Structural and Functional Studies to Investigate Enzymatic Catalysis and Flavin Transfer Mechanism of The Two-component Alkanesulfonate Monooxygenase System from *Escherichia coli*

by

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A dissertation submitted to the Graduate Faculty of Auburn University in partial fulfillments for the requirements for the Degree of Doctor of Philosophy

Auburn, Alabama
August 4, 2012

Keywords: flavin, sulfur, enzymology, protein dynamics, protein-protein interactions

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Abstract

Several bacterial organisms rely on the two-component alkanesulfonate monooxygenase system for the acquisition of organosulfonate compounds when inorganic sulfur is limiting in the environment. This system is comprised of an FMN reductase (SsuE) that supplies reduced flavin to the alkanesulfonate monooxygenase (SsuD). Desulfonation of alkanesulfonates by SsuD is catalyzed through the activation of dioxygen by reduced flavin. The three-dimensional structure of SsuD exists as a TIM-barrel fold with several discrete insertion regions. An extensive insertion region near the putative active site was unresolved in the SsuD structure, suggesting the importance of protein dynamics in the desulfonation mechanism. Three variants containing a partial deletion of the loop region were constructed to evaluate the functional properties of this region. There were no overall gross changes in secondary structure for the three SsuD deletion variants compared to wild-type SsuD, but each variant was found to be catalytically inactive. The deletion variants were unable to undergo the conformational changes necessary for catalysis even though they were able to bind reduced flavin. Results from rapid kinetic analyses suggested that the SsuD deletion variants failed to protect reduced flavin from non-enzymatic oxidation. These studies define the importance of the unresolved loop region for the protection of reduced flavin.
A distinct feature of the alkanesulfonate monooxygenase system is that the flavin is utilized as a substrate instead of a bound prosthetic group. The reduced flavin is transferred from SsuE to SsuD, either through a dissociative or a channeling mechanism. In order to kinetically discern between a dissociative and a channeling flavin transfer mechanism, a method was designed using an SsuD variant with reduced flavin affinity to compete with wild-type SsuD for reduced flavin. Results from this novel competition method suggested that the flavin is transferred within a transient SsuE-SsuD complex during catalysis. In addition, the reductive half-reaction catalyzed by SsuE is affected by SsuD and its substrate under pre-steady-state kinetic conditions. The evidence provided in this study support a channeling flavin transfer mechanism in the two-component alkanesulfonate monooxygenase system.
Acknowledgements

First, I would like to express my deepest appreciation and sincere thanks to my advisor, Dr. Holly R. Ellis, for her invaluable guidance, consistent support and inspiring encouragement throughout my studies here at Auburn University. Not only did she offer me a great deal of knowledge in science and research, she also showed me an upstanding attitude towards carrier and life. Her critical thinking, preciseness, diligence and fairness will be perpetually beneficial in my life. I would also like to thank my Dr. Douglas C. Goodwin, Dr. Evert C. Duin, Dr. Susanne Striegler, and Dr. Sang-Jin Suh for their meaningful discussion and constructive suggestions to my dissertation. I appreciate Dr. Goodwin for letting me use his stopped-flow spectrometer and providing me helpful advice on my research. I wish to thank Dr. Duin for the use of his anaerobic glove box and the -80°C freezer. I would also like to thank Dr. Suh and Dr. Paul A. Cobine for organizing the inter-departmental molecular biology journal club, where I greatly improved my understanding in biological sciences.

Secondly, I would like to thank my former and current lab mates Dr. Xuanzhi Zhan, Dr. Russell A. Carpenter, Dr. Erin M. Imsand, Mary Millwood, John M. Robbins, Catherine Njeri, and Paritosh Dayal for their discussion and help during my studies. I especially want to express my gratitude to my parents Siping Xiong and Min Zhang for their everlasting unconditional love and support.

And last but not least, I wish to thank NSF and EPSCoR for their financial support.
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<tr>
<td>DTNB</td>
<td>5,5-dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>FMNH₂</td>
<td>reduced flavin mononucleotide</td>
</tr>
<tr>
<td>NAD(P)H</td>
<td>nicotinamide adenine dinucleotide (phosphate)</td>
</tr>
<tr>
<td>NTA</td>
<td>Ni-nitritriacetate</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethlysulfonyl fluoride</td>
</tr>
<tr>
<td>SsuE</td>
<td>alkanesulfonate flavin reductase</td>
</tr>
<tr>
<td>SsuD</td>
<td>alkanesulfonate monooxygenase</td>
</tr>
<tr>
<td>TIM</td>
<td>triosephosphate isomerase</td>
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<tr>
<td>LadA</td>
<td>long-chain-alkane monooxygenase</td>
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<tr>
<td>FRP</td>
<td>NADPH-dependent flavin reductase</td>
</tr>
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Chapter 1

Literature Review

1.1 Sulfur metabolism in bacterial organisms

Sulfur is an essential element for the survival and growth of all organisms. Although sulfur is one of the basic elemental building blocks for living organisms, sulfur metabolism is less well-understood compared to other basic elements. In bacteria, sulfur is needed as a component of amino acids cysteine, and methionine. Sulfur is also a crucial component in many enzyme cofactors and participates in maintaining the appropriate redox potential in the cell through sulfur-containing redox-active compounds.

Bacterial organisms primarily assimilate inorganic sulfate for the biosynthesis of sulfur-containing molecules (Figure 1.1) (1, 2). Inorganic sulfate is first assimilated into the cell by sulfate permease (CysU, CysW, CysA and sulfur binding proteins) (3). The intracellular sulfate is utilized to form adenosine phosphate (APS) and pyrophosphate by ATP sulfurylase at the expense of ATP hydrolysis (3). The ATP sulfurylase is a tetramer of heterodimers consisting of a GTPase subunit (CysN) and APS synthesis subunit (CysD). Because the energy required to synthesize APS by CysD is higher than the energy released from the hydrolysis of ATP, another reaction catalyzed by pyrophosphatase is needed to decrease the pyrophosphate concentration in order to shift the reaction equilibrium towards APS production (4). The GTPase subunit (CysN) of the ATP sulfurylase catalyzes the hydrolysis reaction of GTP, providing energy for the APS
synthesis (4). Therefore, the activation of sulfate in the sulfur assimilation pathway is energetically costly, and the recovery of sulfur-containing compounds from reduced-sulfur would be more beneficial in conserving energy. The APS is then activated by APS kinase (CysC) to form 3’-phosphoadenosine-5’-phosphosulfate (PAPS) with the hydrolysis of another ATP and the release of ADP in order for the incorporated sulfur to be reduced. This reaction is thought to further pull the sulfate incorporation reaction towards APS formation (2). The sulfur in PAPS is then reduced to sulfite by thioredoxin dependent PAPS reductase (CysH) in the presence of NADPH, yielding adenosine 3’-5’ diphosphate (PAP) as a by-product. Because PAP is also produced by the transfer of 4-phosphopantetheine from coenzyme A in fatty-acid metabolism and the synthesis of some secondary metabolites, it is speculated that PAP might play a regulatory role in coupling sulfur assimilation with lipid metabolism (5). In the sulfur assimilation pathway, the sulfite is then reduced by NADPH-sulfite reductase (CysJ, CysI and CysG) to generate sulfide by a poorly defined process (2).

The sulfide in the cell reacts with O-acetylserine to form cysteine (3). This reaction is catalyzed by an enzyme complex cysteine synthase, which is composed of serine transacetylase (CysE) and O-acetylserine sulfhydrylase A (CysK) (6). The serine transacetylase catalyzes the condensation reaction of the acetyl group from acetyl CoA onto the hydroxyl group of serine, generating O-acetylserine needed to synthesize cysteine. The O-acetylserine sulfhydrylase A in the cysteine synthase complex then utilizes sulfide as a nucleophile to react with O-acetylserine, forming cysteine and acetate.
Figure 1.1. Sulfate assimilation pathway and cysteine biosynthesis in *Escherichia coli*.

(Adapted from (2)).
The genes responsible for sulfate activation and reduction (cysD, cysN, cysC, cysH, cysI and cysJ) are organized in a cluster in the bacterial chromosome, while other genes involved in cysteine biosynthesis are isolated (Figure 1.2) (7). The operons of all cysteine biosynthetic genes are grouped as the cysteine regulon, which is under central regulation by a LysR-type transcriptional activator CysB (8, 9). The CysB regulator binds upstream of the promoter region in the regulon to stimulate transcription of the cysteine biosynthetic genes (10, 11). Furthermore, the CysB regulator is able to play an additional role in inhibiting transcription from its own promoter (12). The O-acetylserine metabolite serves as an inducer that stimulates the binding of CysB to the positive regulation sites of the cys operon and facilitates the formation of the transcription initiation complex, while inhibiting binding to the negatively autoregulated cysB promoter region (10). O-acetylserine can spontaneously isomerize to N-acetylserine, which is speculated to be a better inducer of the cys regulon. N-acetylserine is suggested to function as a pure inducer because it cannot serve as a sulfur acceptor, and it is 15-fold more efficient in inducing activation compared to O-acetylserine (13). In addition to regulation by the central transcriptional activator CysB, the proteins in the cysteine biosynthetic pathway are also regulated by sulfate, each sulfur intermediate in the pathway, and the end product cysteine (7). The intracellular concentration of cysteine is found to indirectly control the expression of cys genes by feedback inhibition of serine transacetylase (9). The positive regulation by CysB is repressed by sulfide and thiosulfate, which act as anti-inducers that interfere with the binding of the coinducer N-acetylserine (8). Interestingly, even though sulfate is the starting substrate of the cysteine biosynthetic pathway, excess sulfate reduce expression of the cys genes due to its conversion to sulfide and cysteine (9). Therefore,
Figure 1.2. The genetics and biochemistry of sulfate utilization in *Escherichia coli*. The genes involved in cysteine biosynthesis are indicated: *cysTWA* (sulfate permease), *cysDN* (ATP sulfurylase), *cysC* (APS kinase), *cysH* (PAPS reductase), *cysIJ* (sulfite reductase), *cysE* (serine transacetylase), and *cysKM* (O-acetylserine sulphydrylase A or B). (Adapted from (7)).
maximum expression of the cys genes requires an active CysB, coinducer O-acetylserine or N-acetylserine, and the absence of cysteine and excess sulfate (7).

After the sulfur in inorganic sulfate is utilized to synthesize cysteine, the fate of cysteine can be diversified in bacteria. In the methionine biosynthetic pathway cysteine is first converted to cystathionine, which is the precursor of homocysteine. The homocysteine is then methylized to form methionine (14). In the coenzyme A biosynthetic pathway, the cysteine is added to the phosphorylated pantothenate, followed by several steps of decarboxylation, adenylylation, and phosphorylation to generate coenzyme A (15). In iron-sulfur clusters, the sulfur is suggested to be provided by thiocysteine. The thiocysteine may be generated by the lysis of cystine, a dimeric amino acid formed by oxidation of two cysteine residues (16). In the thiamine biosynthetic pathway, cysteine is required to synthesize the thiazole group, which combines with a pyrimidine to yield thiamine monophosphate. The thiamine monophosphate can then be phosphorylated to thiamine pyrophosphate or hydrolyzed to thiamine; however, the enzymatic mechanism of thiazole formation is not fully clear (17). It is also speculated that lipoic acid is synthesized by inserting cysteine sulfur into the hydrocarbon chain of octanoic acid by an enzyme containing iron-sulfur clusters (18). In biotin biosynthesis, the incorporation of sulfur into biotin is catalyzed by iron-sulfur proteins (18, 19).

1.2 Sulfur limitation and bacterial response

Sulfur is an abundant element in the environment, and is commonly found in the earth’s crust, water and atmosphere (20-24). In the atmosphere the sulfur element primarily exists as SO₂ and H₂S, which are eventually oxidized to sulfate (24). The sulfur in aquatic environments is commonly found in the soluble SO₃²⁻ and SO₄²⁻ form (23).
While inorganic sulfate is ubiquitous in the atmosphere and aquatic system, it is relatively poorly represented in aerobic soils, taking up less than 5% of the total sulfur content (21, 25). In addition, not all inorganic sulfur in the soil is easily accessible sulfate that can be readily assimilated by plants and bacteria. Inorganic sulfur can also exist as absorbed sulfate and insoluble sulfate co-precipitated with CaCO₃ (25). Most of the sulfur in aerobic soils is found to be organic sulfur such as sulfonate and sulfate esters, therefore inorganic sulfate and cysteine that are available to bacteria become limiting in aerobic soils (26). In order to survive, bacteria under sulfur limitation condition have developed an alternate process to obtain sulfur from available organic sulfur sources (27-30).

Under sulfur limiting conditions where readily available inorganic sulfate and cysteine are limiting in the environment, bacteria express a set of proteins involved in the uptake and utilization of alternate organic sulfur sources such as alkanesulfonates and taurine (Figure 1.3) (1, 31-34). These specific proteins expressed only in the absence of primary sulfur sources are designated as sulfur starvation induced (ssi) proteins (Table 1.1) (28). In order to meet their sulfur requirements, bacteria employ these ssi proteins to enzymatically liberate sulfite from organic sulfur sources. The released sulfite product can then enter the cysteine biosynthetic pathway to provide bacteria with the necessary precursors for the synthesis of various sulfur-containing compounds (35-36).

Even though *E. coli* is known to utilize organosulfur compounds for growth and these alternate sulfur sources enter the cysteine biosynthetic pathway at the stage of sulfite, *E. coli* has a preference for sulfate over sulfonate if both are available (32). It has been shown that the presence of sulfate represses the expression of ssi proteins. When *E. coli* was cultured in minimal media deprived of sulfate and cysteine, several ssi proteins
were found to be up-regulated (31). These ssi proteins were identified as periplasmic sulfate binding protein (Sbp), periplasmic cysteine binding protein (FliY), O-acetylserine sulfhydrylase A (CysK) and antioxidant alkylhydroperoxide reductase (AhpC) (33). The Sbp and FliY proteins are involved in the initial assimilation of sulfate and cysteine. The CysK enzyme is the last enzyme in the cysteine biosynthetic pathway. Both the substrates (sulfide and O-acetylserine) and the product (cysteine) of CysK are reported to play crucial regulatory roles in cysteine biosynthesis. The ssi proteins involved in the utilization of sulfonate sulfur sources were later identified to be a taurine desulfurization enzyme (TauD) expressed from the \textit{tauABCD} gene cluster and the alkanesulfonate desulfurization enzymes (SsuE and SsuD) expressed from the \textit{ssuEADCB} gene cluster (35-36). Both the \textit{tau} and \textit{ssu} operons are found to encode an oxygenase system (TauD and SsuED) and an ABC (ATP binding cassette)-type transporter system, which includes a periplasmic substrate binding protein, an ATP-binding protein, and a transporter membrane component.

The oxygenase component of the \textit{tau} operon is an $\alpha$-ketoglutarate dependent taurine dioxygenase (TauD), which requires Fe (II) to convert taurine to aminoacetaldehyde and sulfite (Figure 1.4) (35, 37). The oxygenase system of the \textit{ssu} operon contains two components, an NADPH-dependent FMN reductase (SsuE) and an FMNH$_2$-dependent alkanesulfonate monooxygenase (SsuD) (36). The SsuE enzyme catalyzes the reduction of FMN to FMNH$_2$ with NADPH providing reducing equivalents. The SsuD enzyme converts alkanesulfonates to aldehyde and sulfite with the FMNH$_2$ supplied by SsuE (Figure 1.4). While the liberation of sulfite by the \textit{tauABCD} system is performed by
<table>
<thead>
<tr>
<th>Protein</th>
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<th>Function</th>
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<tr>
<td>TauA, TauD</td>
<td>tauABCD</td>
<td>taurine desulfurization</td>
</tr>
<tr>
<td>SsuE, SsuD</td>
<td>ssuEADCB</td>
<td>alkanesulfonate desulfurization</td>
</tr>
<tr>
<td>Sbp</td>
<td>Sbp</td>
<td>periplasmic sulfate binding protein</td>
</tr>
<tr>
<td>FliY</td>
<td>fliY</td>
<td>periplasmic cysteine binding protein</td>
</tr>
<tr>
<td>AhpC</td>
<td>ahpC</td>
<td>alkylhydroperoxide reductase subunit</td>
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Table 1.1. Sulfate-regulated proteins in *Escherichia coli* (28).
Figure 1.3. Assimilation and utilization of organic sulfur sources in *Escherichia coli*.  
(Adapted from (32)).
TauD alone through the activation of dioxygen by ferrous iron, the desulfonation of alkanesulfonates by the ssuEADCB system is dependent on SsuD, SsuE, and a flavin cofactor.

