE was further evaluated through site-directed mutagenesis studies involving Tyr118. Since Tyr118 was observed to participate in a hydrogen bonding network with Ala78 at the tetramer interface, a Y118S SsuE variant was generated. The Y118S SsuE variant contained the hydroxyl group of the serine residue which could still participate in hydrogen bonding with Ala78. In addition to hydrogen bonding, Tyr118 is also involved in π-stacking interactions between Tyr118 residues located at the tetramer interface.[200] A Y118F SsuE variant was generated as a conserved substitution for the tyrosine residue. Lastly, a deletion variant of Tyr118 was

generated since to the presence of a π-helix in different two-component flavin reductases is a result of tyrosine insertion in a conserved α4 helix. The Y118S SsuE variant was purified as a flavin-bound enzyme, similar to what has been observed for the Y118A SsuE variant, whereas the Y118F and the ΔY118 SsuE variants, were flavin-free similar to the wild-type enzyme. [200]

The flavin reductase activity of the Tyr118 SsuE variants was monitored under steady-state conditions following the oxidation of NADPH. The Y118F variant had similar flavin reductase activity as wild-type SsuE, whereas Y118A, Y118S, and ΔY118 SsuE variants did not have any measurable flavin reductase activity. The ability of the Y118 SsuE variant to support desulfonation was also evaluated by monitoring sulfite production in the presence of the monooxygenase, SsuD. Results obtained from the desulfonation assays revealed that, Y118F SsuE had comparable activity as the wild-type enzyme, however the Y118A, Y118S, and ΔY118 SsuE variants did not have any measurable desulfonation activity.[200] Altogether, the results obtained from NADPH oxidase activity and the desulfonation assay revealed that the Y118A, Y118S, and ΔY118 SsuE variants were unable to transfer reduced flavin to SsuD. The comparable kinetic parameters of Y118F SsuE with wild-type SsuE could be attributed to the phenylalanine residue that is capable of π-stacking interactions. Based on the kinetic and structural similarity between wild-type and Y118F SsuE, the presence of π-stacking interactions could be more critical compared to hydrogen-bonding interactions in maintaining the structural and functional integrity. These results further suggested that modifications of the π-helix disable the reduced flavin transfer to the monooxygenase enzyme.[200]

The reduced flavin transfer from SsuE to SsuD is facilitated by protein-protein interactions. The interacting regions in SsuE are located at the tetramer interface and include the π-helix. In previous studies, protein-protein interactions were identified between SsuE and SsuD which correlated to a high binding affinity of the reductase (SsuE) for the monooxygenase (SsuD). The Y118A and Y118F SsuE variants had similar binding affinities for the SsuD as compared to wild-type SsuE, whereas the ΔY118 SsuE variant could not form protein-protein interactions with SsuD.[200] Although protein-protein interactions were observed for the Y118A SsuE variant and SsuD, this variant was unable to transfer reduced flavin to SsuD. Results obtained from competition assays performed in the presence of SsuD, Y118A SsuE, and wild-type SsuE revealed that this SsuE variant was competing with the wild-type enzyme for the SsuD binding sites. With both Y118A SsuE and wild-type SsuE present, a decrease in desulfonation activity was observed due to the inability of the Y118A SsuE variant to transfer reduced flavin to SsuD.[200]

Wild-type SsuE was previously reported to exist in a dimer-tetramer equilibrium.[198] This equilibrium was shifted towards the dimeric state of the enzyme in the presence of flavin. The change in the oligomeric state of SsuE in the presence of flavin was hypothesized to play a role in protein-protein interactions as well as the reduced flavin transfer.[198,200] Wild-type SsuE in the absence of substrates was previously reported to exists as a tetramer.[198] The oligomeric state of the Tyr118 SsuE variants was therefore evaluated to determine if the substitutions influenced the quaternary structure of the enzyme. The Y118A and Y118S SsuE variants that were purified as flavin-bound, were characterized as dimers, whereas Y118F and ΔY118 SsuE that were purified as flavin-free existed as tetramers. Removal of the flavin from the Y118A and Y118S SsuE variants

altered the oligomeric state of these variants revealing their oligomeric state was not dependent on bound flavin. Lastly, the π-helix in SsuE plays a critical role in the oligomeric changes of this enzyme.[200] Flavin binding in SsuE leads to changes in the quaternary structure due to an altered flexibility of the π-helix which increases protein-protein interactions required for the transfer of reduced flavin.[199,200] The presence of π-helices in enzymes is generally associated with an enhanced function due to the energetic destabilization leading to increased protein dynamics.[205-207] Therefore, the presence of a π-helix at the tetramer interface of SsuE could result in a more dynamic region that would facilitate the requisite oligomeric changes followed by protein-protein interactions with SsuD. Taken together, these results suggest that changes in the oligomeric state of SsuE expose the active site to promote catalysis by initiating reduced flavin transfer to SsuD.[200]

In addition to SsuE, other flavin reductases of two-component systems in bacterial sulfur acquisition have been reported to contain a π-helix. These flavin reductases are SfnF and MsuE, which contain a histidine residue (His126) instead of a tyrosine residue.[201] The flavin reductase SfnF supplies reduced flavin to the dimethylsulfone monooxygenase (SfnG), for the oxidation of dimethylsulfone to methanesulfinate.[194] On the other hand, MsuE supplies reduced flavin to the monooxygenases MsuC and MsuD, for the coordinated desulfonation of methanesulfinate and methanesulfonate, respectively.[196,197] Both MsuE and SfnF share ~30% amino acid sequence identity with SsuE, and contain similar structural features.[201] In order to further investigate the role of the π-helices in these flavin reductases, the insertional residues were interchanged to generate the Y118H SsuE and the H126Y MsuE variants. The goal of these studies was to evaluate whether the generated variants had comparable kinetic properties to the wild-

type enzymes. The ability of these variants to reduce FMN was monitored through the NAD(P)H oxidase assay. The H126Y MsuE variant had similar activity as wild-type MsuE, whereas Y118H SsuE had no measurable activity. The kinetic results obtained for Y118H SsuE were similar to that reported previously for the Y118A and ΔY118 SsuE variants.[201] Additionally, desulfonation assays were performed to evaluate the ability of the variants to transfer reduced flavin to SsuD and MsuD. For H126Y MsuE, comparable activity was observed irrespective of what monooxygenase was used, whereas no activity was observed with the Y118H SsuE variants in the presence of either of the monooxygenases. The absence of activity observed with the Y118H SsuE variant suggested that changing the tyrosine to a histidine residue did not result in similar functional properties required for catalysis.[201]

The presence of the π-helix in different flavin reductases involved in sulfur acquisition is hypothesized to play a mechanistic role that is distinct from the FMN-bound reductases.[201] The π-helix in SsuE is located at the tetramer interface and is proposed to be involved in oligomeric changes that facilitate reduced flavin transfer to SsuD.[198-200] Both SfnF and MsuE contain a histidine insertional residue at the bulge site of the π-helix, whereas SsuE has a tyrosine residue. MsuE from *P. aeruginosa* and SsuE from *E. coli* share ~30% amino acid sequence identity. Even though both flavin reductases share a relatively low amino acid sequence identity, the regions surrounding the π-helix in both enzymes are highly similar. The results from these studies revealed that interchanging the insertional residues of SsuE and MsuE, led to an MsuE variant with similar kinetic properties as wild-type MsuE whereas the SsuE variant was incapable of NADPH oxidation and reduced flavin transfer.[201] Although it has been hypothesized that π-helices

emerge due to the presence of an amino acid insertion in an α-helix, an alternate option was proposed from these studies.[201,204,205] The quinone reductase (ChrR) from *E. coli* has a tyrosine residue in the same position as Tyr118 in SsuE, however unlike SsuE ChrR does not form a π-helix.[208] This further indicates that the presence of an aromatic amino acid within an α-helix is not the only structural feature necessary to form a π-helix or facilitate reduced flavin transfer to a two-component monooxygenase.[201]

1.4.3 Mechanism of flavin transfer in two-component flavin-dependent systems

Two-component flavin-dependent enzymes utilize two separate enzymes to catalyze the overall reaction. The flavin reductase performs the reductive reaction by utilizing NADH or NADPH to reduce flavin, while the monooxygenase uses reduced flavin to perform the oxidative reaction.[98] Reduced flavin is extremely oxygen labile and forms different reactive oxygen species when exposed to molecular oxygen. Therefore, the reduced flavin transfer from the reductase to the monooxygenase is extremely critical, considering the instability of reduced flavin as well as the potential formation of reactive oxygen species when exposed to oxygen.[144] In order to efficiently transfer reduced flavin from the reductase to the monooxygenase, different mechanisms have been identified in various two-component enzyme systems.[209] Two-component flavin-dependent systems have been reported to utilize a free diffusion or a channeling mechanism for reduced flavin transfer.[98,209] Certain two-component flavin-dependent system have also been reported to utilize both mechanisms for the transfer of reduced flavin from the reductase to the monooxygenase.[98,209,210] Two-component flavin-dependent systems that utilize free diffusion as a mode of reduced flavin transfer do not require protein-protein interactions

between the reductase and monooxygenase enzymes (Figure **1.32A**). Reduced flavin that is transferred through diffusion could autoxidize when exposed to bulk solvent and

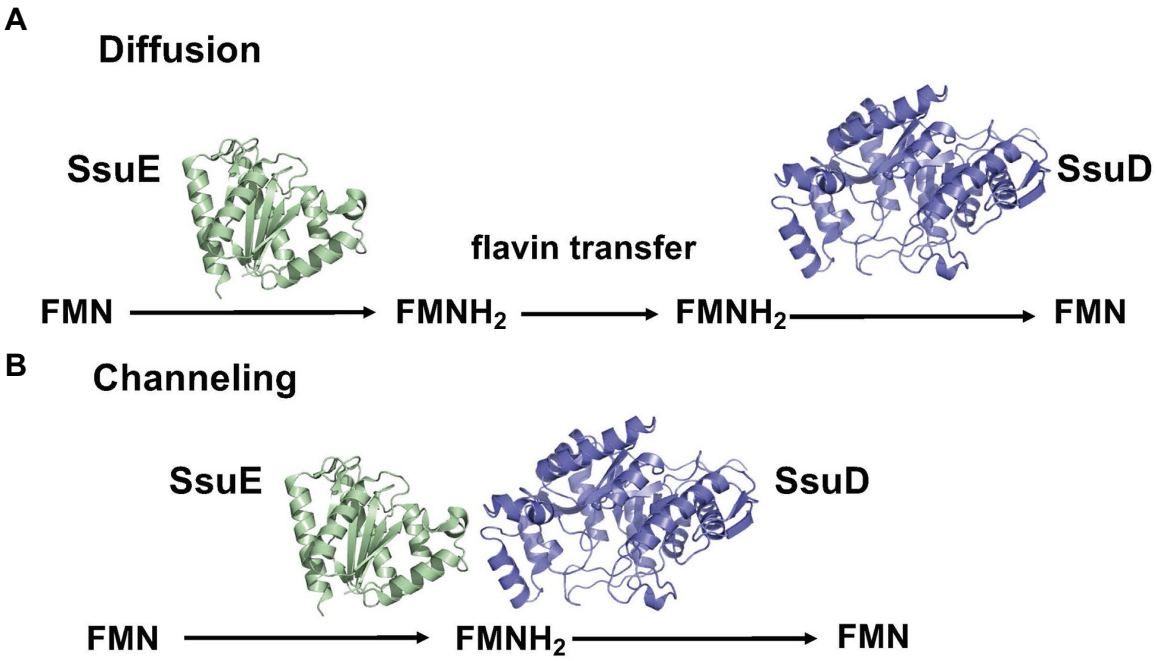

Figure 1.32: The mechanism of reduced flavin transfer between the reductase and the monooxygenase. **A.** The diffusion mechanism does not rely on interactions between the reductase and the monooxygenase. **B.** The channeling mechanism requires interactions between the reductase and the monooxygenase for the transfer of reduced flavin. (Adapted with permission from [99])

produce reactive oxygen species. Additionally, this mode of reduced flavin transfer is considered nonspecific since reduced flavin could bind to various receiver enzymes.[144,209,211] Conversely, reduced flavin transfer can also occur through a channeling mechanism. Enzyme systems that utilize a channeling mechanism require specific interactions between the reductase and the monooxygenase for the efficient transfer of reduced flavin. Given the ability of reduced flavin to form reactive oxygen

species when exposed to oxygen, a channeling mechanism would be beneficial within the cell (Figure **1.32B**).[144,209,211,212]

The mechanism of reduced flavin transfer in bacterial luciferase has been extensively studied. Bacterial luciferase from *Vibrio harveyi* was able to accept reduced flavin from different flavin reductases. These flavin reductases were identified in luminous bacteria and were classified as FRP, FRD, and FRG. Flavin reductases categorized as FRP prefer NADPH, whereas those categorized as FRD show a preference for NADH. Conversely, flavin reductases within the FRG category are able to utilize NADPH and NADH with similar catalytic efficiencies.[151] Kinetic analyses performed only in the presence of the different types of flavin reductases as well as experiments with both the reductase and bacterial luciferase present supported a channeling mechanism for reduced flavin transfer. The results from these kinetics studies revealed an altered K_m when both the reductase and bacterial luciferase are included in the assay as compared to when the assay is performed in the absence of bacterial luciferase. Two-component enzymes systems that utilize a diffusion mechanism for reduced flavin transfer have reported similar K_m values for assays in the absence of the monooxygenase as well as when both the flavin reductase and monooxygenase are present.[151,213-215] For the bacterial luciferase two-component system, there is a slower turnover rate for the luciferase enzyme compared to the reductases. Therefore, the overall catalytic efficiency will be limited when both the reductase and luciferase are included. Lower K_m values for FMN and the reduced pyridine nucleotide substrates were obtained when both the luciferase and flavin reductase are included in the reaction. These findings supported a

channeling mechanism required for reduced flavin transfer from the reductase to the monooxygenase.[146,214,216]

Another example of a two-component flavin-dependent system that utilizes a channeling mechanism for reduced flavin transfer is the EDTA-monooxygenase enzyme system (EmoA/EmoB).[217] The two-component EDTA-monooxygenase system is involved in EDTA-degradation. Initial studies with this system from *Mesorhizobium sp.* BNC1 suggested that reduced flavin was transferred through diffusion from the reductase (EmoB) to the partner monooxygenase (EmoA).[177] The mode of reduced flavin transfer between EmoB and EmoA was re-investigated using various kinetic approaches. Oxidation between NADH and NAD^+ was significantly faster in the presence of the EmoB/EmoA pair as compared to the EmoA/Fre pair. In $FMNH_2$ diffusion experiments, EmoA and EmoB were placed in different diffusion chambers that were separated by dialysis cellulose membrane. The $FMNH_2$ produced by EmoB could diffuse across the membrane, even though there was no physical interaction between EmoB and EmoA. Additionally, the formation of the glyoxylate product was significantly faster in the absence of the membrane barrier. These results suggest that the efficient transfer of reduced flavin from EmoB to EmoA involves a channeling mechanism.[217] Enzymes that follow a channeling mechanism for reduced flavin transfer, require protein-protein interactions between the reductase and the monooxygenase. The reductase and monooxygenase must interact in the right orientation to effectively transfer reduced flavin. Compared to the diffusion mechanism, the channeling mechanism is specific since it requires the presence of protein-protein interactions between two enzymes. Besides its specificity, this

mode of reduced flavin transfer is considered more efficient since it prevents the formation of reactive oxygen species.[211,212]

Although studies with bacterial luciferase and EmoB/EmoA provide strong evidence for a channeling mechanism requiring protein-protein interactions between the reductase and the monooxygenase, the *p*-hydroxyphenyl acetate hydroxylase (HPAH) system has been reported to utilize a diffusion mechanism for reduced flavin transfer. The HPAH two-component enzyme system catalyzes the hydroxylation of *p*-hydroxyphenyl acetate (HPAH) to 3,4-dihydroxyphenyl acetate (DHPA). The mechanism of reduced flavin transfer in HPAH from *E. coli* and *Acinetobacter baumannii* has been investigated using different kinetic approaches. Other flavin reductases were able to effectively replace the *E. coli* reductase involved in the HPAH two-component system. Additionally, the *E. coli* HPAH two-component enzymes were able to catalyze the overall reaction even when both enzymes were separated by a dialysis membrane. Moreover, no complex formation was observed between the reductase and the monooxygenase in gel filtration analyses in the presence and absence of *p*-hydroxyphenyl acetate (HPA) of the HPAH system from *A. baumannii*. Finally, the results obtained from transient state kinetic experiments with cytochrome *c* revealed that the rates of flavin transfer were not dependent on complex formation between the reductase and monooxygenase.[218,219] While most two-component flavin-dependent enzymes have been reported to either only utilize a diffusion mechanism or a channeling mechanism for the transfer of reduced flavin, the two-component styrene monooxygenase system has been reported to utilize both mechanisms.[210]

The transfer of reduced flavin through protein-protein interactions (channeling mechanism) has been observed in different two-component flavin-dependent

systems.[151,211,212,214-217] The mode of reduced flavin transfer between SsuE and SsuD was previously evaluated. Results from affinity chromatography binding assays and chemical cross-linking studies supported a channeling mechanism as a mode of reduced flavin transfer from SsuE to SsuD. In these studies, native SsuE and His-tagged SsuD coeluted in the affinity chromatography binding assays, whereas chemical cross-linking studies showed a protein band (~63 kDa) potentially corresponding to monomers of SsuE and SsuD covalently bound to the sulfo-SBED (sulfo-*N*-hydroxysuccinimidyl-2-(6-[biotinamido]-2-(*p*-azidobenzamido)-hexanoamido) ethyl-1,3'-dithiopropionate cross-linker.[212]

In order to investigate the interaction sites in SsuE and SsuD that are involved in protein-protein interactions, hydrogen-deuterium exchange mass spectrometry (HDX-MS) was performed. There was a ~20% decrease in solvent accessibility for specific regions within the SsuE-SsuD complex. The regions in SsuE that showed decreased deuterium exchange were residues 78-89 (KAAYSGALKTLL) and 118-124 (YALKPVL), whereas for SsuD residues 285-295 (EISPNLWAGVG) showed decreased deuterium exchange (Figure **1.33A**).[211] The protected regions identified on SsuE are part of two different α-helices, whereas those identified on SsuD are part of two α-helices connected by a loop region (Figure **1.33B**). This loop region is located near the active site of SsuD and has been proposed to close over the active site once substrates are bound. This loop region has also been identified in other TIM-barrel enzymes and has been proposed to exclude bulk solvent from the active site thereby stabilizing any reactive intermediates as well as preventing the premature release of bound substrates and products. Due to the presence of the protected regions near the active site, it was further proposed that protein-

protein interactions between SsuE and SsuD facilitate the alignment of the active sites to coordinate reduce flavin transfer.[211]

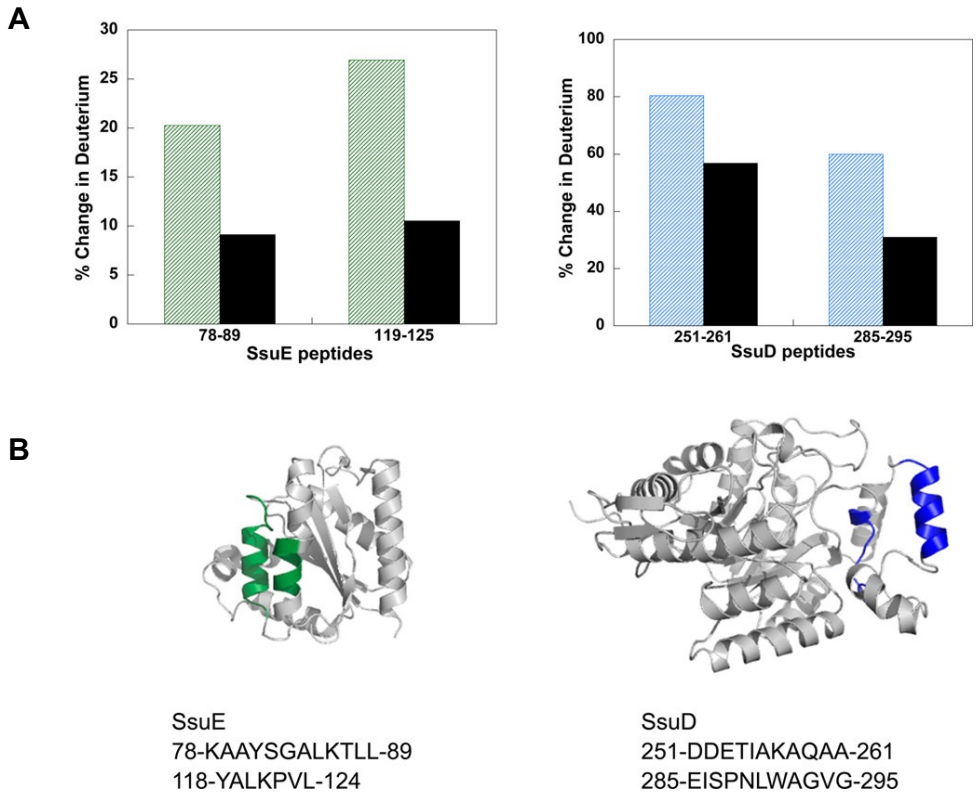

Figure 1.33: Solvent-protected regions in SsuE-SsuD complex identified by HDX-MS. **A.** Changes in solvent accessibility of SsuE (green bars) and SsuD (blue bars) alone and following incubation of both enzymes (black bars). **B.** Regions of SsuE and SsuD with decreased solvent accessibility in the SsuE-SsuD complex. (Adapted with permission from [211]) Copyright (2015) American Chemical Society.

The protected regions located on SsuE contain the positively charged residues Lys78, Lys86, and Lys121, whereas those regions on SsuD contain the negatively charged Asp251, Asp252, and Glu253. These residues were proposed to form salt bridges to facilitate protein-protein interactions between the two enzymes. An SsuD

deletion variant (Δ251-261) was generated to further investigate the role of these residues in protein-protein interactions. The DDE (251/252/253) AAA SsuD variant had a ~4-fold decrease in the k_{cat}/K_m value compared to wild-type SsuD in desulfonation assays, whereas no measurable sulfite production was observed with Δ251-261 SsuD.[211] The binding affinity of the SsuD variants for $FMNH_2$ was also evaluated, and the SsuD variants had a similar binding affinity for $FMNH_2$ as compared to the wild-type enzyme. The affinity of Δ251-261 SsuD for wild-type SsuE was also evaluated through affinity chromatography binding assays. In previous studies His-tagged SsuD co-eluted with native SsuE supporting the presence of protein-protein interactions between the two enzymes.[212] A similar experiment was performed with His-tagged Δ251-261 SsuD and native SsuE, however both enzymes were eluted separately. The inability of Δ251-261 SsuD to produce sulfite in desulfonation assays and to form a complex with native SsuE was attributed to the absence of the interaction region.[211]

The presence of protein-protein interactions between SsuE and SsuD supports a channeling mechanism as a mode for reduced flavin transfer between the reductase and the monooxygenase.[211,212] Both SsuE and SsuD are expressed in a broad range of bacteria enabling these organisms to utilize alternative sulfur sources during sulfur limitation.[4,66,67,75] SsuE and SsuD are expressed on the *ssu* operon that also contains an ABC-type transporter required for the uptake and transport of alkanesulfonates into the bacterial cell.[81] Besides the Ssu enzymes, certain bacteria have a more complex set of enzymes that are expressed during sulfur starvation. In addition to the Ssu enzymes, *Pseudomonas* species also express the enzymes MsuC, MsuD, and MsuE that provide alternative means for sulfur acquisition during sulfur starvation.[4,62,93] In most bacteria, the

genes encoding for SsuE (or MsuE) and SsuD (or MsuC and MsuD) are located on the same operon. However, previous studies with *Bacillus subtilis* revealed that the *ssu* operon does not contain an *ssuE* gene, suggesting that an alternative reductase could be involved in supplying SsuD with reduced flavin during sulfur limitation and that protein-protein interactions may not be required.[72,76] Studies in our lab identified an oxidoreductase within the *ssu* operon that could potentially encode for SsuE. Previous studies demonstrated that SsuD indeed does have a higher affinity for reduced flavin as compared to SsuE, however the concentration of both enzymes has not been determined in the cell.[211,220] Therefore, it is currently unclear how protein-protein interactions would play a role in catalysis under cellular conditions. Although there is strong evidence that SsuD and SsuE utilize a channeling mechanism for reduced flavin transfer *in vitro*, it is currently unclear what mechanism is utilized by MsuE/MsuC and MsuE/MsuD for the transfer of reduced flavin.[211] Since MsuE must supply reduced flavin to two separate monooxygenases, MsuC and MsuD, the overall mechanism could be distinct compared to that observed with SsuE and SsuD. These studies provided a strong basis to further investigate how protein-protein interactions in the alkanesulfonate, methanesulfinate, and methanesulfonate monooxygenase systems are involved in catalysis.

1.4.4 The $FMNH_2$-dependent Monooxygenases in Sulfur Acquisition

Monooxygenases that are part of two-component enzyme systems are provided with reduced flavin by a separate flavin reductase. The alkanesulfonate monooxygenase, SsuD, uses reduced flavin from the flavin reductase, SsuE, to perform the desulfonation of alkanesulfonates.[98] Conversely, in bacteria that express the methanesulfonate monooxygenase consisting of MsuE and MsuD, MsuE supplies reduced flavin to MsuD

for the desulfonation of a broad range of alkanesulfonates.[4,196,201] SsuD and MsuD share ~65% amino acid sequence identity and belong to the bacterial luciferase family. Based on the classification of the flavin-dependent monooxygenases described in previous sections, SsuD and MsuD belong to the class C flavin monooxygenases.[221]

Both SsuD and MsuD adopt the TIM-barrel fold and contain four insertion regions connecting α-helices and β-sheets.[193,196] Enzymes adopting the TIM-barrel fold contain eight alternating α-helices and eight parallel β-strands that are oriented in a doughnut-like shape (Figure **1.34A**).[222] One of the insertion regions of SsuD was largely unresolved in the three-dimensional structure which was attributed to increased flexibility within this region.[193] This flexible loop region is positioned close to the active site which is located at the C-terminal end of the β-barrel for TIM-barrel enzymes.[193,222] This flexible loop is observed in various TIM-barrel enzymes and has been proposed to close over the active site once substrates are bound. MsuD also contains a similar flexible loop region at the C-terminal end of the β-barrel which has been proposed to have a similar function (Figure **1.34B**).[196] The oligomeric state of SsuD (*E. coli*) and MsuD (*P. fluorescens*) has been evaluated through gel filtration analyses and X-ray crystallography, and the results from these studies revealed that both enzymes exist as homotetramer with an active site in each subunit.[66,193,196] Both SsuD and MsuD share conserved structural and active site features and are both involved in bacterial sulfur acquisition.[223] Initial studies have been focused on elucidating the desulfonation mechanism of SsuD as well as how specific structural features are involved in catalysis.

Unlike SsuE, SsuD has a higher affinity for reduced flavin compared to oxidized flavin. The order of substrate binding in SsuD has been previously investigated, and the

results revealed that SsuD follows an ordered-sequential binding mechanism with $FMNH_2$ binding first followed by octanesulfonate or molecular oxygen.[220] Since SsuD and MsuD share a relatively high amino acid sequence identity as well as conserved active site features, it is hypothesized that both enzymes could utilize a similar mechanism for desulfonation.[221,223,224]

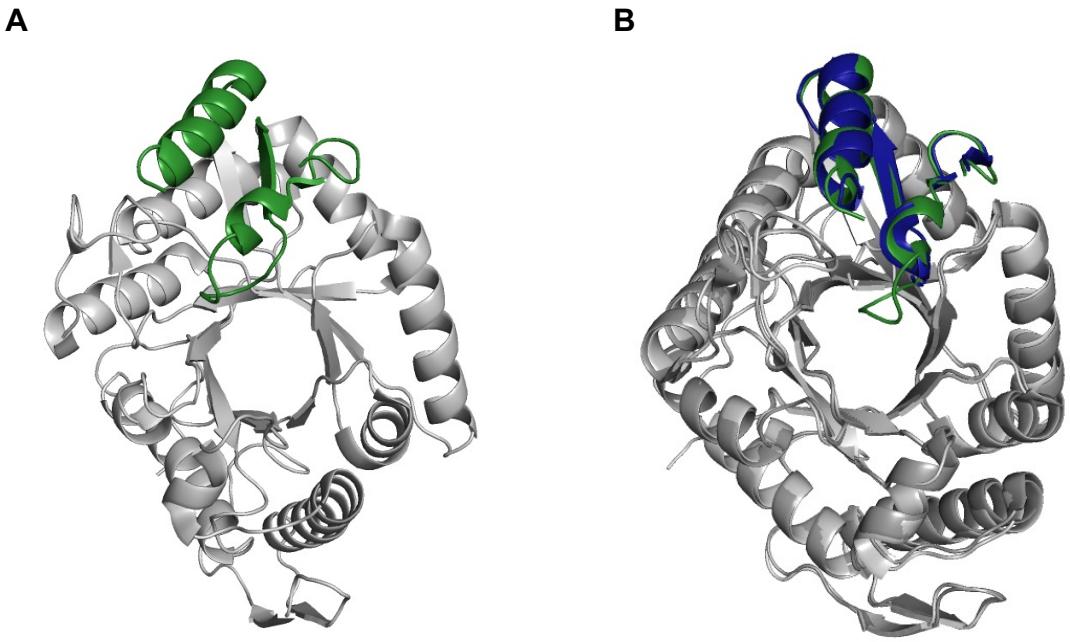

Figure 1.34. Structural features of SsuD **A.** TIM-barrel fold of SsuD with the dynamic loop region portrayed in green. **B.** Overlay of SsuD from *E. coli* (PDB: 1M41) and MsuD from *P. fluorescens* (PDB: 7JV3). The dynamic loop region of SsuD is shown in green, whereas that for MsuD is shown in blue. (Adapted from [193,196]).