Both the tau and ssu operons encode ABC-type transporter genes, but the proteins of the TauABC and SsuABC transport systems are not interchangeable (Figure 1.4) (38). Even though the substrates transported by both systems exhibit chemical similarities, the periplasmic substrate binding protein TauA and SsuA share only 22.7% amino acid identity (38). Some sulfonates can be transported by both TauABC and SsuEADCB systems, while gene deletion studies demonstrated that taurine is exclusively assimilated by the TauABC system and long-chain aliphatic sulfonates are primarily transported by the SsuEADCB system. The substrate range for TauABC and SsuEADCB systems seems to complement each other (Table 1.2) (36). Therefore, the expression of both systems under sulfur limitation conditions constitutes a substrate uptake system that covers the full spectrum of diverse aliphatic sulfonates. While similar ssi proteins found in E. coli were also identified in Pseudomonas sp, the ssu operon from Pseudomonas sp were found to contain one more open reading frame designated as ssuF (Figure 1.5) (39, 40). Because E. coli majorly utilize aliphatic sulfonate whereas Pseudomonas sp can additionally use a wide range of aromatic sulfonates, the molybdopterin protein encoded by ssuF gene is speculated to be specifically involved in desulfonation of aromatic sulfonates as well as alkanesulfonates in Pseudomonas sp (39). In addition to the tau and ssu operon, an msu operon was upregulated in Pseudomonas aeruginosa during sulfur limitation condition (Figure 1.5) (28). This msu operon codes an FMNH2-denpendent methanesulfonate sulfonatase, which liberates sulfite from methanesulfonate (40).
Figure 1.4. Uptake and desulfonation of alkanesulfonates and taurine in *Escherichia coli*. (Adapted from (32)).
<table>
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</table>

a–, not determined

Table 1.2. Substrate ranges and relative activities for SsuD and TauD in *E. coli* (36).
Figure 1.5. Genetic organization of *ssu* and *msu* operons from *Escherichia coli*, *Pseudomonas putita* and *Pseudomonas aeruginosa*. (Adapted from (28)).
1.3 Regulation of sulfonate-sulfur utilization in bacterial organisms

Although the tau and ssu genes are expressed under sulfur limitation as an alternate route to synthesize sulfite and cysteine from sulfonates, the regulation of sulfonate-sulfur utilization by bacteria is linked with the assimilatory sulfate reduction pathway (Figure 1.6) (32). The central transcriptional activator CysB not only positively regulates the cys genes in the cysteine biosynthetic pathway, but also controls the regulation of sulfur assimilation at a global level (3, 32).

The tau and ssu genes were found to be fully repressed when sulfate was present in the growth environment (33). Binding sites for the global transcriptional activator CysB were identified in both the tau and ssu promoter region (41, 42). An additional regulatory protein, Cbl, was also found to regulate expression of the tau and ssu genes as a LysR-type transcriptional activator (41, 42). It has been shown that Cbl binds upstream of the -35 region of the ssu promoter. As both binding sites for CysB and Cbl are present in the tau and ssu promoter region, these binding sites were removed separately to evaluate the effect on gene expression. While Cbl binding was essential for the expression of both tau and ssu, CysB binding was only needed for tau expression (42). Removal of the CysB binding sites in ssu did not have a significant influence on protein expression. The role of the CysB binding sites in the ssu promoter region remains unclear. Interestingly, while CysB expression is autoregulated by self-binding to the cysB promoter region, the expression of Cbl is also positively regulated by CysB (43). As a result, the expression of the tau and the ssu operons are indirectly regulated by CysB and its inducers and anti-inducers.
A coinducer $N$-acetylserine binding cavity was identified in the three-dimensional structure of CysB located at the C-terminal portion of the protein, which is compartmentalized by two $\alpha/\beta$ domains (44). Comparing the amino acid sequence of Cbl to CysB, the Cbl protein contains 45% amino acid sequence identity to CysB and conserved residues that constitute the inducer binding cavity in CysB (32). However, unlike CysB, the binding of Cbl to the $tau$ and $ssu$ promoter regions is not affected by the inclusion of $O$-acetylserine (41, 42). Further transcriptional experiments have shown that $O$-acetylserine is indeed not a coinducer of transcriptional activator Cbl (32). Although sulfate was shown to repress the expression of the $tau$ and $ssu$ genes, it was suggested that sulfate does not function as an anti-inducer of Cbl because the $tau$ and $ssu$ genes were not repressed by sulfate in mutants that were unable to activate sulfate to APS (32). The repression of $tau$ and $ssu$ genes by sulfate becomes effective in mutants that cannot reduce sulfite to sulfide, suggesting that the anti-inducer of Cbl is actually an intermediate of the cysteine biosynthetic pathway (32).

Two-dimensional gel electrophoresis experiments suggested that the Cbl not only regulates the transcription of $tau$ and $ssu$ operon, but also other ssi proteins such as CysK, FliY and Sbp (41). In addition, through transcriptional analysis using DNA arrays, the $cbl$ gene is shown to be cotranscribed and coregulated with the $nac$ gene, which positively regulates the expression of operons for utilization of poor nitrogen sources (45). Therefore, Cbl was suggested to obtain an accessory function in regulating nitrogen assimilation apart from its original function in sulfonate utilization regulation (46).
Figure 1.6. Gene expression regulation of sulfur assimilation in *Escherichia coli*.

(Adapted from (32)).
1.4 Alkanesulfonate monooxygenase system from E. coli

Under sulfur limiting conditions, proteins involved in assimilating sulfonate-sulfur from alternate sources are expressed by the *E. coli ssu* operon (42). Two of these enzymes expressed from the *ssu* operon are responsible for catalyzing the desulfonation reaction of alkanesulfonates (36). The SsuE enzyme catalyzes the reduction of FMN to FMNH$_2$ in the presence of NADPH. The SsuD enzyme utilizes dioxygen and the FMNH$_2$ provided by SsuE to catalyze the oxygenic cleavage of the carbon-sulfur bond in alkanesulfonates, producing sulfite and aldehyde (Figure 1.7) (36, 47). Because the FMNH$_2$ substrate of SsuD is the product of SsuE, the desulfonation reaction catalyzed by SsuD is dependent on SsuE. In addition, SsuE and SsuD are located on the same *ssu* operon and are co-expressed and co-regulated by Cbl (42). Therefore, SsuE and SsuD function as a unit classified as a two-component system. Due to the absolute requirement for flavin by both SsuE and SsuD, this system is known as the flavin-dependent two-component alkanesulfonate monooxygenase system. An interesting feature of this system is that the flavin cofactor does not function as a bound prosthetic group, but serves as a substrate for both SsuE and SsuD.

1.5 Flavins and flavoproteins

The flavin cofactors not only play a vital role in the oxidation of alkanesulfonates, but are involved in catalyzing a wide range of reactions. Proteins associated with flavin cofactors are classified as flavoproteins. Flavins are considered highly versatile compounds because different flavoproteins possess distinctly different and sometimes contradictory functions. It is shown that the flavin environment in the enzyme active site results in the chemical versatility of flavoproteins (48). Flavins are known to participate
Figure 1.7. Overall desulfonation reactions of the flavin-dependent two-component alkanesulfonate monooxygenase system from *Escherichia coli*. (Adapted from (47)).
in both one-electron and two-electron transfer processes (48-67). Some flavoproteins catalyze oxidation and oxygenation reactions by activating dioxygen with reduced flavin (55-62). Other flavoproteins participate in dehydrogenation reactions, flavin reduction reactions or serve as components in the respiratory chain (63-67). Interestingly, flavins are also involved in physiological functions that have opposing effects. For instance, some flavins are able to contribute to oxidative stress by producing superoxide, while others are involved in self-defense mechanism toward oxidative stress by reducing hydroperoxides (48). Ongoing research in the flavin field is to understand the mechanism for the wide range of reactions catalyzed by flavoproteins and how the interactions between the flavin cofactor and the protein mediate these chemical reactions (48).

Flavin is a generic term generally referring to a group of molecules with a heterocyclic isoaalloxazine ring structure (7,8-dimethylisooalloxazine). In nature, flavins usually exist as lumiflavin, riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Figure 1.8) (48). Lumiflavin contains a methyl group attached to the isoaalloxazine ring at the N-10 position. The riboflavin, FMN, and FAD differ from one another by the functional group on the ribityl side chain at the N-10 position. The ribityl sugar side chain is covalently attached to the isoaalloxazine ring in riboflavin. FMN contains a phosphate group attached to the terminal hydroxyl group of the ribityl side chain, while FAD has an adenine dinucleotide group. Flavin can exist freely in the environment or associate with an enzyme to catalyze extremely versatile chemical reactions. Flavin was first discovered and characterized as an active component of old yellow enzyme, the first flavoprotein ever identified in the early 1930s (68-69). Flavin is ubiquitously found and needed in a broad range of biological systems. As the basic unit
Figure 1.8. Numbering system for the flavin isoalloxazine ring and the structures of lumiflavin, riboflavin, FMN and FAD.
of flavin, the isoalloxazine ring cannot be synthesized by humans. Therefore, riboflavin (vitamin B$_2$) is required in the human diet as starting material to synthesize flavin cofactors needed to maintain physiological functions.

Flavins can exist in different redox states that include an oxidized, one-electron reduced (semiquinone), and two-electron reduced (fully reduced hydroquinone) state (Figure 1.9) (49, 70). When flavin is free in solution, a mixture of oxidized and reduced flavin can rapidly form a certain amount of semiquinone radical through equilibrium (48). Approximately 5% of the semiquinone radical is stabilized at pH 7 when flavin is not bound to protein (48). However, the equilibrium can shift towards semiquinone formation when the flavin is associated with some proteins (48, 71). Because the nitrogens in the isoalloxazine ring are able to be protonated or deprotonated, flavin in each of the three redox states can exist in different ionic forms. Because the theoretical cationic forms of flavin are only possible at extremely low pH, the neutral forms and the anionic forms of the flavin are considered physiologically relevant (70). Besides these redox/ionic forms, flavins can adopt other electronic states known as charge-transfer states, which do not belong to any of the three redox states but are defined as the states where partial charge is transferred to or from one of the three redox states (70). The fully reduced hydroquinone can rapidly react with molecular dioxygen auto-catalytically (Scheme 1.1) (48).

\[
\text{F}l_{\text{red}}^{\text{H}}(\uparrow \downarrow) + O_2(\uparrow \uparrow) \rightarrow [\text{Fl}^* \uparrow \uparrow O_2] \rightarrow [\text{Fl}^* \uparrow \downarrow O_2]
\]

\[
\text{FIHOOH} \leftrightarrow \text{FIHOO}^-
\]

Scheme 1.1. Activation of molecular dioxygen by reduced flavin. (Adapted from (48)).
Figure 1.9. Redox and ionic states of flavin. (Adapted from (70)).
Reaction between reduced flavin and molecular dioxygen occurs first through the transfer of an electron from a singlet reduced flavin to triplet $O_2$ to yield a caged radical pair (52). The flavin hydroperoxide is formed when the radical pair is collapsed together followed by spin inversion. The flavin hydroperoxide then dissociates into $H_2O_2$ and oxidized flavin (48, 52). Flavins and flavoproteins are found to exhibit various spectroscopic characteristics based on redox, ionic, and charge-transfer states (Figure 1.10) (48, 71, 72). The oxidized flavin usually absorbs strongly at 450 nm to give a yellow color. When oxidized flavin is reduced by one electron, the yellow color is substituted by a red color representing neutral semiquinone or a light blue color for the anionic semiquinone (71). The neutral semiquinone has a broad absorbance band from 500-650 nm with a maximum near 580 nm, while the anionic form weakly absorbs beyond 550 nm and has an absorbance peak at 370 nm (72). When the flavin is fully reduced to the hydroquinone form, it becomes colorless which correlates with no significant absorbance beyond 400 nm (72). The unique spectroscopic properties of different flavin forms can serve as a useful tool to monitor the flavin oxidation states during catalysis and evaluate the step-wise mechanism of flavoproteins.

Flavoproteins are defined as proteins containing FMN or FAD. Due to the different biological functions and newly identified properties, flavoproteins can be classified according to the type of catalyzed chemical reaction, the substrate involved in catalysis, the number of electrons involved in the catalytic cycle, their physicochemical properties, structural motif, and electron acceptors (70). The most common classification divides the flavoprotein into four categories: oxidases, dehydrogenases, reductases and monooxygenases. The flavin oxidases catalyze the oxidation of substrates by dioxygen,
Figure 1.10. The spectra of flavin in different oxidation states. (48, 72).
producing H$_2$O$_2$. In these reactions, the flavin cofactor is activated by the substrates, and the reduced flavin needs to be stabilized in the oxidases before reacting with O$_2$ (48). Some well characterized flavin oxidases include glucose oxidase, glycine oxidase, and amine oxidase (56-58). The flavin dehydrogenases catalyze reactions with the transfer of a pair of electrons and a proton without the involvement of dioxygen. Examples of classic flavin dehydrogenases include acyl-CoA dehydrogenase, succinate dehydrogenase, and alcohol dehydrogenase (65, 73, 74). While flavoproteins are involved in diverse catalytic reactions, this work is focused on flavin reductases and flavin monooxygenases from the two-component systems.

1.6 Flavin reductases

Flavin reductases, also known as NAD(P)H-dependent flavin oxidoreductase, catalyzes the reduction of the flavin cofactor through the oxidation of a pyridine nucleotide (NADH or NADPH), producing reduced flavin for many diverse enzymatic reactions (Equation 1.1). In the equation, E-Fl represents enzyme associated with oxidized flavin while E-FlH$_2$ represents enzyme bound with reduced flavin.

$$E-\text{Fl} + \text{NAD(P)H} + H^+ \leftrightarrow E-\text{FlH}_2 + \text{NAD(P)}^+ \quad \text{(Equation 1.1)}$$

The pyridine nucleotide substrate specificity may vary depend on the specific flavin reductase. Some flavin reductases preferably utilize NADPH as substrate, while others use NADH as their preferred substrate (75-80). In addition, some flavin reductases are able to utilize both NADPH and NADH with equal efficiency (81, 82). While the flavin reductases have the ability to utilize FMN and/or FAD, they can differ from each other based on the coordination of the flavin content. Some flavin reductases are standard flavoproteins that contain a covalently bound flavin cofactor, which gives a distinct flavin
spectrum after the protein is purified. These flavoproteins include FRase I and FRP associated with bacterial luciferase, EmoB in the EDTA monooxygenase system, NtaB involved in nitrilotriacetate oxidation, C₁ from hydroxyphenylacetate (Hpa) hydroxylase (HpaH), and SnaB in pristinamycin biosynthesis (78, 82-86). Other flavin reductases do not have a bound flavin prosthetic group and utilize flavin as a cosubstrate. These flavin reductases include Fre-like protein and LuxG that are able to provide reduced flavin to bacterial luciferase, SsuE in the alkanesulfonate monooxygenase system, ActVB in actinorhodin biosynthesis, and DszD involved in degradation of dibenzothiophene (81, 87-90).

Bacteria have developed complex systems to utilize the reducing power of flavin reductases. One function of flavin reductases is to provide reduced flavin as an efficient reducing agent or electron transfer mediator in the reduction of iron proteins (91-93). Another important function of flavin reductases is to supply reduced flavin as the substrate for flavin-dependent monooxygenases and metalloproteins (75, 94). The physiological functions of reduced flavin produced by many flavin reductases are not established because the physiological partners of these flavin reductases have not yet been identified. In addition, different enzymes may utilize a general flavin reductase or require specific flavin reductase to ensure catalytic efficiency. How the organisms evolve to benefit from the complex systems of flavin reductases cannot be fully understand until more work is devoted to the study of flavin reductases.