SsuD and MsuD have been characterized as members of the bacterial luciferase family. Enzymes within this family contain a TIM barrel fold and several insertion regions. The active site of enzymes belonging to the bacterial luciferase family is located at the C-terminal end of the β-barrel.[193,196] Both SsuD and MsuD contain an insertion region consisting of a dynamic loop region that is proposed to close over the active site once substrates are bound.[221] Loop closure facilitates the removal of bulk solvent from the

active site, thereby protecting any reactive intermediates that are formed as well as preventing premature release of products.[225-232] In addition to the bacterial luciferase family, this dynamic loop region has been observed in many TIM barrel enzymes.[222,233-236] This dynamic loop region typically contains an arginine or lysine residue that is proposed to interact with the substrates to trigger loop closure. A similar loop region has been identified in bacterial luciferase, and studies evaluating the role of this region revealed that the enzyme was protected from proteolysis in the presence of phosphate and reduced flavin.[233,234] The dynamic loop region has been identified in all SsuD and MsuD homologs and includes an arginine residue which has been thoroughly investigated in previous studies with SsuD.[196,232]

Studies evaluating the role of the dynamic loop region in SsuD as well as the Arg297 residue revealed that substituting the arginine to a lysine or alanine residue resulted in a ~30-fold decrease in the k_{cat}/K_m for the R297K SsuD variant, whereas no activity was observed for the R297A SsuD variant. Both SsuD variants had a comparable binding affinity for $FMNH_2$ as the wild-type enzyme in fluorometric titration analyses; however, no binding affinity was observed for octanesulfonate.[232] The role of the conserved arginine residue in MsuD (Arg296) was similar to that of SsuD were the R296A MsuD variant did not have any measurable activity.[196] Since it has been hypothesized that the binding of substrates induces conformational changes in the form of loop closure over the active of SsuD, the role of the dynamic region containing Arg297 was further investigated using partial trypsin digestion analyses. In the absence of substrates, wild-type SsuD and the Arg297 SsuD variants were rapidly digested with no intact protein remaining after 15 s. Conversely, in the presence of $FMNH_2$ and octanesulfonate, wild-type SsuD had ~20%

intact protein left after 30s, whereas both variants were almost completely digested.[232] The partial digestion of both wild-type SsuD and the variants in the absence of any substrates revealed two major bands that were further analyzed using mass spectrometry. The results obtained from mass spectrometry confirmed the presence of the dynamic loop of SsuD and identified tryptic sites containing Arg263 and Arg271. These two arginine residues located on the flexible loop of SsuD are accessible to proteases (in this case trypsin), however upon substrate binding these sites become partially protected due to the resulting conformational change.[232] In the three-dimensional structure of SsuD, the loop region was seen pointing away from the active site. Since these studies provided evidence that Arg297 is critical for catalysis, it was proposed that this residue could interact with the phosphate group of $FMNH_2$ to initiate conformational changes. The results obtained from these studies highlight the importance of Arg297 in the desulfonation reaction of SsuD.[232]

The mechanistic role of the dynamic loop region in SsuD was further investigated through three deletion variants. A shorter ΔH276-N282 SsuD deletion variant was generated to monitor the effects of the polar residues within the dynamic loop region. Additionally, a second ΔF261-L275 SsuD deletion variant was generated to evaluate the role of twelve conserved residues within this region, and a third ΔF261-N282 SsuD deletion variant was generated that contained both the polar and conserved residues.[237] Results from desulfonation assays revealed that no measurable sulfite production was observed with the generated variants. Since all three SsuD loop deletion variants had comparable binding affinity for $FMNH_2$ as the wild-type enzyme, the absence of activity was not due to $FMNH_2$ binding. The ΔF261-N282 and ΔF261-L275 SsuD deletion variants

showed minimal digestion in the presence of $FMNH_2$ and octanesulfonate as compared to the wild-type enzyme. Even though ΔH276-N282 SsuD still contained both tryptic targets, minimal digestion was observed with this variant, which was attributed to the decreased flexibility because of the partial deletion of the loop.[237] Moreover, a rapid reaction experiment monitoring reduced flavin transfer was performed with the SsuD deletion variants, and the results revealed that reduced flavin was immediately oxidized after the reductive reaction by SsuE. This suggested that the loop region of SsuD protects reduced flavin from autooxidation and could also be involved in protein-protein interactions with SsuE for the efficient transfer of reduced flavin.[237] Lastly, results obtained from affinity binding chromatography experiments revealed that His-tagged ΔF261-N282 SsuD coeluted with native SsuE, indicating that the protein-protein interactions were still intact. Since protein-protein interactions were still intact in the largest SsuD loop deletion variant (ΔF261-N282), the absence of the lag phase in rapid reaction experiments was not due to the absence of protein-protein interactions. The absence of activity with the variants could be due to changes in the active site further affecting the ability of SsuD to exclude bulk solvent and protect reduced flavin from autooxidation. Taken together, these studies provide evidence that SsuD becomes partially protected upon substrate binding due to loop closure over the active site.[232,237]

Both SsuD and MsuD share common structural and active site architectures. The several commonalities between both enzymes have often led to the hypothesis that SsuD and MsuD could utilize a similar mechanism for desulfonation.[196,221,238] Despite extensive research, details surrounding the mechanism of desulfonation by SsuD remain to be elucidated.[220,232,237,239-241] After acquiring reduced flavin from the flavin reductase, flavin-

dependent monooxygenases have been proposed to activate molecular oxygen through the formation of C4a-(hydro)peroxy flavin intermediates.[159,242] The stability of the C4a-(hydro)peroxy flavin intermediates varies between monooxygenases that are part of two-component systems. Generally, a hydrophobic or solvent-free active site environment is required for stabilizing these highly reactive flavin intermediates.[130] Studies with bacterial luciferase have provided evidence that this two-component monooxygenase utilizes the C4a-hydroperoxy flavin intermediate for the oxygenation of long-chain aldehydes to yield to corresponding carboxylic acid. The C4a-hydroperoxy flavin formed by bacterial luciferase was reported to be stable in the absence of the aldehyde substrate at 0 °C.[243] Since SsuD is structurally similar to bacterial luciferase and based on studies with other flavin-dependent monooxygenases, it was proposed that SsuD could utilize similar C4a oxygenating flavin intermediates.[193,239] Two possible desulfonation mechanisms were proposed for SsuD; one involving a C4a-peroxy flavin intermediate and the other one involving a C4a-hydroperoxy flavin intermediate (Figure **1.35**).[239]

In the first proposed mechanism of SsuD, reduced flavin reacts with molecular oxygen to form the C4a-peroxy flavin intermediate which is further proposed to make a nucleophilic attack on the sulfur atom of octanesulfonate (Figure **1.35**, steps 1 and 2).[239] This step is proposed to generate a peroxy flavin-organosulfonate intermediate that undergoes a Baeyer-Villiger rearrangement resulting in the release of sulfite and a peroxyalkane adduct (Figure **1.35**, step 3). An active site base is proposed to abstract a hydrogen from the α-carbon of the peroxyalkane adduct resulting in the heterolytic cleavage of the oxygen-oxygen bond leading to the formation of the corresponding aldehyde and the C4a-hydroxy flavin intermediate (Figure **1.35**, step 4). In the final step,

an active site acid is proposed to donate a proton to the C4a-hydroxy flavin intermediate leading to the formation of water and the regeneration of oxidized flavin (Figure **1.35**, step 5).[239] In the second proposed mechanism involving a C4a-hydroperoxy flavin

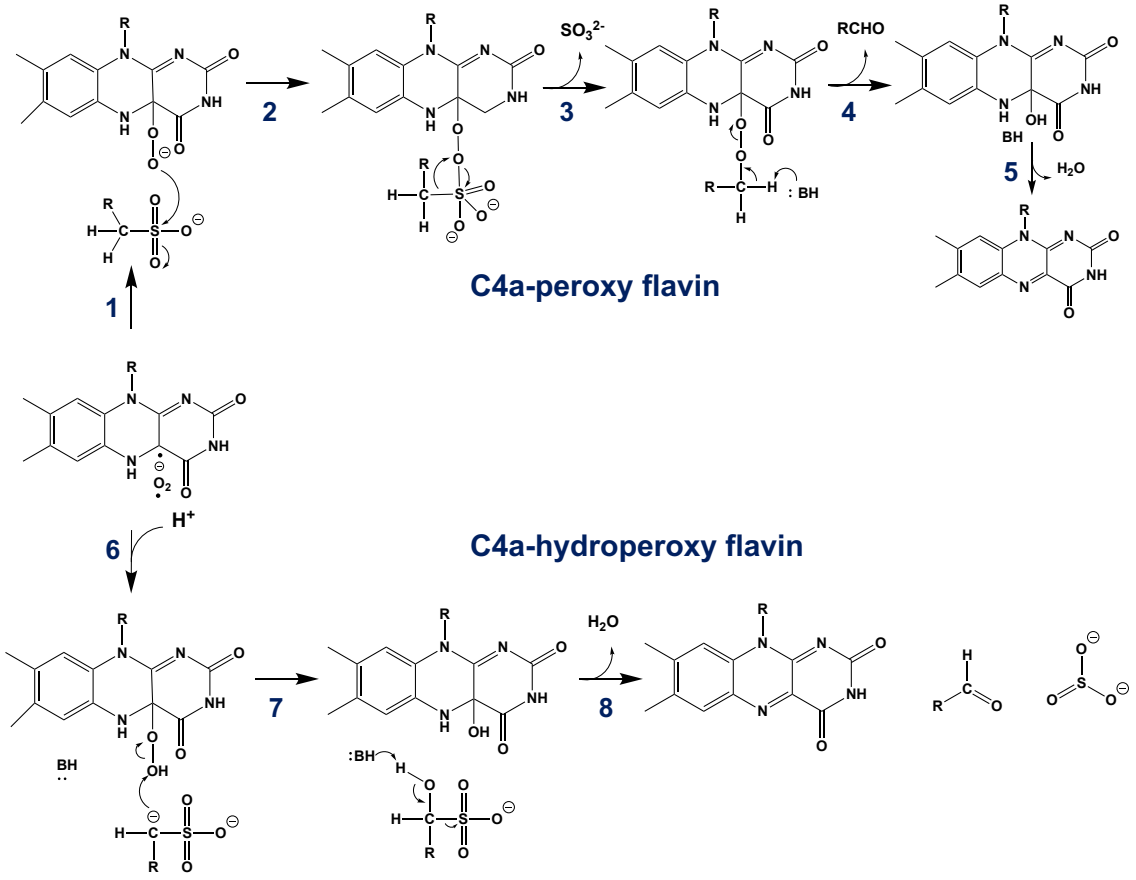

Figure 1.35: Previously proposed mechanisms for SsuD involving a C4a-peroxy flavin intermediate (upper pathway) and a C4a-hydroperoxy flavin intermediate (lower pathway). (Adapted from [239]).

intermediate, an active site base is proposed to abstract a hydrogen from the α-carbon of octanesulfonate generating a carbanion intermediate (Figure **1.35**, step 6). The carbanion intermediate is proposed to make a nucleophilic attack on the C4a-hydroperoxy flavin that would result in an unstable 1-hydroxyalkanesulfonate and the C4a-hydroperoxy flavin intermediate (Figure **1.35**, step 7). The unstable 1-hydroxyalkanesulfonate is

subsequently decomposed to yield the corresponding aldehyde and sulfite (Figure **1.35**, step 8).[239]

Even though SsuD shares a relatively low amino acid sequence identity (~15%) with bacterial luciferase and LadA, SsuD contains several of the conserved amino acids identified within this family of enzymes. Based on structural similarity with these two enzymes, His11, His228, His333, Arg226, Cys54, Tyr331, and Phe7 in SsuD were proposed to play a role in the desulfonation reaction of SsuD (Figure **1.36**).[239] The Cys106

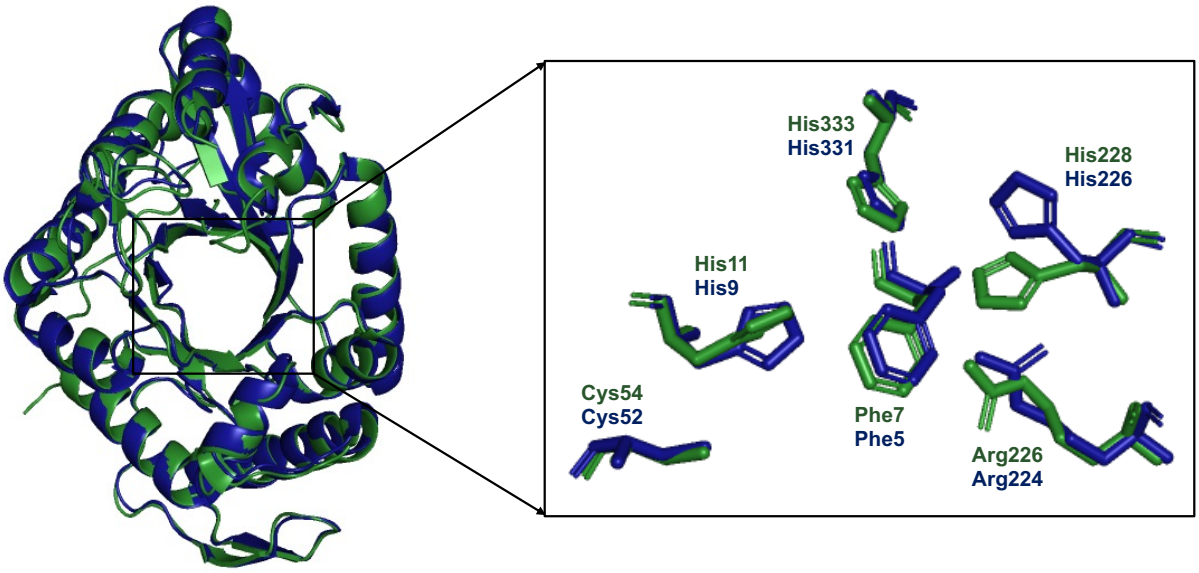

Figure 1.36: Overlay of SsuD from *E. coli* (displayed in dark green; PDB: 1M41) and MsuD from *P. fluorescens* (displayed in dark blue; PDB: 7JV3) portraying the active site residues.

in bacterial luciferase has previously been reported to play a critical role in stabilizing the C4a-(hydro)peroxy flavin intermediate. In previous studies with SsuD, Cys54 was labeled with methylmercury which led to inactivation of the enzyme.[66,193] Since Cys54 is the only cysteine residue in SsuD, it was hypothesized to have an essential role in catalysis.[244]

The ability of the C54S and C54A SsuD variants to produce sulfite was further evaluated through desulfonation assays in the presence of wild-type SsuE. While C54S SsuD had similar activity as wild-type SsuD, a significant decrease in the k_{cat}/K_m was observed with the C54A SsuD variant. These results suggest that the serine residue was able to effectively substitute for the cysteine residue in SsuD. Both variants had similar binding affinities for $FMNH_2$ as compared to wild-type SsuD; however, an increased binding affinity was observed for octanesulfonate. The pK_a value of Cys54 was also evaluated, and a single pK_a of 9.3 ± 0.1 was obtained. This suggested that Cys54 most likely exists in the protonated state during catalysis and could be involved in hydrogen bonding interactions. The similar activity observed with the C54S SsuD variant suggested that a serine residue could effectively substitute for Cys54 and participate in possible hydrogen bonding interactions.[244] Structural modelling of bacterial luciferase, LadA, and SsuD revealed that the corresponding cysteine residues within these enzymes are in close proximity with the pyrimidine portion of the flavin. Even though these structural analyses were performed with oxidized flavin, it was hypothesized that Cys54 in SsuD could be involved in hydrogen bonding interaction with the flavin or could provide for an optimal active site arrangement for catalysis.[244] A single conserved cysteine residue has also been observed in MsuD which could play a comparable functional role.

The mechanistic roles of the other conserved active site residues in SsuD were further investigated through various kinetic approaches. In the proposed reaction for SsuD involving a C4a-peroxy flavin intermediate, an active site base is proposed to abstract a proton from the α-carbon of the peroxyalkane adduct generating the corresponding aldehyde.[239] Previous studies with bacterial luciferase identified His44 as

the active site base in the catalytic reaction, whereas His311 in LadA was reported to be critical for catalysis. Based on the structural similarity with bacterial luciferase and LadA, His11, His228, and His333 were hypothesized to function as the catalytic base in SsuD. Additionally, in the proposed mechanisms of SsuD an active site acid would have to donate a proton to form the C4a-hydroxy flavin intermediate and this role was attributed to Arg226. The roles of these amino acid residues in SsuD were further evaluated through various kinetic approaches.[240] While the R226A and R226K SsuD variants did not have any measurable activity in desulfonation assays, the H11A, H228A, and H333A SsuD variants had similar activity as wild-type SsuD. The results from fluorometric titrations revealed that both Arg226 SsuD variants had similar binding affinity for $FMNH_2$ and octanesulfonate indicating that the absence of activity was not due to substrate binding.[240] SsuD was reported to have optimal activity between pH 7.2 and 8.5 when evaluating its pH profile. Two titratable residues for SsuD with pK_a values 6.6 ± 0.1 and 9.5 ± 0.1 were identified when evaluating the pH dependence of k_{cat}, suggesting that a residue with a pK_a value of 6.6 must be deprotonated, whereas one with a pK_a value of 9.5 must be protonated for catalysis to occur up through product release. It was finally revealed that the guanido functional group of Arg226 may participate in hydrogen-bonding interactions critical for catalysis. MsuD also contains a similar Arg225 residue that was proposed to be play a comparable role in catalysis. Substitution of Arg225 in MsuD to an alanine completely abolished catalytic activity of the enzyme highlighting its importance in catalysis.[240]

Unlike SsuD and MsuD, MsuC is structurally distinct and adopts the acyl-CoA dehydrogenase fold.[197] Enzymes adopting the acyl-CoA dehydrogenase fold generally

have three domains consisting of an N-terminal α-helical domain, a central domain consisting of β-sheets, and C-terminal α-helical domain (Figure **1.37**). Enzymes with this

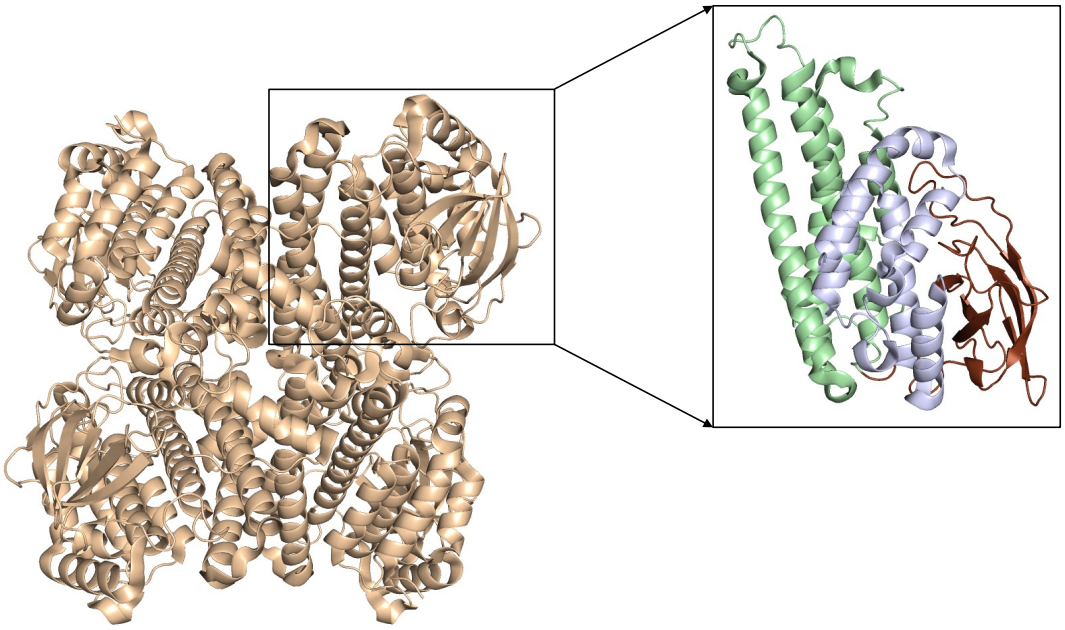

Figure 1.37: Crystal structure of MsuC from *P. fluorescens* (PDB: 6UUG). The different domains are represented by different colors. The C-terminal α-domain is shown in green tints, the N-terminal α-domain is shown in purple tints, and the central β-domain is shown in brown. (Adapted from [197]).

fold generally exist as homotetramers. MsuC has been characterized as a monooxygenase that is part of the two-component methanesulfinate monooxygenase system and belongs to the class D flavin-dependent monooxygenases. The methanesulfinate monooxygenase system contains the enzymes MsuE and MsuC. MsuE supplies reduced flavin to MsuC for the oxidation of methanesulfinate to methanesulfonate.[197] Previous studies with MsuC from *P. fluorescens* reported that this enzyme exists as homotetramer in gel filtration analyses which was also in agreement with the results obtained from structures obtained through X-ray crystallography. The

quaternary structure of MsuC resembles that of DszC as well as other flavin-dependent monooxygenases that adopt the acyl-CoA dehydrogenase fold. MsuC shares 42% amino acid sequence identity with DszC. Since MsuC is structurally most similar to DszC, it was hypothesized that MsuC could have a similar functional role. The ability of MsuC to oxidize methanesulfinate and yield methanesulfonate using reduced flavin provided by MsuE was further demonstrated by ^{1}H NMR spectroscopy.[197]

SsuD, MsuC, and MsuD have been characterized as monooxygenases belonging to two-component enzyme systems. These monooxygenases rely on a separate flavin reductase for reduced flavin required to perform the oxidative half reaction.[4] SsuD and MsuD are structurally similar and have been proposed to utilize a similar mechanism for desulfonation.[196,221,238] Contrarily, MsuC is structurally distinct and has therefore been hypothesized to utilize a different catalytic mechanism.[197] Although SsuD from *E. coli* has been extensively studied, details regarding the overall catalytic mechanism remain to be elucidated. Previous studies with MsuC and MsuD from *P. fluorescens* have provided structural insight into these enzymes; however, extensive research on the catalytic mechanism of these enzymes has not been performed.[196,197] The mechanistic details of SsuD, MsuC, and MsuD will provide a strong foundation and a better understanding of how bacteria are able to utilize alternative sulfur when deprived from inorganic sulfate. Since sulfur is an extremely important element for metabolism and survival in bacteria including pathogens, mechanistic information regarding these enzymes could represent a notable drug target.

1.5 Physiological Relevance of These Studies

Previous sections in this dissertation have extensively highlighted the importance of sulfur in various metabolic and physiological processes in all organisms. Since bacteria often find themselves in environments with low sulfur bioavailability, the synthesis of various two-component flavin-dependent systems enable bacteria to obtain sulfur in the form of organosulfonates.[4] The two-component flavin-dependent systems expressed by several bacteria include the alkanesulfonate monooxygenase system, the methanesulfinate monooxygenase system, and the methanesulfonate monooxygenase system. The alkanesulfonate monooxygenase system is common in a broad range of bacteria.[4,66,75,196,197,239] Certain bacteria also express the methanesulfinate monooxygenase system and methanesulfonate monooxygenase system in addition to the alkanesulfonate monooxygenase system.[4]

The aforementioned two-component systems are also found in pathogenic bacteria including *P. aeruginosa*.[4,9,48,245] *P. aeruginosa* is a Gram-negative bacterium as well as known opportunistic human pathogen. *P. aeruginosa* is ubiquitous in the environment and has been the main cause of mortality in patients with cystic fibrosis (CF) and patients with a compromised immune system.[246-249] *P. aeruginosa* has often been linked to hospital acquired infections and has become a major health threat globally.[249-251] Treatment of infections caused by *P. aeruginosa* have become extremely difficult to treat due to its natural resistance to several antibiotics.[246-249,252,253] In addition to its natural resistance to antibiotics, *P. aeruginosa* also expresses a variety of genes for virulence, survival, and adaptation.[246-249]

The global emergence of antibiotic resistance encourages the development of new therapeutic strategies to treat infections caused by bacterial pathogens.[253,254] The fact that bacterial pathogens require sulfur for various metabolic needs could be utilized as a strategy for drug development. As previously discussed in earlier sections of this dissertation, bacteria utilize inorganic sulfate as their main sulfur source.[4] In addition to sulfur, transition metals are also required for various biological processes. Previous studies have reported that upon bacterial infection, the host limits the availability of several transition metals to the pathogen. This process has been referred to as nutritional immunity.[255,256] It is currently unclear whether there is a time frame during bacterial infection that leads to sulfur limitation within the host. Sulfur limitation would enable the expression of the *ssi* genes by the bacteria which encode for the alkanesulfonate monooxygenase system, the methanesulfinate monooxygenase system, and the methanesulfonate monooxygenase system.[4,9,48,64,65,75,93] These enzyme systems would allow the pathogen to utilize alternative sulfur sources. The two-component enzymes have only been identified in bacteria, and the absence of these enzymes in mammals makes these enzyme systems an excellent drug target. Investigating the catalytic mechanism of these two-component systems in bacteria including pathogens, has therefore been proposed to be a promising strategy which could be further exploited in biotechnology as wells as in the design of potential inhibitors.

1.6 Summary

Sulfur-containing biomolecules fulfil many essential roles in all domains of life. Unlike mammals, bacteria often find themselves deprived from inorganic sulfate in their habitat.[4] Due to the limited availability of their preferred sulfur sources, bacteria must rely on

organosulfonates to meet their sulfur needs.[4,62,63,67] In order to utilize organosulfonates, bacteria have evolved to express the sulfate-starvation-induced (*ssi*) genes. The *ssi* genes encode for ABC-type transporters as well as enzymes that perform the desulfonation of organosulfonates.[66,67,69,75,81,257] The desulfonation reaction involves the cleavage of the C-S bond of the organosulfonates to yield sulfite which is further incorporated in the biosynthesis of other sulfur-containing biomolecules. The *ssi* genes are expressed during sulfate starvation and initially included taurine dioxygenase and the alkanesulfonate monooxygenase system.[66,69] Following initial characterization of these two enzyme systems, other SSI enzymes have been identified which include the methanesulfinate monooxygenase system and the methanesulfonate monooxygenase system.[4,62,93,196,197]

The alkanesulfonate (SsuE/SsuD), the methanesulfinate (MsuE/MsuC), and the methanesulfonate (MsuE/MsuD) monooxygenase systems are part of two-component flavin-dependent systems that enable bacteria to utilize a broad set of organosulfonates during sulfur starvation.[4] The two-component flavin-dependent monooxygenase systems consists of a flavin reductase and a partner monooxygenase that are required to catalyze the overall reaction. The role of the flavin reductases (SsuE and MsuE) is to supply reduced flavin to the partner monooxygenase (SsuD, MsuC, and MsuD) to perform the oxidative half reaction.[98] Both the flavin reductases and monooxygenases are generally expressed on the same operon that often includes an ABC-type transporter system. An interesting feature of these two-component flavin-dependent systems is that the enzymes do not utilize flavin as a prosthetic group, instead flavin is utilized as a co-substrate in the

overall reaction.[98] The involvement of flavin as a co-substrate in the overall reaction brings new challenges when studying these enzyme systems.[99]

The alkanesulfonate monooxygenase, SsuD has been extensively studied. SsuD is a TIM-barrel enzyme belonging to the bacterial luciferase family.[66,193] Despite substantial research for decades, the catalytic mechanism of SsuD remains elusive.[220,232,237,240,241] Based on structural similarity with bacterial luciferase, SsuD was initially hypothesized to utilize a similar catalytic mechanism involving the C4a-(hydro)peroxy flavin intermediates.[239] Even after years of research, no spectral evidence for the formation of the C4a-(hydro)peroxy flavin intermediates in SsuD has been provided. Since no spectral evidence has been provided for the C4a-(hydro)peroxy flavin intermediates in SsuD, it has been hypothesized that most likely these intermediates are not stabilized by SsuD or that the enzyme utilizes another flavin intermediate. Recent studies with different bacterial flavin monooxygenases including enzymes belonging to two-component systems have provided spectral evidence for another flavin-adduct, namely the flavin-N5-oxide which is formed as the oxygenating flavin intermediate or as the final product during catalytic turnover.[166-170,175] Studies reported in this dissertation will focus on further elucidating the desulfonation mechanism of SsuD and how structural features play a role in catalysis.

In addition to the two-component alkanesulfonate monooxygenase system (SsuE/SsuD), *Pseudomonas* species express a more complex set of enzymes during sulfur starvation. These enzymes include the methanesulfinate monooxygenase system (MsuE/MsuC) and the methanesulfonate monooxygenase system (MsuE/MsuD).[4] Both SsuD and MsuD are structurally similar and have been hypothesized to utilize a common mechanism of desulfonation. Although previous studies have been focused on the

structural characterization of MsuD, details regarding the catalytic mechanism are currently unknown.[196] Unlike the structurally similar monooxygenases SsuD and MsuD, MsuC is structurally distinct. MsuC from *P. fluorescens* has been structurally characterized, however kinetic and mechanistic characterization of this enzyme are yet to be performed.[197]

The focus of this dissertation is to elucidate the mechanistic strategies of the two-component $FMNH_2$-monooxygenases SsuD (*E. coli*), MsuD (*P. aeruginosa*), and MsuC (*P. aeruginosa*) which all play key roles in bacterial sulfur acquisition. Additional studies are also focused on understanding how the structural features of these enzymes are involved in catalysis. The studies presented in this dissertation utilize structural, kinetic, and spectroscopic analyses to evaluate the catalytic mechanism of SsuD, MsuD, and MsuC in bacterial sulfur acquisition. This work provides a foundation for future studies focused on exploring the catalytic mechanism of two-component enzymes involved in bacterial sulfur acquisition. Finally, the results obtained from these studies provide a better understanding of the functional role of these enzymes in the overall global sulfur cycle.

CHAPTER TWO

Shorter Alkanesulfonate Carbon Chain Destabilize the Active Site of SsuD for Desulfonation

2.1 Introduction

Sulfur plays a key role in diverse metabolic process that occur in all organisms. Bacteria predominantly rely on inorganic sulfate or cysteine as their main sulfur sources.[4,10] However, due the limited availability of inorganic sulfate in many environments, bacteria need to utilize alternative sulfur sources to obtain this critical element. During sulfur starvation, bacteria can express specific proteins that provide an alternative means of obtaining sulfur through organosulfur uptake, sulfur acquisition from organic compounds, and protection against reactive oxygen species (ROS).[4,10,64] The type of alternative sulfur source utilized by bacteria is not universal and varies between bacterial groups. *E. coli* expresses enzymes during sulfur starvation that catalyze the desulfonation of taurine (TauD) and alkanesulfonates (SsuE/SsuD) as sulfur sources. *P. aeruginosa* has a complex system for sulfur acquisition due its presence in diverse environments and is able to utilize alkanesulfonates, sulfate esters, and aromatic sulfonates as sulfur sources.[4,9,93,258] In addition to the alkanesulfonate monooxygenase system, *P. aeruginosa* also contains a methanesulfonate monooxygenase system (MsuE/MsuD) that allows the organism to utilize a broad range of alkanesulfonate (C1-C12) substrates for sulfur acquisition.[93] Both the alkanesulfonate and methanesulfonate monooxygenase systems belong to the group C flavin monooxygenases that consist of a

flavin reductase and a separate $FMNH_2$-dependent monooxygenase to catalyze the desulfonation reaction.[98,155]

SsuD from *E. coli* and MsuD from *P. aeruginosa* share ~60% amino acid sequence identity (Figure **2.1A**) and have a similar active site architecture (Figure **2.1B**).[193] Both

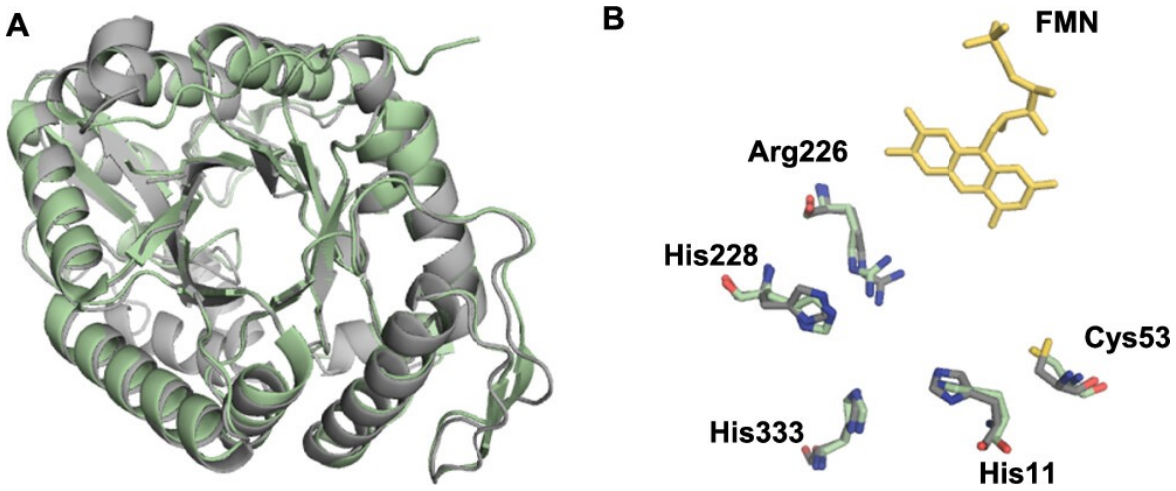

Figure 2.1: Structural comparison of SsuD from *E. coli* (PDB: 1M41) and MsuD from *P. aeruginosa*. **A.** Overlay of SsuD (green) and MsuD (gray). Both proteins have a TIM-barrel fold and similar insertion regions that diverge from the classic TIM-barrel structure. The MsuD structure was generated with AlphaFold.[322] The C-terminal region (356-381) showed low confidence level and was not included in the structure. **B.** Active site of SsuD (green) and MsuD (gray). The numbering of the active site amino acids is for the SsuD structure. The flavin highlighted is bound in the MsuD structure from *P. fluorescens* (PDB: 7JW9).

monooxygenases are TIM-barrel proteins that contain a dynamic loop region near their active site that is located at the C-terminal end of the β-barrel.[193,196,232] This dynamic loop region is found in different bacterial SsuD and MsuD enzymes and is proposed to close over the active site with the ordered binding of reduced flavin and the alkanesulfonate.

Loop closure over the active site would exclude the bulk solvent and stabilize reactive intermediates that are formed during catalysis, as well as prevent the premature release of any intermediates and bound substrates.[222,236] Previous studies provided evidence that SsuD was protected from proteolysis in the presence of $FMNH_2$, suggesting that $FMNH_2$ binding promotes loop closure over the active site. The subsequent binding of octanesulfonate (OCS) has also been hypothesized to induce a second conformational change in SsuD.[196,232,237] For MsuD, $FMNH_2$ binding organizes the active site for the binding of the sulfonated substrate.[196] The binding of methanesulfonate (MES) initiates a second conformational change that fully encloses over the active site.

Although both monooxygenases have a similar active site arrangement, SsuD from *E. coli* has reduced relative activity with MES.[66] MsuD from *P. aeruginosa* showed a clear preference for MES monitoring relative activity, while the MsuD homolog from *P. fluorescens* showed a broader alkanesulfonate range with higher relative activity observed for octanesulfonate.[93,196] The substrate preferences provide a wide range of alternative alkanesulfonate substrates for bacteria that are susceptible to limiting sulfur conditions. Given the similarity of the active site structure, it is unclear what contributes to the substrate specificity of SsuD and MsuD. Differences in the dynamic loop that closes over the active site may contribute to the specificity, and recent studies suggest that the C-terminal region is also involved in substrate recognition.[196] These studies directly compare the substrate specificity and conformational changes that occur with the binding of substrates to SsuD and MsuD. Complementary kinetic and structural analyses were performed to establish how enzymes with similar active site are able to both recognize and discern between similar substrates.

2.2 Experimental Procedures

2.2.1 Materials

All chemicals for protein purification and enzyme assays were purchased from Sigma-Aldrich, Fischer, Bio-Rad, or Fluka.

2.2.2 Expression and Purification of Recombinant Proteins

SsuD, SsuE, MsuD, and MsuE enzymes were purified as described previously.[179,201] The concentrations of SsuD and SsuE proteins were determined from A_{280} measurements using molar extinction coefficients of 47.9 and 20.3 M^{-1} cm^{-1}, respectively. The concentrations of MsuD and MsuE proteins were determined from A_{280} measurements using molar extinction coefficients of 49.4 and 7.54 M^{-1} cm^{-1}, respectively.[179,201]

2.2.3 Steady-state Kinetic Analyses

A coupled assay monitoring sulfite production was used to determine the steady-state kinetic parameters of SsuD and MsuD. The reactions were initiated with the addition of NADPH (500 μM) into a reaction mixture containing SsuD or MsuD (0.2 μM), SsuE or MsuE (0.6 μM), FMN (2 μM), and varying concentrations of the sulfonated substrates (0-1000 μM) in 25 mM Tris-HCl (pH 7.5) and 0.1 M NaCl at 25 °C. The reaction was quenched after 3 min with 8 M urea followed by the addition of DTNB (1 mM). After the addition of DTNB, the reaction was allowed to develop at room temperature for 2 min, and the absorbance was measured at 412 nm using the molar extinction coefficient for the TNB anion of 14.1 M^{-1} cm^{-1}. All assays were performed in triplicate, and the steady-state kinetic parameters were determined by fitting the data to the Michaelis-Menten equation.

2.2.4 Spectrofluorometric Titrations

The affinity of wild-type SsuD and MsuD for oxidized flavin (FMN) was monitored by spectrofluorometric titration. The binding of FMN to SsuD and MsuD (0.5 μM) was performed under aerobic conditions by adding aliquots of oxidized flavin (5-100 μM) to the enzyme solution in a fluorescence cuvette. Spectral changes were monitored at 344 nm (excitation at 280 nm) after each addition of FMN following a 2 min incubation.

For the titration of reduced flavin ($FMNH_2$), an anaerobic solution of SsuD and MsuD (0.5 μM) in 25 mM potassium phosphate (pH 7.5) and 100 mM NaCl (1.0 mL total volume) was titrated with a solution of $FMNH_2$. The enzyme solution was transferred inside the glovebox and treated with glucose (10 mM) and glucose oxidase (0.1 μM) to remove trace amounts of oxygen after which it was diluted in anaerobic 25 mM potassium phosphate (pH7.5) and 100 mM NaCl buffer. The buffer was made anaerobic by bubbling with ultrahigh-purity argon gas for 1 h before being transferred inside the anaerobic glovebox. Reduced flavin was prepared in 25 mM potassium phosphate (pH 8.0), 20 mM EDTA, and 100 mM NaCl. The flavin solution was bubbled with ultrahigh-purity argon for 30 min before being transferred to the anaerobic glovebox. After the addition of glucose (10 mM) and glucose oxidase (0.1 μM) to both protein and flavin, the solutions were incubated in an aerobic glovebox for 2 h to remove traces of dioxygen. The anaerobic flavin solution was photoreduced inside a gastight titrating syringe with a long wavelength UV lamp, after which the anaerobic cuvette was assembled inside the anaerobic glovebox. Wild-type SsuD or MsuD was titrated with 15 aliquots of $FMNH_2$ (0.08-1.20 μM). The fluorescence spectrum at 344 nm (excitation at 280 nm) was recorded following

a 2 min incubation after each addition of reduced flavin. Bound $FMNH_2$ was determined using equation 1.

$$[FMNH_2]_{bound} = [E]\frac{I_0 - I_c}{I_0 - I_f} \quad \textbf{Equation 1}$$

In equation 1, $[FMNH_2]_{bound}$ represents the concentration of the $FMNH_2$-bounds enzyme. [E] represents the initial concentration of the enzyme, I_0 is the initial fluorescence intensity of the enzyme prior to the addition of the substrate, I_c is the fluorescence intensity of the enzyme following the addition, and I_f is the final fluorescence intensity. The concentration of $FMNH_2$ bound was plotted against the free substrate to obtain the dissociation constant (K_d) according to equation 2.

$$Y = \frac{B_{max}X}{K_d + X} \quad \textbf{Equation 2}$$

In equation 2, Y and X represent the concentration of bound and free substrates, respectively, following each addition. B_{max} is the maximum binding at equilibrium with the maximum binding of substrate.

The affinity of $FMNH_2$-bound SsuD and MsuD for sulfonated substrates was investigated by a similar fluorometric titration method employed for reduced flavin binding. The sulfonated substrates were transferred inside the glovebox and dissolved in 25 mM potassium phosphate (pH 7.5) and 100 mM NaCl buffer that was made anaerobic as described. The buffer was made anaerobic by bubbling with ultrahigh-purity argon gas for 1 h before being transferred inside the anaerobic glovebox. Aliquots of an anaerobic solution of the sulfonated substrates (2.5-50 μM) in an airtight syringe were added to an anaerobic solution of either SsuD and MsuD (1.0 μM) and $FMNH_2$ (2.0 μM) in 25 mM potassium phosphate (pH 7.5) and 100 mM NaCl (1.0 mL total volume). Reduced flavin

was prepared as described but was added to the titration cuvette with the enzymes prior to the assembly of the anaerobic titration syringe containing the sulfonated substrates.

2.2.5 Limited Proteolytic Analysis

The susceptibility of SsuD and MsuD to proteolysis was investigated with trypsin in the absence and presence of FMN. Trypsin stock solution (1 mg/mL) was prepared in 1 mM HCl/ 1 mM $CaCl_2$ (pH 8.4). Samples of SsuD or MsuD (15 μM) were prepared in 200 mM ammonium bicarbonate/ 1 mM $CaCl_2$ (pH 8.4) and treated with TPCK-treated trypsin (10 μg/mL). To monitor the effects of oxidized flavin on proteolytic susceptibility, FMN (20 μM) in 25 mM potassium phosphate (pH 7.5) and 10% glycerol was included. After the addition of trypsin, samples (10 μL) were taken at various times (15 s, 30 s, 45 s, 1 min, and 3 min) and quenched through heat denaturation for 2 min at 90 °C. The degree of proteolysis of each sample was analyzed by SDS-PAGE. The protein band was quantified with ImageJ software (NIH, Maryland, United States of America) to determine the percent digestion.

To measure the degree of proteolysis in the presence of $FMNH_2$, an anaerobic solution of FMN (200 μM) was prepared in 25 mM potassium phosphate (pH 8.0), 20 mM EDTA, 10% glycerol. The FMN solution was bubbled with ultrahigh-purity argon gas for 30 min before being transferred to an anaerobic chamber. The SsuD or MsuD solutions were prepared in a glovebox with 200 mM ammonium bicarbonate (pH 8.4) and 1 mM $CaCl_2$. The buffer was made anaerobic by bubbling with ultrahigh-purity argon gas for 1 h before being transferred inside the anaerobic glovebox. After the addition of glucose (10 mM) and glucose oxidase (0.1 μM), both the enzyme and FMN solution were

incubated in anaerobic glovebox to remove trace amounts of dioxygen. The $FMNH_2$ solution was photoreduced inside a gastight syringe with a long-wavelength UV lamp. The proteolytic susceptibility of SsuD and MsuD (15 μM) was also evaluated in the presence of the sulfonate substrate (200 μM). The sulfonated substrate was resuspended in anaerobic 25 mM potassium phosphate (pH 7.5) and 10% glycerol. The buffer was made anaerobic by bubbling with ultrahigh-purity argon gas for 1 h before being transferred inside the anaerobic glovebox. The reaction was started with the addition of trypsin, and samples (10 μL) were taken at various times (15 s, 30 s, 45 s, 1 min, and 3 min). The reaction was quenched through heat denaturation for 2 min at 90 °C. The degree of proteolysis of each sample was analyzed by SDS-PAGE. The protein band was quantified with ImageJ software (NIH, Maryland, United States of America) to determine the percent digestion.

2.2.6 Enzyme Preparation

The Cartesian coordinates for the SsuD structures were constructed using a crystal structure at 2.3 Å resolution (PDB: 1M41). The reported structure was lacking internal residues 250-282 and C-terminal residues 362-380. Therefore, residues 250-282 were inserted using a comparative modelling program MODELLER 9.10. The program generated a refined 3D model of the given protein sequence (target) based primarily on its alignment to one or more proteins of known structure (templates). The templates used were the SsuD structure (PDB: 1NQK) and the structure of the luciferase-like monooxygenase from *Bacillus cereus* (PDB: 3RAO). The $FMNH_2$ ligand was inserted into the active site region of SsuD based on superposition with coordinates from LadA enzyme with a bound FMN (PDB: 3B90).

The methanesulfonate and octanesulfonate substrates were inserted into SsuD using AutoDock Vina. A grid box was fit to encompass the proposed active site, where between 10 and 20 binding modes were analyzed to determine the most probable structure based on previously proposed catalytic sites. Standard flexible protocols of AutoDock Vina using the Iterated Local Search global optimizer algorithm were employed to evaluate the binding affinities of the substrates within SsuD. All active site residues, as defined by the box size used for the receptors, were set to be rotatable. Calculations were performed using the exhaustiveness of the global search set to 100, a number of generated binding modes set to 20, and a maximum energy difference between the best and the worst biding modes set to 5.

2.2.7 aMD Protocol

Accelerated molecular dynamics (aMD) simulations were performed on the wild-type SsuD monomer with reduced flavin, octanesulfonate, and methanesulfonate. The coordinate and parameter files of the SsuD system was generated using the leap module of the Amber16 package, where the appropriate hydrogen atoms were added.[259] The system was solvated with TIP3P water molecules using a box that extended to 10 Å beyond the enzyme. Sodium ions were added to maintain system charge neutrality. The Amber force field was applied to construct the topology files for the protein, while the generalized Amber force field (GAFF) was used to generate the related parameters for the ligands.[260-262]

For the SsuD complex, the initial structure was conjugate gradient (CG) minimized for 5000 steps for water molecules only followed by 10,000 steps of CG optimization for the entire system. After minimization, the system was slowly heated from 0 to 300 K using

an NVT ensemble for 50 ps using the weak-coupling algorithm with a temperature coupling value of 2.8 ps. Then, the system was switched to an NPT ensemble at 300 K and 1 atm for 500 ps using a Berendsen barostat and the weak-coupling algorithm with a coupling value of 2.0 ps for both pressure and temperature. Next, the system was equilibrated for an additional 500 ps with an NVT ensemble. After this equilibration procedure, 10 ns of production data at NVT using unbiased MD was collected to derive the boost parameters needed for the subsequent aMD simulation using a best-practices procedure. These aMD bias potentials allow the system to overcome high energy barriers and only require the evolution of a single copy of the system. The bias boost potential function, $V(r)$, which is a continuous positive value, is utilized whenever the potential value $V(r)$ gets below a certain chosen energy value E.[263] Finally, the aMD simulation was caried out in the NVT ensemble for 1000 ns. All aMD simulations utilized the GPU-accelerated version of AMBER 16 featuring the particle mesh Ewald method to compute the long-range Coulomb force, periodic boundary conditions (PBC) with a nonbonded cutoff distance of 12 Å, and a time step of 1.0 fs.

2.3 Results

2.3.1 Evaluating the Kinetic Parameters of Wild-type SsuD and MsuD

Steady-state kinetic analyses monitoring sulfite production were performed to evaluate the range of alkanesulfonate carbon chain lengths utilized by SsuD and MsuD (Table **2.1** and Figures **2.2** and **2.3**). The kinetic parameters of SsuD have been evaluated with OCS based on the initial characterization of the enzyme, but the kinetic parameters have not been reported for alkanesulfonate substrates with varied carbon chain lengths. SsuD gave similar k_{cat}/K_m values with C6-C10 alkanesulfonate; however, no detectible

sulfite production was observed with MES or ethanesulfonate (ETS). MsuD gave a k_{cat}/K_m value of 0.83 x 10^4 M^{-1} s^{-1} with MES, but the activity decreases with C2 and C4 sulfonate substrates. However, as the carbon length increases, the k_{cat}/K_m values were comparable to MES.

Table 2.1: Steady-state kinetic parameters for SsuD and MsuD using sulfonated substrates of varying carbon lengths.

SsuD	**k_{cat} (s^{-1})**	**K_m (M × 10^{-6})**	**k_{cat}/K_m (M^{-1} s^{-1}, × 10^{-6})**
Methanesulfonate	-[a]	-	-
Ethanesulfonate	-	-	-
Butanesulfonate	0.56 ± 0.14	363 ± 193	0.15 ± 0.09
Hexanesulfonate	1.20 ± 0.06	123 ± 17	0.98 ± 0.14
Octanesulfonate	1.06 ± 0.04	62 ± 8	1.7 ± 0.2
Decanesulfonate	1.01 ± 0.03	30 ± 4	3.4 ± 0.5
MsuD	**k_{cat} (s^{-1})**	**K_m (M × 10^{-6})**	**k_{cat}/K_m (M^{-1} s^{-1}, × 10^{-6})**
Methanesulfonate	0.30 ± 0.01	36 ± 7	0.83 ± 0.16
Ethanesulfonate	0.48 ± 0.09	275 ± 159	0.17 ± 0.10
Butanesulfonate	0.30 ± 0.16	235 ± 200	0.13 ± 0.13
Hexanesulfonate	0.64 ± 0.04	118 ± 20	0.54 ± 0.10
Octanesulfonate	0.59 ± 0.02	49 ± 6	1.20 ± 0.15
Decanesulfonate	0.47 ± 0.02	39 ± 5	1.21 ± 0.16

[a]Value could not be determined under the experimental conditions used.

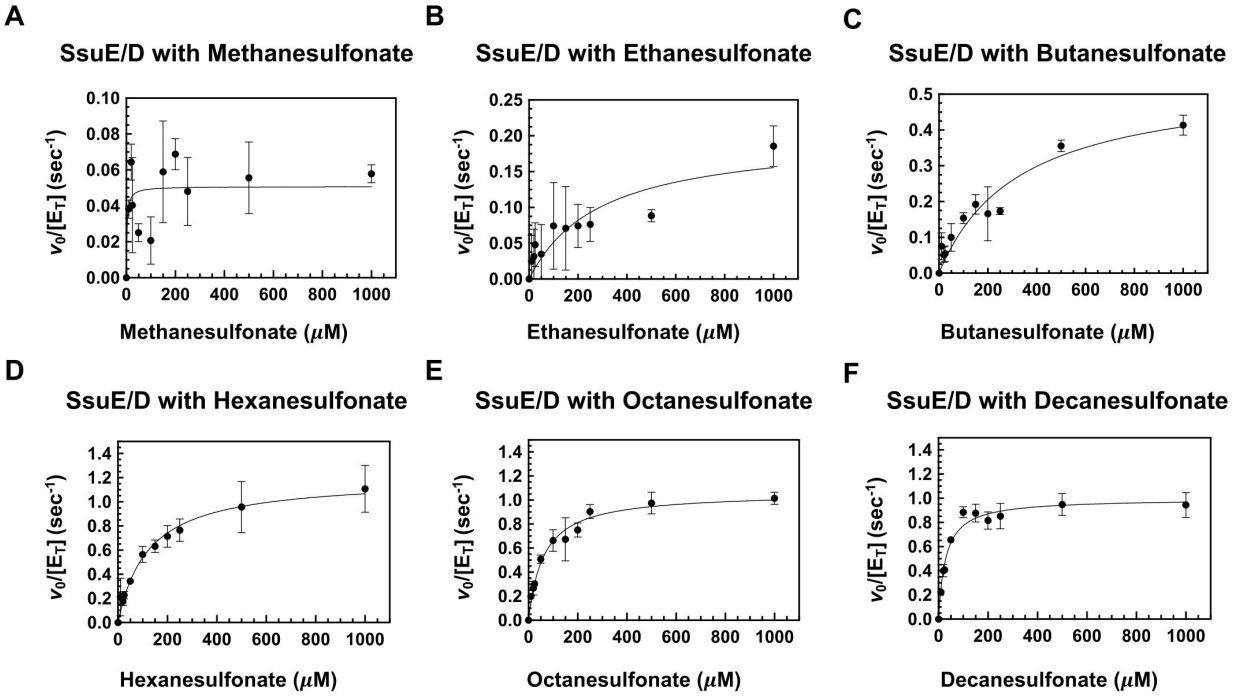

Figure 2.2: Steady-state kinetic analyses of SsuD with different alkanesulfonate substrates: **A.** Methanesulfonate. **B.** Ethanesulfonate. **C.** Butanesulfonate. **D.** Hexanesulfonate. **E.** Octanesulfonate. **F.** Decanesulfonate.

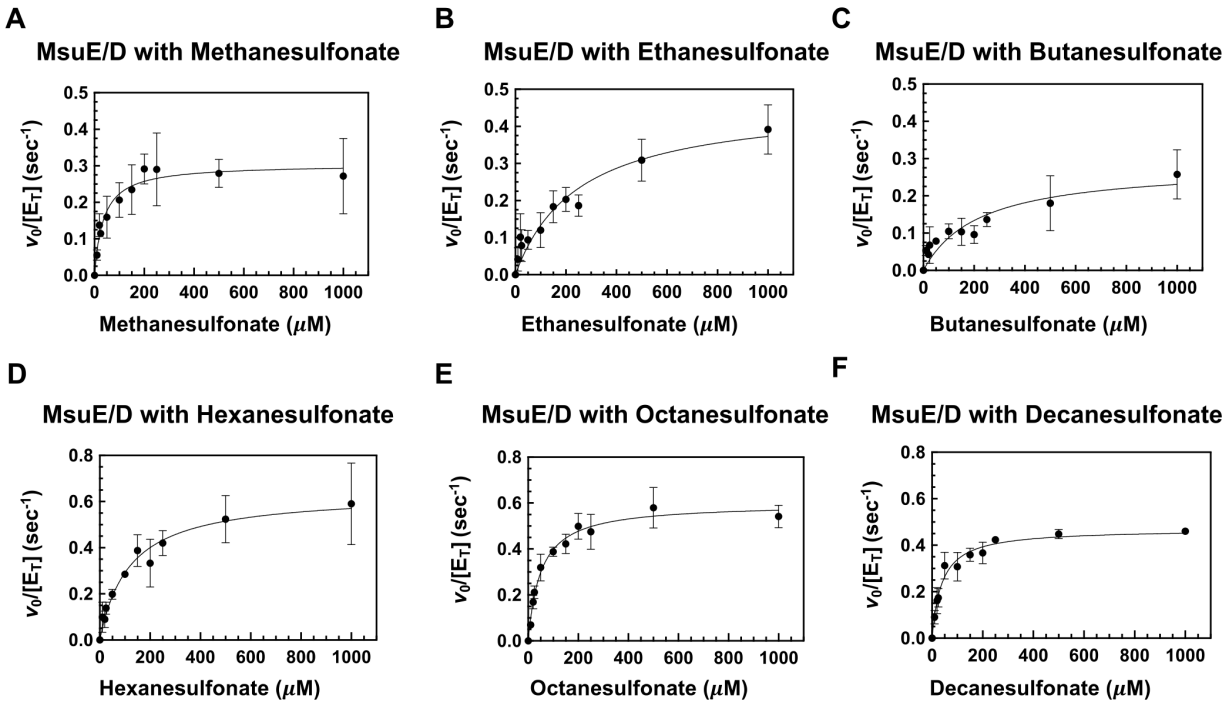

Figure 2.3: Steady-state kinetic analyses of MsuD with different alkanesulfonate substrates: **A.** Methanesulfonate. **B.** Ethanesulfonate. **C.** Butanesulfonate. **D.** Hexanesulfonate. **E.** Octanesulfonate. **F.** Decanesulfonate.

The difference in the kinetic parameters of SsuD and MsuD with methanesulfonate (MES) as a substrate suggested that there may be a difference in the binding affinity of each enzyme. Therefore, the binding affinities of SsuD and MsuD were evaluated through spectrofluorometric titration experiments using different sulfonated substrates. Binding studies with oxidized and reduced flavin to SsuD and MsuD were also performed to determine if the dissociation constants (K_d) for oxidized and reduced FMN to SsuD and MsuD were comparable (Table **2.2**). Both SsuD and MsuD showed a ~100-fold higher affinity for $FMNH_2$ (Figure **2.4C** and **D**) compared to FMN (Figure **2.4A** and **B**). The similar K_d values for MES and OCS suggest that both SsuD and MsuD might utilize similar binding modes for reduced flavin (Figure **2.5**). Additionally, the absence of SsuD activity

with MES was not due to overt changes in the binding affinity, as SsuD had similar K_d values for MES and OCS (Figure **2.5A** and **B**). The K_d values obtained for MsuD with MES and OCS (Figure **2.5C** and **D**). were comparable to SsuD.

Table 2.2: Dissociation constants for SsuD and MsuD

	K_d, FMN ($M \times 10^{-6}$)	K_d, $FMNH_2$ ($M \times 10^{-6}$)	K_d, OCS ($M \times 10^{-6}$)	K_d, MES ($M \times 10^{-6}$)
SsuD	15 ± 2	0.19 ± 0.02	8.7 ± 0.7	15 ± 1
MsuD	15 ± 4	0.20 ± 0.03	7.2 ± 2.1	12 ± 1

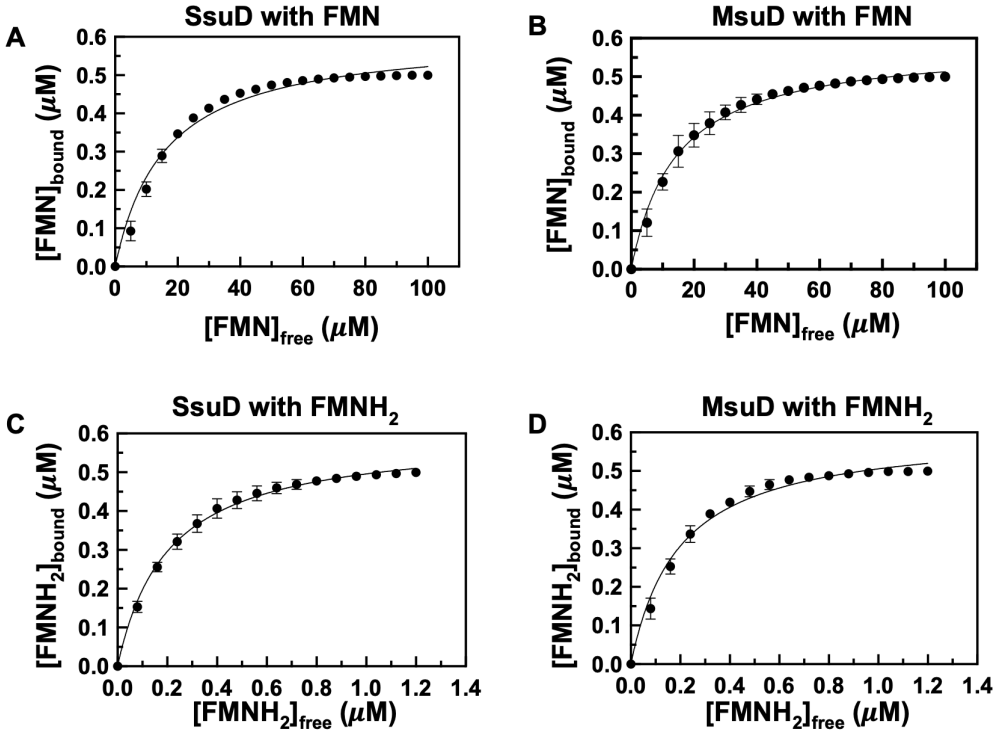

Figure 2.4: Fluorometric titrations of SsuD and MsuD with oxidized and reduced FMN. **A.** SsuD with FMN. **B.** MsuD with FMN. **C.** SsuD with $FMNH_2$. **D.** MsuD with $FMNH_2$.

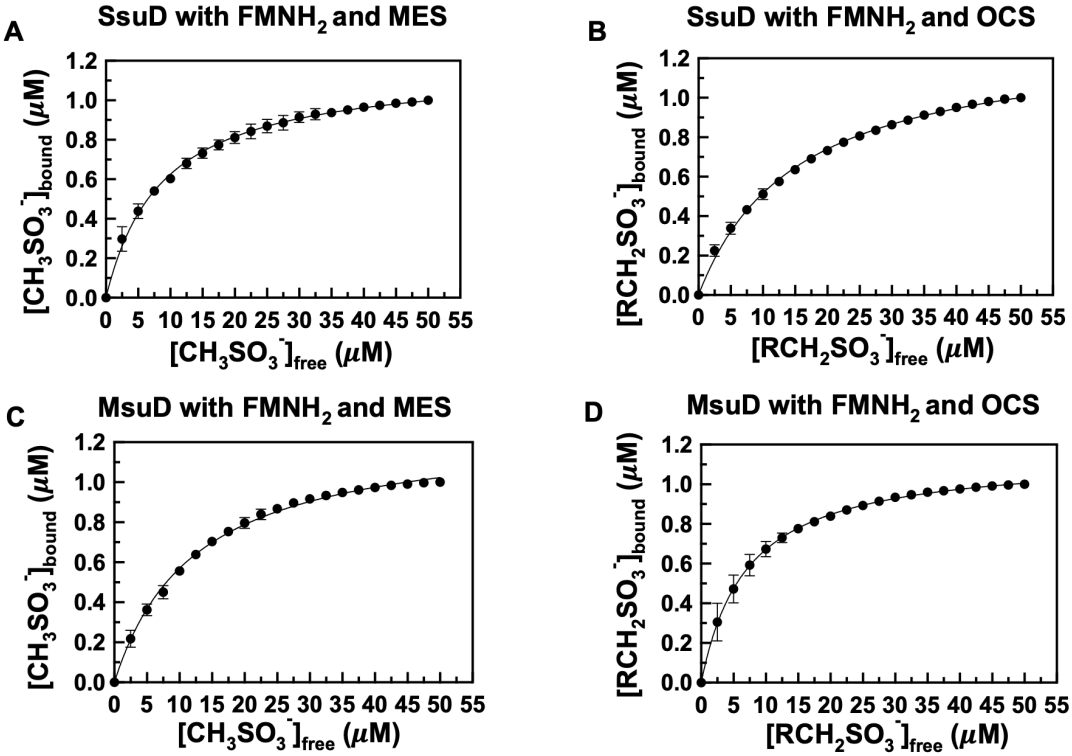

Figure 2.5: Fluorometric titrations of SsuD and MsuD with methanesulfonate and octanesulfonate. **A.** SsuD with methanesulfonate. **B.** SsuD with octanesulfonate. **C.** MsuD with methanesulfonate. **D.** MsuD with octanesulfonate.