1.6.1 Flavin reductase (SsuE) in the alkanesulfonate monooxygenase system from E. coli

The first gene in the E. coli ssu operon encodes a flavin reductase SsuE that is expressed when sulfur is limiting (42). The SsuE enzyme catalyzes the reduction of FMN
in the presence of a pyridine nucleotide, generating FMNH2 as the product. Because the genes of SsuE and SsuD are located on the same ssu operon, the physiological function of SsuE is to provide reduced flavin to SsuD (42). Based on initial characterization, SsuE exists as a homodimer with a 21.3 kDa monomeric molecular weight (36). The SsuE enzyme does not contain a sulfur-containing amino acid residue, suggesting that the SsuE enzyme is evolutionally efficient in sulfur conservation when sulfur is limiting. Even though the initial characterization suggested that SsuE utilizes NADPH as the preferred substrate, further steady-state kinetic analyses revealed similar $K_m$ value for NADPH and NADH (36, 81). The SsuE enzyme is found to reduce both FAD and FMN as well, but the $K_m$ value for FAD is 130-fold higher than the $K_m$ value for FMN, suggesting FMN is the preferred flavin substrate for SsuE (81). The purified SsuE enzyme does not show a characteristic flavin absorbance spectrum, suggesting that SsuE does not possess any covalently or tightly bound flavin cofactor (36). Although the SsuE enzyme showed little sequence identity to most two-component flavin reductases, it showed a 36% overall sequence similarity compared to EmoB in the EDTA monooxygenase system (95). Further analysis of the two sequences suggests that a highly conserved motif exists at the N-terminal region, and this motif is speculated to assist in flavin binding and protein-protein interactions (36).

Following the initial characterization of SsuE, detailed kinetic and mechanistic investigations were performed (81). The affinity of SsuE for FMN was determined by monitoring the decrease in intrinsic fluorescence emission with the titration of FMN substrate. The dissociation constant for FMN binding was $0.015 \pm 0.004 \mu M$ with one FMN molecule bound per monomer of SsuE. The $K_d$ for FMNH2 binding to SsuE was
1000-fold higher than the $K_d$ for FMN, suggesting that the SsuE enzyme preferentially binds oxidized flavin. Studies to evaluate the steady-state kinetic mechanism of SsuE suggested a sequential mechanism with an apparent $k_{cat}$ value of $116.0 \pm 6.3 \text{ min}^{-1}$, $K_m$ values of $0.016 \pm 0.002 \mu M$ for FMN and $5.4 \pm 0.9 \mu M$ for NADPH. The $k_{cat}/K_m$ values were $5153 \pm 25 \text{ min}^{-1}\mu M^{-1}$ for FMN and $5.5 \pm 0.4 \text{ min}^{-1}\mu M^{-1}$ for NADPH. In addition, inhibition studies using NADP$^+$ as an inhibitor showed a competitive inhibition with a $K_i$ value of $13.6 \pm 6 \mu M$. It is also found that saturating concentrations of substrate NADPH can reverse the inhibiting effect by competing with the product NADP$^+$, strongly suggesting an ordered sequential mechanism with NADPH binding first and NADP$^+$ dissociates last.

Because the physiological function of SsuE is to supply reduced flavin to SsuD during sulfur limitation, the steady-state kinetics was also evaluated in the presence of SsuD and the octanesulfonate substrate (81). The SsuD enzyme will not compete with SsuE for FMN due to the 600-fold higher affinity of SsuE for FMN. There were no significant changes in the kinetic parameters of SsuE in the presence of SsuD alone, which confirmed that SsuD does not have any impact on SsuE catalysis. Interestingly, when both SsuD and octanesulfonate were present, the steady-state kinetic mechanism of SsuE was altered to a rapid equilibrium ordered mechanism with NADPH binding first followed by FMN (Figure 1.11) (81). While the apparent $k_{cat}$ value for SsuE was not significantly affected, rapid equilibrium between free SsuE and NADPH bound SsuE resulted in a $K_m$ value of $0.13 \pm 0.01 \mu M$ for FMN. The change in the kinetic mechanism of SsuE in the presence of SsuD and its substrate octanesulfonate suggested that SsuE catalysis is only affected by the SsuD when it is actively catalyzing the desulfonation
reaction. The 10-fold increase in the $K_m$ value for FMN suggested that SsuE has a lower affinity for FMN when there is a demand of reduced flavin for the desulfonation reaction. The presence of SsuD and octanesulfonate ensures that the flavin reduction reaction is driven forward even when the concentration of NADPH is low.

The altered mechanism and FMN affinity for SsuE is speculated to efficiently facilitate the critical flavin transfer step in the two-component alkanesulfonate monooxygenase system (81). If flavin reduction cannot appropriately synchronize with desulfonation by SsuD, excess reduced flavin would be nonproductively oxidized generating damaging hydrogen peroxide. An alternative explanation for the change in the SsuE mechanism and decrease in FMN affinity is that there might be two FMN binding sites, the first binding site would be involved in nonproductive flavin reduction which would correspond to the ordered sequential mechanism and higher FMN affinity, while the second binding site involved in flavin transfer has a low FMN affinity (81). However, more investigations have to be performed in order to fully understand how SsuE collaborate with SsuD during catalysis.

Microscopic steps involved in flavin reduction by SsuE were also defined by rapid reaction kinetic analyses (96). Three distinct phases were observed after the initial substrate binding step that forms a ternary Michaelis complex (MC-1). The first phase is a fast interaction between NADPH and FMN with a rate of 241 s$^{-1}$ representing the first charge transfer complex (CT-1). In CT-1, the hydride is leaving NADPH and is shared between NADPH and FMN. The second phase is a slow conversion (11 s$^{-1}$) to the second charge transfer complex (CT-2) between NADP$^+$ and FMNH$_2$, which corresponds with the deuterium-sensitive rate-limiting electron/hydride transfer step from NADPH to FMN.
Figure 1.11. Altered reaction mechanisms of SsuE in the absence and presence of SsuD and alkanesulfonate (81).
The third phase is the decay of the charge transfer complex to product bound SsuE (MC-2) or product release with a rate of 19 s\(^{-1}\). These detailed characterizations of the individual steps in reductive half-reaction have built the basis for understanding the mechanism of the two-component alkanesulfonate monooxygenase system.

1.7 Flavin monooxygenases

Another class of flavoproteins, flavin monooxygenases (FMO), catalyzes the insertion of dioxygen into a wide range of substrates. The flavin monooxygenases utilize a reduced flavin to activate dioxygen, and then oxygenate the substrate by splitting of the oxygen-oxygen bond, resulting in the incorporation of one oxygen atom in the substrate and the other oxygen atom incorporated in water (Equation 1.2 and 1.3). In the equation, E-Fl represents enzyme associated with oxidized flavin, E-FlH\(_2\) represents enzyme with reduced flavin bound, S represents the substrate, and SO represents the oxidized substrate (product).

\[
\text{E-Fl} + \text{NAD(P)H} + \text{H}^+ \leftrightarrow \text{E-FlH}_2 + \text{NAD(P)}^+ \quad \text{(Equation 1.2)}
\]
\[
\text{E-FlH}_2 + \text{S} + \text{O}_2 \leftrightarrow \text{E-Fl} + \text{SO} + \text{H}_2\text{O} \quad \text{(Equation 1.3)}
\]

Flavin monooxygenases either contain a covalently or tightly bound flavin prosthetic group, or utilize the flavin as a substrate. For the flavin monooxygenases with bound flavin, the flavin cofactor is usually reduced by NAD(P)H in the same polypeptide that accomplishes the oxygenation reaction. For the flavin-dependent monooxygenases, the reduced flavin is usually supplied by a flavin reductase. Current research regarding flavin monooxygenases is generally focused on evaluating the oxygen-activation strategies, catalytic mechanisms, and protein conformational changes that are important for catalysis.
Flavin monooxygenases activate dioxygen by reduced flavin prior to catalyzing the oxygenation reaction (Figure 1.12) (48). The reduced flavin transfers one electron to dioxygen, producing a flavin semiquinone radical and a superoxide radical respectively. These two radical intermediates rapidly form a caged superoxide-semiquinone pair before they diffuse apart. An oxygen-carbon bond between the superoxide and the semiquinone forms at the C4a position of the flavin isoalloxazine ring, producing a flavin C4a-peroxide (Fl-OO') intermediate (97-104). This flavin C4a-peroxide intermediate is in equilibrium with flavin C4a-hydoperoxide (Fl-OOH) through protonation and deprotonation. In the absence of protein stabilization, the flavin C4a-hydrperoxide intermediate is rapidly deprotonated at the N-5 position, and the proton is transferred to the oxygen leaving group resulting in the elimination of H₂O₂ in this buffer-catalyzed reaction. Conversely, when the flavin is associated with protein, the intermediate is found to be stabilized by the active site environment and/or protein conformational changes (105).

Depending on the type of reaction catalyzed, flavin monooxygenases utilize two types of flavin intermediates (59, 98, 106-108). The C4a-hydroperoxyflavin usually performs an electrophilic attack to hydroxylate aromatic compounds, while the C4a-peroxyflavin often serves as a nucleophile in the oxidation of non-aromatic compounds (47, 109-113). Alternatively, both flavin intermediates have the ability to eliminate hydrogen peroxide to yield oxidized flavin. The flavin monooxygenases that catalyze aromatic hydroxylations through a hydroperoxyflavin intermediate include \( p \)-hydroxybenzoate hydroxylase (PHBH), \( p \)-hydroxyphenylacetate 3-hydroxylase (\( p \)HPAH), kynurenine 3-monoxygenase (KMO), and a large aromatic hydroxylase
Figure 1.12. Reactions of reduced flavin with dioxygen (48).
complex called Molecule Interacting with CasL (MICAL) (98, 99, 114, 115). The flavin monooxygenases that utilize a peroxylflavin intermediate to catalyze non-aromatic oxidation include cyclohexanone monooxygenase, bacterial luciferase, and alkanesulfonate monooxygenase (47, 109, 111). While some of the extensively studied flavin monooxygenases accomplish their function as a single component monooxygenase and use flavin as a bound prosthetic group, more two-component flavin monooxygenase systems have been identified (80-90, 116). Based on the nature of this study, the following review will focus on flavin monooxygenases in two-component systems.

The recently identified \( p \)-hydroxyphenylacetate 3-hydroxylase (\( p \)HPAH) from a two-component hydroxylase system catalyzes the hydroxylation reaction of \( p \)-hydroxyphenylacetate (85). The first step of the proposed reaction mechanism of \( p \)HPAH involves the binding of reduced flavin to the monooxygenase, and reaction of dioxygen with reduced flavin to form a stable C4a-hydroxyflavin intermediate (Figure 1.13) (99). Following the binding of \( p \)-hydroxyphenylacetate, the bound substrate performs an electrophilic attack on the flavin intermediate. The next step is product release and formation of an enzyme bound hydroxyflavin, which finally decays to oxidized flavin and \( \text{H}_2\text{O} \). Even though both \( p \)HPAH and single-component monooxygenase PHBH catalyze hydroxylation of aromatic compound through the hydroperoxyflavin intermediate, the overall mechanisms and substrate binding order are different. Flavin reduction and flavin intermediate formation by \( p \)HPAH precedes the substrate binding, supported by the observation of a stabilized hydroxyflavin intermediate in \( p \)HPAH (99).
Figure 1.13. Catalytic mechanism of PHPAH using hydroperoxyflavin as an electrophile. (Adapted from (99)).
A well characterized flavin-dependent monooxygenase that utilizes a C4a-peroxyflavin intermediate as a nucleophile is bacterial luciferase (Figure 1.14) (111). Initially the enzyme bound peroxylflavin intermediate was thought to perform a nucleophilic attack on long-chain aldehyde substrates, followed by a Baeyer-Villiger rearrangement to generate corresponding carboxylic acid and green blue light (102-104). However, a general Baeyer-Villiger rearrangement would not produce the excited state of the flavin intermediate responsible for light production. An alternative mechanism involving a dioxirane intermediate was then proposed based on observed isotope effects and theoretical calculations (113). An interesting feature of bacterial luciferase is the ability to stabilize the C4a-(hydro)peroxyflavin intermediate for several hours (111). The flavin intermediate is found to be protected by several active site residues and a flexible loop near the active site (117-119). The gradual decay of the flavin intermediate results in the bioluminescence of bacterial luciferase (119).

One of the challenges for flavin monooxygenases is to catalyze a diverse range of reactions while controlling the stabilization of reaction intermediates. The highly reactive reduced flavin can form toxic hydrogen peroxide by-products. In order to avoid wasteful consumption of NAD(P)H and the formation of hydrogen peroxide during oxygenation reaction, flavin monooxygenases have developed two mechanistic strategies (105). One strategy is described as “cautious” due to the requirement of the bound substrate for flavin reduction. Therefore, flavin intermediate formation is triggered by the binding of the substrate to be oxygenated. Because the substrate is already present, the oxygenation reaction out-competes the $\text{H}_2\text{O}_2$ elimination side reaction and ensures the productive catalysis (105). Flavin monooxygenases that catalyze the hydroxylation of
Figure 1.14. Proposed reaction mechanism of bacterial luciferase. The intermediate responsible for light production is indicated. (Adapted from (113)).
aromatic compound usually belong to this “cautious” category. In contrast, the second strategy is described as “bold”, which does not necessarily require the presence of substrate in order for the rapid flavin reduction and flavin intermediate formation to proceed (105). These flavin monooxygenases are able to effectively stabilize flavin intermediates through interactions with active site residues and/or conformational changes that control the accessibility of the active site (97, 120). The stabilized flavin intermediate then catalyzes the oxygen insertion reaction after a competent substrate is encountered. This “bold” strategy is often associated with flavin monooxygenases that oxidize the non-aromatic compounds, as well as two-component systems including ρHPAH, bacterial luciferase, and alkanesulfonate monooxygenase (47, 85, 111). Since the active sites of these flavin monooxygenase have to be open for the substrate, the strategies used to provide necessary protection of the labile flavin intermediate from the bulk solvent are particularly interesting to fully understand these “simple” yet complex flavoproteins.

1.7.1 Alkanesulfonate monooxygenase SsuD from E. coli

The SsuD enzyme is a flavin-dependent monooxygenase that catalyzes the oxygenolytic cleavage of the carbon-sulfur bond in 1-substituted alkanesulfonates in the presence of FMNH₂ and dioxygen (36, 47). The initial characterization of purified SsuD suggested that the desulfonation activity is absolutely dependent on the presence of SsuE, NADPH, FMN, and dioxygen, and maximum activity was obtained for molecular ratio of SsuE/SsuD between 2.1 and 4.2 (36). The SsuD enzyme was found to strictly use FMNH₂ as the substrate due to the absence of activity when FMN was substituted by FAD (36). The products of the desulfonation of alkanesulfonate are confirmed to be the
corresponding alkanealdehyde and sulfite (36). While the *E. coli* SsuD enzyme cannot utilize taurine, L-cysteic acid, methanesulfonate, and aromatic sulfonates, it is able to desulfonate a broad range of unsubstituted 1-alkanesulfonates with chain lengths longer than two carbons (36). In addition, substituted ethanesulfonic acid, *N*-phenyltaurine, 4-phenyl-1-butanesulfonic acid, and sulfonated buffers can also serve as substrates for SsuD (36). This broad substrate range of SsuD is thought to compliment the TauD desulfonation system to correspond to the full sulfonate sources available. However, further structural and mechanistic characterization of SsuD is needed to illustrate how the SsuD manages to utilize such a diverse range of substrates.

The three-dimensional structure of *E. coli* SsuD in the absence of substrates was solved at a resolution of 2.3 Å (Figure 1.15) (121). The SsuD monomer was shown to contain a single domain with an eight-parallel-stranded β/α barrel motif, which is denoted as a TIM (triose phosphate isomerase) barrel structure. The TIM-barrel motif represents a large family of proteins with distinct functions. The structure of SsuD differs from the typical TIM-barrel proteins by four insertion regions connecting β strands with α helices and an extension region at the C-terminus end of the polypeptide chain (Figure 1.16) (121). Insertion region 4 is the most extensive insertion region containing 75 residues. It is located over the C-terminal end of the TIM-barrel and covers part of the inner core. In the crystal structure, insertion region 4 contains an unresolved loop region from residue 250 to 282 (121). The poorly defined electron density of this region in the crystal structure was speculated to be caused by the high mobility of this loop region. Despite the low level of amino acid sequence identity, the SsuD enzyme is structurally related to TIM-barrel proteins bacterial luciferase and long-chain alkane monoxygenase
Figure 1.15. Three-dimensional structure of SsuD (PDB entry: 1M41) (121). Two identical subunits are shown in gray and light blue.
Figure 1.16. Topology diagram showing secondary structural elements of each SsuD monomer, represented as arrows for β-strands and cylinders for α-helices (121).
(LadA), classified as the bacterial luciferase structural family (116, 122, 123). Both bacterial luciferase and LadA belongs to the two-component monooxygenase systems that utilize reduced flavin as a substrate instead of bound prosthetic group.