2.3.2 Proteolytic Susceptibility of Wild-type SsuD and MsuD

Both SsuD and MsuD contain a dynamic loop region that has been proposed to undergo conformational changes with the binding of substrates/products. SsuD showed decreased proteolytic susceptibility in the presence of reduced flavin compared to FMN. The proteolytic sites cleaved first were located on the mobile loop, and the decrease in the proteolysis was attributed to conformational changes of the mobile loop with the binding of reduced flavin.[232] The proteolytic susceptibility of MsuD was also evaluated in the presence of $FMNH_2$ to determine if MsuD was more protected from proteolytic digestion upon reduced flavin binding. Digestion of MsuD quenched at various incubation

times showed a decreased proteolytic susceptibility with reduced flavin (Figure **2.6B**) compared to MsuD alone (Figure **2.6A**). The proteolytic susceptibility of both enzymes was similar with 45 ± 4% of SsuD/$FMNH_2$ and 46 ± 5% of MsuD/$FMNH_2$ remaining after 180 s (Figure **2.6B**). There were more bands observed with MsuD/$FMNH_2$ compared to SsuD/$FMNH_2$ due to a greater number of basic amino acids (Figure **2.6B**). Additionally,

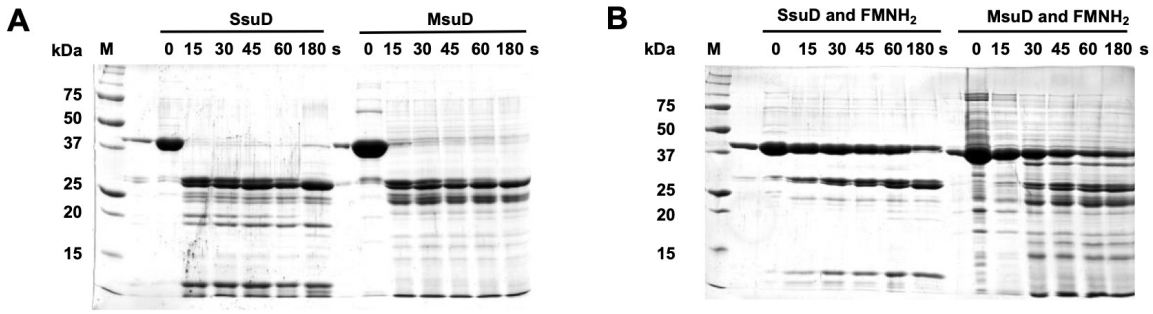

Figure 2.6: Limited proteolytic digestion of SsuD and MsuD in the absence and presence of $FMNH_2$. **A.** SsuD and MsuD in the absence of substrates. **B.** SsuD and MsuD in the presence of $FMNH_2$. Gel lanes: molecular weight marker (M), SsuD and MsuD standard (0 s); aliquots were removed and quenched through heat denaturation after 15, 30, 45, 60, and 180 s.

differences in the proteolytic susceptibility of MsuD and SsuD with $FMNH_2$ were evaluated in the presence of MES and OCS (Figure **2.7**). Digestion of the SsuD/$FMNH_2$/OCS complex was similar to $FMNH_2$ only with 51 ± 7% undigested protein remaining (Figure **2.7A**). Even though MES was not a substrate, SsuD showed similar proteolytic susceptibility in the presence of MES and $FMNH_2$ (53 ± 8% undigested) compared to $FMNH_2$/OCS (Figure **2.7B**). These results further support the ability of SsuD/$FMNH_2$ to bind MES in an active ternary complex. While SsuD showed a similar proteolytic susceptibility with $FMNH_2$ alone or with $FMNH_2$/alkanesulfonates, MsuD demonstrated altered proteolysis under comparable conditions. There was a decrease in the proteolytic

susceptibility of MsuDFMNH$_2$/MES (64 ± 10% undigested) and MsuD/FMNH$_2$/OCS (66 ± 12% undigested) compared to the MsuD/FMNH$_2$ complex only, suggesting that MsuD is in an altered conformation with both reduced flavin, and alkanesulfonates bound.

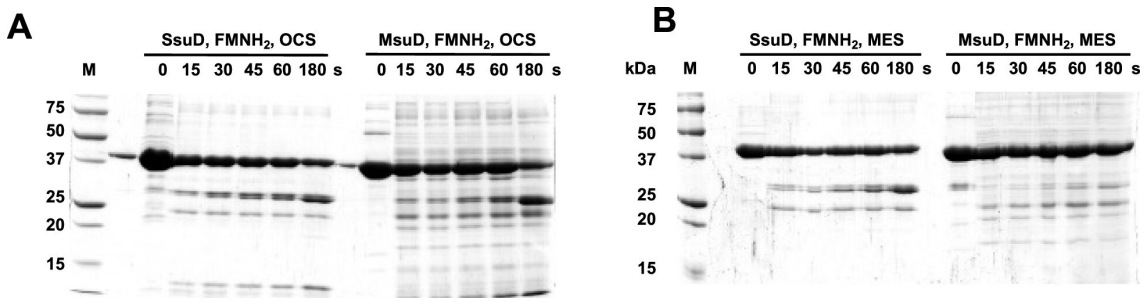

Figure 2.7: Limited proteolytic digestion of SsuD and MsuD in the presence of FMNH$_2$/OCS and FMNH$_2$/MES. **A.** SsuD and MsuD with FMNH$_2$ and OCS. **B.** SsuD and MsuD with FMNH$_2$ and MES. Digestions were performed under anaerobic conditions. Gel lanes: molecular weight marker (M), SsuD and MsuD standard (0 s); aliquots were removed and quenched through heat denaturation after 15, 30, 45, 60, and 180 s.

2.3.3 Structural Analyses from Computational Studies

Structures of SsuD and MsuD with substrates bound were obtained from accelerated molecular dynamics (aMD) simulations, since there are currently no crystal structures solved of these enzymes that have the substrates bound. The structures obtained for SsuD/FMNH$_2$/MES revealed variable clusters due to alterations in the position of MES. The top 10 structures from the clustering analyses of the SsuD/FMNH$_2$/MES complex showed three overall conformations for MES relative to SsuD/FMNH$_2$: MES: (1) bound in the active site (Figure **2.8A**), (2) at the opening of the active site (Figure **2.8B**), and (3) outside of the active site (Figure **2.8C**). MES bound to the opening of the active site was the dominant structure contributing to 52.8% of the total structures, while MES bound at the active site contributed to 33.6% of the clusters.

Although not as dominant, MES was found at various positions outside of the active site in 13.6% of the structures. Therefore, MES was never stably bound to the active site over the 1000 ns trajectory.

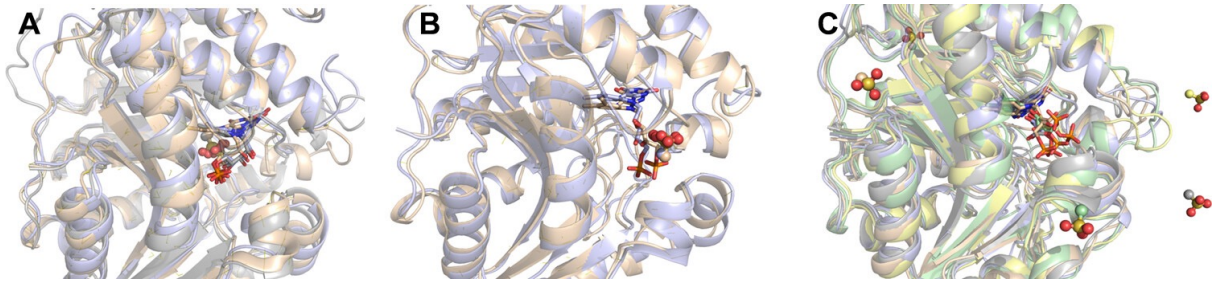

Figure 2.8: Cluster comparison of the SsuD/$FMNH_2$ structure with MES. **A.** MES bound at the active site. **B.** MES bound at the opening of the active site. **C.** MES bound outside of the active site. The SsuD structures represent overlays of the predominant locations of MES from the top 10 clusters.

The results from the kinetic analyses of SsuD suggested that the enzyme was unable to catalyze the desulfonation of alkanesulfonates with shorter alkyl chain lengths. An overlay of SsuD/$FMNH_2$/OCS with the SsuD/$FMNH_2$/MES (bound to the active site) clusters shows that $FMNH_2$ has shifted position, but the sulfonate groups of OCS and MES remain in a similar position. For the SsuD/$FMNH_2$/MES complex, there is a restructuring of the active site that leads to an overall shift in $FMNH_2$ compared to the enzyme with $FMNH_2$/OCS bound (Figure **2.9A**). More notable are the variable positions of the MES molecule in the active site of the SsuD/$FMNH_2$/MES clusters (bound in the active site), which suggests that the MES substrate bound in the active site is unstable (Figure **2.9B**). Interestingly, the sulfonate groups of OCS and MES are in a similar position (Figure **2.9C**); however, the repositioning of the active site shifts the flavin N5 in close proximity to C1 of MES leaving limited space for the activation of dioxygen. The alkyl

chain of the OCS substrate in the SsuD/$FMNH_2$/OCS complex is packed against the dimethylbenzene portion of the flavin on one side of the alkyl chain and a hydrophobic pocket distal from the active site opening on the other side of the alkyl chain (Figure **2.9C**). This hydrophobic packing is critical in stabilizing the octanesulfonate to properly position the sulfonate group for the reaction with the flavin hydroperoxide. One potential reason for the binding instability of MES in the SsuD active site is the slight collapse of the active site in the absence of the alkyl chain. This leads to the observed conformation flexibility of the bound MES in the active site of SsuD leading to the eventual displacement of MES.

RutA is a group C two-component monooxygenase that catalyzes the oxidation of pyrimidine nucleotides and shares similar structural features as SsuD and MsuD.[264] In the O_2-pressurized crystal structure of RutA with oxidized flavin bound, the dioxygen is located within a cavity surrounded by conserved amino acid residues on the *re*-side of the flavin isoalloxazine ring.[170] Several of these amino acids have been proposed to be involved in the binding of dioxygen and stabilization of the superoxyanion.[170] Conversely, the substrate binding site is located on the *si*-side of the isoalloxazine ring. The peroxy group on the N5 is proposed to undergo an inversion from the *re*-side to react with the substrate in the *si*-side of the flavin. Both SsuD and MsuD also share many of these conserved residues for oxygen binding and stabilization of the superoxide.[170] The conserved residues and substrates are both on the *re*-side of the flavin for the SsuD and MsuD structures with $FMNH_2$ and their preferred alkanesulfonate substrate; therefore, a nitrogen inversion would not be necessary to catalyze the desulfonation reaction (Figure **2.9D**).

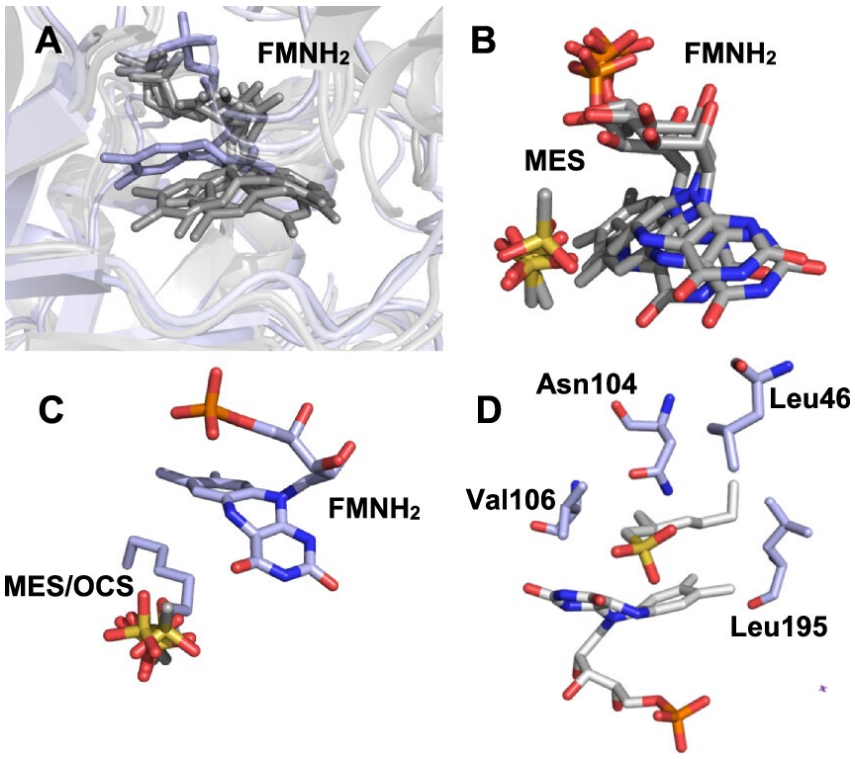

Figure 2.9: Computational analyses of the active site organization of SsuD with $FMNH_2$ and the sulfonate substrates bound. **A.** SsuD active site showing a shift in $FMNH_2$ between OCS (purple tints) and the clusters with MES (gray) bound at the active site. SsuD/$FMNH_2$ complexes with the alkanesulfonate substrates are not shown for clarity. **B.** SsuD active site with $FMNH_2$ and MES. SsuD clusters of MES bound at the active site. **C.** SsuD active site with OCS (purple tints) and MES bound clusters. The SsuD/$FMNH_2$/OCS bound structure is overlaid with the clusters of MES bound at the active site. **D.** SsuD active site with OCS and $FMNH_2$. The flavin and OCS substrates are highlighted in white. Active site amino acids proposed to stabilize the superoxyanion are highlighted in purple tints.

2.4 Discussion

Bacteria are adequately equipped to manage metabolic fluctuations using alternative pathways to maintain their viability. Sulfur is a critical element for the survival of bacteria, and there are alternative mechanisms to ensure usable forms of sulfur are readily used.[4,10] In some bacteria, both the *msu* and *ssu* operons are expressed together during sulfur limiting conditions to ensure that all available alkanesulfonate substrates can be utilized by the cell.[4,93,258] Sulfonate compounds are produced as intermediates in diverse metabolic pathways and serve as synthetic surfactants in industrial applications. Therefore, these compounds are prevalent in both bacterial hosts and the environment. The two-component $FMNH_2$-dependent alkanesulfonate monooxygenases share a high structural similarity but have a distinct catalytic function.[93,170,196] SsuD showed a clear substrate preference for C4 to C10 alkanesulfonates and was not able to desulfonate methanesulfonate and ethanesulfonate substrates. MsuD showed a broader range of substrate specificity (C1-C10). Even though a broader substrate range was observed, the MsuD enzyme is part of a metabolic pathway that includes the MsuC monooxygenase.[93,197] MsuC catalyzes the oxidation of methanesulfinate to methanesulfonate, and MsuD catalyzes the desulfonation of methanesulfonate to formaldehyde and sulfite.[196,197] The mechanism for the transfer of methanesulfonate from MsuC to MsuD is not known and could involve complex formation. Therefore, the ability of MsuD to utilize longer alkanesulfonate substrates may not be relevant under physiological conditions when coupled with MsuC.

SsuD could not utilize methanesulfonate as a substrate, but the enzyme had a similar binding affinity for both MES and OCS in the presence of reduced flavin. We had

previously shown increased protection from proteolysis for SsuD in the presence of reduced $FMNH_2$ compared to apo SsuD.[232,238] This was attributed to movement of a dynamic loop region over the active site that is part of an insertion sequence that diverges from the classic TIM-barrel structure.[232,238] The loop protection did not change when both $FMNH_2$ and OCS substrates were included with SsuD, and the SsuD/$FMNH_2$ complex with MES showed similar protection. MsuD had similar K_d values as SsuD for the binding of both alkanesulfonate substrates, and there was increased proteolytic protection seen with the MES and OCS substrates compared to MsuD/$FMNH_2$. It is interesting that MsuD and SsuD have greater than 60% amino acid sequence identity, but there was a clear difference in the loop conformation with MsuD. It has been proposed that the mobile loop may play a role in conveying substrate specificity.[98,196] Although the mobile loop of SsuD and MsuD shows ~80% amino acid sequence similarity, different conformations of the mobile loop for SsuD and MsuD in the tertiary complex could be important in arranging the active site architecture for alkanesulfonate binding and stabilization. For SsuD and MsuD, the binding of $FMNH_2$ is needed for the preferred alkanesulfonate substrate to bind.[220] Therefore, the binding of reduced flavin must organize the active site for alkanesulfonate binding. Once the alkanesulfonate is bound; a global reorganization of the structure is required for catalytic turnover. Unlike apo SsuD, the mobile loop of apo MsuD showed increased motion compared to SsuD in computational simulations. MsuE, MsuC, and MsuD are involved in a similar metabolic pathway. Under physiological conditions, MsuD would have to accept the methanesulfonate substrate from MsuC and the reduced flavin from MsuE.[196,197] The transfer of reduced $FMNH_2$ from MsuE may be linked to protein-protein interactions between multiple monooxygenase partners.

Conversely, SsuD does not rely on another enzyme for the sulfonate product to catalyze the desulfonation reaction.

Currently, there are no structures of SsuD with reduced flavin and substrates bound. SsuD/$FMNH_2$ and MES or OCS structures obtained from our computational studies showed a difference in the stability of the bound substrate. Even when bound to the active site the MES substrate was not stable and existed in multiple conformations. These results seem conflicting with the similar affinity observed for SsuD with both MES and OCS. The K_d values were obtained under equilibrium conditions; therefore, a decrease in fluorescence was observed with the addition of MES even though the substrate was not stably bound in computational investigations. The titrations are performed anaerobically to prevent the oxidation of reduced flavin and may not represent the active conformation during catalytic turnover. We had previously proposed that SsuD/$FMNH_2$/OCS may form an inactive complex in the absence of oxygen that undergoes a slow conformational change to an active form in the presence of oxygen.[220] In the comparable structure of SsuD/$FMNH_2$/OCS the alkane chain of OCS is stabilized in a hydrophobic pocket that places the sulfonate substrate near the N5 of the flavin. The lack of a long alkyl chain with the MES substrate prevents this stabilization and leads to a collapse of the flavin and a restructuring of the active site, which explains the observed protection seen in proteolytic studies. Activity is not observed for SsuD with the alkanesulfonate substrates until a chain length of C4 is reached providing a chain length conducive to hydrophobic stabilization. The sulfonate group likely contributes to initial substrate recognition, but the alkyl chain length plays a critical role in stabilizing the binding of the alkanesulfonate in SsuD. Previous structural work to evaluate substrate

binding with MsuD was performed with the oxidized flavin product alone or with the MES substrate by itself.[196] There is a 100-fold increase in the binding affinity for FMN compared to reduced flavin for both SsuD and MsuD Therefore, conformational changes may lead to alterations in the binding specificity for a specific redox form of the flavin. Structural investigations into the binding specificity for both SsuD and MsuD should be performed with both the $FMNH_2$ substrate and FMN to detect subtle conformational changes. For the three-dimensional structure of the MsuD/FMN/MES complex both the mobile loop and C-terminal end of the enzyme played a role in alkanesulfonate specificity with the oxidized flavin product.[196] The C-terminal end of the enzyme formed intersubunit hydrogen-bonds with an adjacent subunit. Similar interactions with the reduced flavin substrate were not observed in these studies as the simulations were performed with a single subunit. An unexpected result from these studies was the difference in reduced flavin binding between the three-dimensional crystal structure of MsuD with FMN/MES and the computational investigations with $FMNH_2$ and MES. There were increased contacts made with the phosphate group in the MsuD/$FMNH_2$/MES complex. The binding site around the phosphate makes additional electrostatic and hydrogen-bonding contacts in the MsuD/$FMNH_2$/MES complex that would lead to the increased binding affinity observed with reduced flavin compared to oxidized flavin.

The activation of dioxygen by several two-component monooxygenase enzymes has been proposed to occur through the formation of a flavin N5 oxygenating intermediate.[166,167,169-171,175,265-267] An N5 oxide is often formed as an intermediate in the reaction or as a final product. For the group C two-component monooxygenase YxeK, the enzyme utilizes an N5-peroxyflavin to salvage *S*-(2-succino)cysteine; however, a stable

N5-oxide intermediate is not observed.[171] Similar to YxeK, a flavin N5 has been proposed to facilitate electron transfer from the isoalloxazine ring to oxygen to form a superoxide intermediate for both SsuD and MsuD.[170,171,196] The superoxide is stabilized by conserved polar amino acids to promote radical coupling between the superoxide radical and N5 position of the isoalloxazine ring of the flavin to form the N5-(hydro)peroxyflavin. Three-dimensional structures of two-component monooxygenases with bound substrates showed a delineation between the oxygen activation site and the site of substrate oxidation. It was proposed that an N5-inversion would need to occur to move the N5 peroxyflavin to the substrate binding site for oxidation.[170,171] Neither the MsuD or SsuD structure with $FMNH_2$ and alkanesulfonates bound show a separation between the proposed oxygen activation and substrate oxidation site. The reduced flavin observed in computational structures had moved relative to the oxidized flavin to allow sequential oxidation which was not dependent on a spatial separation. Therefore, the N5-peroxyflavin would not need to undergo an inversion to react with the alkane sulfonated substrate in the computational models. Given the differences in binding affinity between the different redox forms of the flavin for some two-component monooxygenases, structural analyses of both the oxidized and reduced forms of the flavin will need to be performed.

CHAPTER THREE

Investigating the Roles of Conserved Amino Acids in the Desulfonation Reaction of SsuD

3.1 Introduction

The sulfur-starvation induced (SSI) proteins allow bacteria to utilize a broad range of sulfur sources during sulfur starvation. These alternative sulfur sources include organosulfonates, sulfate esters, and oxidation products of dimethyl sulfide.[4,9,48,64,65,75] Of specific interest here is the alkanesulfonate monooxygenase (SsuD), a flavin-dependent enzyme that performs the oxygenolytic cleavage of 1-alkanesulfonates ranging from C4 to C10 in carbon chain length to produce the corresponding aldehyde and sulfite. SsuD is part of a two-component flavin-dependent enzyme system, and relies on a separate NAD(P):FMN reductase, SsuE for the supply of $FMNH_2$.[66,67,221,239] The higher binding affinity of SsuD for $FMNH_2$ (K_d of 0.32 ± 0.15 μM) compared to SsuE (K_d of 15.5 ± 1.3 μM) would promote an immediate release and transfer of the reduced flavin from SsuE.[220] Protein-protein interactions between SsuE and SsuD have also been identified to promote direct transfer of $FMNH_2$ from SsuE to SsuD.[211,212]

SsuD is structurally similar to the bacterial luciferase family despite a relatively low amino acid sequence identity. All members within the bacterial luciferase family adopt a triosephosphate isomerase (TIM)-barrel fold with the active site located at the C-terminal end of the β-barrel.[193,268-270] In addition to its TIM-barrel structure, SsuD contains several insertion regions. One of the insertion regions in SsuD contains a dynamic loop region located near the active site (Figure **3.1A**).[193] A similar loop region has also been identified

in other TIM-barrel proteins including bacterial luciferase and orotidine 5'-monophosphate decarboxylase. These mobile loops have been proposed to protect reactive intermediates from bulk solvent and prevent the release of reactive catalytic intermediates once substrates are bound.[222,226,235,271,272] The SsuD dynamic loop region is highly conserved based on sequence alignments of different SsuD homologues. Partial deletion of the

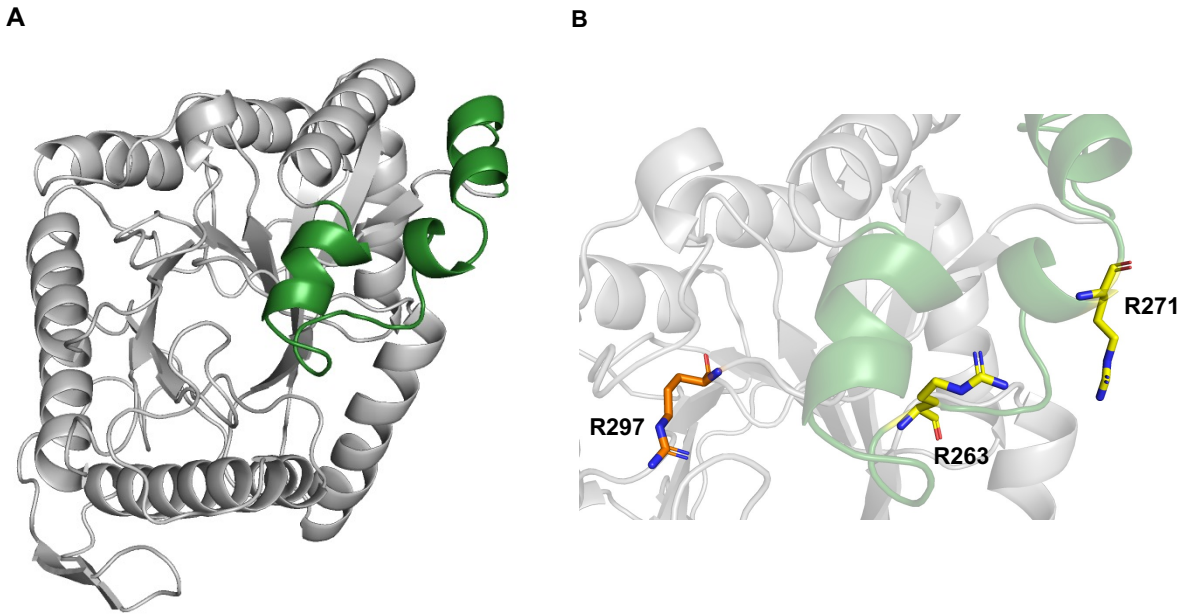

Figure 3.1: Structure of SsuD (PDB: 1M41).[193] **A.** SsuD adopts the TIM-barrel fold and has several insertion regions. One of the insertion regions in SsuD contains a dynamic loop region (displayed in green) that closes over the active site upon substrate binding. The dynamic loop region in SsuD was modelled using computational simulations. **B.** Arginine residues in SsuD that have been proposed to play a role in salt bridge formation.

dynamic loop region in SsuD has previously yielded catalytically inactive enzymes despite no overall gross changes in the secondary structure and the continued ability to bind reduced flavin.[237] Similar to bacterial luciferase, the SsuD loop has been proposed to undergo a lid-gating conformational change following the binding of substrates. A conformational change induced by the binding of $FMNH_2$ is essential for the subsequent

binding of octanesulfonate in SsuD. The dynamic loop region in SsuD was also shown to prevent unproductive oxidation of reduced flavin.[220,232,237,273]

A conserved arginine residue (Arg297) located in the mobile loop insertion sequence contributes to catalysis, since the R297C and R297A SsuD variants had no observable activity and the R297K SsuD variant had a 30-fold lower k_{cat}/K_m relative to wild-type SsuD.[193,232] Interestingly, Arg297 is positioned away from the active site in the reported crystal structure, requiring a conformational change in order to be catalytically relevant.[193] Previous computational studies have proposed that the active site accessibility might be driven by salt bridge formation involving Arg297 and Glu20 or Asp111.[274] However, similar k_{cat}/K_m values as wild-type SsuD were reported for the E20A and D111A SsuD variants bringing into question the exact role of salt bridges during the desulfonation mechanism.[275] Extensive analysis has been carried out to evaluate the role of amino acid residues located within the mobile loop region and how these are involved in catalysis.[211,232,237] Interestingly, two arginine residues (Arg263 and Arg271) located on the mobile loop region surrounding the active site of SsuD were hypothesized to be involved in salt bridge formation with Asp111 and Glu205 upon binding of substrates (Figure **3.1B**). The functional roles of Arg263 and Arg271 in SsuD were evaluated using site-directed mutagenesis and kinetic approaches.

The two-component alkanesulfonate monooxygenase system consisting of SsuE and SsuD are commonly found in a broad range of bacteria, highlighting its importance in enabling bacteria to utilize alternative sulfur sources during sulfur starvation.[4,64,75,239] Despite extensive research, details regarding the desulfonation mechanism of SsuD remain elusive.[240] SsuD utilizes the reduced flavin obtained from SsuE to activate

molecular oxygen which results to the subsequent formation of oxygenating flavin intermediates. SsuD has been previously hypothesized to utilize a C4a-(hydro)peroxy flavin intermediate to perform the oxidative half reaction.[220,232,237,239-241] While the C4a-(hydro)peroxy flavin intermediates have been considered the universal intermediates in flavoenzymology, recent studies with various bacterial flavin-dependent enzymes have provided evidence for the formation of another flavin adduct, namely, the flavin-N5-oxide.[129,166-171,175,264,266,267] The flavin-N5-oxide has been reported to be utilized as an oxygenating flavin intermediate or formed as the final product during catalysis.[166-171,175,264,266,267] Even though the flavin-N5-oxide has initially been reported as an oxygenating intermediate or a final flavin product, studies with the bacterial flavin-dependent enzyme YxeK, an enzyme involved in the salvaging of *S*-(2-succino) cysteine, have proposed an alternative mechanism in which the oxidative reaction could be performed by an N5-peroxyflavin rather than a flavin-N5-oxide.[171]

The flavin-N5-oxide is formed as the final flavin product by different bacterial enzymes including RutA, a flavin-dependent enzyme involved in uracil catabolism.[167,170,267] Similar to SsuD, RutA is also part of a two-component system and adopts a TIM-barrel structure.[170] Different amino acids have been identified in RutA that are involved in stabilizing the flavin-N5 adduct. Structural and amino acid sequence alignment revealed that several of these residues are also conserved in SsuD, indicating that these residues could have a similar function in SsuD.[170] The roles of Asn106, Val108, and Thr109 in SsuD were further evaluated using site-directed mutagenesis and kinetic analyses (Figure **3.2** and Table **3.1**). Altogether, these studies were performed to explore how the dynamic loop region and the conserved amino acids are involved in the activation

of oxygen as well as in the overall desulfonation reaction catalyzed by SsuD. Given the importance of the alkanesulfonate monooxygenase system (SsuE/SsuD) in bacterial sulfur acquisition, these studies provide valuable insight into the kinetic mechanism utilized by this enzyme system.

Table 3.1: Amino acid sequence alignment of RutA with SsuD and MsuD showing the residues that are involved in the stabilization of the flavin-N5 adduct.

RutA	**L65**	**T105**	**N134**	**V136**	**A206**	**F224**
SsuD	**L48**	**T109**	**N106**	**V108**	**G117**	**L195**
MsuD	**L46**	**T107**	**N104**	**V106**	**G175**	**L139**

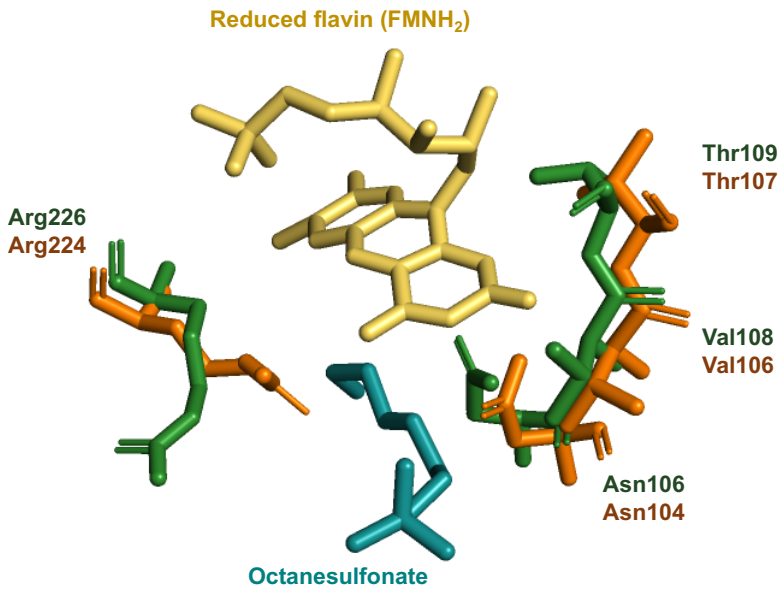

Figure 3.2: Proposed active site residues of SsuD (displayed in green) and their alignment with the structurally similar flavin-dependent monooxygenase, MsuD (displayed in orange). Since there are currently no crystal structures of SsuD and MsuD with reduced flavin and the sulfonated substrate, the structure displayed was obtained from computational simulations.

3.2 Experimental Procedures

3.2.1 Materials

All chemicals for protein purification and enzyme assays were purchased from Sigma-Aldrich, Fischer, Bio-Rad, or Fluka.

3.2.2 Construction, Expression, and Purification of Recombinant Proteins

A recombinant pET21a plasmid containing the *ssuD* gene was used to construct variants of the SsuD enzyme. Primers for each variant were designed as 33 base oligonucleotides containing the desired substitution and were purchased from Invitrogen (California, United States of America). The CGG codon and the CGA codon for Arg263 and Arg271, respectively, were replaced by GCG (R263A) and GCA (R271A). For Asn106, the AAC codon was replaced by CTG (N106L); whereas the GTC codon for Val108 was replaced by ACC (V108T). Finally, the ACA codon of Thr109 was replaced by GCG (T109A). The Qiagen kit plasmid purification protocol was utilized to prepare the SsuD plasmid for site-directed mutagenesis. The constructed variants were confirmed through DNA sequencing analysis by Eurofins Genomics (Kentucky, United States of America). Each plasmid containing the substituted *ssuD* gene was transformed into *E. coli* BL21(DE3) supercompetent cells (Invitrogen, California, United States of America) for protein expression. The expression and purification of the SsuD variants, wild-type SsuD, and wild-type SsuE enzymes were performed as previously described.[179] Following purification, stocks of the variants, wild-type SsuD, and wild-type SsuE enzymes were stored in 25 mM potassium phosphate (pH 7.5), 100 mM NaCl, and 10% glycerol at -80 °C. The concentration of SsuD and SsuE proteins were determined from

A_{280} measurements using molar extinction coefficients of 47.9 and 20.3 M^{-1} cm^{-1}, respectively.

3.2.3 Circular Dichroism Spectroscopy

Far-UV circular dichroism (CD) spectra of wild-type SsuD and variants were obtained by mixing 5 μM of enzyme in 10 mM potassium phosphate buffer (pH 7.5) at 25 °C. Spectra were recorded on an Applied Photophysics Chirascan V100 Spectropolarimeter (Leatherhead, United Kingdom). The spectropolarimeter was purged with nitrogen a day in advance before turning on the light source (Xenon lamp). Measurements were taken in 1.0 nm increments from 300 to 185 nm in a 0.5 mm path length cuvette with a bandwidth of 1 nm. Each spectrum is the average of five scans. Background subtraction was performed using the default parameters within the Chirascan software. Final data were plotted using GraphPad Prism 9 software.

3.2.4 Steady-state Kinetic Analyses

A coupled assay monitoring sulfite production was used to determine the steady-state kinetic parameters of the variants and wild-type SsuD. The reactions were initiated with the addition of NADPH (500 μM) into a reaction mixture containing wild-type or the SsuD variants (0.2 μM), SsuE (0.6 μM), FMN (2 μM), and varying concentrations of the sulfonated substrates (0-1000 μM) in 25 mM Tris-HCl (pH 7.5) and 0.1 M NaCl at 25 °C. The reaction was quenched after 3 min with 8 M urea followed by the addition of DTNB (1 mM). After the addition of DTNB, the reaction was allowed to develop at room temperature for 2 min, and the absorbance was measured at 412 nm using the molar extinction coefficient for the TNB anion of 14.1 M^{-1} cm^{-1}. All assays were performed in

triplicate, and the steady-state kinetic parameters were determined by fitting the data to the Michaelis-Menten equation.

3.2.5 Limited Proteolytic Analysis

The susceptibility of the SsuD variants (R263A, R271A, and R263A/R271A SsuD) and wild-type SsuD to proteolysis was investigated at room temperature with chymotrypsin in the absence and presence of FMN and $FMNH_2$. A chymotrypsin stock solution (1 mg/mL) was prepared in 1 mM HCl/ 1 mM $CaCl_2$ (pH 8.4). Individual samples of R263A, R271A, R263A/R271A, and wild-type SsuD (15 μM) were prepared in 200 mM ammonium bicarbonate/1 mM $CaCl_2$ (pH 8.4) and treated with TLCK-treated chymotrypsin (10 μg/mL). To monitor the effects of oxidized flavin on proteolytic susceptibility, FMN (20 μM) in 25 mM potassium phosphate (pH 7.5) and 10% glycerol was included. After the addition of chymotrypsin, samples (10 μL) were taken at various times (0 s, 1 min, 5 min, 7 min, and 10 min) and added to 2 μL of PMSF prepared in 100% isopropanol to quench the reaction. The degree of proteolysis of each sample was analyzed by SDS-PAGE.

To measure the degree of proteolysis in the presence of $FMNH_2$, an anaerobic solution of FMN (200 μM) was prepared in 25 mM potassium phosphate (pH 8.0), 20 mM EDTA and 10% glycerol. The FMN solution was bubbled with ultrahigh-purity argon gas for 30 min before being transferred to an anaerobic chamber. The solutions for both the SsuD variants and wild-type SsuD were prepared in a glovebox with 200 mM ammonium bicarbonate (pH 8.4) and 1 mM $CaCl_2$. The buffer was made anaerobic by bubbling with ultrahigh-purity argon gas for 1 h before being transferred inside the anaerobic glovebox.

After the addition of glucose (10 mM) and glucose oxidase (0.1 μM), both the enzyme and FMN solution were incubated in anaerobic glovebox to remove trace amounts of dioxygen. The $FMNH_2$ solution was photoreduced inside a gastight syringe with a long-wavelength UV lamp. The proteolytic susceptibility of the SsuD variants and wild-type SsuD (15 μM) was also evaluated in the presence of octanesulfonate (150 μM). The octanesulfonate substrate was resuspended in anaerobic 25 mM potassium phosphate (pH 7.5) and 10% glycerol. The buffer was made anaerobic by bubbling with ultrahigh-purity argon gas for 1 h before being transferred inside the anaerobic glovebox. After the addition of chymotrypsin, samples (10 μL) were taken at various times (0 s, 1 min, 5 min, 7 min, and 10 min) and added to 2 μL of PMSF prepared in 100% isopropanol to quench the reaction. The degree of proteolysis of each sample was analyzed by SDS-PAGE.

3.2.6 Enzymatic Assay for the Determination of the Final Flavin Product

Prior to performing the enzymatic assay, wild-type SsuE and SsuD were exchanged into 25 mM potassium phosphate (pH 7.5) and 0.1 M NaCl at 4 °C using an Amicon Ultra-4 centrifugal filter (Millipore) with a 10K molecular weight cut off. Enzymatic assays (500 μL) were performed in 25 mM Tris-HCl (pH 7.5) and 0.1 M NaCl at room temperature and included wild-type SsuD (0.2 μM), wild-type SsuE (0.6 μM), FMN (20 μM) and octanesulfonate (500 μM). The reactions were initiated with the addition of NADPH (300 μM) and were allowed to develop for 3 min at 25 °C. After centrifugation for 20 min at 13,000 rpm using an Amicon Ultra-0.5 mL centrifugal filter, samples were filtered through a 0.22 μm Millex Hydrophillic Durapore (PVDF) membrane. Filtered samples (30 μL), were analyzed by reversed phase HPLC comprising a Waters 1525 binary pump and

a Waters 2489 UV-Vis detector. A Waters Delta Pak C18 column (150 x 3.9 mm internal diameter (ID), 5 μm, 300 Å) was used for separation at a flow rate of 0.5 mL/min. The mobile phase consisted of 10 mM ammonium acetate (pH 6.5) (solvent A) and 100% HPLC grade methanol (solvent B). The following gradient was used: 0 to 3 min (98% A and 2 %B), 3 to 5 min (85% A and 15% B), 5 to 10 min (50% A and 50% B), 10 to 15 min (30% A and 70% B), 15 to 20 min (98% A and 2% B), and 20 to 22 min (98% A and 2% B). All chromatographic steps were performed at room temperature. Products were detected using absorbance at 450 nm. Data were processed using the Breeze 2 software. Final data were plotted using GraphPad Prism 9 software.

3.3 Results

3.3.1 Evaluating the Steady-state Kinetic Parameters of Wild-type SsuD and Variants

Results from circular dichroism spectroscopy experiments revealed that there were no obvious perturbations in the overall secondary structure between wild-type and the SsuD variants (Figure **3.3**). These results suggested that all the SsuD substitutions that were made, did not cause any major alterations to the overall secondary structure of SsuD. A coupled assay monitoring sulfite production was used to determine the steady-state kinetic parameters of the variants and wild-type SsuD. This assay was performed in order to evaluate whether the amino acid substitutions in SsuD altered the kinetic activity of the variants as compared to wild-type SsuD. Single and double substitutions of Arg263 and Arg271 to alanine (R263A, R271A, R263A/R271A SsuD) were generated to determine if the desulfonation activity of SsuD was affected by alterations in the salt

bridge interactions. The single and double arginine SsuD variants showed similar kinetic parameters as wild-type SsuD (Table **3.2** and Figure **3.4 A-D**). Steady-state kinetic

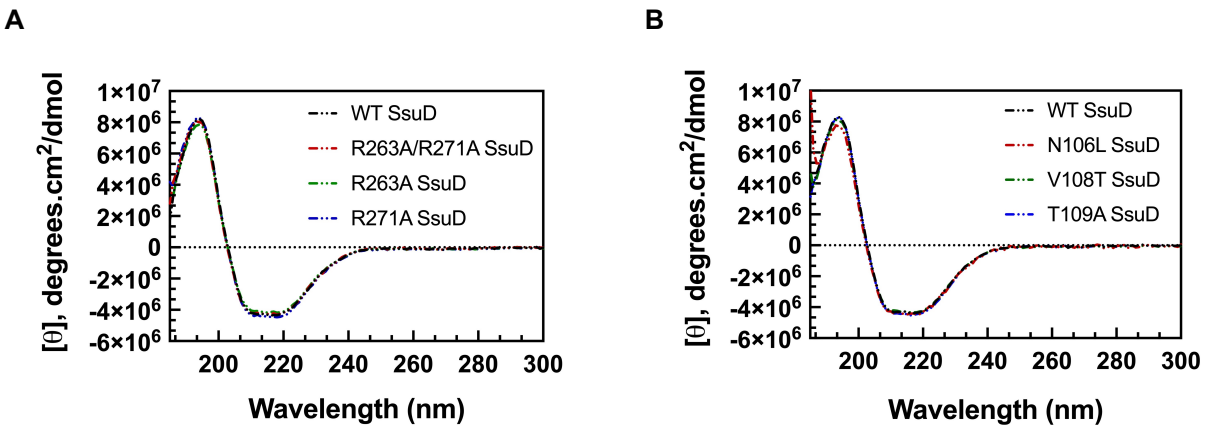

Figure 3.3: Circular dichroism spectra of wild-type SsuD and the variants. **A.** Wild-type SsuD and the arginine variants involved in salt bridge formation. **B.** Wild-type SsuD and variants made of the proposed residues involved in the formation of the flavin-N5-oxide by SsuD.

analyses were also performed with the N106L, V108T, and T109A SsuD variants to determine if substitution led to an alteration in the kinetic parameters of these SsuD variants compared to the wild-type enzyme. The polar Asn106 and Thr109 in SsuD have been proposed to stabilize the superoxide anion, whereas the nonpolar Val108 has been proposed to control the interaction with molecular oxygen. In order to further investigate the roles of these residues in SsuD, the polar residues were substituted with nonpolar residues, whereas the nonpolar valine was substituted with the polar threonine. In addition to its chemical properties, threonine was also selected as a substitute for valine given its similarity in the size and shape of the branched side chain. Results from desulfonation assays revealed that the V108T and T109A SsuD variants had similar

kinetic parameters as wild-type SsuD; however, no measurable desulfonation activity was observed for the N106L SsuD variant (Table **3.2** and Figure **3.4 A, E-G**).

Table 3.2: Steady-state kinetic parameters for the variants and wild-type SsuD

	k_{cat} (s^{-1})	K_m ($M \times 10^{-6}$)	k_{cat}/K_m ($M^{-1} s^{-1}, \times 10^{-6}$)
WT SsuD	0.84 ± 0.06	50 ± 13	1.7 ± 0.5
R263A SsuD	1.40 ± 0.05	57 ± 8	2.5 ± 0.4
R271A SsuD	1.03 ± 0.14	235 ± 76	0.44 ± 0.14
R263A/R271A SsuD	1.10 ± 0.10	113 ± 31	1.0 ± 0.3
N106L SsuD	-[a]	-	-
V108T SsuD	0.78 ± 0.07	61 ± 19	1.3 ± 0.4
T109A SsuD	1.14 ± 0.03	45 ± 5	2.5 ± 0.3

[a]Value could not be determined under the experimental conditions used.

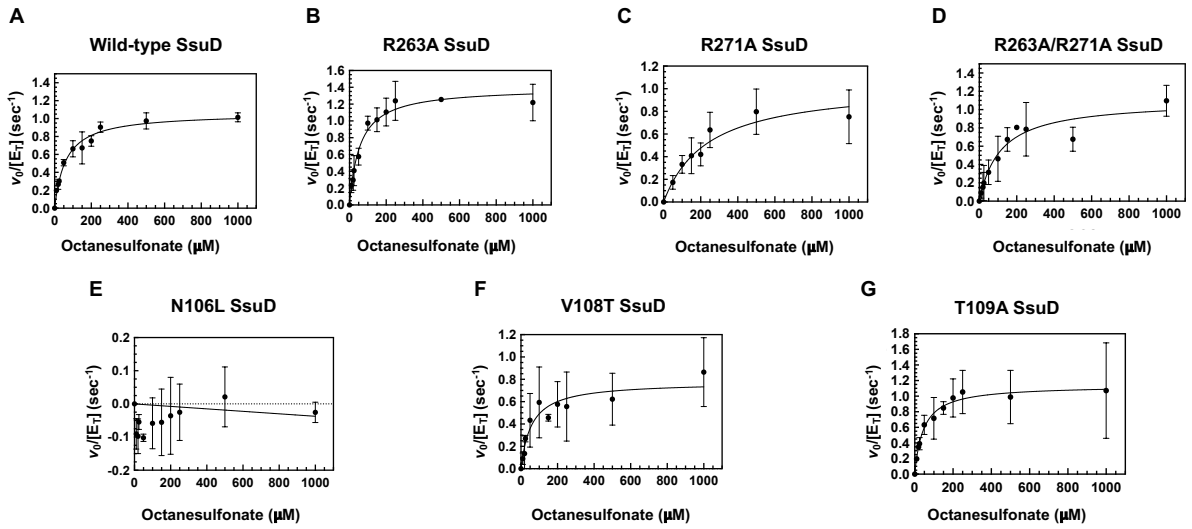

Figure 3.4: Steady-state kinetic analyses of wild-type and the variants of SsuD in the presence of octanesulfonate as the sulfur substrate. A. Wild-type SsuD. B. R263A SsuD. C. R271A SsuD. D. R263A/R271A SsuD E. N106L SsuD. F. V108T SsuD. G. T109A SsuD.

3.3.2 Proteolytic Susceptibility of Wild-type SsuD and Variants

Wild-type SsuD is protected from proteolytic digestion in the presence of $FMNH_2$ due to loop closure. In the absence of $FMNH_2$, Arg297 is readily accessible to proteolytic digestion with trypsin. However, there was no change in the degree of protection with $FMNH_2$ and octanesulfonate.[232] Partial proteolytic digestion experiments were performed with wild-type SsuD and the generated arginine variants in order to evaluate the proteolytic susceptibility in the presence of FMN and $FMNH_2$. Limited proteolytic digestion of the R263A SsuD variant and $FMNH_2$ with chymotrypsin showed comparable protection as wild-type SsuD (Figure **3.5A** and **B**). However, the R271A and R263A/R271A SsuD variants were not similarly protected (Figure **3.5C** and **D**). The lack of protection is likely due to the Arg271 to alanine substitution, since the same level of protection was observed

in the double variant. In the presence of both $FMNH_2$ and octanesulfonate, R271A SsuD was more protected as compared to $FMNH_2$ alone (Figure **3.6B**). Therefore, although the kinetic parameters for the arginine variants were the same as wild-type SsuD, the Arg271 SsuD variants were not equally protected.

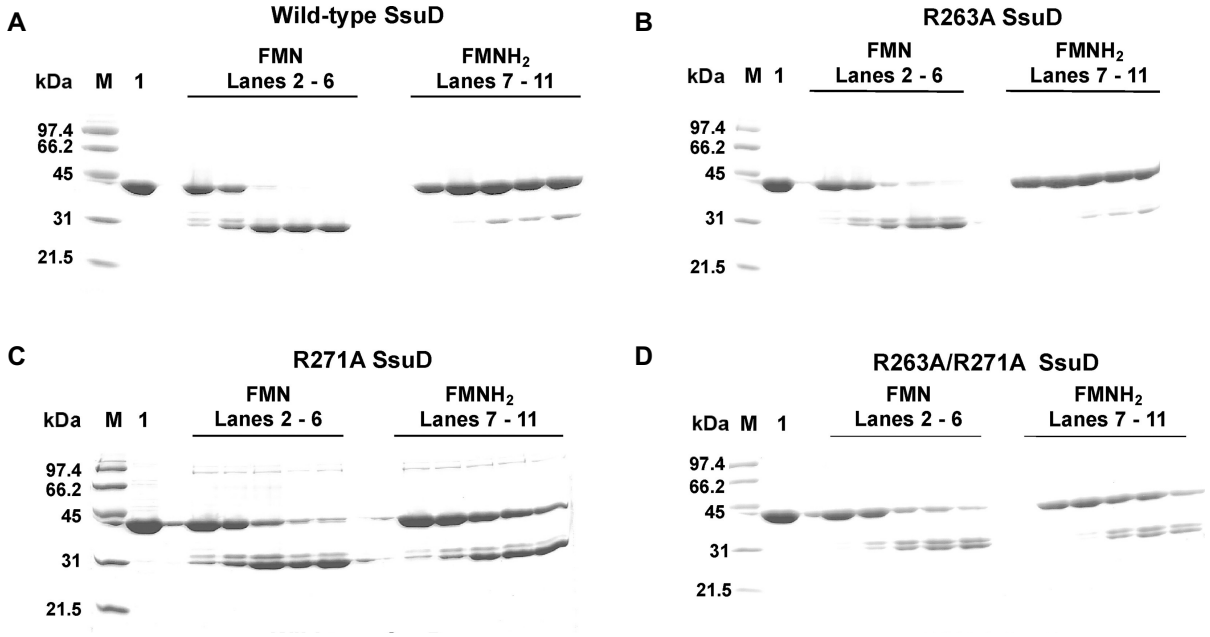

Figure 3.5: Limited proteolytic digestion of wild-type SsuD and the arginine variants in the presence of FMN and $FMNH_2$. **A.** Wild-type SsuD with FMN and $FMNH_2$. **B.** R263A SsuD with FMN and $FMNH_2$. **C.** R271A SsuD with FMN and $FMNH_2$. **D.** R263A/R271A SsuD with FMN and $FMNH_2$.Digestions were performed under anaerobic conditions for $FMNH_2$. Gel lanes: molecular weight marker (M), SsuD standard (lane 1); aliquots were removed and quenched with PSMF after 0 s (lanes 2 and 7), 1 min (lanes 3 and 8), 5 min (lanes 4 and 9), 7 min (lanes 5 and 10), 10 min (lanes 6 and 11).

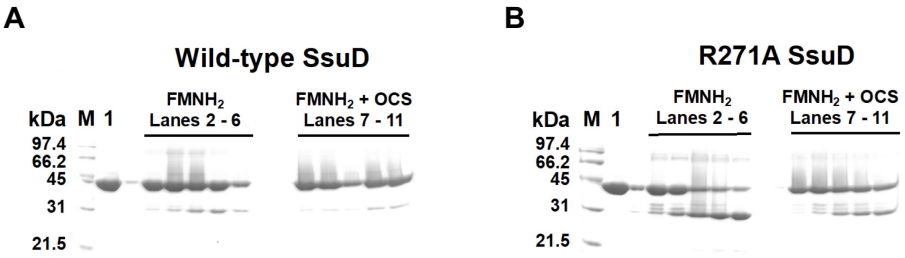

Figure 3.6: Limited proteolytic digestion of wild-type SsuD and R271A SsuD in the presence of $FMNH_2$ and $FMNH_2$/OCS. **A.** Wild-type SsuD with $FMNH_2$ and $FMNH_2$/OCS. **B.** R271A SsuD with $FMNH_2$ and $FMNH_2$/OCS. Digestions were performed under anaerobic conditions. Gel lanes: molecular weight marker (M), SsuD standard (lane 1); aliquots were removed and quenched with PSMF after 0 s (lanes 2 and 7), 1 min (lanes 3 and 8), 5 min (lanes 4 and 9), 7 min (lanes 5 and 10), 10 min (lanes 6 and 11).

3.3.3 Evaluating the Final Flavin Product in the Desulfonation Reaction of SsuD

Flavin-dependent monooxygenases perform oxidative reactions which involve the formation of flavin intermediates due to the reaction between reduced flavin and molecular oxygen.[129,144,159] The flavin adducts that have been spectrally identified in the reactions catalyzed by flavin-dependent monooxygenases are the C4a-(hydro)peroxy flavin intermediates and the flavin-N5-oxide. The C4a-(hydro)peroxy flavin intermediates are formed due to the reaction of molecular oxygen at the C4a position of the reduced flavin. Conversely, the flavin-N5-oxide is formed due to the reaction of molecular oxygen at the N5 position of the reduced flavin. Previous studies with various bacterial flavin-dependent monooxygenases provided evidence that the flavin-N5-oxide could be utilized as an oxygenating intermediate or formed as the final product during catalytic turnover.[166-171,266] High-performance liquid chromatography (HPLC) analyses were performed to determine the final flavin product in the desulfonation reaction of SsuD. Results from

HPLC analyses confirmed that oxidized FMN is the final flavin product (Figure **3.7B**). A concurrent desulfonation assay monitoring sulfite production was performed to confirm that the final flavin product observed was due to enzymatic turnover rather than a nonenzymatic reaction.

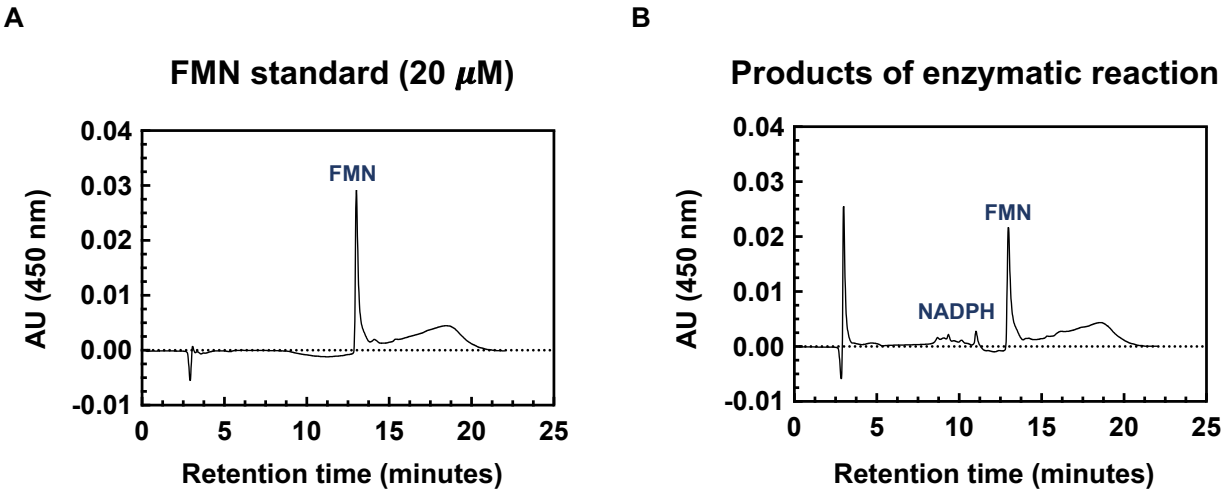

Figure 3.7: HPLC analysis of the SsuE/SsuD enzymatic reaction evaluating the final flavin product. **A.** The FMN standard elutes at 13.5 min **B.** The SsuE/SsuD enzymatic reaction mixture confirms the presence of NADPH and FMN in HPLC analysis. FMN was the only flavin product identified in these experiments.

3.4 Discussion

Bacteria often find themselves deprived of essential nutrients in the environment which also includes sulfur.[4] The two-component alkanesulfonate monooxygenase enzyme system (SsuE/SsuD) provides an alternative means for bacteria to obtain sulfur using alkanesulfonates.[4,48,64,75] SsuD relies on SsuE for the supply of reduced flavin to perform the oxidative half reaction.[193,239] Previous studies with both SsuE and SsuD provided evidence that the reduced flavin transfer involves protein-protein interactions between the reductase and the monooxygenase.[211,212] The protein-protein interactions

between SsuE and SsuD are facilitated by structural features from both enzymes.[199,200,211,232,237] Studies evaluating the oligomeric state have reported that SsuE is in a dimer-tetramer equilibrium and favors the dimeric form in the presence of flavin or SsuD. These changes in the oligomeric state of SsuE have also been hypothesized to affect protein-protein interactions with SsuD.[198] Unlike SsuE that undergoes changes in the oligomeric state, SsuD induces conformational changes in the form of loop closure over the active site upon substrate binding. The dynamic loop region in SsuD is part of an insertion region that was previously shown to protect reduced flavin from autooxidation, thereby indicating its potential role in protein-protein interactions.[232,237]

Protein dynamics in SsuD involving conformational changes induced by the dynamic loop region have previously been shown to protect the enzyme from proteolysis in the presence of substrate binding.[232] Moreover, the conformational changes of the dynamic loop region in SsuD were proposed to be driven by salt bridge formation involving Arg297/Asp111 and Arg297/Glu20.[274] It was hypothesized that the Arg297/Asp111 salt bridge in SsuD is formed upon the binding of $FMNH_2$. The Arg297/Asp111 salt bridge would result in a “closed” conformation preventing the entry of bulk solvent into the active site. A second salt bridge between Arg297 and Glu20 was proposed to be formed sporadically, allowing SsuD to be in a “open” conformation facilitating the entry of substrates into the active site.[274,275] Similar k_{cat}/K_m values for the E20A and D111A SsuD variants as compared to wild-type SsuD were previously reported. Finally, molecular dynamic simulations also revealed that the salt bridges between Arg297/Asp111 and Arg297/Glu20 were not found to be dominant, suggesting that these salt bridges are not formed or not critical for desulfonation.[275]

While previous molecular dynamic simulations lacked the mobile loop residues 250-282, recent computational analyses modelled in these missing SsuD loop residues and proposed that Arg263 and Arg271 could be involved in salt bridge formation.[238] Molecular dynamic simulations predicted two salt bridges between Arg263/Asp111 and Arg271/Glu205 when the dynamic loop region of SsuD is in a "closed" conformation (Figure **3.8**). The roles of these two arginine residues were further investigated and the

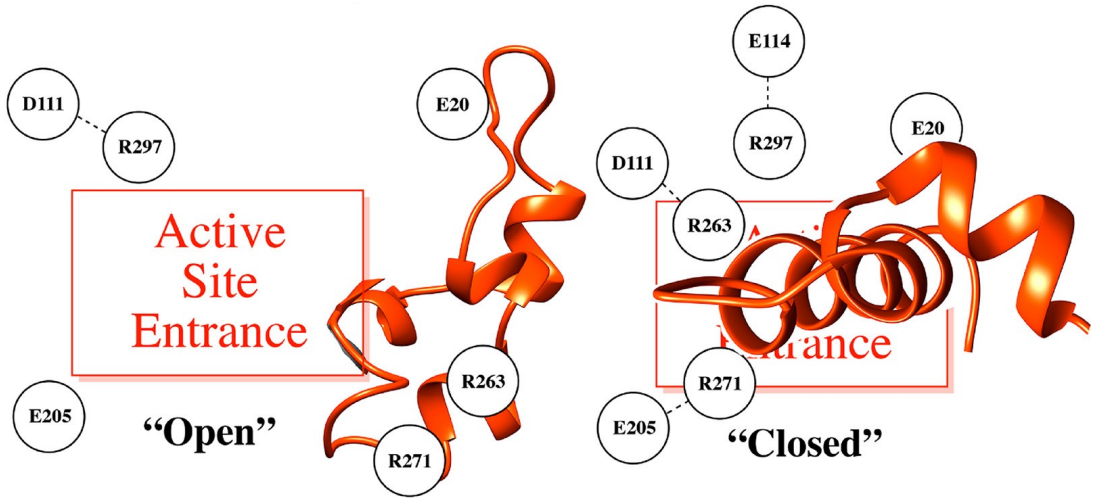

Figure 3.8: Representation of the active site of SsuD and the mobile loop residues in the "open" and "closed" conformations. The insertion sequence of SsuD containing the mobile loop is displayed in orange. (Adapted with permission from [238]). Copyright (2020) American Chemical Society.

results revealed that single and double substitutions of Arg263 and Arg271 to an alanine resulted in similar k_{cat}/K_m values as wild-type SsuD. Since the dynamic loop region of SsuD is susceptible to proteolysis, the susceptibility of the arginine SsuD variants was evaluated in the presence of chymotrypsin. While R263A SsuD showed similar protection as wild-type SsuD, R271A and R263A/R271A SsuD were more susceptible towards proteolysis. The increased proteolytic susceptibility was attributed to the Arg271 to

alanine substitution, causing an increased distance between Glu205 and Arg271 as well as the disruption of the salt bridge. This increased distance could subsequently result in a more "open" conformation leading to increased proteolysis. In the presence of both $FMNH_2$ and octanesulfonate, the proteolytic susceptibility of R271A SsuD was similar as wild-type SsuD. The binding of octanesulfonate to SsuD has been proposed to induce a second conformational change that would result in a "semiclosed" conformation. The proteolytic experiments were performed under anaerobic conditions and may not represent the correct conformational state in the presence of dioxygen. In the presence of dioxygen, the closed conformation observed with $FMNH_2$ may not be formed as this conformational state would slow down catalysis. In the "semiclosed" conformation, the active site of SsuD may be appropriate for accepting dioxygen into the binding pocket, and for ensuring its proper orientation for promoting subsequent oxygenolytic cleavage.

The structural features and conformational changes induced by SsuD are essential for creating a solvent free environment required for the stabilization of reactive intermediates. Previous studies with SsuD proposed that this enzyme utilizes C4a-(hydro)peroxy flavin intermediates based on the structural similarity with bacterial luciferase.[220,232,237,239-241,273,275] Even after decades of extensive research, no spectral evidence has been obtained for the formation of the C4a-(hydro)peroxy flavin intermediates by SsuD. It has been reported that the increased electrostatic environment within the active site could prevent SsuD from adequately stabilizing these C4a-(hydro)peroxy flavin intermediates.[239] Additionally, it may be speculated that the C4a-(hydro)peroxy flavin intermediates are short-lived and highly unstable, thereby preventing its capture using rapid reaction kinetic experiments.