Steady-state and presteady-state kinetic studies were performed to investigate the mechanism of desulfonation. While SsuD was able to utilize a broad range of substrate, 1-octanesulfonate was one of the substrates with which SsuD showed optimal activity (47). Thus, 1-octanesulfonate was used to obtain the kinetic parameters. The substrate affinity of SsuD was evaluated through fluorimetric titration by monitoring the decrease of intrinsic protein fluorescence upon addition of substrate (47). The dissociation constant for FMNH$_2$ binding to SsuD was 0.032 ± 0.15 μM, while the dissociation constant for FMN binding to SsuD was 10.2 ± 0.4 μM, confirming that SsuD preferentially binds reduced flavin (47). Interestingly, there was no observable change in the intrinsic fluorescence with the addition of octanesulfonate to the free enzyme. In fact, the decrease in protein fluorescence was only observed when octanesulfonate was added to a solution of pre-mixed SsuD and FMNH$_2$. Therefore, it was suggested that an SsuD-FMNH$_2$ protein complex has to form prior to the binding of octanesulfonate (47). This ordered substrate binding indicates that different conformations exist between substrate-free SsuD and SsuD-FMNH$_2$ complex, and the conformational change necessary for octanesulfonate binding may be induced by FMNH$_2$ binding (47). The FMNH$_2$-bound SsuD then reacts with dioxygen to form a C4a-(hydro)peroxyflavin intermediate, which was identified by rapid reaction kinetic studies monitoring reduced flavin oxidation at 370 nm. Based on rapid kinetic analyses alternating the substrate mixing order, the protein complex formed by premixing SsuD and FMNH$_2$ may not represent the true
enzyme conformation during catalysis. It is speculated that an inactive complex forms when SsuD is premixed with FMNH₂, with the observation of decreased octanal product formation with premixed SsuD-FMNH₂ (47). This may be due to the slow conversion of the inactive complex back to the active form in the presence of octanesulfonate and dioxygen (47). In addition, the proposed C4a-(hydro)peroxyflavin intermediate observed without pre-mixing SsuD and FMNH₂ was absent in the kinetic trace, suggesting that the flavin intermediate cannot accumulate to a significant level due to the formation of inactive complex (47). While it is established that FMNH₂ binds to SsuD prior to octanesulfonate, the inactive complex is only formed in the absence of dioxygen. Because the SsuD requires dioxygen to catalyze the desulfonation reaction, the formation of this inactive complex may be due to in vitro anaerobic experimentation and is not physiologically relevant. Alternate mixing experiments suggested that reduced flavin oxidation is faster if the SsuD, FMNH₂ are pre-mixed with octanesulfonate instead of dioxygen (47). Therefore, it was concluded that during catalysis FMNH₂ binds to SsuD first, followed by immediate octanesulfonate binding and dioxygen binds last to ensure the coupling of intermediate formation and desulfonation. Therefore, an ordered substrate binding mechanism is suggested for the alkanesulfonate monooxygenase (Scheme 1.2) (47).

In the proposed mechanism of SsuD, a C4a-peroxyflavin intermediate is suggested to make a nucleophilic attack on the sulfonate functional group (Figure 1.17) (47). The alkanesulfonate peroxyflavin adduct then undergoes a Baeyer-Villiger rearrangement and proton abstraction by an active site base, leading to the generation of hydroxyflavin, alkanaldehyde, and sulfite. For the well studied flavin monooxygenases adopting similar
Scheme 1.2. Order of substrates binding for SsuD. (Adapted from (47)).
mechanistic strategies through peroxyflavin intermediate formation, the reactive flavin peroxyflavin intermediate is usually stabilized by the protein during catalysis. Cyclohexanone monooxygenase can stabilize the C4a-peroxyflavin intermediate for a maximum of five minutes with the NADP⁺ bound, while bacterial luciferase is able to stabilize its intermediate for several hours at low temperature (97, 111). Although bacterial luciferase and SsuD belong to the same structurally related FMNH₂-dependent two-component monooxygenase family, the proposed peroxyflavin intermediate is not as stable in SsuD (47). While the flavin intermediate for bacterial luciferase can be stabilized for several hours, the flavin intermediate for SsuD is much more susceptible to elimination than the intermediate for bacterial luciferase. Therefore, SsuD must offer a more precise and delicate intermediate stabilization network in order to ensure profitable and efficient catalysis.

One of the stabilization strategies developed by SsuD is to provide hydrogen bonding interactions through a conserved Cys54 residue (124). In the three-dimensional structure of SsuD, Cys54 is located in the putative catalytic center at the C-terminal end of the TIM-barrel (Figure 1.18) (121). The SsuD lost activity upon cysteine labeling with methylmercury, suggesting that Cys54 is important for catalytic function (121). By comparison with structurally related flavin-dependent two-component monooxygenases, the SsuD Cys54 residue is found in a similar spatial arrangement as Cys106 from bacterial luciferase and Cys14 from LadA (116, 122). Chemical modification of the reactive thiol group of bacterial luciferase Cys106 abolished enzymatic activity (100). Bioluminescence was reduced with the substitution of Cys106, indicating that Cys106 may be involved in intermediate stabilization in bacterial luciferase (103). The activity of
Figure 1.17. Proposed mechanism of the desulfonation reaction by SsuD (47).
Figure 1.18. Putative active site of the alkanesulfonate monooxygenase SsuD (PDB entry: 1M41) (121). Cys54 is shown in yellow, Ser179 is shown in orange, Tyr331 is shown in red, Arg226 and Arg297 are shown in green, His228 and His333 are shown in blue.
LadA was also found to be diminished with the substitution of Cys14 (116). When Cys54 of SsuD was substituted with serine, the substrate affinity was not significantly altered and the $k_{cat}/K_m$ value increased 3-fold (124). In addition, an increased C4a-(hydro)peroxyflavin intermediate formation rate was observed for the C54S SsuD variant in the absence of octanesulfonate. The accumulation of the C4a-(hydro)peroxyflavin intermediate was not observable even at low concentration of octanesulfonate, suggesting that catalysis happens faster than the accumulation of the flavin intermediate, which correlates with the increased catalytic efficiency (124). The conservative serine substitution appears to effectively take the place of cysteine in catalysis. However, when Cys54 was substituted with alanine, the $k_{cat}/K_m$ value decreased 6-fold even though the affinity for both substrates was not impaired (99). No C4a-(hydro)peroxyflavin intermediate accumulation was observed, suggesting that Cys54 of SsuD is involved in stabilizing the C4a-(hydro)peroxyflavin (124). Although the catalytic efficiency of C54A SsuD is 6-fold lower than the wild-type SsuD, the catalytic activity of C54A SsuD is not fully diminished, indicating that the Cys54 residue is not directly involved in catalysis as an active site base (124). When the thiol functional group of Cys54 was substituted with the serine hydroxyl functional group, the increase in intermediate formation rate and overall catalytic activity suggested that the Cys54 participates directly or indirectly in intermediate stabilization through hydrogen bonding interactions with the flavin intermediate.

1.8 Protein dynamics and flexible loop

In order for enzymes to stabilized reactive intermediates and facilitate efficient catalysis, another commonly practiced strategy is adopting an optimal protein
conformation. It is generally accepted that the diverse biological functions of proteins rely on their structural fluctuations. Different segments of protein can move in relation to one another with only small expenditures of energy due to the intrinsic flexibility of proteins (125). These structural motions that alter the protein conformations are the principle type of protein dynamics. At the molecular level, protein conformational changes are realized via the changes in polypeptide chain torsional angles and side chain orientations (125). Small changes in a few critical placed residues can often lead to a large dislocation of tertiary structural components, known as domain motions (125). Domain motions mainly exist in two forms: shear motions that occur parallel to the interface of closely packed segments of polypeptide, and hinge motions of secondary structural components that are not constrained by tertiary packing forces (125). While shear motions occur predominantly between α-helices, hinge motions are found in β-strands, β-sheets and α-helices that are connected through a hinge outside of the interface (125).

The commonly found hinge structures in many globular proteins are often located at the surface (125). Due to their high intrinsic mobility, these surface hinges are generally referred to as flexible loops. Since the flexible loop usually lies between the protein/solvent interface, it is found to play a critical role in contributing to protein function. In relation to the inner core of proteins, the flexible loops can easily assume an open/closed conformation with the torsion of only a few residues (126). The lid-gated movements of these flexible loops are able to create concealed compartments for precise catalysis (126). In addition, flexible loops can also be involved in ligand binding, signal recognition, and catalysis by providing essential catalytic residues (125). Therefore, it is
intriguing to understand the role of flexible loops in various proteins with distinct functions. Protein dynamics including flexible loop movements can be induced by many factors: temperature, pH, voltage, ion concentration, covalent labeling, hydrogen bonding, and ligand binding etc (127). The biophysical and biochemical evaluations of the flexible loop movements of the proteins related to the current study are reviewed in the following subsections.

1.8.1 Flexible loop of Triose phosphate isomerase (TIM)

Triose phosphate isomerase (TIM) was the first enzyme identified to contain the classical 8-stranded β/α barrel structure, a conserved motif also found in flavin-dependent monooxygenases SsuD and bacterial luciferase (128). Triose phosphate isomerase catalyzes the reversible isomerization of D-glyceraldehyde 3-phosphate (GAP) to dihydroxyacetone phosphate (DHAP) through an enediolate intermediate (Figure 1.19) (129). A substantial conformational change was observed in the three-dimensional structure with the binding of substrate (129). In the substrate-free conformation, a loop near the C-terminal end of the barrel extends outward from the protein inner core, leaving the proposed active site freely accessible to solvent (130). Therefore, the substrate-free TIM exists in the “open” conformation. When the substrate DHAP is bound, the loop folds over the active site, resulting in the “closed” conformation (129). The loop is referred to as a phosphate gripper loop because it interacts with the phosphate dianion of the substrate (129). The reactions catalyzed by TIM occur at a rate approaching the diffusion limit (131). This fast reaction rate is mainly due to the stabilization of the transition state with the phosphate intrinsic binding energy provided by the closure of the
Figure 1.19. The isomerization reaction catalyzed by TIM (A) and the crystal structures of the loop-open and loop-close forms of TIM from chicken muscle (B). The aqua ribbons are for the open unliganded TIM [PDB entry 1TIM] and the green ribbons show the closed TIM liganded with 2-phosphoglycolohydroxamate (PGH) [PDB entry 1TPH]. (Adapted from (128)).
loop and associated conformational changes (128). Based on a series of kinetic analyses, the substrate binding and corresponding loop closure is proposed to be rate determining in the isomerization from GAP to DHAP, while loop opening and product release is rate limiting in the conversion of DHAP to GAP (131-138). In addition, studies have also shown that the interaction between the flexible loop and substrate phosphate group activates proton transfer. Therefore, it is believed that the motion of the flexible loop in TIM not only anchors the substrate, but also actively controls the rate of catalysis upon interaction with substrate (128).

Another important role of the TIM flexible loop is to sequester the substrate and intermediate from solvent by closing over the active site. In order to perform efficient catalysis, TIM requires strong transition state stabilization activated through loop closure (128). The TIM flexible loop not only interacts with substrate to supply binding energy, it also provides a compartmentalized active site for optimal catalysis (128). The enediolate intermediate was found to be sequestered from bulk solvent through studies evaluating partitioning of the intermediate in D$_2$O (139-141). The percentage of intramolecular hydrogen transfer was much higher when the TIM-catalyzed isomerization of GAP proceeded in D$_2$O, suggesting the active site and the intermediate is less accessible to bulk solvent. In addition, the substrate is transferred from solvent to a confined catalytically favorable protein environment by loop closure, providing additional contacts between the protein and the substrate shown in the three-dimensional structure (142). A highly conserved Lys12 residue situated adjacent to the TIM flexible loop was shown to directly interact with the substrate phosphate group (143). Substituting Lys12 to glycine resulted in a 6×10$^5$-fold decrease in activity that corresponded to a 7.8 kcal/mol
stabilizing energy loss, which was originally provided by the interaction between wild-type Lys12 and the substrate (144). The same interaction of free lysine with phosphate is much weaker in aqueous solution, suggesting that movement of the flexible loop in the solvent-free protein active site environment enhances the interaction between the lysine side chain and the substrate (145). Furthermore, the closed conformation occludes bulk solvent and displaces several active site water molecules, which induces the desolvation of the TIM catalytic base Glu165 (146-148). This ligand induced conformational change shifts Glu165 2 Å into its catalytically active position (149). Loop closure is also found to cause the hydrophobic side chains of an isoleucine and a leucine to fold over Glu165, shielding the active site base from bulk solvent (150). This loop-closure induced hydrophobic active site environment leads to an increase in the basicity of the Glu165 side chain, which ensures efficient catalytic activity.

1.8.2 Flexible loop of Bacterial luciferase

Bacterial luciferase is a flavin dependent-monoxygenase that belongs to the family of TIM-barrel proteins. Bacterial luciferase employs reduced flavin as a substrate to activate dioxygen and catalyzes the oxidation of a long-chain aliphatic aldehyde to carboxylic acid with the emission of blue-green light (102). The proposed reaction mechanism of bacterial luciferase proceeds through several flavin intermediates (Figure 1.14) (113). The breakdown of the flavin intermediate is believed to be responsible for light emission (119). In order to achieve maximum bioluminescence, specific stabilization of the flavin intermediate followed by gradual decay is required. Therefore, the mechanism for intermediate stabilization in bacterial luciferase is particularly intriguing.
Bacterial luciferase is comprised of two TIM-barrel subunits designated as \( \alpha \) and \( \beta \) (122). The two subunits are expressed by \( luxA \) and \( luxB \) under the same \( lux \) operon and display high sequence homology (151). Although the \( \alpha \) and \( \beta \) subunits are similar in overall structure and share 32% amino acid sequence similarity, the \( \alpha \) subunit contains the active site that binds one reduced flavin substrate while the \( \beta \) subunit is not associated with flavin binding or product formation (122). However, studies have shown that the \( \beta \) subunit is essential for a high bioluminescent activity of the \( \alpha \) subunit despite its inability to bind reduced flavin (112). The main structural component that distinguishes the \( \alpha \) subunit from the \( \beta \) subunit of bacterial luciferase is an unresolved loop in an insertion region between an \( \alpha \)-helix and \( \beta \)-sheet, indicating high mobility of this region (122). Similar to the flexible loop of TIM and the unresolved loop of \( \text{SsuD} \), the unresolved loop of bacterial luciferase is located above the active site at the C-terminal end of the barrel. It is reported that the unresolved loop of bacterial luciferase is highly susceptible to proteolysis (152, 153). However, the presence of the \( \text{FMNH}_2 \) substrate was shown to inhibit the proteolysis of this region, indicating that the association of reduced flavin immobilizes and protects this mobile loop region (154). In the three dimensional structure of the bacterial luciferase/FMN complex, localized electron density was observed in the portion of the mobile loop that is distal to the flavin binding site, suggesting this loop is partially ordered and exists in alternative conformations with the binding of FMN (Figure 1.20) (123). Apparently, substrate binding initiates substantial conformational changes including loop movement, which is speculated to play an important role in bacterial luciferase bioluminescence activity.

The functional and mechanistic role of the bacterial luciferase mobile loop region
was first evaluated by deleting the 29-residue loop region of the α subunit that was unresolved in the original three dimensional structure without flavin bound (119). The loop-deleted bacterial luciferase variant $\alpha\Delta_{262-290}\beta$ was shown by far-UV circular dichroism to contain a higher percentage of α-helical structure than the wild-type enzyme, which was thought to be caused by the deletion of the unresolved loop region comprising 10% of the protein (119). Based on the results from both far- and near-UV circular dichroism spectroscopic analyses, it was believed that deletion of the unresolved loop in bacterial luciferase did not cause significant perturbations in overall secondary structural and the environment of the aromatic side chains. However, the $\alpha\Delta_{262-290}\beta$ heterodimer showed a subunit equilibrium dissociation constant of $1.32 \pm 1.25 \mu$M, whereas the wild-type heterodimer did not dissociate (119). Therefore, the unresolved loop region of bacterial luciferase was proposed to be involved in inter-subunit interactions. Kinetic characterization of the $\alpha\Delta_{262-290}\beta$ variant showed similar substrate affinity for reduced flavin and aldehyde as the wild-type enzyme, suggesting the unresolved loop region does not participate in substrate binding (119). Although the $\alpha\Delta_{262-290}\beta$ variant was able to form the carboxylic acid product with similar catalytic efficiency as the wild-type enzyme, the bioluminescence quantum yield of the reaction catalyzed by $\alpha\Delta_{262-290}\beta$ variant was 100-fold lower than the wild-type enzyme (119). It was postulated that without the unresolved loop region of the α subunit the exited flavin emitter responsible for bioluminescence either cannot efficiently populate or is quenched by the solvent that can easily access the active site without protection from the loop region (119). Therefore, the functional role of the bacterial luciferase unresolved loop region is believed to protect and stabilize the excited flavin emitter intermediate.
Figure 1.20. Three-dimensional structures of bacterial luciferase \( \alpha \) subunit with two observed conformations of the mobile loop (PDB entry: 1BRL and 3FGC). The mobile loop of \( \alpha \) subunit observed in a complex with FMN is shown in red. The mobile loop of flavin-free \( \alpha \) subunit is shaded in blue (122, 123).
Several conserved amino acids located on the bacterial luciferase unresolved loop region were substituted through alanine-scanning mutagenesis to determine the role of these residues in catalysis (117). Lys283 and Lys286 substitutions were found to result in a loss of bioluminescent activity comparable to the loop deletion variant (117). Both variants yielded the carboxylic acid product at the same efficiency as wild-type bacterial luciferase. Experiments evaluating the kinetic stability of the flavin intermediate suggested that Lys283 participates in C4a-hydroperoxylavin stabilization (117). In order to probe the accessibility of the flavin intermediate to the bulk solvent, a dynamic quencher potassium iodide was used. Potassium iodide is able to quench the flavin excited state and increase the rate constant for the decay of bioluminescence. It was reported that potassium iodide had a greater effect on the decay of bioluminescence for the Lys286 variant (117). It seems that substituting the Lys286 to alanine increased the exposure of the excited flavin intermediates to solvent. When residue Gly275 on the unresolved loop region of the α subunit was substituted to proline, the intrinsic torsional flexibility of glycine was replaced by the more constrained proline ring structure (118). As a result, the bioluminescence activity of the αG275P variant was reduced by 4 orders of magnitude compared to the wild-type enzyme (118). Because the αG275P variant can bind both substrates and form flavin intermediates at the same efficiency as the wild-type enzyme, the decrease in bioluminescence activity was attributed to the loss of flexibility of the unresolved loop. When residue Phe261 on the unresolved loop region of α was mutated to aspartic acid, the hydrophobic nature of the phenylalanine was substituted to a hydrophilic positively charged side chain (118). The αF261D variant showed a decrease in bioluminescence production by 6 orders of magnitude, while maintaining similar...
substrate affinity and catalytic efficiency toward product formation (118). It was suggested that the bulky hydrophobic phenylalanine side chain may be involved in providing a solvent inaccessible hydrophobic active site environment for the flavin emitter intermediate to accumulate. These results support the role of the bacterial luciferase unresolved loop as a mobile lid in shielding critical reaction intermediates from exposure to the bulk solvent.

1.8.3 Flexible loop of alkanesulfonate monooxygenase SsuD

The flavin dependent monooxygenase SsuD belongs to the bacterial luciferase structural family. Although SsuD shares a relatively low amino acid sequence identity with bacterial luciferase, the three dimensional structure of the two enzymes show common overall structural features including a TIM-barrel fold and an unresolved flexible loop region above the active site (121). However, structural variations exist between SsuD and bacterial luciferase. Bacterial luciferase is a heterodimer with one active site per dimer, while SsuD is a homotetramer with one active site per monomer. In addition, SsuD may differ from bacterial luciferase in the functional role of the unresolved flexible loop region despite their similar location (121-123). The flexible loop of bacterial luciferase was suggested to stabilize the flavin emitter responsible for bioluminescence (117-119). Deletion of this region does not affect product formation, suggesting the flexible loop may not be directly involved in bacterial luciferase catalysis (119). On the other hand, a random mutation of SsuD Arg297 to cysteine in the initial cloning process resulted in the loss of SsuD activity (Figure 1.21) (36). It was speculated that the Arg297Cys may form a disulfide bond with Cys54, affecting the flexibility of the loop region. Therefore, it is suggested that the SsuD unresolved loop and its motions may
be required for efficient catalysis (155). In the structure of SsuD without substrate bound, the conserved Arg297 resides near the unresolved loop that protrudes over the putative active site, but the functional group of this arginine is found to point away from the active site (121). Therefore, conformational changes of Arg297 would be necessary for catalytic involvement of this residue.

In order to understand the role of the conserved Arg297, various biochemical and mechanistic evaluations were performed on two SsuD variants with the Arg297 substituted by alanine or lysine (155). Although the reduced flavin substrate affinity was not altered significantly, the \( k_{cat}/K_m \) value was decreased 30-fold by the substitution of Arg297 with lysine. The R297A SsuD variant showed no detectable activity with a 5-fold increase in the \( K_d \) value for FMNH\(_2\) compared to wild-type SsuD (155). In addition, both arginine variants had similar affinity for octanesulfonate as wild-type SsuD. The results suggested that Arg297 may interact with another amino acid or substrate functional group to stabilize conformational changes induced by reduced flavin binding that correlates with flexible loop closure (155). Rapid reaction kinetic analyses of the R297A and R297K SsuD variants showed no accumulation of C4a-(hydro)peroxyflavin intermediate, and there was no observable dependence on the \( k_{obs} \) with increasing concentrations of octanesulfonate as previously observed for wild-type (47). These results suggested that conformational changes of the flexible loop cannot be realized without the crucial Arg297 residue, and the disrupted conformational changes would leave the active flavin intermediate accessible to solvent and susceptible to non-enzymatic oxidation (155). The requirement of Arg297 to undergo essential conformational changes
Figure 1.21. Three-dimensional structures of SsuD with the sequence of the unresolved region indicated [PDB entry 1M41]. The conserved Arg297 is shown in green (121).
was further supported by studies evaluating proteolytic susceptibility of both wild-type and arginine variants (155). The SsuD wild-type and Arg297 variants were rapidly (<15 s) digested in the absence of substrates, while there was 20% of the wild-type SsuD remaining after extended proteolysis (30 s) in the presence of reduced flavin substrate (155). It was speculated that substrate binding provided some level of protection towards proteolysis. Based on results from mass spectrometric analysis of the digested wild-type SsuD, the susceptible proteolytic sites were located at Arg263 and Arg271 on the insertion region containing the unresolved flexible loop (155). Therefore, the binding of reduced flavin substrate initiated conformational changes that immobilized the flexible loop. As a result, the proteolytic susceptible sites Arg263 and Arg271 on the ordered loop region were less accessible to tryptic digestion upon FMNH₂ binding. The same level of protection was not observed with R297A or R297K SsuD in the presence of reduced flavin substrate (155). In fact, the R297A and R297K SsuD variant exhibited a similar susceptibility toward proteolysis regardless of the appearance of the substrates FMNH₂ and octanesulfonate. It was concluded that Arg297 variants are not able to undergo conformational changes observed with wild-type SsuD, and that Arg297 is critical in stabilizing the conformational changes associated with loop movement (155). Substitution of Arg297 results in a more solvent accessible active site due to the inability of the loop to undergo the requisite conformational changes. The flavin intermediates are not adequately stabilized in the less protected active site in the Arg297 variants, leading to deleterious effects on catalysis. These kinetic and structural studies of the conserved Arg297 residue indicate that the SsuD unresolved loop region may be involved in stabilizing and protecting the reaction intermediates from bulk solvent, which is similar
to the functional role of the bacterial luciferase flexible loop. However, direct evaluation of the structural and functional role of the SsuD flexible loop was not performed. Since deviations exist between the flexible loops of bacterial luciferase and SsuD, the SsuD flexible loop may play additional functional roles in the desulfonation reaction.

Because the SsuD flexible loop is located above the active site and functions as a mobile lid, another possible role of this loop could be assisting in flavin transfer between SsuE and SsuD. The reduced flavin needs to be transferred from the SsuE active site to SsuD active site. In order for FMNH$_2$ to access the active site, the SsuD flexible loop would need to assume an extended conformation before flavin transfer. Following the flavin transfer events, the SsuD flexible loop would close over the active site for effective catalysis. Since the phosphate gripper loop of TIM is known to bind the substrate phosphate group, the SsuD flexible loop may also interact with the phosphate group of flavin and assist in flavin transfer.

1.9 Flavin transfer mechanism in two-component systems

The SsuD enzyme belongs to a family of two-component monooxygenase systems that utilize reduced flavin as a substrate. Although identified only in bacterial organisms, these flavin dependent two-component monooxygenase systems are capable of catalyzing a wide range of reactions such as oxidation of aromatic and polycyclic compounds, biosynthesis of antibiotics, bioluminescence, oxidation of long-chain alkanes, and desulfurization of sulfonate compounds (36, 80-90, 116). In these two-component systems, the monooxygenase activity relies on a flavin reductase that supplies reduced flavin to activate dioxygen. Because the reduced flavin is extremely labile, a controlled flavin transfer mechanism is necessary for these monooxygenase to efficiently utilize the
reduced flavin substrate.

Reduced flavin transfer could either occur through a dissociative or channeling mechanism (Figure 1.22). In the dissociative mechanism, the reduced flavin is released by the reductase into solution, followed by passive diffusion to the monooxygenase. In the dissociative flavin transfer process, no protein-protein interactions are required and the diffusion is driven by the affinity of the monooxygenase enzyme for reduced flavin. In the channeling mechanism, a transient reductase/monooxygenase complex is formed prior to flavin transfer, and the flavin reduced by the reductase is actively shuffled to the monooxygenase within the reductase/monooxygenase complex. Typically, the flavin reductases have a higher affinity for oxidized flavin while the monooxygenases preferentially bind reduced flavin (47, 81, 94). This difference in flavin affinity facilitates flavin transfer from the reductase to the monooxygenase. However, reduced flavin can also be oxidized rapidly in solution, generating toxic reactive oxygen species and damaging cellular components. Therefore, it still remains to be explored whether protein-protein interactions are critical to avoid in vivo non-enzymatic flavin oxidation in bacterial organisms. Among the characterized flavin-dependent monooxygenases, many are capable of utilizing reduced flavin supplied by flavin reductases from other two-component systems, supporting a dissociative flavin transfer mechanism (78, 82, 87, 88). However, the flavin reductases from different systems could share similar structural motifs. Although structural information is limiting, if flavin reductases contain similar structural motifs, it is possible that protein-protein interactions could be formed between these flavin reductases and monooxygenases. Then, the ability of some monooxygenases to couple with different flavin reductase in vitro is not a convincing argument to support
Figure 1.22. Schematic representation of flavin transfer mechanisms in two-component systems. The blue square represents flavin reductase, the yellow triangle represents reduced flavin, and the green circle represents the flavin monooxygenase.
a dissociative flavin transfer mechanism. Both dissociative and channeling flavin transfer mechanisms have been reported in two-component systems depending on the specific enzymes under investigation.

1.9.1 Flavin transfer mechanism in bacterial luciferase system

Even though bacterial luciferase is the most extensively studied FMN-dependent two-component monooxygenase system, the physiological relevant flavin reductase component was suggested to be LuxG in *Photobacterium leiognathi* (88). *In vitro*, bacterial luciferase can utilize reduced flavin supplied by various flavin reductases including FRP, FRase I, Fre-like reductase, and LuxG (78, 82, 87, 88). Alternative results describing the flavin transfer mechanism of bacterial luciferase have been reported depending on the flavin reductase and the techniques used for evaluation. Therefore, the *in vivo* flavin transfer mechanism in bacterial luciferase system is still under debate.

There are two major arguments against a channeling flavin transfer mechanism in the bacterial luciferase system. One is that the flavin reductase component is not specific in the system. When bacterial luciferase was assayed with various flavin reductases, it was able to accept reduced flavin provided by different flavin reductases and still maintain full activity (78, 82, 87, 88). Based on the assumption that these flavin reductases have no structural similarities, it was suggested that no protein-protein complex is formed between the two components, and a dissociative flavin transfer mechanism is highly likely (156-160). The other argument against a channeling mechanism is that studies designed specifically to identify protein-protein interactions between bacterial luciferase and some flavin reductases showed no evidence for complex formation (156-160). The gene responsible for expressing flavin reductase LuxG is located on the same operon with
luciferase lux genes in *Photobacterium leiognathi* TH1 (160). In this specific organism, LuxG is believed to be the functioning flavin reductase for bacterial luminescence based on *in vitro* and *in vivo* studies (160). In the *in vitro* coupled assays, LuxG was able to supply reduced flavin to bacterial luciferase for bioluminescence activity. When the luxG gene was knocked out from *Photobacterium leiognathi* TH1, the organism showed much dimmer bioluminescence, indicating that LuxG provides the most significant source of reduced flavin for the bioluminescence reaction *in vivo*. When gel filtration chromatography was utilized to evaluate if LuxG interacts with bacterial luciferase there were no protein-protein interactions identified, suggesting a dissociative flavin transfer mechanism (160). Similarly, investigation of interactions between bacterial luciferase and flavin reductase Fre or FRP via pull-down assays have also shown that there is no evidence for protein complex formation (161). However, based on the author’s speculation, the possibility of transient protein-protein interactions cannot be ruled out simply by the negative results from one or two techniques. More aspects need to be investigated before a final conclusion can be made.

Evidence demonstrating a channeling flavin transfer mechanism in bacterial luciferase system has also been identified (75, 162-164). A transient protein complex between bacterial luciferase and monomeric FRP was identified by fluorescence anisotropy (164). This study also suggested that dimeric FRP cannot form a complex with bacterial luciferase as efficiently as monomeric FRP. Therefore, the negative results in previous attempts to identify protein-protein interactions could have been due to the formation of dimeric FRP, which is not effective in complexing with luciferase. It was established that 94% of FRP in the cell is in complex with luciferase when *V. harveyi*
exhibits maximum *in vivo* bioluminescence (164). Direct channeling of reduced flavin within the complex of FRP and luciferase would save energy, avoid toxifications, and maintain efficient bioluminescence at low levels of FMN and NADPH (162). A channeling flavin transfer mechanism between FRP and bacterial luciferase was also supported by kinetic evidence (162). In the absence of bacterial luciferase, flavin reduction reaction catalyzed by FRP proceeds through a ping-pong mechanism with a $K_m$ value of 8 μM for FMN and a $K_m$ value of 20 μM for NADPH (162). However, in the presence of bacterial luciferase, the FRP mechanism was altered to a sequential mechanism with a $K_m$ value of 0.3 μM for FMN and a $K_m$ value of 0.02 μM for NADPH (162). The change in kinetic mechanism and $K_m$ values strongly supported complex formation between FRP and bacterial luciferase, suggesting a channeling flavin transfer mechanism. In addition, an engineered fusion protein of FRP and luciferase closely mimics a single-component bifunctional monooxygenase, exhibiting efficient activities with minimum FMN supply (165). The fusion protein does not need exogenously added flavin because the enzyme-bound flavin is shuffled and recycled within the fusion protein. Thus, in order for the two-component bacterial luciferase system to maintain efficient activity without consuming excess flavin, a direct transfer of flavin would be more favorable.