While the C4a-(hydro)peroxy flavin intermediates have been accepted as the classical oxygenating flavin intermediates in flavoenzymology, recent studies have provided spectral evidence for the formation of the flavin-N5-oxide.[166-171,175,266,267] The flavin-N5-oxide is a flavin adduct that was first identified as the oxygenating flavin intermediate in the flavin-dependent EncM, which is involved in the biosynthesis of the polyketide antibiotic enterocin.[169,175] After EncM, the flavin-N5-oxide has been reported in several other bacterial flavin-dependent enzymes. The flavin-N5-oxide has been reported as the oxygenating flavin intermediate or as the final product during catalytic turnover in various bacterial flavin-dependent enzymes.[166-171,175,266,267] Since the flavin-N5-oxide has also been observed in two-component flavin-dependent monooxygenases that contain a TIM-barrel structure (RutA), we currently propose that SsuD could utilize the flavin-N5-oxide as an oxygenating intermediate or form this flavin adduct as the final product (Figure **3.9**). In order to form the flavin-N5-oxide as the final product in SsuD, reduced flavin is proposed to activate molecular oxygen followed by the subsequent formation of the N5-peroxy flavin intermediate (Figure **3.9**, step 1 and 4). This intermediate is further proposed to perform a nucleophilic attack on the α-carbon of the alkanesulfonate substrate resulting in cleavage of the C-S bond followed by the dissociation of sulfite and the formation of an N5-peroxy alkane intermediate (Figure **3.9**, step 2). An active site base is proposed to abstract a proton from the α-carbon of the N5-peroxy alkane intermediate yielding the flavin-N5-oxide and the corresponding aldehyde (Figure **3.9**, step 3). Conversely, the formation of the flavin-N5-oxide as the oxygenating intermediate by SsuD has also been proposed to occur through the formation of an N5-peroxy flavin intermediate followed by

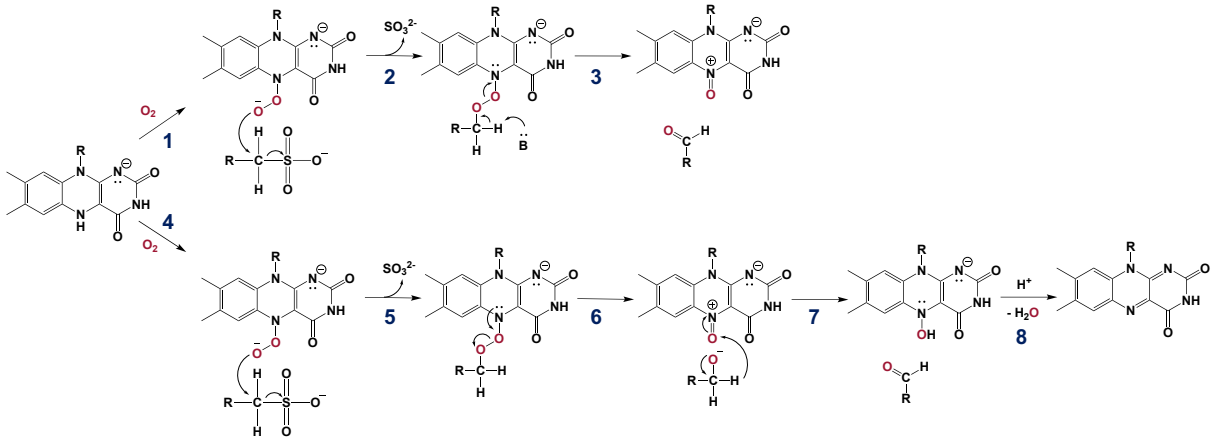

Figure 3.9: Proposed mechanisms for SsuD involving a flavin-N5-oxide. The upper pathway represents the formation of the flavin-N5-oxide as the final product, whereas the lower pathway shows the formation of the flavin-N5-oxide as one of the oxygenating intermediates.

an N5-peroxy alkane intermediate (Figure **3.9**, step 5). An internal rearrangement of the N5-peroxy alkane intermediate results in the formation of the flavin-N5-oxide (Figure **3.9**, step 6) followed by the formation of an N5-hydroxy flavin intermediate with the release of the corresponding aldehyde (Figure **3.9**, step 7). Dehydration of the N5-hydroxy flavin intermediate in the final step results in regeneration of oxidized FMN (Figure **3.9** step 8). Even though the mechanisms proposed for SsuD are based the formation of the flavin-N5-oxide as the final product or as one of the oxygenating intermediates, we also hypothesize that a flavin-N5-oxide might not be stabilized by SsuD. Therefore, we suggest that a concerted reaction might occur where the N5-peroxy flavin intermediate performs the oxidative half reaction, similar to what has been proposed for YxeK.[171]

Different amino acids have been reported to play a role in the stabilization of the flavin-N5 adduct.[170] While distinct nonpolar amino acids have been proposed to control

the interaction with the hydrophobic dioxygen, the polar amino acids are involved in stabilizing the transiently formed superoxide anion through strong hydrogen bonding.[170] The roles of the conserved Asn106 and Thr109 in SsuD were evaluated by substituting these polar amino acids with a nonpolar residue. While T109A SsuD had similar activity as the wild-type enzyme, no activity was observed with the N106L SsuD variant indicating that this residue could play a potential role in stabilizing the transiently formed superoxide anion. A similar result has been observed in RutA where substitution of Asn134 to a leucine completely abolished the activity of the enzyme as well as its ability to form the flavin-N5-oxide. Even though T109A SsuD still had similar activity as wild-type SsuD, substitution of Thr105 with an alanine in RutA significantly lowered the activity of the enzyme as well as the amount of flavin-N5-oxide that was formed as the final product.[170]

In addition to EncM and RutA, YxeK has also been proposed to employ a flavin-N5-adduct.[171] Similar to RutA, substitution of Thr96 with an alanine and substitution of Asn125 with a leucine in YxeK resulted in a significant decrease in the catalytic rate of these variants compared to wild-type YxeK. These results suggested that Thr96 and Asn125 in YxeK had a comparable role as the conserved residues in RutA.[170,171] Lastly, the role of the nonpolar Val108 in SsuD was also investigated by substituting this residue with a polar threonine. The V108T SsuD variant had similar activity as wild-type SsuD, suggesting that Val106 might not be involved in controlling the interaction with the hydrophobic dioxygen. While this is contrasting with what has been reported for the conserved Val136 in RutA, this result could also suggest that other nonpolar amino acids in SsuD could be involved in interacting with dioxygen.

Based on its occurrence as either the oxygenating flavin intermediate or as the final product in the reaction of different bacterial flavin-dependent enzymes, two mechanisms of SsuD have been proposed where the flavin-N5-oxide can be formed as the final product or employed as one of the oxygenating flavin intermediates (Figure **3.9**).[166-171,175,267] The enzymatic reaction of SsuE and SsuD analyzed by HPLC confirmed the presence of oxidized flavin (FMN) as the final flavin product. This suggests that the flavin-N5-oxide might not be formed or stabilized by SsuD under the given experimental conditions. Alternatively, these results could also indicate that the flavin-N5-oxide is resolved as one of the oxygenating intermediates rather than formed as the final product.

Different studies have provided evidence that the flavin-N5-oxide can be spectrally observed at 462 nm.[166-170,267] Based on results obtained for flavin-dependent monooxygenases that employ C4a-(hydro)peroxy flavin intermediates, there could be a possibility that the absorption of the flavin-N5-oxide is influenced by the protein environment which would result in a shift from the proposed 462 nm. Pre-steady state kinetic experiments monitoring changes in the overall flavin spectrum could be extremely useful for the evaluation of the N5-peroxy flavin as well as the flavin-N5-oxide in different flavin-dependent enzymes. Altogether, these studies were able to provide insight on how conserved amino acids are involved in the desulfonation reaction catalyzed by SsuD. However, further studies need to be performed to provide evidence for the formation of the oxygenating flavin intermediates by SsuD, in order to support the proposed desulfonation mechanisms.

CHAPTER FOUR

Evaluating the Structural and Mechanistic Properties of MsuC from *Pseudomonas aeruginosa*

4.1 Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium that is ubiquitous in the environment.[246,249] This bacterium is an opportunistic human pathogen capable of causing life-threatening infections in immunocompromised patients. *P. aeruginosa* is also the main cause of mortality in patients with cystic fibrosis and known as one of the leading pathogens causing nosocomial infections.[246,247,249,252,276] Treatment of infections caused by *P. aeruginosa* are extremely challenging due to the natural resistance of this bacterium to a broad range of antibiotics as well as the increasing number of multidrug- and pan-drug-resistant strains.[246-249,276] *P. aeruginosa* utilizes diverse mechanisms that are critical for its survival, persistence, and pathogenesis in various environments. Examples of adaptation mechanisms utilized by this pathogen include biofilm and persistence formation, quorum sensing, and resistance to antibiotics.[246-249]

Similar to other organisms, bacteria including *P. aeruginosa* require different nutrients including sulfur for numerous metabolic purposes.[255,256] While inorganic sulfate is the preferred sulfur source of bacteria, this sulfur compound is poorly represented in soil, and therefore bacteria often find themselves deprived of their preferred sulfur source.[4] In order to make sure that sulfur is readily available, bacteria express different proteins that enable these microorganisms to utilize alternative sulfur sources.[4,48,64,65,69,75] Even though there are various alternative sulfur sources, this chapter will focus on the

ability of bacteria to utilize dimethyl sulfide and its oxidation products as a potential sulfur source during sulfur starvation.

Dimethyl sulfide (DMS) is a common sulfur source in marine environments which is primarily obtained from the microbial degradation of dimethylsulfoniopropionate (DMSP).[277-280] DMSP is an osmolyte that is synthesized by phytoplankton and marine algae. The DMS formed as a consequence of microbial degradation can be released into the atmosphere where it is subsequently oxidized to dimethyl sulfone ($DMSO_2$), methanesulfinate, and methanesulfonate. The oxidation products of DMS are eventually deposited in terrestrial environments through precipitation.[280-283] In addition to using DMS and its oxidation products as alternative sulfur sources, bacteria also utilize these compounds as a source of carbon and energy.[277,282] In order to utilize DMS and its oxidation products as a source of sulfur during sulfur limitation, different bacteria in particular *Pseudomonas* species express the *sfn* and *msu* enzymes.[4,9,93,95-97] Of specific interest in this chapter is the flavin-dependent monooxygenase, MsuC, which allows different *Pseudomonas* species to utilize methanesulfinate as the sole source of sulfur.[197]

MsuC is part of a two-component flavin-dependent enzyme system and is proposed to rely on a separate NAD(P)H:FMN reductase, MsuE, for the supply of reduced flavin to perform the oxidative half reaction.[197] Additionally, MsuC is involved in a similar metabolic pathway as SfnG and MsuD.[194,196,197] The substrate in the reaction of MsuC is methanesulfinate, which is proposed to be the product in the reaction catalyzed by SfnF and SfnG. The methanesulfonate product from the MsuE/MsuC reaction serves as the substrate in the desulfonation reaction catalyzed by MsuE and MsuD (Figure

4.1).[194,196,197] The crystal structure of MsuC from *P. fluorescens* was previously solved,

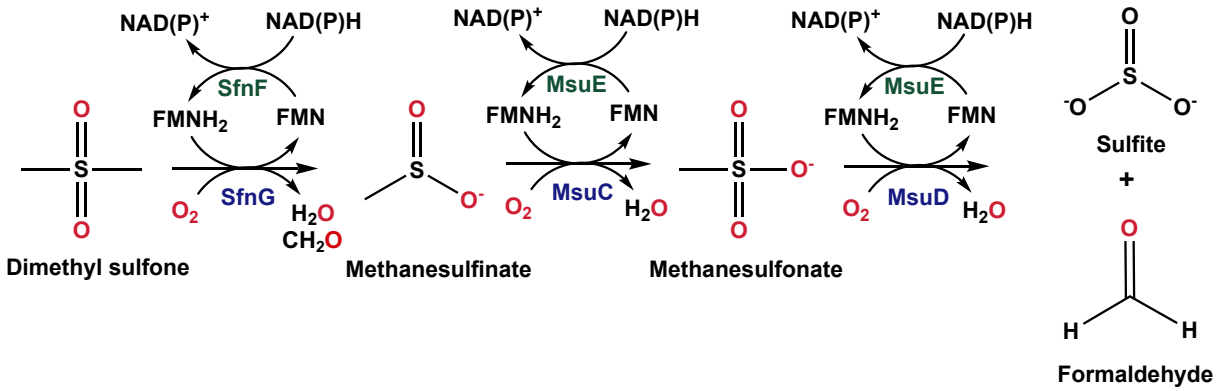

Figure 4.1: Two-component flavin-dependent enzymes that enable *Pseudomonas* species to utilize oxidation products of dimethyl sulfide as alternative sulfur sources. (Adapted from [95,194]).

and this enzyme was characterized as a homotetramer adopting the acyl CoA dehydrogenase fold (Figure **4.2A**).[197] The ability of MsuC from *P. fluorescens* to oxidize methanesulfinate and yield methanesulfonate was also confirmed through ^{1}H NMR experiments.[197] Even though there is evidence that MsuC from *P. fluorescens* catalyzes the oxidation of methanesulfinate to methanesulfonate, details regarding the oligomeric state and mechanistic properties of this enzyme have not been elucidated. Two-component flavin-dependent enzymes have been reported to undergo changes in the oligomeric state in order to facilitate protein-protein interactions and reduced flavin transfer between the reductase and the monooxygenase enzyme.[198-200]

Besides *P. fluorescens*, MsuC is also conserved in *P. aeruginosa*. MsuC from *P. fluorescens* and *P. aeruginosa* share ~70% amino acid sequence identity (Figure **4.2B**). MsuC from *P. aeruginosa* has not been structurally or mechanistically characterized; however, the relatively high amino acid sequence identity and similarity with MsuC from

P. fluorescens suggests a comparable functional role. The studies described in this chapter are the first focused on investigating the structural and mechanistic features of MsuC from *P. aeruginosa* in order to understand its function during bacterial sulfur starvation. In addition to kinetic experiments, bacterial growth assays were performed to evaluate the viability of MsuC within the cell.

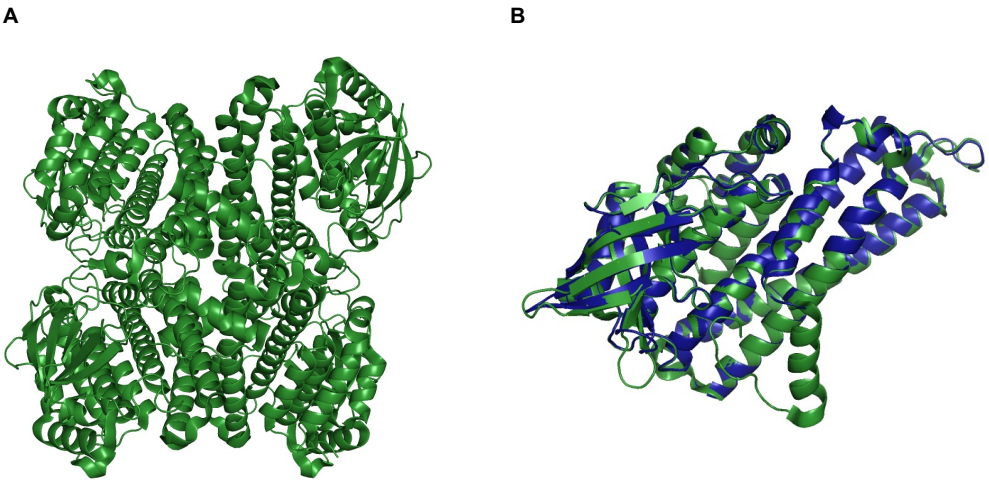

Figure 4.2: MsuC has been characterized as a homotetramer adopting the acyl CoA dehydrogenase fold.[197] **A.** Crystal structure of MsuC from *P. fluorescens* (PDB: 6UUG). **B.** Overlay of the monomeric structure of MsuC from *P. fluorescens* (displayed in green) and *P. aeruginosa* (displayed in blue). The monomeric structure of MsuC from *P. aeruginosa* was generated with AlphaFold.[322]

4.2 Experimental Procedures

4.2.1 Materials

All chemicals for purification and protein assays were purchased from Sigma-Aldrich, Fischer, Bio-Rad, or Fluka. *E. coli* strains (XL-1 and BL21(DE3)) were purchased from Stratagene (California, United States of America). Plasmid vectors and pET21a were

obtained from Novagen (Wisconsin, United States of America). DNA primers were synthesized by Invitrogen (California, United States of America).

4.2.2 Cloning of the *msuC* Gene from *Pseudomonas aeruginosa*

The gene encoding the methanesulfinate monooxygenase (*msuC*) was obtained from genomic DNA and prepared directly from the *P. aeruginosa* PAO1 strain. The *msuC* gene was PCR-amplified with Herculase II Fusion DNA polymerase using the primers (5'-G AAA CCC CAT ATG AAT GCG AAG AC-3') and (5'-TAT GGG TAG CTC GAG TCA TGA GTA G-3') that included *Nde*I and *Xho*I restriction sites. Ligation into the pet21a expression vector was performed with T4 DNA ligase. DNA vectors containing representative clones were submitted for sequence analysis at Eurofins Genomics (Kentucky, United States of America). After successful cloning of the *msuC* gene into the pet21a vector, bacterial transformation was performed into *E. coli* XL-1 and BL21(DE3) super-competent expression cells. Bacterial cultures were stored as 40% glycerol stocks at -80 °C.

4.2.3 Expression and Purification of MsuC

Cells from frozen stocks were isolated on LB-agar plates containing 100 μg/mL ampicillin (LB-Amp). A single colony of *E. coli* BL21(DE3) containing the appropriate expression plasmid was used to inoculate 5 mL LB-Amp media which were incubated 7 h at 37 °C. A 1% inoculum of the 5 mL culture was used to inoculate 100 mL LB-Amp media which was incubated overnight at 37 °C and used to inoculate nine 1 L flasks of LB-Amp media. When the OD_{600} value reached 0.5–0.6 at 37 °C, the flasks were moved from the shaker and kept on ice. The temperature was decreased from 37 to 25 °C, and isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.4 mM. The

cultures were incubated at 25 °C for 6 h. After 6 h, the cells were harvested by centrifugation at 5000 rpm for 20 min and store at -80 °C.

The cell pellets were resuspended in 200 mL of 25 mM potassium phosphate (pH 7.5) and 10% glycerol buffer supplemented with lysozyme (0.02 g/mL) and lysed by sonication (45 sec on, 1 min off, 20% amplitude, 10 cycles). After sonication, cell debris was removed by centrifugation at 10,000 rpm for 20 min at 4 °C. Nucleic acids were removed by adding 1.5% streptomycin sulfate (w/v) to the supernatant while the solution was slowly stirred for 1 hour at 4 °C. Precipitated nucleic acids were pelleted via centrifugation at 10,000 rpm at 4 °C for 20 min. Ammonium sulfate precipitation was performed from 20% to 60%. The pelleted protein from the 60% ammonium sulfate precipitation was resuspended in 45 mL of 25 mM potassium phosphate (pH 7.5) and 10% glycerol and dialyzed in the same buffer at 4 °C. The next day, the protein was further diluted to 200 mL with 25 mM potassium phosphate (pH 7.5) and 10% glycerol buffer and loaded onto a Macro-Prep High Q anion exchange column. After washing the column with 25 mM potassium phosphate (pH 7.5) and 10% glycerol buffer, the protein was eluted with a linear gradient from 0-300 mM NaCl in 25 mM potassium phosphate (pH 7.5) and 10% glycerol buffer. Fraction purity was determined by A_{280} values and SDS-PAGE (5% stacking and 12% resolving). Fractions determined to be of highest purity were pooled and precipitated with 60% ammonium sulfate and resuspended in 25 mM potassium phosphate (pH 7.5), 10 % glycerol and 100 mM NaCl. The resuspended pellet was dialyzed against 25 mM potassium phosphate (pH 7.5), 10 % glycerol and 100 mM NaCl buffer. After dialysis the protein was centrifuged at 10,000 rpm at 4 °C for 20 min to

remove any precipitated protein. Protein aliquots were flash frozen with liquid nitrogen and stored at -80 °C.

4.2.3 Spectrofluorometric Titrations

The affinity of wild-type MsuC for oxidized flavin (FMN and FAD) was monitored by spectrofluorometric titration. The binding of FMN and FAD to MsuC (0.5 μM) was performed under aerobic conditions by adding aliquots of oxidized flavin (5-100 μM) to the enzyme solution in a fluorescence cuvette. Spectral changes were monitored at 344 nm (excitation at 280 nm) after each addition of FMN or FAD following a 2 min incubation.

For the titration of reduced flavin ($FMNH_2$ and $FADH_2$), an anaerobic solution of wild-type MsuC (0.5 μM) in 25 mM potassium phosphate (pH 7.5) and 100 mM NaCl (1.0 mL total volume) was titrated with a solution of $FMNH_2$ or $FADH_2$. The enzyme solution was transferred inside the glovebox and treated with glucose (10 mM) and glucose oxidase (0.1 μM) to remove trace amounts of oxygen after which it was diluted in anaerobic 25 mM potassium phosphate (pH7.5) and 100 mM NaCl buffer. The buffer was made anaerobic by bubbling with ultrahigh-purity argon gas for 1 h before being transferred inside the anaerobic glovebox. Reduced flavin was prepared in 25 mM potassium phosphate (pH 8.0), 20 mM EDTA, and 100 mM NaCl. The flavin solution was bubbled with ultrahigh-purity argon for 30 min before being transferred to the anaerobic glovebox. After the addition of glucose (10 mM) and glucose oxidase (0.1 μM) to both protein and flavin, the solutions were incubated in an anaerobic glovebox for 2 h to remove traces of dioxygen. The anaerobic flavin solution was photoreduced inside a gastight titrating syringe with a long wavelength UV lamp, after which the anaerobic cuvette was assembled inside the anaerobic glovebox. Wild-type MsuC was titrated with

20 aliquots of $FMNH_2$ (0.5-10 μM) or $FADH_2$ (2.0-40 μM). The fluorescence spectrum at 344 nm (excitation at 280 nm) was recorded following a 2 min incubation after each addition of reduced flavin. Bound $FMNH_2$ or $FADH_2$ was determined using equation 1.

$$[FMNH_2]_{bound} = [E]\frac{I_0 - I_c}{I_0 - I_f} \quad \textbf{Equation 1}$$

In equation 1, $[FMNH_2]_{bound}$ represents the concentration of the $FMNH_2$-bounds enzyme. [E] represents the initial concentration of the enzyme, I_0 is the initial fluorescence intensity of the enzyme prior to the addition of the substrate, I_c is the fluorescence intensity of the enzyme following the addition, and I_f is the final fluorescence intensity. The concentration of $FMNH_2$ bound was plotted against the free substrate to obtain the dissociation constant (K_d) according to equation 2.

$$Y = \frac{B_{max}X}{K_d + X} \quad \textbf{Equation 2}$$

In equation 2, Y and X represent the concentration of bound and free substrates, respectively, following each addition. B_{max} is the maximum binding at equilibrium with the maximum binding of substrate.

4.2.4 Thermal Melt Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy measurements as a function of temperature and wavelength were performed using an Applied Photophysics Chirascan V100 Spectropolarimeter (Leatherhead, United Kingdom) to evaluate the thermal stability of MsuC in the presence and absence of methanesulfinate and flavin (FMN and FAD). MsuC (1 μM) in 10 mM potassium phosphate (pH 7.5) buffer was placed in a sealed quartz cuvette with a 1 mm pathlength that contained the temperature probe immersed in the sample. CD spectra were collected over a wavelength range of 185-300 nm with a step

size of 1 nm, and a temperature range of 20-94 °C. The temperature was increased at a step interval of 2 °C using a Quantum Northwest peltier-controlled temperature cell holder (Washington, United States of America). The thermal melting was measured at a heating rate of 2 °C/min following a 5 min equilibration period at each temperature. When substrates were included, a concentration of 200 μM was utilized for FMN and FAD, whereas a concentration of either 250 μM or 1 mM of methanesulfinate was utilized. All experiments were performed in triplicate.

The CD thermal melting profiles were analyzed using global thermodynamic analysis. A plot of the CD signal against wavelength yields an unfolding curve as function of temperature. Each melting curve is fitted with a sigmoidal function which is derived from the Gibbs-Helmholtz equation shown in equation 3.

$$\mathrm{CD(T)} = \frac{(\mathrm{m_F T + b_F}) - (\mathrm{m_U T + b_U})}{1 + \mathrm{e}^{\frac{\Delta H_{vH}}{\mathrm{R}}(\frac{1}{\mathrm{T_m}} - \frac{1}{\mathrm{T}})}} + \mathrm{m_U} + \mathrm{b_U} \qquad \textbf{Equation 3}$$

Equation 3 contains both local and global fitting variables. The thermodynamic parameters, melting temperature (T_m) and van't Hoff's enthalpy (ΔH_{vH}), are global variables which are consistent for each wavelength. The local fitting parameters, b_F and b_U describe CD signals corresponding to the completely folded and unfolded conformation of the protein at a certain wavelength. The m_F and m_U variables stand for baseline at pre and post transition regions when baseline correction is required. Finally, *T* in equation 3 describes the absolute temperature, whereas *R* is the gas constant (8.314 J/mol/K).

4.2.5 Native-PAGE Analysis

The oligomeric state of MsuC in the presence and absence of methanesulfinate as well as flavin (FMN and FAD) was analyzed by native-PAGE. In the absence of methanesulfinate or flavin, various concentrations of MsuC (5, 10, 15, 20, 30, 50 μM) were mixed with native sample buffer to a final volume of 15 μL. When methanesulfinate and flavin (FMN and FAD) were included, the following concentrations were utilized: MsuC (10 μM), FMN (200 μM), FAD (200 μM), and methanesulfinate (250 μM or 1 mM). Protein samples (10 μL) were loaded onto a 5% stacking and 10% resolving gel which was made a day in advance and stored at 4 °C. After loading, protein samples were separated at 118 V for 16 hours at 4 °C. After 16 hours, gels were removed and stained with Brilliant Blue.

4.2.6 Sedimentation Velocity Analytical Ultracentrifugation

The oligomeric state of MsuC in the absence and presence of methanesulfinate was also evaluated by sedimentation velocity on an Optima XL-A analytical ultracentrifuge with an An-60 Ti 4-hole rotor (Beckman Coulter, California, United States of America). Samples of MsuC were exchanged 3 times into 25 mM potassium phosphate (pH 7.5) and 100 mM NaCl (total volume of 4 mL) using an Amicon Ultra-4 filter (Millipore) with a 10 kDa molecular weight cutoff to remove glycerol. MsuC (8 μM) was loaded into double-sectored cells and equilibrated to 20 °C. Sedimentation data at 280 nm were collected at a rotor speed of 40,000 rpm and a radial step size of 0.003 cm. When methanesulfinate was included, a concentration of 250 μM or 1 mM was loaded with MsuC (8 μM). The partial specific volume of 0.7309 cm^3/g was calculated using the amino acid composition in the program SEDNTERP.[284] The buffer density (1.00574 g/mL) and

buffer viscosity (0.010183 Poise) were also determined using SEDNTERP.[284] Plots and fits of the data were generated with SEDFIT (version 16.1c) software using every 3rd out of the 300 scans for the analysis.[285,286] Continuous sedimentation coefficient distributions [*c*(*s*)] were calculated using SEDFIT that deconvolutes diffusion effects based on the direct boundary modelling with distributions of Lamm equation.

4.2.7 Rapid-reaction Kinetic Analyses

Stopped-flow kinetic experiments monitoring both the reductive and oxidative half-reactions were performed at room temperature with aerobic solutions of wild-type MsuC (35 μM), wild-type MsuE (35 μM), and FMN (30 μM) in 25 mM potassium phosphate (pH 7.5) and 100 mM NaCl buffer, mixed against NADPH (30 μM) in air-saturated 10 mM Tris-HCl (pH 8.5) and 100 mM NaCl. When included, methanesulfinate (250 μM) was present in the drive syringe with NADPH. 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 360, 370, and 450 nm over 60 s. Spectra were recorded on an Applied Photophysics Chirascan V100 Spectropolarimeter attached to a SF.3 stopped-flow accessory (Leatherhead, United Kingdom). Control experiments were also performed as described above in the absence of wild-type MsuC in order to monitor flavin reduction by wild-type MsuE, and subsequent non-enzymatic flavin oxidation. All rapid-reaction experiments were performed in triplicate. Kinetic traces were averaged using the default Chirascan parameters, and final data were plotted using GraphPad Prism 9 software.

4.2.8 Strain Constructions for Bacterial Growth Studies

Wild-type *P. aeruginosa* and transposon insertion mutants of *cysB*, *sfnG*, and *msuC* were obtained from the University of Washington Transposon Mutant Collection (Manoil Laboratory).[287] Transposon insertion in each gene was verified by PCR with a transposon specific primer (Hah-138) (5'-CGG GTG CAG TAA TAT CGC CCT-3') and a primer specific for the gene of interest.

4.2.9 Bacterial Growth Studies

Growth of wild-type *P. aeruginosa* and the transposon insertion mutants was performed in sulfur free medium (SFM). SFM contained 50 mM Tris-HCl (pH 7.3), 25 mM sodium succinate, 20 mM ammonium chloride, 500 μM potassium phosphate monobasic, 1.2 mM magnesium chloride, and 50 μg/mL of the amino acids Pro, Arg, Val, Leu, Ile, Asp, Glu, Ala, His, Gly, Phe, Ser, Tyr, Trp, Lys, Asn, and Gln. SFM was also supplemented with 10 μM of ferric chloride, manganese chloride, calcium chloride, and zinc chloride. SFM medium was stored at 4 °C and was sterilized through filtration using a 150 mL Corning filter system (New York, United States of America) prior to being included in the bacterial growth assays.

Wild-type *P. aeruginosa* and the transposon insertion mutants were isolated on LB-agar plates without any antibiotics. A single colony from these plates was selected and inoculated into 5 mL LB medium and incubated with shaking at 37 °C overnight. Overnight cultures were diluted 1:100 into SFM in a Corning Costar 48 well flat bottom cell culture plate (New York, United States of America) and grown for 16 hours with continuous shaking in an Agilent BioTek Synergy H1 microplate reader (California, United States of America). Cell growth was monitored every 10 minutes at an OD of 600 nm.

The growth behavior of wild-type *P. aeruginosa* and the transposon insertion mutants was also monitored in the presence of different sulfur sources. The sulfur sources tested were sodium sulfate, L-cysteine, dimethyl sulfoxide, dimethyl sulfone, methanesulfinate, methanesulfonate, and octanesulfonate (each at a concentration of 500 μM supplemented in SFM).

4.3 Results

4.3.1 Evaluating the Binding Affinity of MsuC for Different Flavins

Spectrofluorometric titrations were performed to determine the binding affinity of MsuC for the oxidized and reduced forms of FMN and FAD (Figure **4.3 A-D**, Table **4.1**).

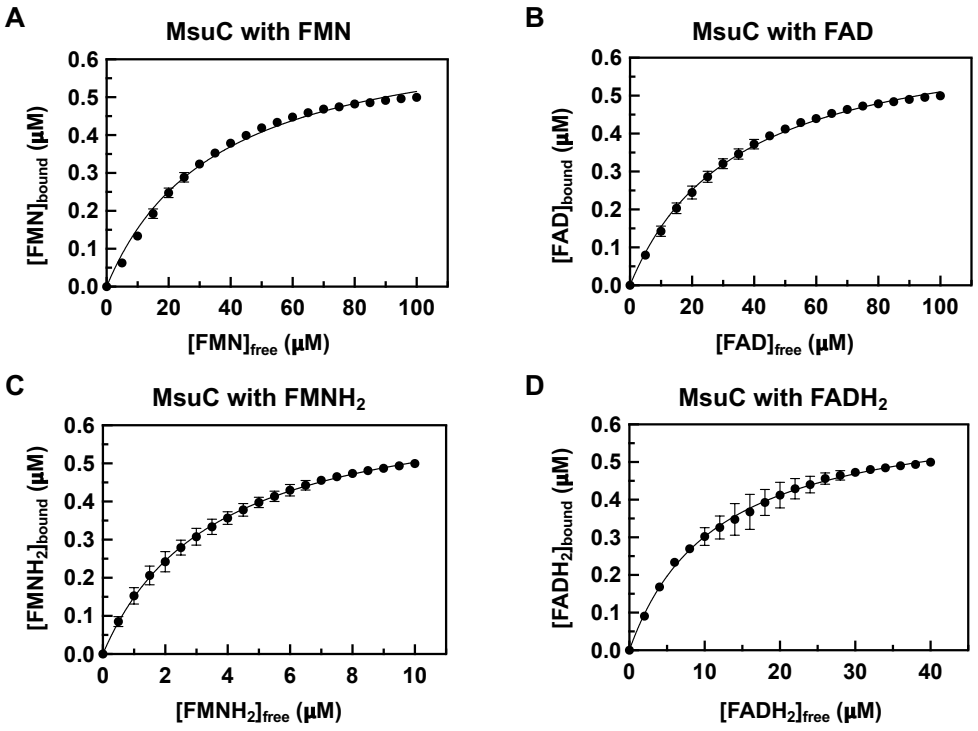

Figure 4.3: Fluorometric titrations of MsuC with oxidized and reduced flavins. **A.** MsuC with FMN. **B.** MsuC with FAD. **C.** MsuC with $FMNH_2$. **D.** MsuC with $FADH_2$.

MsuC had a similar binding affinity for both the oxidized forms of FMN and FAD (Figure **4.3A** and **B**, Table **4.1**); however, a ~10-fold higher binding affinity was observed with $FMNH_2$ as compared to FMN (Figure **4.3C**, Table **4.1**). Additionally, these results also revealed that MsuC had a similar binding affinity for $FMNH_2$ and $FADH_2$ (Figure **4.3C** and **D**, Table **4.1**). The similar binding affinity of MsuC for $FMNH_2$ and $FADH_2$ suggests that MsuC could utilize both forms of reduced flavins to catalyze the oxidative half-reaction.

Table 4.1: Dissociation constants for MsuC

	K_d, FMN ($M \times 10^{-6}$)	K_d, $FMNH_2$ ($M \times 10^{-6}$)	K_d, FAD ($M \times 10^{-6}$)	K_d, $FADH_2$ ($M \times 10^{-6}$)
MsuC	36 ± 4	3.5 ± 0.9	35 ± 5	11.9 ± 2.5

4.3.2 Determining the Oligomeric State and Thermal Stability of MsuC

The oligomeric state of MsuC in the absence of flavin (FMN or FAD) and methanesulfinate was first evaluated using native-PAGE analysis. Different concentrations of MsuC were evaluated to determine if the oligomeric state of MsuC was concentration dependent. The results from the native-PAGE analyses revealed that MsuC likely exists as a tetramer. Based on its amino acid composition, MsuC has a monomeric molecular weight of 44.8 kDa. Therefore, MsuC as a tetramer would have a molecular weight of 179 kDa which would run in between the protein standards containing a molecular weight of 146 and 242 kDa. Moreover, these results also revealed that the oligomeric state of MsuC is not dependent on the concentration (Figure **4.4**). The presence of FMN, FAD, and methanesulfinate did not alter the oligomeric state of MsuC in native-PAGE studies (Figure **4.5A** and **B**).

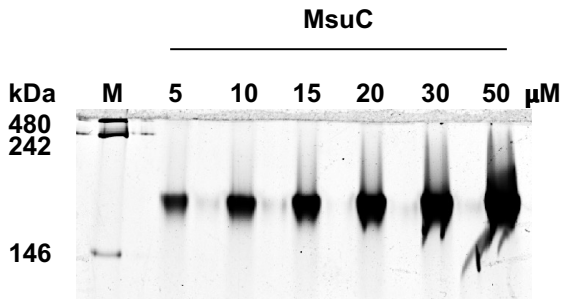

Figure 4.4: Native-PAGE analysis of MsuC using different concentrations. The lane containing the native protein marker is labeled with M.

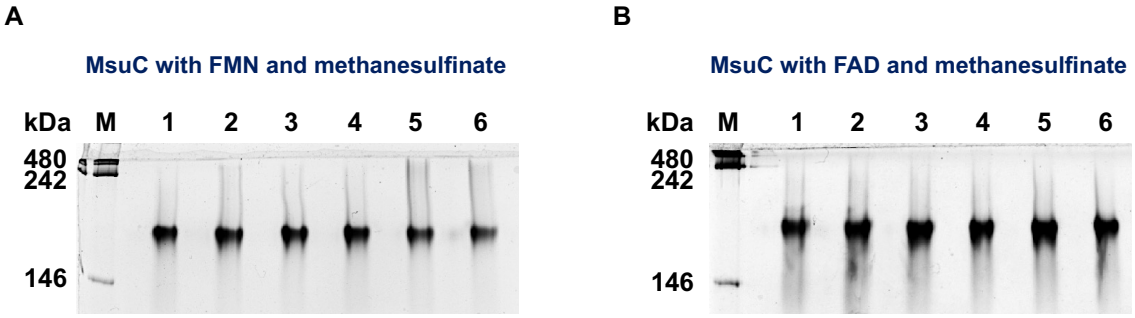

Figure 4.5: Native-PAGE analyses of MsuC in the presence and absence of different flavins and methanesulfinate. **A.** MsuC (10 μM) in the presence and absence of FMN (200 μM) and methanesulfinate (250 μM or 1 mM). **B.** MsuC in the presence and absence of FAD and methanesulfinate. Gel lanes for A and B: native protein marker (M), MsuC (Lane 1), MsuC and flavin (Lane 2), MsuC and 250 μM of methanesulfinate (Lane 3), MsuC and 1 mM of methanesulfinate (Lane 4), MsuC with flavin and 250 μM of methanesulfinate (Lane 5), MsuC with flavin and 1 mM of methanesulfinate (Lane 6).

The native-PAGE analyses as well as the sedimentation velocity and thermal melt studies were performed with two concentrations of methanesulfinate. A concentration of 1 mM methanesulfinate was utilized in previous studies with MsuC from *P. fluorescens*, whereas a concentration of 250 μM would represent the cellular concentration of

methanesulfinate. The oligomeric state of MsuC was also determined through sedimentation velocity analytical ultracentrifugation experiments in order to evaluate its quaternary structure in solution (Figure **4.6**). The resulting continuous sedimentation distribution profiles of MsuC confirmed the presence of a single species in solution with a sedimentation coefficient ($s_{20,w}$) of 8.0 S and a molecular weight of 171 ± 1 kDa (Figure **4.6A**, Table **4.2**). The experimental molecular weight (171 ± 1 kDa) obtained from sedimentation velocity experiments divided by the calculated monomeric molecular weight (44.8 kDa) of MsuC confirmed that MsuC exists as a tetramer in solution. In the presence of methanesulfinate, a similar sedimentation coefficient and experimental molecular weight (167 ± 3 kDa) was obtained as compared to apo MsuC, confirming that the presence of methanesulfinate does not affect the oligomeric state of MsuC in solution (Figure **4.6B** and **C**, Table **4.2**).

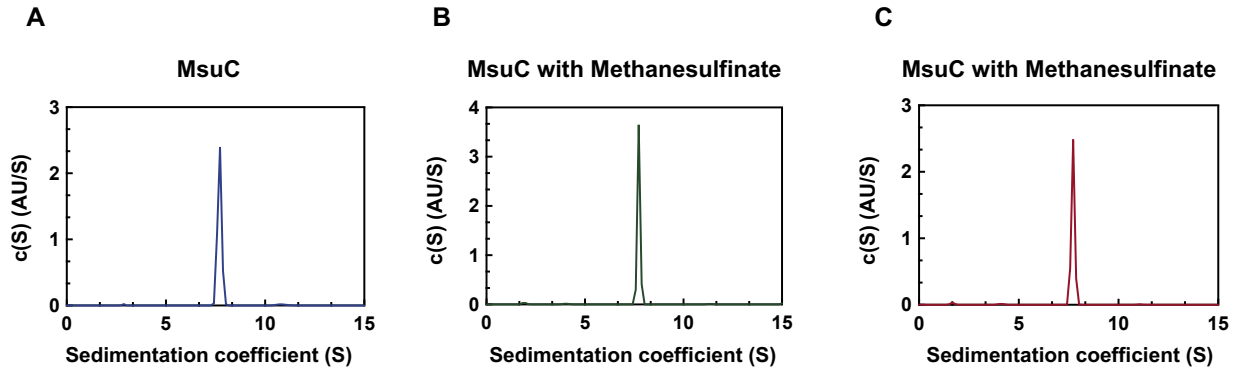

Figure 4.6: Continuous sedimentation distribution profiles of MsuC in the presence and absence of methanesulfinate. **A.** MsuC in the absence of methanesulfinate. **B.** MsuC with 250 μM methanesulfinate. **C.** MsuC with 1 mM methanesulfinate.

Table 4.2: Sedimentation velocity analyses of MsuC in the presence and absence of methanesulfinate

	MsuC	MsuC with 250 μM methanesulfinate	MsuC with 1 mM methanesulfinate
$s_{20,w}$ (S)	8.0 ± 0.1	8.0 ± 0.1	8.0 ± 0.1
Monomeric M_w (kDa)	44.8	44.8	44.8
Experimental M_w (kDa)	171 ± 1	167 ± 3	165 ± 1
Oligomeric state	Tetramer	Tetramer	Tetramer

The thermal stability of MsuC in the presence and absence of flavin (FMN or FAD) and methanesulfinate was evaluated using thermal melt circular dichroism spectroscopy experiments (Figure **4.7 A-G**, Table **4.3**). The results obtained from these experiments revealed that apo MsuC has a melting temperature (T_m) of 43.5 ± 0.1 °C. When substrate and/or product were included, no significant changes were observed in the melting temperature of MsuC (Table **4.3**). Taken together, these results suggest that the presence or absence of FMN, FAD, and methanesulfinate does not affect the thermal stability and quaternary structure of MsuC.

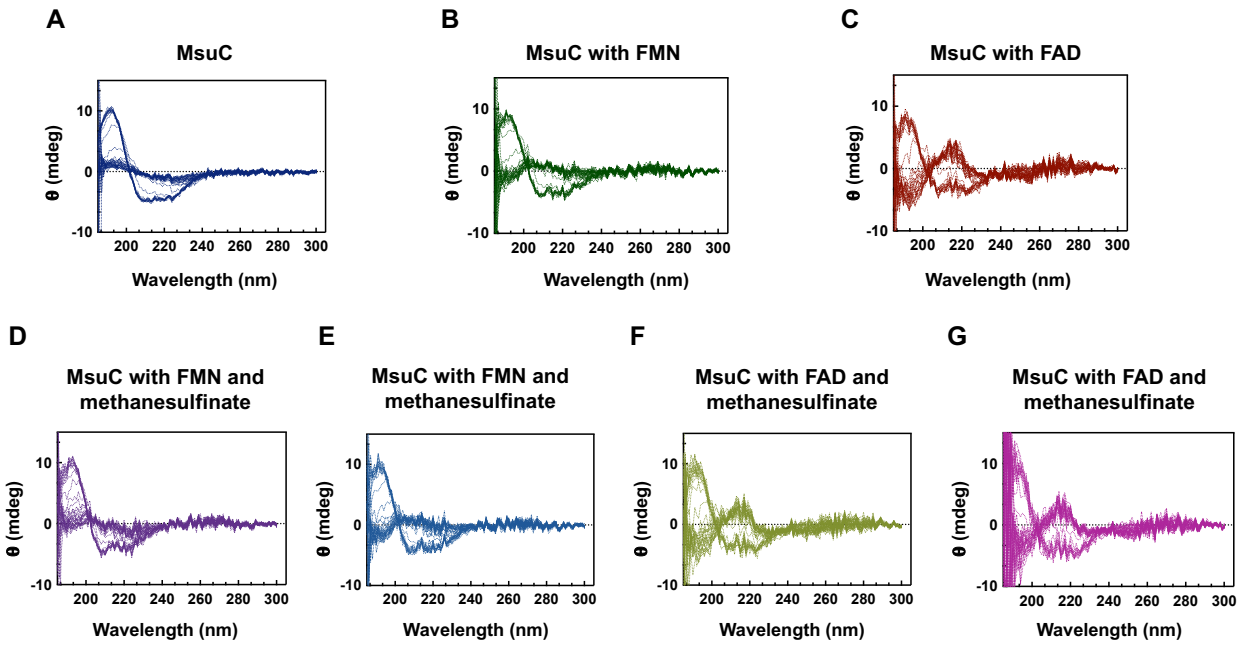

Figure 4.7: Thermal melt circular dichroism profiles of MsuC in the presence and absence of FMN, FAD, and methanesulfinate. **A.** MsuC in the absence of flavin and methanesulfinate. **B.** MsuC with FMN. **C.** MsuC with FAD. **D.** MsuC with FMN and 250 μM methanesulfinate. **E.** MsuC with FMN and 1 mM methanesulfinate. **F.** MsuC with FAD and 250 μM methanesulfinate. **G.** MsuC with FAD and 1 mM methanesulfinate.

Table 4.3: Melting temperatures (T_m) of MsuC in the presence of flavin (FMN and FAD) and methanesulfinate

	T_m (°C)
MsuC	43.5 ± 0.1
MsuC with FMN	43.5 ± 0.1
MsuC with FAD	44.5 ± 0.6
MsuC with FMN and 250 μM methanesulfinate	43.8 ± 0.2
MsuC with FAD and 250 μM methanesulfinate	43.7 ± 0.4

4.3.3 Rapid-reaction Kinetics of Flavin Oxidation

Flavin-dependent monooxygenases that are part of two-component enzyme systems rely on a separate reductase to supply reduced flavin.[288] Reduced flavin provided by the reductase to the monooxygenase is subsequently utilized for the activation of molecular oxygen which involves the formation of oxygenating flavin intermediates.[129,144,159] Unlike SsuD and MsuD that have been proposed to utilize a flavin-N5 oxygenating intermediate, we hypothesized that MsuC utilizes a classical C4a-(hydro)peroxy flavin intermediate which can be spectrally observed between 360-400 nm (Figure **1.20**).[129,130] Rapid reaction kinetic analyses were performed in the presence of MsuE and MsuC to determine if MsuC forms a C4a-(hydro)peroxy flavin intermediate in the presence and absence of methanesulfinate.

The formation of the C4a-(hydro)peroxy flavin intermediate by MsuC was monitored at 360 and 370 nm in the presence and absence of methanesulfinate. In the presence of methanesulfinate, the traces observed at 360 and 370 nm showed a decrease in absorbance over the course of 60 seconds (Figure **4.8A**). A similar result was also obtained in the absence of methanesulfinate (Figure **4.8B**) and even when the concentration of MsuE was doubled. From the results obtained under the given conditions, we were unable to provide spectral evidence for the formation of a C4a-(hydro)peroxy flavin intermediate by MsuC.

Rapid reaction kinetic studies were also performed with MsuE and MsuC to investigate reduced flavin transfer at 450 nm. The reduction and oxidation of flavin by MsuE and MsuC in the presence and absence of methanesulfinate was monitored at 450 nm. In the presence of methanesulfinate a decrease in the absorbance was observed followed by a subsequent increase in the absorbance. This decrease in absorbance corresponds to the reduction of flavin by MsuE followed by the subsequent oxidation by MsuC which is represented by the increase in absorbance (Figure **4.8A**). Conversely, in the absence of methanesulfinate there was a decrease in flavin absorbance, but the flavin was not oxidized by MsuC (Figure **4.8B**). Therefore, the flavin was protected from oxidation by MsuC in the absence of substrate. In order to make sure that the observed result was due to an enzymatic reaction between MsuE and MsuC, rapid reaction kinetic experiments were also performed without MsuC. In the absence of MsuC, flavin was seen to be reduced by MsuE in the initial phase followed by its rapid oxidation in the second phase. A similar result was also observed when methanesulfinate was included. The rapid oxidation of reduced flavin in the absence of MsuC is suggested to occur

nonenzymatically due to its release into bulk solvent or due to the lack of protection coming from MsuC.

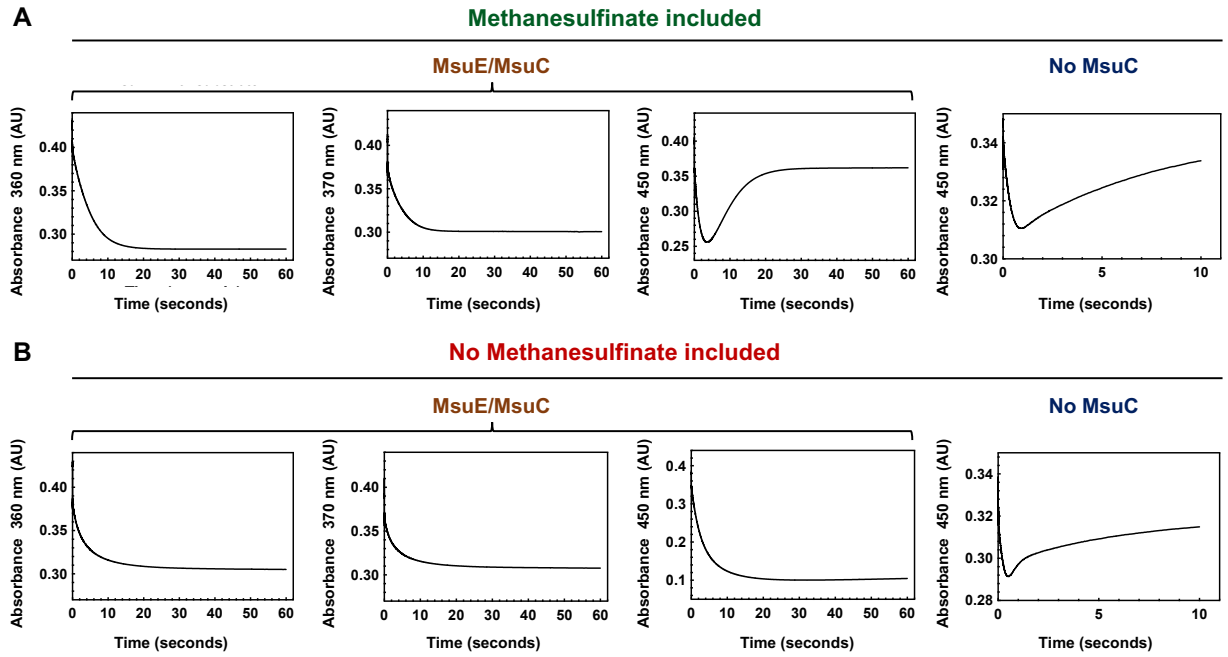

Figure 4.8: Rapid reaction kinetic traces of wild-type MsuE and MsuC in the presence and absence of methanesulfinate. **A.** Rapid reaction kinetic traces obtained at 360, 370, and 450 nm in the presence of methanesulfinate. **B.** Rapid reaction kinetic traces obtained at 360, 370, and 450 nm in the absence of methanesulfinate. The formation of the C4a-(hydro)peroxy flavin intermediate by MsuE/MsuC was monitored at 360 and 370 nm, whereas reduced flavin transfer between the two enzymes was monitored at 450 nm.

4.3.4: Growth of Wild-type *P. aeruginosa* and Transposon Mutants in the Presence of Various Sulfur Sources

SfnG and MsuC are part of two-component flavin-dependent enzyme systems that are expressed by *Pseudomonas* species during sulfur limitation.[194,197] In order to evaluate the viability of these enzymes within the cell in the presence of their preferred sulfur

source, bacterial growth studies were performed with transposon mutants of SfnG and MsuC from *P. aeruginosa* in the presence of SFM, sodium sulfate, dimethyl sulfone and methanesulfinate. The growth of *P. aeruginosa* in the presence of the aforementioned sulfur sources was also monitored and used as a positive control in the bacterial growth assays. The highest OD_{600} for wild-type *P. aeruginosa* was observed when SFM was supplemented with sodium sulfate. In the presence of dimethyl sulfone and methanesulfinate a slightly lower OD_{600} was observed compared to that of sodium sulfate with wild-type *P. aeruginosa* (Figure **4.9A**). A similar result was obtained for the SfnG transposon mutant in the presence of sodium sulfate and methanesulfinate. Moreover, when the SfnG transposon mutant was grown in SFM and in SFM that was supplemented with dimethyl sulfone, the OD_{600} values were similar to that of wild-type *P. aeruginosa* grown in SFM (Figure **4.9B**). After 5 hours, a slight increase in the OD_{600} was observed for the SfnG transposon mutant in the presence of dimethyl sulfone. Lastly, similar results were obtained for the MsuC transposon mutant as compared to wild-type *P. aeruginosa* when monitoring the OD_{600} (Figure **4.9C**).

The growth of wild-type *P. aeruginosa* was further evaluated in the presence of other sulfur sources such as L-cysteine, dimethyl sulfoxide, methanesulfonate, and octanesulfonate. In addition to wild-type *P. aeruginosa*, the growth of the CysB transposon mutant was also evaluated in the presence of different sulfur sources. CysB is known as the master regulator of genes involved in sulfur assimilation and sulfur starvation, which also includes the genes *sfnG* and *msuC*.[4,289-291] In order to further evaluate role of CysB during sulfur starvation, the OD_{600} of a CysB transposon mutant

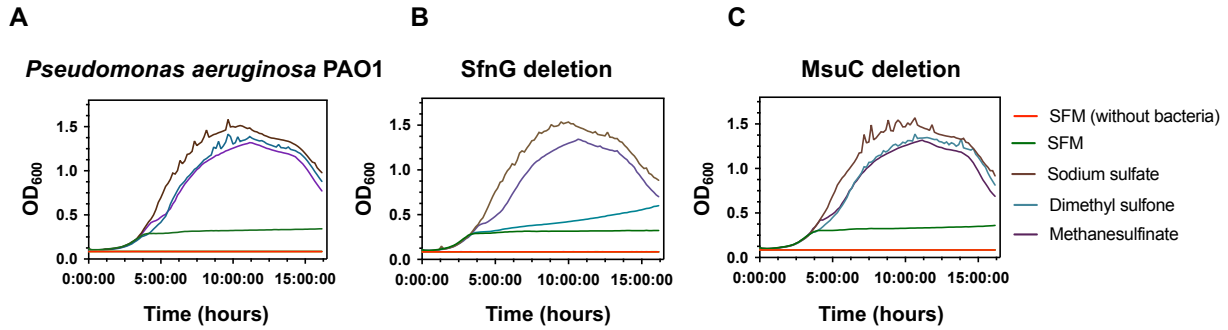

Figure 4.9: Growth studies with *P. aeruginosa*, SfnG, and MsuC transposon mutants in the presence of SFM, sodium sulfate, dimethyl sulfone, and methanesulfinate. **A.** Wild-type *P. aeruginosa*. **B.** SfnG transposon mutant. **C.** MsuC transposon mutant. When supplemented with the sulfur source, 500 μM of the respective sulfur source was included in SFM.

was monitored in the presence of different sulfur sources. The highest OD_{600} for wild-type *P. aeruginosa* was observed for SFM that was supplemented with sodium sulfate and L-cysteine, whereas slightly lower OD_{600} values were observed when SFM was supplemented with dimethyl sulfoxide, dimethyl sulfone, methanesulfinate, methanesulfonate, and octanesulfonate (Figure **4.10A**). These results suggest that wild-type *P. aeruginosa* utilizes sodium sulfate and L-cysteine as its preferred sulfur sources. Conversely, the CysB transposon mutant took approximately 5 hours to reach the exponential phase in the presence of the different sulfur sources, whereas the exponential phase for wild-type *P. aeruginosa* could be observed after 2 hours. From the obtained results, a higher OD_{600} was obtained when the CysB transposon mutant was grown in SFM that was supplemented with L-cysteine (Figure **4.10B**). Moreover, slightly lower OD_{600} values were observed when the CysB transposon mutant was grown in the presence of sodium sulfate and dimethyl sulfoxide. Finally, similar OD_{600} values were

observed when the CysB transposon mutant was grown in SFM or SFM that was supplemented with dimethyl sulfone, methanesulfinate, methanesulfonate, or octanesulfonate.

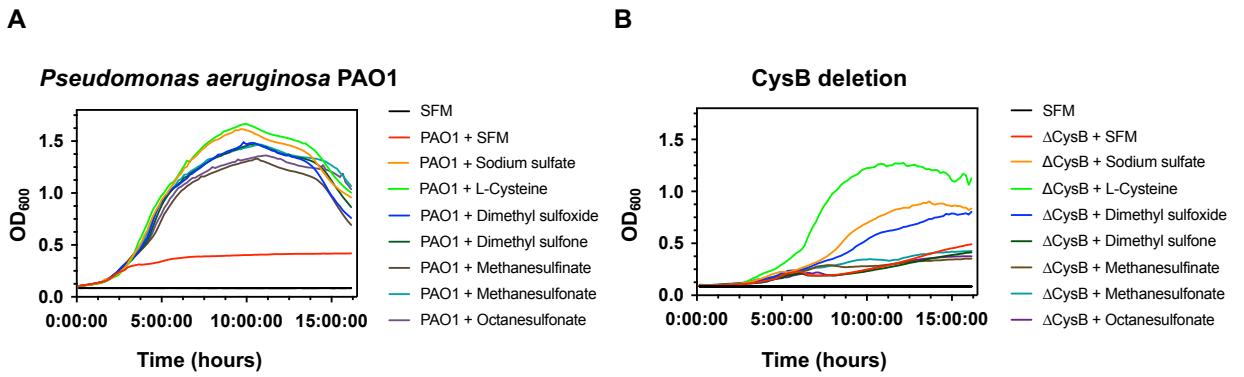

Figure 4.10: Growth studies of *P. aeruginosa* and CysB transposon mutants in the presence of SFM and SFM supplemented with different sulfur sources. **A.** Wild-type *P. aeruginosa*. **B.** CysB transposon mutant. When supplemented with the sulfur source, 500 μM of the respective sulfur source was included in SFM. The sulfur sources tested were sodium sulfate, L-cysteine, dimethyl sulfoxide, dimethyl sulfone, methanesulfinate, methanesulfonate, and octanesulfonate.

4.4 Discussion

P. aeruginosa is a known human pathogen that is widespread in diverse environments and requires sulfur for both survival and pathogenicity.[246-249] The limited availability of inorganic sulfate in the environment has resulted in bacteria expressing different sets of enzymes that allow these microorganisms to utilize alternative sulfur sources.[4,9,64,69,75,93] One specific set of enzymes expressed by bacteria in particular *Pseudomonas* species is the multi two-component methanesulfinate and methanesulfonate monooxygenase system consisting of MsuE/MsuC and MsuE/MsuD,

respectively.[4,93,196,197] The methanesulfinate monooxygenase system has been proposed to allow bacteria to utilize methanesulfinate, an oxidation product of DMS, in order to obtain sulfur during sulfur limitation.[197] In the three-dimensional structure, MsuC from *P. fluorescens* exists as a homotetramer adopting the acyl CoA dehydrogenase fold. Results from these studies also confirmed that the MsuE/MsuC system was able to oxidize methanesulfinate to yield methanesulfonate.[197] The presence of the MsuE/MsuC systems in diverse bacteria suggests that this enzyme system performs a specific role during sulfur starvation.

These studies were focused on the structural and mechanistic characterization of MsuC from *P. aeruginosa*. MsuC from *P. fluorescens* has been previously characterized as a class D flavin-dependent monooxygenase.[197] In addition to adopting the acyl CoA dehydrogenase fold, enzymes within this class have also been reported to utilize both $FMNH_2$ and $FADH_2$ as co-substrates.[155] Results from spectrofluorometric titrations revealed that MsuC had a similar binding affinity for both $FMNH_2$ and $FADH_2$. Even though there was no significant difference in the binding affinity with FAD and $FADH_2$, two-component flavin-dependent monooxygenases typically have a higher affinity for the reduced form of the flavins.[98] The higher affinity of reduced flavin for the monooxygenase ensures an immediate transfer of reduced flavin from the reductase followed by tight binding to the monooxygenase.[98] Under cellular conditions, MsuC would have to rely on its partner reductase, MsuE, to supply reduced flavin.[197] Results obtained from spectrofluorometric titrations with MsuE have also confirmed that this enzyme binds both FMN and FAD with similar binding affinities (unpublished data from the Ellis laboratory), suggesting that MsuC could utilize both $FMNH_2$ and $FADH_2$ for the subsequent oxidative

half reaction. Further studies with both MsuE and MsuC from *P. aeruginosa* are warranted to confirm whether this enzyme system catalyzes the oxidation of methanesulfinate to yield methanesulfonate *in vitro* and *in vivo*.

Protein oligomerization plays an important role in metabolic function. Approximately 50% of the proteins identified have been reported to exist in different oligomeric states.[292-294] Oligomerization enables proteins to form larger structures without affecting the genome size and can also result in the increase of protein stability. Additionally, the ability of different proteins to undergo changes in their oligomeric state has been reported to be essential in regulation of apoptosis and tumor regulation.[292,294-297] Protein oligomers fulfill critical roles in different metabolic pathways and are also involved in gene regulation and expression, enzyme activity, ion channels, and cell-cell adhesion processes.[292,294-298]

Studies with MsuE from *P. aeruginosa* have provided evidence that this enzyme exists in a dimer-tetramer equilibrium. The binding of FMN to MsuE shifts this equilibrium favoring the tetrameric form in sedimentation velocity experiments. Moreover, in the presence of FMN, MsuE becomes more thermally stable as compared to the apo form (unpublished data from Ellis laboratory). Since MsuE supplies reduced flavin to MsuC, native-PAGE and sedimentation velocity experiments were performed to determine if MsuC also undergoes oligomeric changes in the presence and absence of methanesulfinate, FMN, and FAD. Even though results from studies confirmed that MsuC exists as tetramer in the presence and absence of methanesulfinate, FMN, and FAD, further studies will have to be performed in the presence of the $FMNH_2$ and $FADH_2$ substrates. Additionally, results from previous studies with SsuE revealed that in addition

to FMN, the presence of SsuD also influenced the oligomeric state of the reductase.[198,200] Despite the low amino acid sequence identity between these enzymes, a similar approach could be utilized to further investigate the oligomeric state of both MsuE and MsuC. Lastly, the presence or absence of methanesulfinate, FMN, and FAD did not affect the thermal stability of MsuC. Taken together, the results obtained from the native-PAGE and sedimentation velocity studies as well as the thermal denaturation experiments revealed that substrate or product binding did not alter the structural and thermal stability of MsuC.