1.9.2 Flavin transfer mechanism in the HPAH and ActVA-ActVB systems

The *p*-hydroxyphenylacetate hydroxylase (HPAH) system from *Acinetobacter baumannii* is a flavin-dependent two component system containing an FMN reductase (C₁) and an oxygenase (C₂) (85, 99). A unique feature of the HPAH system is that the HPA not only serves as the substrate of C₂, it also stimulates flavin reduction and release
by C₁ (99). A dissociative flavin transfer mechanism was proposed in the HPAH system based on kinetic evidence (166). It was suggested that in a mixture of C₁ and C₂, the reduced flavin substrate bound more tightly to C₂ and the reduced flavin associated with C₁ was rapidly transferred to C₂ before it can react with dioxygen in solution. It was shown that the dissociation of reduced flavin from C₁ was the rate-limiting step in the intermolecular transfer of flavin from C₁ to C₂. The flavin transfer event was found to be regulated by the presence of HPA. It was speculated that the absence of HPA, production of H₂O₂ was avoided by reducing the rate of flavin release. Therefore, no protein-protein interaction between C₁ and C₂ was necessary for efficient flavin transfer and a dissociative flavin transfer mechanism was possible.

The ActVA-ActVB system from *Streptomyces coelicolor* involved in antibiotic actinorhodin biosynthesis is also an FMN-dependent two-component system that transfers reduced flavin between the reductase ActVB and monooxygenase ActVA (159). It was demonstrated by kinetic analysis that the reduced flavin can be transferred efficiently without the formation of any protein-protein complexes (159). The higher reduced flavin affinity of ActVA ensured that the reduced flavin was rapidly acquired upon release by ActVB and was tightly bound to ActVA. It was found that the rate of flavin transfer was controlled by NAD⁺ product release from ActVB and the concentration of NAD⁺, which was associated with the formation of a strong charge transfer complex between NAD⁺ and reduced flavin in the ActVB active site. It was speculated that the reductase component ActVB serves as a regulatory component of the monooxygenase activity by controlling the flavin transfer process. When NADH concentration was low, the reduced flavin would remain in the reductase preventing
further flavin reduction and H₂O₂ production. Therefore, the studies on the ActVA-ActVB system demonstrate a dissociative flavin transfer mechanism and the modulation of flavin transfer by the kinetics of the reductase component.

1.9.3 Flavin transfer mechanism in the alkanesulfonate monooxygenase system

Although different flavin transfer mechanisms were suggested for bacterial luciferase from different organisms, the flavin transfer event for the alkanesulfonate monooxygenase system in *E. coli* has always been speculated to occur through a substrate channeling mechanism. The ordered sequential mechanism of SsuE in the absence of SsuD was altered to a rapid equilibrium ordered mechanism in the presence of SsuD and octanesulfonate (81). It would be difficult for the SsuD enzyme to affect the flavin reductive reaction catalyzed by SsuE without protein-protein interactions. Therefore, a channeling flavin transfer mechanism has been proposed. The static SsuE-SsuD interaction was later identified through several experimental approaches (167). Results from pull-down assays using a Ni-NTA column showed coelution of SsuE and His-tagged SsuD. This result suggested that the SsuE forms a stable complex with His-tagged SsuD. The possibility of random protein aggregation was excluded by a control experiment with His-tagged SsuD and an unrelated protein (167). Since the His-tagged does not significantly alter the structure and activity of SsuD, the interactions between SsuE and His-tagged SsuD should presumably resemble the interactions between SsuE and wild-type SsuD (167). The identification of static interactions between SsuE and His-tagged SsuD provide evidence for a channeling flavin transfer mechanism.

In addition, the direct interactions between SsuE and wild-type SsuD were identified through chemical crossing-linking and visible circular dichroism (CD) spectroscopy. It
was established that perturbations in the flavin environment within the active site could be monitored through visible CD spectroscopy (167). Because SsuE binds FMN 100-fold tighter than SsuD, the change of the FMN-bound SsuE active site flavin environment with the addition of SsuD was attributed to specific SsuE/SsuD protein-protein interactions. Furthermore, the dissociation constant for SsuD binding to SsuE was determined to be 0.0022 ± 0.0010 μM by fluorescence spectroscopy (167). These static interactions between SsuE and SsuD highly support a channeling flavin transfer mechanism in the alkanesulfonate monooxygenase system. However, whether the SsuE/SsuD complex is formed during catalysis still remains unclear. A channeling flavin transfer mechanism would be more convincing if direct kinetic evidence were obtained during the course of catalysis.

1.10 Summary

Flavin is a vital cofactor for the survival of all organisms. Flavin can be easily reduced to transport electrons or to catalyze a variety of reactions. The reduced flavin is volatile under aerobic conditions because it is rapidly oxidized by dioxygen, generating reactive oxygen species that could be toxic to cellular components. In order to avoid production of these unwanted toxic molecules, the concentration of free reduced flavin must be cautiously controlled.

The reduced flavin is considered a key catalytic force in the two-component alkanesulfonate monooxygenase system that catalyzes the desulfonation of 1-substituted alkanesulfonates. The SsuE enzyme reduces FMN to FMNH₂ in the presence of NADPH. The reduced flavin is supplied by SsuE to SsuD for the desulfonation reaction. The SsuD enzyme utilizes reduced flavin as substrate to activate dioxygen to form an enzyme
bound C4a-flavin peroxide intermediate, which serves as an oxygenating agent to directly insert an oxygen atom into the alkanesulfonate substrates (47). The oxygenolytic cleavage of the sulfur carbon bond results in the liberation of inorganic sulfite, which is a crucial sulfur source for bacteria when inorganic sulfate is not readily available.

In the desulfonation process, both the reduced flavin transfer from SsuE to SsuD and formation of the flavin peroxide intermediate have the potential to generate toxic H₂O₂ by undergoing non-enzymatic flavin oxidation. How the alkanesulfonate monooxygenase system is designed to prevent non-enzymatic flavin oxidation during catalysis becomes a rather intriguing question. Even through previous studies have suggested that an unresolved loop region closes over the SsuD active site when substrates bind, the detailed mechanistic role of the SsuD unresolved loop region was not evaluated (155). In the following chapter, the functional roles of this unresolved loop region in the desulfonation reaction will be fully discussed. Another common way for the flavin-dependent two-component systems to avoid non-enzymatic flavin oxidation is by providing a cautiously controlled flavin transfer mechanism. The identification of static SsuE-SsuD interactions have indicated that the flavin might be transferred through a channeling mechanism in the alkanesulfonate monooxygenase system to reduced non-enzymatic flavin oxidation by limiting the accessibility of reduce flavin to bulk solvent (167). Nevertheless, static SsuE-SsuD complex does not fully convey a channeling mechanism during catalysis. In chapter three, the flavin transfer mechanism in alkanesulfonate monooxygenase will be evaluated in detail through direct kinetic analyses.
Chapter 2
Deletional Studies to Investigate the Functional Role of a Dynamic Loop Region of Alkanesulfonate Monooxygenase

2.1 Introduction

Sulfur is an essential element for the survival and growth of all living organisms. Being a primary component of several essential biological molecules, sulfur plays an important role in various metabolic events. The principal sulfur source for many bacterial organisms is cysteine or inorganic sulfate. Sulfur exists in various forms in the environment and the majority of sulfur-containing compounds in aerobic soils are organic sulfonates and sulfate esters that are not readily assimilated by bacteria (28). These organic sulfonate compounds are an alternate sulfur source metabolized by bacteria that are able to express sulfonate-sulfur-utilization (ssu) proteins (31). In *E. coli*, induction of the *ssuEADCB* operon during sulfur limitation leads to the expression of ABC-type transport proteins as well as an NAD(P)H-dependent flavin mononucleotide (FMN) reductase (SsuE) and FMNH₂-dependent alkanesulfonate monooxygenase (SsuD) (36). The SsuE enzyme catalyzes the reduction of FMN, and SsuD catalyzes the desulfonation of alkanesulfonates. The alkanesulfonate monooxygenase system is capable of utilizing a diverse range of alkanesulfonates with varying carbon lengths as substrates including sulfonated buffers (36). The *ssu* operon provides bacteria with a mechanism for obtaining sulfur from a broad range of organosulfonate compounds during periods of sulfur limitation.
The alkanesulfonate monooxygenase system belongs to a family of two-component enzymes that utilize flavin as a substrate (32). The flavin is not a tightly bound prosthetic group, but is released and transferred to SsuD following reduction by SsuE. Once the reduced flavin is transferred, the SsuD enzyme activates dioxygen with the reduced flavin cofactor. Similar to many other two-component flavin-dependent monooxygenases, the SsuD enzyme catalyzes the desulfonation reaction through a C4a-(hydro)peroxyflavin intermediate (47, 102, 113, 168, 169). The C4a-(hydro)peroxyflavin intermediate catalyzes the oxygenolytic cleavage of the carbon-sulfonate bond of alkanesulfonate to generate the corresponding aldehyde and sulfite product (Scheme 2.1) (47). The sulfite product of the alkanesulfonate monooxygenase system is then assimilated into various sulfur-containing compounds for the survival and growth of the organism (36). Evaluation of the SsuD desulfonation reaction through steady-state and rapid reaction kinetics analysis suggests that the reduced flavin binds to SsuD before the octanesulfonate substrate can bind, indicating a possible conformational change induced by the binding of reduced flavin (47).

Scheme 2.1. Reaction mechanism of the alkanesulfonate monooxygenase system (47).
The three-dimensional structure of the SsuD enzyme is composed of a triose phosphate isomerase (TIM)-barrel fold, with a dynamic loop region located in an insertion sequence that deviates from the (β/α)$_8$ barrel core (Figure 2.1) (121). In several TIM-barrel proteins a mobile loop located over the active site protects reactive intermediates from bulk solvent and prevents the premature release of catalytic intermediates (170, 171). The SsuD enzyme shares similar structural properties with the flavin-dependent monooxygenase enzymes bacterial luciferase and LadA. As observed with other TIM-barrel proteins, members of the bacterial luciferase family contain a loop region located over the active site. The loop region of bacterial luciferase was shown to display time-dependent proteolytic resistance with the binding of substrates that correlated with conformational changes (172). Conserved amino acid residues on the mobile loop are crucial to bacterial luciferase activity suggesting that the loop contributes to the active site architecture (117-119). Amino acid sequence alignment of *E. coli* SsuD with homologues from different organisms show that the dynamic loop region is highly conserved (Figure 2.2). Studies suggest a conformational change of the SsuD dynamic loop is initiated by the binding of substrates and protects the peroxylavlin intermediate generated in the desulfonation mechanism (155). Three variants containing partial deletions of the dynamic loop of SsuD were constructed to evaluate how the loop region of SsuD correlates with catalytic function. The reported results define the mechanistic roles of the dynamic loop region of SsuD in the desulfonation reaction. Based on these results, the roles of the loop region in SsuD appear more diverse than in bacterial luciferase, suggesting mechanistic differences exist between members of the bacterial luciferase structural family.
Figure 2.1. Three-dimensional structures of SsuD with the sequence of the dynamic region indicated (PDB entry 1M41) (121). The insertion region 4 is shaded in blue.
Figure 2.2. Sequence alignment of SsuD homologues. Sequence containing the dynamic region of *E. coli* SsuD (SSUD_ECOLI) is aligned with its homologues from *Pseudomonas putida* (SSUD_PSEPU), *Pseudomonas* sp. (SSUD_PSESP), *Pseudomonas aeruginosa* (SSUD_PSEAE), *Yersinia pestis* (SSUD_YERPE), *Shigella flexneri* (SSUD_SHIFL) and *Erwinia tasmaniensis* (SSUD_ERWT9). The underlined amino acid residues are conserved in all seven proteins. Dashed line indicates the dynamic region in the *E. coli* SsuD three-dimensional structure. The figure was prepared with CLUSTALW.

<table>
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<th>Amino Acid Sequence</th>
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<td>SSUD_ERWT9</td>
<td>QAADRLISHLDDDETIAKAQAFARTDSVGGQRMAALHGGKRDNLEISPNLWAGVGLVRGG 300</td>
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2.2 Material and Methods

2.2.1 Materials

Potassium phosphate (monobasic and dibasic), flavin mononucleotide phosphate (FMN), nicotinamide adenine dinucleotide phosphate (NADPH), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), glucose, glucose oxidase, ammonium sulfate, ammonium bicarbonate, calcium chloride, phenylmethylsulfonyl fluoride (PMSF), ampicillin, streptomycin sulfate, lysozyme, trypsin, EDTA, and imidazole were purchased from Sigma (St. Louis, MO). Glycerol, calcium chloride, isopropanol, and sodium chloride were obtained from Fisher (Pittsburgh, PA). Octanesulfonate was from Fluka (Milwaukee, WI). QIAPrep Spin Miniprep Kit, QIAquick PCR Purification Kit and Ni-nitrilotriacetate (NTA) Superflow resin were from Qiagen (Valencia, CA). Super-competent E. coli BL21 (DE3) cells were from Stratagene (La Jolla, CA). Oligonucleotide primers were from Invitrogen (Carlsbad, CA).

2.2.2 Construction, expression, and purification of recombinant proteins

The SsuD deletion variants were generated using the pET21a plasmid containing the ssuD gene as the template for PCR-amplification. The primers used to construct the SsuD deletion variants were (5’ ATAACGGAGGAGGCGACAATCTGGAGATCAGCCCCCAAT) and (5’ CTACGGAGGAGGAGTTGCTGGGCTGCTGCT) for ΔF261-N282 SsuD, (5’ ATAACGGAGGAGGCGACAATCTGGAGATCAGCCCCCAAT) and
(5’GAGTGTGAGGAGGTTCAGTAACCGCCCATTCGC) for ΔH276-N282 SsuD, (5’GCAACAGAGGAGGCAGCTCACATAACGGAAGCGC) and (5’ACGAAGGAGGAGCGGAGATGCGGCCTGTGCTTT) for ΔF261-L275 SsuD that includes a BseRI recognition site. The PCR products were purified with the QIAquick PCR Purification Kit, and digested with BseRI for 1 hr at 37 °C. Following digestion, the truncated vectors were ligated generating the specified deletions. The C-terminal His-tagged ΔF261-N282 SsuD variant was constructed with the Stratagene QuikChange site-directed mutagenesis kit. The stop codon (TAA) of ΔF261-N282 ssuD was replaced with the alanine codon (GCG). All deletion and 6×His-tagged variants were confirmed by sequence analysis at Davis Sequencing (Davis, CA). The vectors containing the correct gene sequence for the SsuD variant was transformed into chemically-competent E. coli BL21 (DE3) cells for protein expression. The expression and purification of the variants and wild-type SsuD proteins were performed as previously described (81).

2.2.3 Circular dichroism spectroscopic analysis

The far-UV circular dichroism (CD) spectra of the deletion variants and wild-type SsuD enzymes were obtained from 300 nm to 190 nm with an enzyme concentration of 1.2 μM in 25 mM potassium phosphate buffer, pH 7.5, at 25 °C as previously described (167). The visible CD spectrum of reduced flavin was obtained under anaerobic conditions with 20 μM FMNH₂ in 25 mM potassium phosphate buffer, pH 7.5, at 25 °C. The reduced flavin solution was prepared as previously described (47). The visible CD spectra of the deletion variants and wild-type SsuD in the presence of reduced flavin were obtained with 20 μM enzyme and 20 μM FMNH₂ in 25 mM potassium phosphate buffer, pH 7.5, at 25 °C. Measurements were taken in 1.0-nm increments from 550 to 300 nm in
a 1-cm-path-length cuvette with a bandwidth of 1.0 nm and a scanning speed of 50 nm/min. All CD spectra were obtained on a Jasco J-810 Spectropolarimeter (Easton, MD). Each spectrum is the average of eight scans.

2.2.4 Proteolytic and mass spectrometric analyses

The proteolytic digestion of the deletion variants and wild-type SsuD was performed at room temperature with trypsin (10 μg/ml) and SsuD (24 μM) in 200 mM ammonium bicarbonate/1 mM calcium chloride reaction buffer adjusted to pH 8.4. Trypsin stock solution (2 mg/mL) was prepared in 1 mM HCl/1 mM calcium chloride, pH 8.4. After the addition of trypsin, 10 μl aliquots of the reaction mixture were taken out at 30 s, 60 s, and 120 s, and immediately added to 2 μl PMSF in isopropanol (6 mg/ml) to quench the digestion reaction. The degree of proteolysis of each sample was analyzed by SDS-PAGE. For analysis of the tryptic digests of wild-type SsuD by mass spectrometry, 2% formic acid (final concentration) was added to the sample prior to direct injection into a Waters Q-TOF Premier mass spectrometer. The multiply charged protein was deconvoluted using MaxEnt 1 to obtain the protein molecular mass. Peptide mapping was performed through database analysis with GPMAW software (Lighthouse, Odense, Denmark).

2.2.5 Affinity chromatography binding assays

The native SsuE and His-tagged ΔF261-N282 SsuD were expressed as previously described (81). A 1.0 liter cell culture containing 6×His-tagged ΔF261-N282 SsuD was harvested by centrifugation at 5000 g for 15 min and resuspended in 100 ml 25 mM potassium phosphate, pH 7.5, and 10% glycerol containing 4 μg/ml lysozyme. Cell lysis was performed by sonication followed by centrifugation (20 min, 15,000 g). Solid streptomycin sulfate (1.5% w/v) was added to the cell supernatant and stirred slowly for

- 80 -
one hour at 4 °C to precipitate nucleic acids. The treated supernatant was centrifuged (20 min, 15,000 g), and the clarified supernatant was loaded onto a Ni-NTA column and washed with 100 ml of 25 mM potassium phosphate, pH 7.5, and 10% glycerol. A 1.0 liter cell culture of expressed SsuE was treated similarly to ΔF261-N282 SsuD and the resulting clarified supernatant loaded onto the Ni-NTA column, followed by an additional wash with 100 ml of 25 mM potassium phosphate, pH 7.5, and 10% glycerol to remove unbound proteins. Adventitiously bound proteins were removed from the column with 200 ml of 25 mM potassium phosphate, pH 7.5, 10% glycerol, and 125 mM imidazole. His-tagged ΔF261-N282 SsuD and interacting proteins were eluted with 100 ml 25 mM potassium phosphate, pH 7.5, 10% glycerol, and 300 mM imidazole. The collected fractions from the 125 and 300 mM imidazole wash were collected and analyzed by SDS-PAGE.

2.2.6 Kinetic analysis

The kinetic parameters for wild-type SsuD and the deletion variants were determined based on previously reported methods (47). Reactions were initiated by the addition of NADPH (500 μM) into a solution containing SsuD (0.2 μM), SsuE (0.6 μM), FMN (2.0 μM), and varying concentrations of octanesulfonate (5-1000 μM) in 25 mM potassium phosphate buffer, pH 7.5, 10% glycerol at 25 °C. Because of undetectable activity, a final protein concentration of 1.0 μM was used in assays with the SsuD deletion variants.

A previously described spectrofluorimetric titration method was used to determine the $K_d$ values of wild-type SsuD and the deletion variants for reduced flavin with slight modifications (47, 81). Protein solutions in the absence of SsuE were prepared in a glass titration cuvette by at least 15 cycles of evacuation followed by equilibration with
ultrahigh purity argon gas. Flavin prepared in 25 mM potassium phosphate, pH 7.5, and 10% glycerol was bubbled with ultrahigh purity argon gas for 15 min. Both protein and flavin solution were then incubated in an anaerobic glovebox for 20 min after adding glucose (20 mM) and glucose oxidase (10 Units) to remove trace amounts of dioxygen. The anaerobic FMN solution was photoreduced by two consecutive 15 min irradiations in the presence of EDTA (10 mM). For the titration of ΔF261-N282 SsuD with reduced flavin, aliquots of reduced flavin (0.2-9.1 μM) were added to a 1 mL solution of ΔF261-N282 SsuD (0.5 μM) in 25 mM potassium phosphate, pH 7.5, and 10% glycerol and the fluorescence spectra were recorded following a 5 min incubation after each addition. The addition of reduced flavin was performed in an anaerobic glovebox to ensure anaerobiosis. Protein samples were exited at 280 nm and the emission intensity recorded at 344 nm on a Perkin-Elmer LS 55 luminescence spectrometer (Palo Alto, CA). The $K_d$ value was determined as previously described (47). Corrections for inner filter effects were not included because the absorbance contribution of reduced flavin at the excitation and emission wavelengths was negligible at the flavin concentrations used in these studies (181).

Rapid reaction kinetic analyses monitoring flavin reduction and oxidation were performed by mixing SsuE (35 μM), wild-type SsuD or the deletion variants (35 μM), and FMN (25 μM) in 25 mM potassium phosphate, pH 7.5, and 10% glycerol against NADPH (250 μM) and octanesulfonate (50 μM) in air-equilibrated 10 mM Tris-HCl buffer, pH 8.5, and 10% glycerol. Control experiments were also performed as described above in the absence of the deletion variants or wild-type SsuD in order to monitor flavin reduction by SsuE and subsequent non-enzymatic flavin oxidation.
For the FMNH₂ oxidation experiments, FMNH₂ (25 μM) in 25 mM potassium phosphate, pH 7.5, and 10% glycerol was mixed against deletion variants or wild-type SsuD (35 μM) and octanesulfonate (25 μM) in air-equilibrated 25 mM potassium phosphate, pH 7.5, 10% glycerol, and 100 mM NaCl. The single wavelength traces of each deletion variant at 450 nm were fitted to the following equations using KaleidaGraph software (Abelbeck Software, Reading, PA):

\[
A = A_1 e^{(-k_1 t)} + A_2 e^{(-k_2 t)} + C
\]  

(1)

where \( k_1 \) and \( k_2 \) are the apparent rate constants for the different phases, \( A \) is the absorbance at time \( t \), \( A_1 \) and \( A_2 \) are amplitudes of each phases, and \( C \) is the absorbance at the end of the reaction.
2.3 Results

2.3.1 Functional and structural evaluation of the SsuD deletion variants

In order to evaluate the structural and functional role of the SsuD dynamic loop region, three partial loop deletion variants were constructed. The shorter ΔH276-N282 deletion region was generated to evaluate the effect of populated charges or polar residues on enzyme function. The ΔF261-L275 SsuD variant was constructed to evaluate the effect of the 12-amino acid conserved sequence on enzyme activity. The ΔF261-N282 SsuD variant contains a 22-amino acid deletion of the dynamic loop region that included the 12-amino acid conserved sequence of the dynamic loop as well as the charged and polar amino acid region. The individual CD spectra of the SsuD deletion variants were obtained in the far UV region to examine if there were any observable variations in secondary structure (Figure 2.3). The CD spectra of the SsuD deletion variants showed no significant changes in the negative ellipticity in the 200-240 nm region and each spectrum essentially overlapped with wild-type. Interestingly, the positive ellipticity in the 190-200 nm region showed some small perturbations. However, the overall similarities between the far-UV CD spectra of the deletion variants and wild-type SsuD suggest that partial deletion of the dynamic loop region did not affect the gross secondary structural fold of SsuD.

Steady-state kinetic studies were performed to evaluate the kinetic properties of the deletion variants. The SsuE-coupled steady-state kinetic assay monitors the reaction of DTNB with the sulfite product at 412 nm (47). All three SsuD deletion variants showed no observable sulfite production at standard protein concentrations. Increasing the concentration of each variant 5-fold (1 μM) with increased octanesulfonate
concentrations (5 to 5000 μM), while still maintaining steady-state conditions, did not result in detectable activity indicating that the absence of activity was not due to an increase in the $K_m$ value. The absence of the loop region may result in the inability of the flavin to bind. Fluorescence titration experiments were performed to determine if reduced flavin binding was affected with the longer ΔF261-N282 SsuD deletion variant. The $K_d$ value for reduced flavin binding to the ΔF261-N282 SsuD was 0.31 ± 0.90 μM similar to the value obtained for wild-type SsuD (0.32 ± 0.15 μM) (Figure 2.4). These results indicate that although there was no general disruption of the overall secondary structure, partial deletion of the SsuD dynamic loop region abolished the ability of the enzyme to catalyze the desulfonation reaction. However, the resulting lack of activity was not due to a decrease in the reduced flavin binding affinity.
Figure 2.3. The far-UV circular dichroism spectra of wild-type SsuD and the deletion variants. Wild-type SsuD (○), ΔF261-N282 SsuD (□), ΔH276-N282 SsuD (◇) and ΔF261-L275 SsuD (△) were obtained with 0.05 mg/ml of SsuD in 25 mM potassium phosphate buffer, pH 7.5, at 25 °C. Measurements were taken in 1 nm increments from 300 nm to 190 nm in a 0.1 cm path length cuvette. Each spectrum is the average of eight accumulated scans.
Figure 2.4. Fluorimetric titration experiments determining the $K_d$ value of FMNH$_2$ binding to ΔF261-N282 SsuD. The ΔF261-N282SsuD (0.5 μM) in anaerobic buffer was titrated with photoreduced FMNH$_2$ (2.63-56.24 μM). Emission spectra were obtained with an excitation wavelength of 280 nm. The fluorescence intensity maxima at 344 nm of each spectrum were used to obtain the $K_d$ value for reduced flavin binding. The $K_d$ value was obtained through the average of three separate experiments.
2.3.2 Evaluation of the SsuD deletion variants active site environment

It was previously reported that in the visible region, a change in the flavin CD spectra could be observed due to changes in the flavin environment following binding to wild-type SsuD (167). Flavin binding has been proposed to induce a conformational change that modifies the active site environment (47). Visible CD experiments were performed with the deletion variants to determine if the integrity of the flavin environment was affected by the loop deletion. The individual CD spectra were obtained by scanning reduced flavin incubated with standard buffer alone, wild-type SsuD, or the SsuD deletion variants from 300 to 550 nm. A characteristic weak CD spectrum was observed for free reduced flavin (Figure 2.5, ●). When wild-type SsuD was incubated with reduced flavin (Figure 2.5, ○), an increase in the negative (300-350 nm) and positive (350-500 nm) ellipticity was observed that correlated with flavin binding. Conversely, the spectra with reduced flavin and the SsuD deletion variants showed only minimal changes in ellipticity compared to wild-type SsuD (Figure 2.5). These results suggest that the presence of the intact loop region is essential to maintain the integrity of the active site flavin environment. Although the SsuD deletion variants were still able to bind reduced flavin, the flavin environment was altered compared to wild-type SsuD.
Figure 2.5. Visible CD spectra of the FMNH$_2$ with and without wild-type SsuD and the deletion variants. FMNH$_2$ in the absence of SsuD (●), in the presence of wild-type SsuD (○), ΔF261-N282 SsuD (□), ΔH276-N282 SsuD (◇) and ΔF261-L275 SsuD (△) were obtained with 20 µM FMNH$_2$ in 25 mM potassium phosphate buffer, pH 7.5, at 25 °C. Measurements were taken in 1 nm increments from 550 nm to 300 nm in a 1 cm path length cuvette. Each spectrum is the average of eight accumulated scans.
2.3.3 Proteolytic susceptibility of the SsuD deletion variants

The wild-type SsuD enzyme was susceptible to time-dependent limited trypsin digestion in the absence of FMNH$_2$ and octanesulfonate. There was a decrease in proteolytic susceptibility with the addition of substrates suggesting SsuD undergoes conformational changes with the binding of substrates (155). Digestion of wild-type SsuD (42 kDa) resulted in the appearance of two bands with nearly equal intensity resolving at approximately 30 kDa. Results from mass spectrometric analysis of partially digested wild-type SsuD identified two peptide fragments with a high relative abundance at 29.3 and 30.1 kDa (Figure 2.6 A). These peptide fragments contained the tryptic digestion site on the loop region following Arg263 (29.3 kDa peptide fragment) and Arg271 (30.1 kDa peptide fragment) (Figure 2.6 B). The presence of a tryptic target on the loop region indicates the loop region is the accessible proteolytic site. The tryptic sites become partially protected during substrate binding suggesting conformational changes associated with loop movement are important in catalysis (155). Time-dependent limited trypsin digestion experiments were performed with the deletion variants to evaluate the role of the loop in conferring proteolytic resistance (Figure 2.7). The ΔF261-N282 and ΔF261-L275 SsuD variants that lacked the primary tryptic digestion sites at Arg263 and Arg271 showed minimal digestion even with longer incubation times. Interestingly, while wild-type enzyme (41.7 kDa) in lane 2, 6 and 10 underwent gradual proteolysis over time, the ΔH276-N282 SsuD variant that still contained the two primary trypsin recognition sites were resistant to proteolysis (lane 4, 8, and 12). The shortened loop could alter the accessibility of trypsin to the Arg263 and Arg271 recognition sites.
With the shortened unresolved loop, the flexibility of the loop would likely be decreased and the motion constrained leading to an overall decrease in proteolytic susceptibility.
Figure 2.6. Mass spectrometric analysis of wild-type SsuD following limited tryptic digestion. A. Peptide fragments with the highest relative abundance from limited trypsin proteolysis. B. Peptide mapping of the peptides peaks identified by mass spectrometry with the highest relative abundance using the known SsuD amino acid sequence.
Figure 2.7. Limited trypsin digestion of wild-type SsuD and the deletion variants.

Wild-type SsuD (lane 2, 6 and 10), ΔF261-N282 SsuD (lane 3, 7 and 11), ΔH276-N282 SsuD (lane 4, 8 and 12) and ΔF261-L275 SsuD (lane 5, 9 and 13) were incubated with 10.0 µg/ml TPCK-treated trypsin in 200 mM ammonium bicarbonate, 1 mM CaCl₂, pH 8.4, at 25 °C. At various times (30-120 s) 0.01 ml aliquots were removed and added to 2 µl of 6 mg/ml phenylmethylsulfonyl fluoride in isopropyl alcohol to quench the reaction. The resolved proteins near 45 kDa marker represent intact wild-type SsuD or deletion variants. Lane 1 is protein standard.
2.3.4 Rapid reaction kinetic analysis of the SsuD deletion variants

In the alkanesulfonate monooxygenase system, the flavin reductive and oxidative half-reactions occur on separate enzymes, and there is a defined separation between these two events (155). Following transfer from SsuE, the binding of reduced flavin to SsuD induces a conformational change that facilitates the binding of octanesulfonate (47). The loop region of SsuD may assist in flavin transfer or trigger the conformational changes necessary for the desulfonation reaction. Rapid reaction kinetic studies were performed with the SsuD deletion variants in the presence of SsuE to investigate if flavin transfer or protein dynamics were affected by the loop deletion. Under the experimental conditions, higher concentrations of SsuE ensured that the majority of FMN was associated with SsuE and subsequently reduced before being transferred to SsuD. The kinetic traces of the oxidative half-reaction by the deletion variants were altered from wild-type SsuD. There was a distinct lag phase in the kinetic trace for wild-type SsuE and SsuD, where the flavin remains reduced between the reductive and oxidative half-reaction (Figure 2.8, ○). This lag phase likely represents the transfer of reduced flavin from SsuE to SsuD or a conformational change that occurs with the binding of substrates to SsuD. Conversely, the kinetic traces for the SsuD deletion variants showed no apparent lag phase, and the reduced flavin was oxidized immediately after the reductive half-reaction (Figure 2.8). While reduced flavin oxidation by wild-type SsuD was previously shown to be coupled with sulfite product formation, reduced flavin oxidation for the SsuD deletion variants did not result in detectable sulfite production (47). The immediate oxidation of the reduced flavin observed with the SsuD deletion variants was comparable to the kinetic trace for the oxidation of free flavin obtained in the absence of SsuD (Figure 2.8, ●).
Therefore, the reduced flavin was not protected from unproductive flavin oxidation. Although the rates for flavin reduction were similar between the SsuD deletion variants and wild-type SsuD (27 s\(^{-1}\) for wild-type SsuD), the kinetic trace for flavin reduction in the presence of the SsuD deletion variants were delayed by 0.004 s compared to the kinetic trace for flavin reduction in the presence of wild-type SsuD. When SsuE was coupled with wild-type SsuD, the kinetic trace for flavin reduction started at 0.002 s and was complete at 0.14 s (Figure 2.8, □). When SsuE was coupled with the SsuD deletion variants, the kinetic trace for flavin reduction started at 0.006 s and was complete by 0.3 s (Figure 2.8). The kinetic traces for flavin reduction in the presence of the deletion variants were similar to the kinetic trace for flavin reduction without SsuD. In addition to affording protection of the reduced flavin, the loop region of SsuD may also play a role in enhancing the SsuE reaction through protein-protein contacts.