MsuC has been proposed to rely on MsuE to supply reduced flavin for the oxidative half-reaction.[197] Reduced flavin is required to activate molecular oxygen which typically proceeds through the formation of oxygenating flavin intermediates.[106,109,129,144,159] MsuC shares 42% amino acid sequence identity with the two-component flavin-dependent monooxygenase DszC, which forms a C4a-(hydro)peroxy flavin intermediate.[197,299] Based on the amino acid sequence identity as well as the structural and mechanistic similarity with DszC, we hypothesized that MsuC utilizes a C4a-(hydro)peroxy flavin intermediate which can be spectrally observed between 360-400 nm.[106,109,129,144,159,197,299] Results from rapid reaction kinetic analyses with both MsuE and MsuC in the presence and absence of methanesulfinate revealed that there was no C4a-(hydro)peroxy flavin observed in the oxidative half-reaction of MsuC. Since the rapid reaction experiments were performed at room temperature, there could be a possibility that the C4a-(hydro)peroxy flavin intermediate was not stable enough to accumulate under the given experimental conditions, which resulted in the inability to provide spectral evidence. Given that the C4a-(hydro)peroxy flavin is not stabilized by MsuC at room temperature, future rapid reactions experiments should be performed at a lower temperature in order to

stabilize the formation of this flavin intermediate. Lastly, from the results obtained at 360 and 370 nm both in the presence and absence of methanesulfinate, a decrease in absorbance was observed followed by a constant absorbance after 20 seconds. An isosbestic point at or around the measured wavelengths could lead to no observable change in absorbance. For future rapid reaction kinetic studies with MsuC, a photo-diode array detection could be extremely advantageous, given its ability to monitor changes in the overall flavin spectrum.

The mechanism of reduced flavin transfer was also monitored through rapid reaction kinetic analyses at 450 nm. Enzymes that are part of two-component flavin-dependent systems can either utilize a diffusion or channeling mechanism for the transfer of reduced flavin from the reductase to the partner monooxygenase.[98,99,209,214] The reduced flavin transfer between MsuE and MsuC was investigated in the presence and absence of methanesulfinate. In the presence of methanesulfinate, the reduction and oxidation phases of flavin were spectrally observed at 450 nm. Conversely, in the absence of methanesulfinate the reduction of flavin was observed followed by a constant absorbance after 20 seconds. The absence of any changes in the absorbance after 20 seconds could be due to the protection of reduced flavin by MsuC after it was transferred from MsuE. In the control experiments that were performed in the absence of MsuC, both the reductive and oxidative phase of flavin where observed, supporting the transfer of reduced flavin from MsuE to MsuC. The absence of a similar protection in other two-component enzyme systems such as the alkanesulfonate and the dibenzothiophene monooxygenase systems suggests that interactions specific to MsuC could be responsible for the increased protection of reduced flavin observed in the absence of

methanesulfinate.[211,212,237,240,241,299] Further studies with MsuC alone will need to be performed in order to evaluate the nature of protein-protein interactions between these enzymes.

Since *P. aeruginosa* is ubiquitous in nature, this bacterium has found several ways to survive under various conditions.[246-249] The presence of different sets of SSI proteins in *P. aeruginosa* also equip the bacterium with a "sulfur survival strategy" during sulfur limitation.[4,9,64,69,75,93] Some examples of the SSI proteins expressed by *P. aeruginosa* include the two-component dimethyl sulfone monooxygenase system consisting of SfnF and SfnG, and the methanesulfinate monooxygenase system consisting of MsuE and MsuC.[4,9,93,194,196,197] The roles of the monooxygenases SfnG and MsuC were further evaluated by monitoring the growth of their respective transposon mutants in growth assays in the presence of SFM that was supplemented with different sulfur substrates. The SfnG transposon mutant was able to grow significantly slower in the presence of SFM and SFM that was supplemented with dimethyl sulfone as compared to sodium sulfate and methanesulfinate when monitoring the OD_{600}. This result supported the assigned role of SfnG in dimethyl sulfone utilization during sulfur limitation.[194] Conversely, the MsuC transposon mutant was able to grow in the presence of SFM supplemented with either sodium sulfate, dimethyl sulfone, or methanesulfinate. The ability of the MsuC transposon mutant to grow in the presence of methanesulfinate with an almost similar efficiency as sodium sulfate, brings into the question the previously assigned role of MsuC serving as a methanesulfinate monooxygenase.[197] However, it could be possible that methanesulfinate is being oxidized nonenzymatically to methanesulfonate under the given experimental conditions. Methanesulfonate would then serve as the substrate of

the flavin-dependent monooxygenase, MsuD (which is still intact), in order to yield sulfite and formaldehyde.[4,194,196,197] However, given that the cell is a highly reducing environment, the nonenzymatic oxidation of methanesulfinate would be unlikely to occur within the cellular environment. It could also be possible that the oxidation of methanesulfinate was an artifact of the experimental conditions. Lastly, the ability of the MsuC transposon mutant to grow on methanesulfinate could also be due to another enzyme component that is able to utilize this sulfur source as a potential substrate, which remains to be identified.

The ability of wild-type *P. aeruginosa* to grow in the presence of different sulfur sources was also evaluated through bacterial growth assays monitoring the OD_{600}. The obtained results revealed that *P. aeruginosa* was able to grow on different sulfur sources which are commonly found in nature as well as in the cellular environment. The highest OD_{600} was obtained when *P. aeruginosa* was grown in SFM media supplemented with sodium sulfate or L-cysteine suggesting that these sulfur sources are preferred by this bacterium. The growth of a CysB transposon mutant was also evaluated in the presence of different sulfur sources. The CysB transposon mutant took a longer time to reach the exponential phase as compared to wild-type *P. aeruginosa*. The results obtained from the growth studies also revealed that the highest OD_{600} was obtained from SFM that was supplemented with L-cysteine. Since CysB regulates the genes involved in sulfate assimilation and cysteine biosynthesis, the presence of L-cysteine in SFM explains the ability of this transposon mutant to have a faster growth rate as compared to the other sulfur sources.[87,89,90,96,290] Results from previous studies focused on evaluating the role of CysB on bacterial virulence in *P. aeruginosa* also revealed that CysB regulates the

expression of different virulence factors produced by this bacterium. It was also revealed that the deletion of CysB reduced virulence expression and bacterial pathogenesis; however, biofilm formation was increased.[300]

P. aeruginosa utilizes different survival strategies in nature as well as in the host.[246,247,249] Some of these survival strategies include the production of several secondary metabolites as well as virulence factors by this bacterium.[301-304] A very interesting feature of *P. aeruginosa* is its blue-green appearance which is caused by the production of the secondary metabolite, pyocyanin.[301] Pyocyanin is a common phenazine pigment which is produced by ~95% of *P. aeruginosa* strains. This blue-green pigment is of particular interest due to its ability to cross biological cell membranes and generate reactive oxygen species (ROS) in host cells, ultimately resulting in DNA damage.[301,302,305-307] Additionally, the production of pyocyanin by this bacterium has also been linked to iron uptake and sequestering.[302,303,308-310] When the growth of wild-type *P. aeruginosa* was evaluated in the presence of different sulfur sources, different intensities of the blue-green color were observed. The darkest blue-green colors were observed when wild-type *P. aeruginosa* was grown in SFM that was supplemented with sodium sulfate or L-cysteine (Figure **4.11**). Conversely, a lighter shade was observed for all other sulfur sources tested; however, the blue-green color was not observed when wild-type *P. aeruginosa* was grown in SFM medium that did not have any sulfur source present. In the oxidized form, pyocyanin has a maximal absorption between 230 and 380 nm as well as a lower absorption band around 700 nm.[305,306] Due to the absorption of pyocyanin around 700 nm, there could be a possibility of contributing absorption effects when monitoring the OD_{600}. The production of pyocyanin by *P. aeruginosa* has been reported to be influenced

by several environmental factors including the availability of nutrients such as iron, magnesium, sulfate, and phosphate which could explain the difference in blue-green intensity observed when tested with the other sulfur sources.[311]

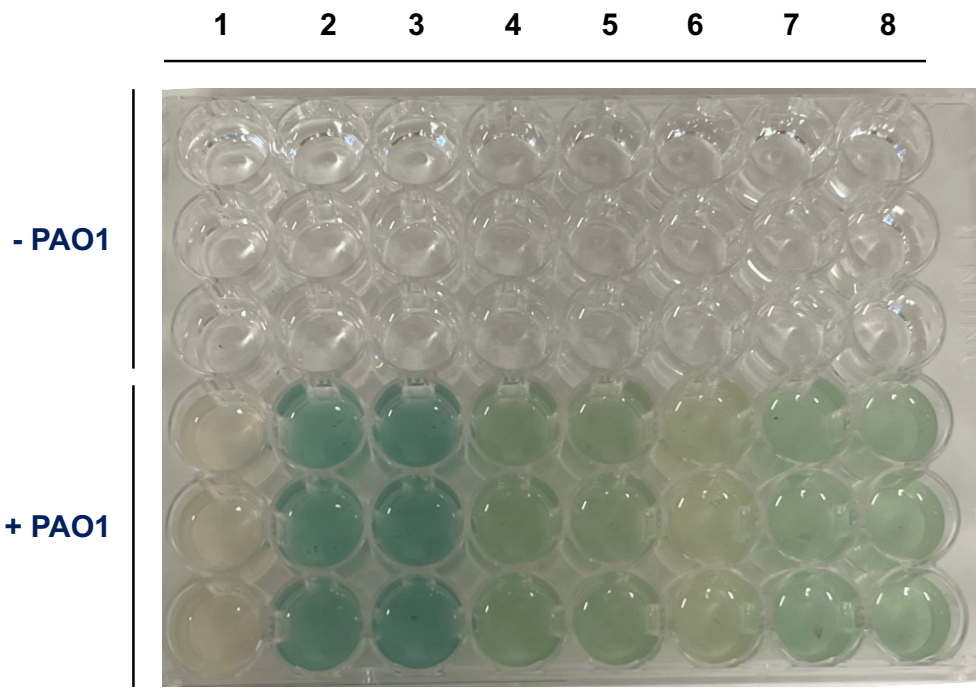

Figure 4.11: Pyocyanin formation by wild-type *P. aeruginosa* in the presence of SFM supplemented with various sulfur sources. The negative control (-PAO1) represents SFM with 500 μM of the respective sulfur source. Lanes: SFM (Lane 1), sodium sulfate (Lane 2), L-cysteine (Lane 3), dimethyl sulfoxide (Lane 4), dimethyl sulfone (Lane 5), methanesulfinate (Lane 6), methanesulfonate (Lane 7), and octanesulfonate (Lane 8).

All together the results obtained from these studies provide a strong foundation for the further mechanistic and structural elucidation of MsuC from *P. aeruginosa*. Studies focused on how the presence of different sulfur sources affect biofilm formation as well as the formation of virulence factors by wild-type and the transposon mutants of *P.*

aeruginosa have yet to be performed. Finally, future studies focused on evaluating how the decrease in virulence factors affects the pathogenicity of *P. aeruginosa* are warranted.

CHAPTER FIVE

Conclusions

Sulfur is an essential element required by all organisms for various metabolic purposes. While humans rely on the amino acids cysteine and methionine to meet their sulfur needs, bacteria primarily utilize inorganic sulfate as their sulfur source.[1,2,4,7,8,16,46,312] Since inorganic sulfate is poorly represented in soil, bacteria express a diverse set of SSI proteins that allow them to utilize a broad range of alternative sulfur sources.[4,9,48,64,65,69] Some of these SSI proteins expressed by bacteria are part of two-component flavin-dependent enzyme systems.[4,66,194,196,197,239] Of specific interest in this dissertation are the alkanesulfonate monooxygenase (SsuE/D), methanesulfinate monooxygenase (MsuE/C), and the methanesulfonate monooxygenase systems (MsuE/D). All three aforementioned two-component enzyme systems require two separate enzymes to catalyze the overall reaction.[4,66,98,194,196,197,239] The reductases SsuE and MsuE, supply reduced flavin to their respective partner monooxygenases, SsuD, MsuC, and MsuD. The research described in this dissertation is focused on elucidating the mechanistic strategies of these two-component flavin-dependent monooxygenases using various kinetic, structural, computational, and bacterial growth studies.

The monooxygenases SsuD and MsuD share ~65% amino acid sequence identity and are both TIM-barrel enzymes belonging to the bacterial luciferase family.[193,196,221] Despite the overall structural similarity between the two monooxygenases, SsuD has been previously characterized as an alkanesulfonate monooxygenase that is unable to use methanesulfonate as a sulfur substrate, and instead utilizes alkanesulfonates ranging

from a C2-C10 in carbon chain length to yield the corresponding aldehyde and sulfite.[66,193] Conversely, MsuD has been previously characterized as a methanesulfonate monooxygenase.[196] In order to investigate why two structurally similar flavin-dependent monooxygenases have different substrate specificities, a combination of kinetic, biochemical, and computational experiments were performed. Results from desulfonation assays revealed that SsuD was unable to utilize methanesulfonate and ethanesulfonate as sulfonated substrates but had comparable kinetic activity with C4-C10 alkanesulfonates. Conversely, MsuD had a similar activity with all sulfonated substrates tested in the desulfonation assays. Additionally, both SsuD and MsuD had similar binding affinities for FMN and $FMNH_2$ in fluorometric titrations. A ~100-fold higher binding affinity for $FMNH_2$ as compared to FMN was observed for both SsuD and MsuD. Moreover, SsuD and MsuD were also able to bind both methanesulfonate and octanesulfonate.

SsuD and MsuD contain several insertion regions in addition to the eight α-helices and β-sheets that are part of the TIM-barrel fold. One of these insertion regions contains a dynamic loop that closes over the active site upon substrate binding.[193,196,221,232] Previous studies with SsuD demonstrated that the dynamic loop was susceptible to proteolysis; however, this region became partially protected due to substrate binding.[232,237] In order to determine if the dynamic loop in SsuD and MsuD influenced their respective substrate preferences, partial proteolytic digestion experiments were performed in the presence and absence of substrates. SsuD and MsuD showed decreased susceptibility in the presence of $FMNH_2$ and methanesulfonate or octanesulfonate. Additionally, tighter loop closure was observed with MsuD in the

presence of $FMNH_2$ and methanesulfonate or octanesulfonate as compared to SsuD under similar conditions.

Since the kinetic analyses did not reveal why SsuD and MsuD had distinct substrate preferences, structural analyses using computational studies revealed that methanesulfonate exists in different conformations, indicating that this substrate is unstable within the active site of SsuD. Moreover, the results from these analyses revealed that the absence of a longer alkyl chain with methanesulfonate leads to a shift in the conformation of $FMNH_2$ due to a collapse within the active site of SsuD. Therefore, the active site is not in the correct conformation to support desulfonation. Even though SsuD is unable to catalyze the desulfonation reaction using methanesulfonate as a sulfonated substrate, both SsuD and MsuD could utilize a common desulfonation mechanism due to their similar structural features.

Structural features of enzymes often play a critical in the overall reaction catalyzed. For SsuD, the dynamic loop was previously shown to be involved in protein-protein interactions with SsuE in the absence of substrates.[211] Additionally, the dynamic loop of SsuD was revealed to undergo a conformational change in the form of loop closure over the active site once substrates were bound.[232,237] Loop closure over the active site of SsuD would create a hydrophobic environment optimal for the stabilization of any reactive intermediates and would also prevent the premature release of substrates and products.[226-231] In order to maintain the SsuD dynamic loop in a closed conformation, Arg263 and Arg271 were hypothesized to form salt bridges with Asp111 and Glu205. The roles of these two arginine residues were further evaluated through site-directed mutagenesis and kinetic studies. Three arginine variants were generated, namely,

R263A, R271A, and R263A/R271A SsuD. All three SsuD variants had comparable activity as the wild-type enzyme when monitoring sulfite production through desulfonation assays. The susceptibility of these SsuD variants was also evaluated in the presence of chymotrypsin. Even though R263A SsuD was similarly protected as wild-type SsuD, the R271A and R263A/R271A SsuD variants were more susceptible to proteolysis. The increased susceptibility observed with R271A and R263A/R271A SsuD was attributed to the substitution of Arg271 with alanine which disrupted the salt bridge resulting in a more "open" conformation of the dynamic loop region. Taken together, the results from these studies revealed that substitution of Arg263 and Arg271 did not affect the ability of these SsuD variants to produce sulfite; however, substitution of Arg271 with an alanine resulted in a more "open conformation", therefore explaining the increased proteolytic susceptibility.

SsuD and MsuD utilize reduced flavin from their respective partner reductase to perform the oxygenolytic cleavage of a broad range of alkanesulfonates.[66,193,196,239] Due to several commonalities these two monooxygenases share, we propose that SsuD and MsuD could utilize a similar desulfonation mechanism.[221] The mechanism of SsuD has been studied for decades; however, details regarding specific catalytic steps and the formation of flavin intermediates remain elusive.[211,212,220,232,237,239-241,244,275] While SsuD has previously been proposed to utilize the classical C4a-(hydro)peroxy flavin intermediate, recent studies provided evidence for the formation of the flavin-N5-oxide in various bacterial flavin-dependent monooxygenases.[166-171,266] Based on structural similarity with enzymes employing a flavin-N5-oxide, we proposed two mechanisms for

SsuD in which the flavin-N5-oxide can be formed as the final product or as one of the resolving oxygenating flavin intermediates.

Different amino acids have been identified that play a critical role in stabilizing the formation of the flavin-N5 adduct in flavin-dependent enzymes. Structural and amino acid alignment revealed that these residues are also conserved in SsuD and MsuD.[170] While different polar amino acids (Asn106 and Thr109 in SsuD) have been proposed to stabilize the transiently formed superoxide anion, the nonpolar amino acids (Val108 in SsuD) are involved in controlling the interaction of reduced flavin with molecular oxygen.[170] The polar Asn106 and Thr109 in SsuD were substituted with a leucine and alanine, respectively, whereas the nonpolar Val108 was substituted with a threonine. The results obtained from desulfonation assays revealed that V108T and T109A SsuD had similar activity as compared to wild-type SsuD; however, no measurable activity was observed with N106L SsuD variant. The lack of activity observed with the N106L SsuD variants suggests that Asn106 could be involved in stabilizing the transiently formed superoxide anion. These residues are also conserved in MsuD and are hypothesized to play a comparable role.[170]

In the proposed mechanisms for SsuD, the flavin-N5-oxide can be formed as the final flavin product or as one of the resolving oxygenating flavin intermediates.[166-171] Results obtained from HPLC analysis confirmed the presence of FMN as the final flavin product during enzymatic turnover, and there was no flavin-N5-oxide observed. These results suggest that the flavin-N5-oxide is not formed as the final product but, could be formed as one of the oxygenating flavin intermediates in the reaction catalyzed by SsuD. Alternatively, it could also be possible that SsuD does not form any flavin-N5-oxide at all but instead utilizes the N5-peroxy flavin to perform the half-reaction similar to what has

been previously proposed for YxeK.[171] Future studies using rapid reaction kinetics could be useful for the identification of any proposed N5-oxygenating flavin intermediates utilized by SsuD.

In addition to the alkanesulfonate monooxygenase (SsuE/D) and methanesulfonate monooxygenase systems (MsuE/D), bacteria also express the methanesulfinate monooxygenase system (MsuE/C) during sulfur starvation. The *msu* enzymes enable bacteria, in particular *Pseudomonas* species, to utilize oxidation products from dimethyl sulfide as alternative sulfur sources.[4,9,48,64,93] While SsuD and MsuD share similar structural features, MsuC is structurally distinct.[197,221] Previous studies have been successful in solving the three-dimensional structure of MsuC from *P. fluorescens* and providing evidence that MsuC utilizes reduced flavin from MsuE to catalyze the oxidation of methanesulfinate to yield methanesulfonate.[197] MsuC is conserved in various *Pseudomonas* species including the opportunistic human pathogen *P. aeruginosa*.[4,197] Even though initial studies have been performed with MsuC from *P. fluorescens*, details regarding the oligomeric state and mechanism of MsuC from *P. aeruginosa* are currently unknown. The studies presented in this dissertation are the first focused on evaluating the oligomeric state and mechanism of MsuC from *P. aeruginosa*.

Since MsuC has been proposed to rely on MsuE to supply reduced flavin, the binding affinity of MsuC for different flavins (FMN and FAD) was evaluated using fluorometric titrations under aerobic and anaerobic conditions. MsuC had a similar binding affinity for FMN as FAD; however, a 10-fold higher binding affinity was obtained for $FMNH_2$. Moreover, similar binding affinities were obtained for FAD and $FADH_2$. MsuC is involved in the same metabolic pathway as MsuE/MsuD. Studies with MsuE have

demonstrated that this enzyme exists in a dimer-tetramer equilibrium, favoring the tetrameric form in the presence of FMN (unpublished data Ellis laboratory). Since MsuC relies on MsuE for the supply of reduced flavin, the oligomeric state of MsuC was evaluated in the presence and absence of FMN, FAD, and methanesulfinate through native-PAGE and sedimentation velocity studies. Results from these studies revealed that MsuC exists as a tetramer in the presence and absence of FMN, FAD, and methanesulfinate. Finally, results from thermal melt circular dichroism spectroscopy revealed that the presence or absence of FMN, FAD, and methanesulfinate do not affect the thermal stability of MsuC.

MsuC has been characterized as a two-component flavin-dependent monooxygenase; however, details regarding its mechanism are currently unknown.[197] To obtain more insight into its catalytic mechanism, the formation of flavin intermediates by MsuC was investigated through rapid reaction kinetic experiments. Since MsuC has a 42% amino acid sequence identity with DszC, a two-component flavin-dependent monooxygenase involved in dibenzothiophene oxidation, we hypothesized that MsuC could utilize a similar flavin intermediate as DszC.[197] DszC has been reported to utilize a C4a-(hydro)peroxy flavin intermediate for the oxidation of dibenzothiophenes.[299] Similar to DszC, we proposed that MsuC could utilize a C4a-(hydro)peroxy flavin intermediate which can be spectrally observed between 360-400 nm.[106,109,129,144,159] Results obtained from rapid reaction kinetic experiments in the presence and absence of methanesulfinate, monitoring the formation of the C4a-(hydro)peroxy flavin intermediates at 360 and 370 nm revealed a decrease in absorbance followed by no observable change in the absorbance after 20 seconds. We were unable to provide spectral evidence for the

formation of a C4a-(hydro)peroxy flavin intermediate by MsuC under the given experimental conditions used in these studies. The inability to provide spectral evidence for the formation of a C4a-(hydro)peroxy flavin intermediate by MsuC could be due to the instability of this intermediate at room temperature. Alternatively, the presence of an isosbestic point around the measured wavelengths could result in the inability to monitor changes in the absorbance.

In two-component flavin-dependent systems the reductase supplies the monooxygenase with reduced flavin.[98] These group of enzymes have been reported to utilize a diffusion or a channeling mechanism for the transfer of reduced flavin. While diffusion does not require any interaction between the reductase and the monooxygenase, the channeling mechanism utilizes protein-protein interactions between both enzymes for the efficient transfer of reduced flavin.[98,99,209,212,214] Previous studies with the alkanesulfonate monooxygenase system (SsuE/D) provided evidence that reduced flavin transfer proceeds through the formation of protein-protein interaction between the two enzymes.[211,212,237] The reduction and subsequent oxidation of flavin by MsuE and MsuC in the presence and absence of methanesulfinate were monitored at 450 nm using rapid reaction kinetic experiments. In the presence of methanesulfinate a decrease followed by a subsequent increase in absorbance was observed which corresponded to the reduction and oxidation of flavin, respectively. Conversely, in the absence of methanesulfinate a decrease in absorbance was observed followed by a constant absorbance after 20 seconds. This result suggested that reduced flavin is protected from unproductive oxidation in the absence of methanesulfinate by MsuC. The autooxidation of reduced flavin in the absence of substrate could be harmful for the

bacterial cell due to the production of reactive oxygen species (ROS).[313] The reduced flavin transfer between the reductase and the monooxygenase has been previously evaluated for SsuE and SsuD. Flavin oxidation at 450 nm could be observed even in the absence of octanesulfonate.[237] Even though the reduced flavin transfer has not been evaluated for the dibenzothiophene monooxygenase system, studies have provided evidence for the formation of a C4a-(hydro)peroxy flavin intermediate when air saturated DszC was reacted with $FMNH_2$ in stopped-flow kinetic experiments.[299] Therefore, the constant absorbance observed at 450 nm with MsuE/MsuC in the absence of methanesulfinate suggests that MsuC provides better protection against auto-oxidation of reduced flavin compared to other two-component flavin-dependent monooxygenases.

The alkanesulfonate monooxygenase, methanesulfinate monooxygenase, and methanesulfonate monooxygenase systems are SSI proteins and help bacteria including pathogens acquire sulfur when deprived from inorganic sulfate.[4] *P. aeruginosa* is an opportunistic human pathogen that also expressed the aforementioned SSI proteins. Due to its prevalence in the environment, *P. aeruginosa* has been proposed to utilize several alternative sulfur sources when inorganic sulfate is limiting.[4,9,48,93,246-249] The ability of wild-type *P. aeruginosa* to grow on various sulfur sources was evaluated in bacterial growth assays monitoring the OD_{600}. The highest OD_{600} absorbance values were obtained when wild-type *P. aeruginosa* was grown in SFM that was supplemented with either sodium sulfate or L-cysteine, suggested that this bacterium utilizes these sulfur compounds as its preferred sulfur sources. An interesting observation during these experiments was the occurrence of an intense blue-green color which was predominant when wild-type *P. aeruginosa* was grown in SFM that was supplemented with sodium sulfate or L-cysteine.

The blue-green color observed is due to the production of pyocyanin, a secondary metabolite produced by *P. aeruginosa* which has also linked to play a role in its virulence.[248,302,314-317]

The growth of some *P. aeruginosa* transposon mutants was also monitored through similar experiments in the presence of different sulfur sources. The transposon mutants tested consisted of CysB, SfnG, and MsuC deletions. CysB plays an important role in the regulation and expression of genes involved in sulfur assimilation and sulfur starvation. These genes encode for various enzymes that are involved in cysteine biosynthesis.[73,87-90,290] The CysB transposon mutant was grown in SFM that was supplemented with various sulfur sources and the OD_{600} was monitored through bacterial growth assays. The CysB transposon mutant took a longer time to reach the exponential phase as compared to wild-type *P. aeruginosa*. Moreover, the highest OD_{600} was observed when this mutant was grown in the presence of L-cysteine.

The SfnG and MsuC transposon mutants were grown in SFM as well as SFM that was supplemented with sodium sulfate, dimethyl sulfone, and methanesulfinate. When the SfnG transposon mutant was grown in SFM that was supplemented with dimethyl sulfone, a lower OD_{600} was observed suggesting that the deletion of this gene results in the inability of *P. aeruginosa* to utilize dimethyl sulfone as an alternative sulfur source. Conversely, similar OD_{600} values were obtained when the MsuC transposon mutant was grown in SFM that was supplemented with dimethyl sulfone or methanesulfinate. The ability of the MsuC transposon mutant to grow on methanesulfinate was interesting since MsuC has been previously characterized as a methanesulfinate monooxygenase.[197] The unexpected result of the MsuC transposon mutant to grow in the presence of

methanesulfinate, raises questions considering the assigned role and substrate preference of MsuC. Further mechanistic and growth studies will have to be performed to understand the function of MsuC.

In summary, the studies described in this dissertation provide structural, kinetic, and mechanistic understanding on how bacteria utilize a diverse set of SSI proteins during sulfur limitation. The occurrence of SSI proteins in pathogenic bacteria including but not limited to *P. aeruginosa*, makes these enzymes a promising therapeutic target. Infections caused by multidrug resistant bacteria are a global health concern, and scientists from different research areas are extensively exploring various therapeutic strategies to combat this major health issue. The results from these studies further explain the roles of these SSI proteins in bacterial sulfur metabolism. Structural and mechanistic understanding of these enzymes could be utilized as a promising strategy for rational drug design (Figure **5.1**).

In addition to its antibiotic resistance mechanisms, *P. aeruginosa* also produces several virulence factors that are part of its adaption mechanism.[247-249,310] One of the virulence factors produced by *P. aeruginosa* is pyocyanin which is responsible for its characteristic dark blue-green color.[248,302,314-317] The results of the bacterial growth assays performed within this study revealed different intensities of the blue-green color when wild-type *P. aeruginosa* was grown in SFM and SFM that was supplemented with various sulfur sources. The darkest blue-green colors were observed with wild-type *P. aeruginosa* that was grown in SFM supplemented with either sodium sulfate or L-cysteine, whereas no blue-green color was observed when the bacterium was grown in SFM. Even though the formation of pyocyanin by *P. aeruginosa* has been previously reported to be affected

by the availability of nutrients including sulfur, future studies are warranted to investigate how the amount of virulence factors (including but not limited to pyocyanin) influences the pathogenicity of *P. aeruginosa*.

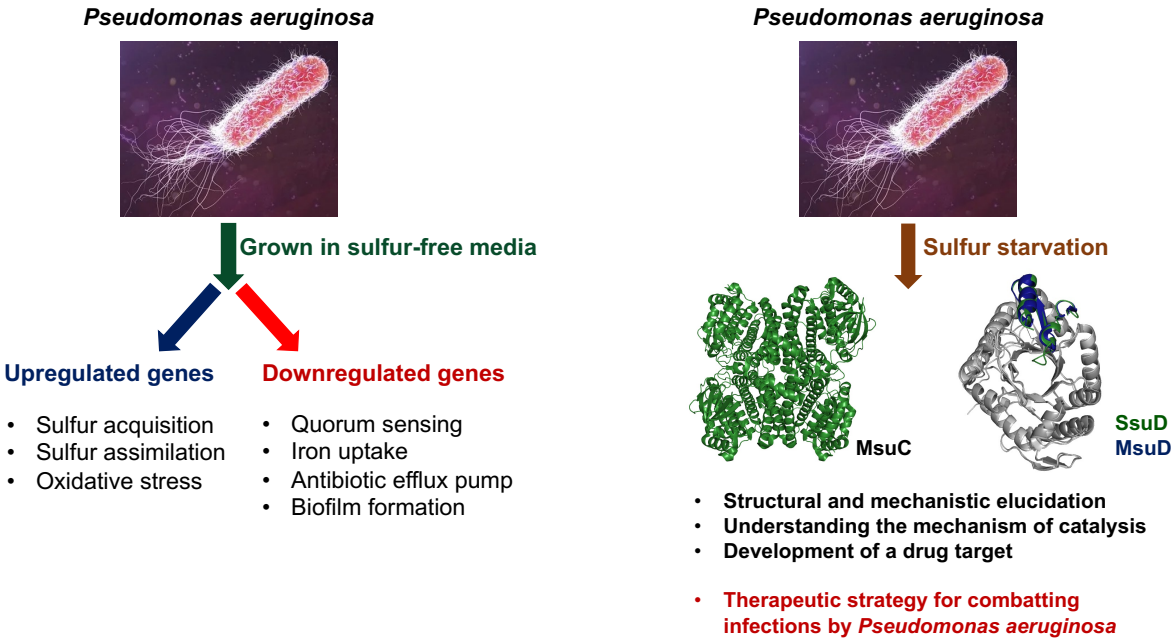

Figure 5.1: A conceptual representation of the long-term goals of these studies. The studies presented in this dissertation evaluate the mechanism of various two-component flavin-dependent monooxygenases that are critical in bacterial sulfur acquisition. The mechanistic information obtained from these studies could represent a notable target for drug development. (Adapted from [193,196,197,323]).

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