The dynamic loop region of SsuD may function as a gated lid to protect reduced flavin from bulk solvent following the transfer event. In order to firmly establish the accessibility of the SsuD active site in the SsuD loop deletion variants, stopped-flow kinetic experiments monitoring only flavin oxidation were performed (Figure 2.9). The kinetic traces obtained at 450 nm for the SsuD deletion variants and wild-type SsuD were best fit to a double exponential equation (eq 1). The rate constants for flavin oxidation for the shorter deletion variants (ΔF261-L275 and ΔF276-L282 SsuD) were similar to wild-type (Table 2.1). While the longer deletion (ΔF261-N282 SsuD) showed a similar \(k_1\) value as wild-type SsuD, there was a 4-fold increase in the \(k_2\) value. Although the rate constants were similar between the SsuD deletion variants and wild-type, the amplitudes for the two phases showed distinct differences. In fact, the amplitudes for \(k_1\) and \(k_2\)
obtained for the SsuD deletion variants were similar to the amplitudes obtained with non-enzymatic oxidation of free flavin (182-184). The comparable amplitudes obtained with the SsuD deletion variants and free flavin suggests that the reduced flavin substrate is accessible to dioxygen. As a result, the deletion variants may not be able to fully protect the reduced flavin from oxidation uncoupled from sulfite production.
Figure 2.8. Rapid reaction kinetic analysis of wild-type SsuD and deletion variants. \( \text{FMNH}_2 \) (25 µM) in anaerobic buffer potassium phosphate buffer, pH 7.5 and 10% glycerol was mixed against NADPH (250 µM), octanesulfonate (50 µM), SsuE (35 µM), FMN (25 µM), in the absence of SsuD (●), wild-type SsuD (○), \( \Delta F261\text{-N282} \) SsuD (□), \( \Delta H276\text{-N282} \) SsuD (◇), and \( \Delta F261\text{-L275} \) SsuD (△) (35 µM). Kinetic traces were obtained by following flavin reduction and oxidation at 450 nm. All the reactions were carried out in 25 mM phosphate buffer, pH 7.5, 10% glycerol at 4 °C.
Figure 2.9. Rapid reaction kinetic analysis of wild-type SsuD and deletion variants. FMNH$_2$ (25 µM) in anaerobic buffer potassium phosphate buffer, pH 7.5 and 10% glycerol was mixed against octanesulfonate (50 µM), in the absence of SsuD (●), wild-type SsuD (○), ΔF261-N282 SsuD (□), ΔH276-N282 SsuD (◇) and ΔF261-L275 SsuD (△) (35 µM). Kinetic traces were obtained by following flavin reduction and oxidation at 450 nm. All the reactions were carried out in 25 mM phosphate buffer, pH 7.5, 10% glycerol at 4 °C.
<table>
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<th>$A_1$</th>
<th>$k_2$ (s$^{-1}$)</th>
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<td>1.66</td>
<td>94.96</td>
<td>0.191</td>
<td>6.76</td>
</tr>
</tbody>
</table>

Table 2.1. Rate constants for FMNH$_2$ oxidation monitored at 450 nm.
2.3.5 Protein-protein interactions between SsuE and ΔF261-N282 SsuD

For the flavin-dependent two-component systems, it has been postulated that reduced flavin can be transferred through a channeling or dissociative mechanism (156-164). Previous studies have identified static interactions between SsuE and SsuD, supporting a channeling mechanism for flavin transfer (167). The rapid reaction kinetic evaluation of the SsuD deletion variants with SsuE suggested that flavin reduction could be altered due to the disruption of protein-protein interactions. In order to examine if the dynamic loop region of SsuD is involved in static protein-protein interactions, affinity chromatography experiments with His-tagged ΔF261-N282 SsuD and native SsuE were performed. Analysis of the eluted protein by SDS-PAGE showed that the unbound native SsuE was removed following the 125 mM imidazole wash (Figure 2.10 A, lane 3-10). With the 300 mM imidazole wash, native SsuE coeluted with His-tagged ΔF261-N282 SsuD (Figure 2.10 B, lane 8-15), suggesting that ΔF261-N282 SsuD was able to form static interaction with native SsuE. Because ΔF261-N282 SsuD contains the largest deletion of the dynamic loop region, the protein-protein interactions between ΔH276-N282 and ΔF261-L275 SsuD with SsuE would likely still be intact. Therefore, the apparent flavin oxidation and altered kinetic traces for SsuE flavin reduction observed in rapid reaction kinetic experiments with the SsuD deletion variants were not due to the disruption of protein-protein interactions.
Figure 2.10. SDS-PAGE (12% acrylamide) from the affinity chromatography experiments with SsuE and the 6×His-tagged ΔF261-N282 SsuD. 50 ml cell lysate containing 6×His-tagged ΔF261-N282 SsuD was loaded onto Ni-NTA column followed by loading 50 ml of native SsuE cell lysate. A: SDS-PAGE of fractions after 125 mM imidazole wash, lane 1 is protein standard, lane 2 is wild-type SsuD (41.7 kDa) and SsuE (21.3 kDa), lane 3-15 represent fractions after 125 mM imidazole wash over time. B: SDS-PAGE of fractions after 300 mM imidazole wash, lane 1 is protein standard, lane 2 is wild-type SsuD (41.7 kDa) and SsuE (21.3 kDa), and 3-15 represent fractions after 300 mM imidazole wash over time.
2.4 Discussion

The SsuD enzyme belongs to the bacterial luciferase family that includes bacterial luciferase and long-chain alkane monooxygenase (LadA). Enzymes in this family are defined as flavin-dependent two-component monooxygenases that rely on a flavin reductase to provide reduced flavin to the monooxygenase enzyme (94). A mechanistic challenge for the two-component monooxygenase systems is the transfer of reduced flavin from the flavin reductase to the monooxygenase (94, 156). The monooxygenase active site would need to be in an open conformation for flavin transfer, leaving the reduced flavin susceptible to unproductive oxidation (173). Protection of the active site from bulk solvent would be essential in order to prevent flavin intermediates from unproductive oxidations. Members of the bacterial luciferase structural family have the characteristic TIM-barrel conformation with several extended regions that deviate from the typical (β/α)8 barrel fold (116, 121-123). The α subunit of the heterodimeric structure of bacterial luciferase contains a loop region located in a conserved insertion sequence that was unresolved in the initial three-dimensional crystal structure of the enzyme (122). A partial ordering of this region was observed with flavin bound (123). Detailed mechanistic studies of the bacterial luciferase loop region demonstrate the vital importance of this region in maintaining catalytic function (117-119). In the three-dimensional structure of LadA with FMN bound, a similar insertion sequence protrudes over the proposed active site (116). This region was proposed to be involved in providing functionality to LadA by assisting in FMN binding (116). Unlike bacterial luciferase, there were no obvious conformational changes observed between the flavin-bound and apoenzyme in LadA (116). The three-dimensional structure of SsuD
also contains a dynamic insertion region located over the putative active site that is distinct from the barrel (121). This region is highly conserved among the SsuD enzymes from different organisms suggesting a potential role of this region in maintaining catalytic function. A conserved arginine residue (Arg297) located near the dynamic loop region in SsuD was shown to be involved in facilitating loop closure over the active site (115). These results established the dynamic movement of the loop with the binding of substrates.

In order to investigate the structural and functional role of the loop region of SsuD, three deletion variants were constructed based on the grouping of amino acid properties of this region. The gross secondary structure of the SsuD deletion variants was not markedly altered by partial deletion of the dynamic loop region. Similarly the deletion of the mobile loop in bacterial luciferase and triosephosphate isomerase did not disrupt the overall fold of the TIM barrel core (119, 174). All three of the SsuD deletion variants were unable to generate the sulfite product in steady-state kinetic assays, suggesting the loop region is essential for effective catalytic activity. A variant of bacterial luciferase with a loop deletion in the α subunit showed a decrease in bioluminescence by 2-orders of magnitude (119). Active site hydrophobicity was suggested to be critical to the bioluminescence activity (175). Although bioluminescence was decreased, the loop deletion variant of bacterial luciferase was still able to form the carboxylic acid product suggesting the overall catalytic function was not considerably affected (119). The loss of activity observed with the SsuD deletion variants indicates that the loop region likely plays an important role in vital catalytic steps related to desulfonation. Although SsuD
and bacterial luciferase are classified within the same structural family, the SsuD dynamic loop appears to play a critical role in maintaining catalytic function.

The interaction between the loop region and substrate phosphate group is necessary for the isomerization reaction of several TIM-barrel enzymes, even though the phosphate is not directly involved in catalysis (135, 144, 146, 171, 174, 176-178). A change in the conformation of the dynamic loop region of bacterial luciferase was observed with the addition of phosphate and with the binding of FMNH$_2$ (123). An insertion region containing the loop region was also shown to coordinate the phosphate group of FMN in the three-dimensional structure of LadA with flavin bound (116). The SsuD deletion variants were still able to bind FMNH$_2$ with similar $K_d$ values as wild-type SsuD. Further studies to evaluate the reduced flavin environment in the active site by visible CD spectroscopy demonstrated that the flavin environment of the SsuD deletion variants were similar to the reduced flavin spectrum in the absence of enzyme indicating the loop region assists in maintaining the integrity of the active site architecture. Although, the reduced flavin was able to bind to the active site of the SsuD deletion variants and form loose interactions with active site residues, the loop was required to close over the active site to for a stabilized flavin environment in order to promote catalysis for wild-type SsuD.

The proteolytic resistance of the bacteria luciferase mobile loop with the binding of reduced flavin was correlated with a closed conformation where the mobile loop was positioned over the active site and protected from proteolysis by the main enzyme structure (154). Interestingly, similar resistance to time-dependent limited trypsin digestion was observed for wild-type SsuD when substrates were present, indicating
possible conformational changes were induced by substrate binding (155). In the absence of substrates, the susceptible proteolytic sites were located at Arg263 and Arg271 on the loop region (155). Proteolytic susceptibility experiments on the SsuD deletion variants in the absence of substrates showed limited digestion compared to wild-type SsuD. The ΔF261-N282 and ΔF261-L275 SsuD variants were not susceptible to proteolytic trypsin digestion because both variants lacked the primary digestion sites. Although ΔH276-N282 SsuD contained both cleavage sites, this deletion variant showed minimal proteolytic digestion in the absence of substrates compared to wild-type SsuD. Decreasing the length of the loop likely decreases the mobility of this region altering protease accessibility. The conformational changes necessary for catalysis that corresponds with loop closure requires the full-length loop region, and a shortened region would not induce the requisite conformational changes.

In the alkanesulfonate monooxygenase system the reduced flavin must be transferred from SsuE to SsuD. Therefore, the oxidative and reductive half-reactions are partitioned between the two enzymes, which allows for the evaluation of the reduced flavin transfer step followed by binding to SsuD. Rapid reaction kinetic traces obtained at 450 nm with both SsuE and wild-type SsuD in the reaction show a lag phase between the reductive and oxidative half-reactions (155). The observed lag phase may be associated with reduced flavin transfer and/or a conformational change associated with reduced flavin binding to SsuD. The kinetic traces representing reduced flavin oxidation by the SsuD deletion variants were essentially the same as the kinetic traces for free flavin oxidation. Additionally, flavin reduction by SsuE was initiated 0.004 s later in the presence of the SsuD deletion variants compared to the wild-type enzyme. These studies suggest that the
SsuD loop region may be involved in promoting flavin transfer to SsuD. Previous studies have demonstrated protein-protein interactions between SsuE and SsuD, suggesting that flavin transfer occurs through a channeling mechanism in the alkanesulfonate monooxygenase system (167). If the loop region were involved in promoting the required contacts between SsuE and SsuD, then deletion of the loop would ultimately disrupt flavin transfer. However, protein-protein interactions were observed between the 6×His-tagged ΔF261-N282 SsuD variant and native SsuE in pull-down experiments. The possibility of SsuE coeluting with His-tagged ΔF261-N282 SsuD due to non-specific protein-protein interaction was excluded since all non-specifically bound native SsuE was removed with lower imidazole concentrations. Because the deletion regions of ΔH276-N282 SsuD and ΔF261-L275 SsuD were located within the deletion region of ΔF261-N282 SsuD, it was assumed that the ability of these variants to interact with SsuE was still intact. The preservation of protein-protein interactions between SsuE and the SsuD deletion variant suggest that the altered flavin reduction may be due to a disruption in the synchronization of activities between the SsuE and SsuD active sites. Synchronization of active sites has been observed in enzymes with multiple active sites that tunnel intermediates between active sites (185-187). In these enzymes, the reaction at a catalytic site is often enhanced by chemical or substrate binding events occurring at a distant catalytic site. While the loop was not shown to play a major role in protein-protein interactions, the loop may be involved in subtle contacts that promote active site synchronization between SsuE and SsuD.

In the coupled rapid reaction kinetic studies, evaluation of reduced flavin oxidation was complicated by the flavin transfer and binding event. Rapid reaction kinetic analyses
in the absence of SsuE provided additional support regarding the importance of the loop in protecting the reduced flavin substrate. The shorter SsuD deletion variants (ΔF261-N282 and ΔF261-L275 SsuD) had similar rate constants for flavin oxidation at 450 nm as wild-type SsuD, while the longer deletion showed a 4-fold decrease in the $k_2$ value. However, the amplitudes obtained from a fit of the kinetic traces for the deletion variants were in fact very similar to those for non-enzymatic flavin oxidation. The reduced flavin may be able to bind to the active site in the SsuD deletion variants, but cannot trigger the conformational changes needed for catalysis leading to unproductive flavin oxidation. Therefore flavin oxidation is no longer effectively coupled to the formation of the sulfite product.

Many TIM barrel enzymes are not dependent on the transfer of substrates between two proteins; rather loop movement is associated with the initial binding of substrates (128, 135, 170, 171, 174). In contrast, the alkanesulfonate monooxygenase enzymes are expressed from the same operon and appear to be tightly associated with each other (31). The reduced flavin must be transferred from the reductase to the alkanesulfonate monooxygenase enzyme, and the loop may assist with flavin binding following the flavin transfer event. Deletion of the bacterial luciferase loop region resulted in the flavin being effectively transferred in in vivo studies, and there was no disruption in flavin binding or generation of the carboxylic acid product (119). The decrease in bacterial luciferase bioluminescence was attributed to the flavin emitter being exposed to solvent (119). Conversely, deletion of the dynamic loop of SsuD resulted in a complete loss of activity due to the altered active site environment that was not able to protect reactive reduced
flavin from bulk solvent. These distinct differences suggest different mechanistic roles of the loop region between members of the bacterial luciferase family.
3.1 Introduction

Sulfur is an essential element for the survival and growth of all organisms. Bacteria require inorganic sulfur sources to synthesize sulfur-containing metabolites and cofactors. Over 95% of the sulfur in the aerobic soils exists as sulfonate and sulfate esters, therefore bacteria must have an alternative mechanism to obtain sulfur when inorganic sulfur is poorly represented (28). During sulfur limitation when inorganic sulfur in the environment is limiting, bacteria express a set of proteins in order to utilize alternate sulfur sources (31). Alkanesulfonates are an alternate sulfur source that can be metabolized by bacteria that are capable of expressing sulfonate-sulfur-utilization (ssu) proteins. Induction of the ssu operon during sulfur limiting conditions leads to the expression of the two-component alkanesulfonate monooxygenase system, that includes an NAD(P)H-dependent flavin mononucleotide (FMN) reductase (SsuE) and FMNH$_2$-dependent alkanesulfonate monooxygenase (SsuD). The SsuE enzyme catalyzes the reduction of FMN to FMNH$_2$ with NAD(P)H providing the reducing equivalents. The reduced flavin is transferred from SsuE to SsuD for the desulfonation of alkanesulfonate. Once the reduced flavin is transferred, the SsuD enzyme activates dioxygen with the reduced flavin cofactor. Many two-component flavin-dependent monooxygenases catalyze the enzymatic oxidation reaction via a C4a-(hydro)peroxyflavin intermediate.
Similarly, the SsuD enzyme is proposed to utilize the C4a-(hydro)peroxyflavin intermediate to catalyze the oxygenolytic cleavage of the carbon-sulfonate bond of alkanesulfonate to generate the corresponding aldehyde and sulfite product (Scheme 3.1) (47). This highly reactive flavin peroxide intermediate is thought to be stabilized and protected by an active site cysteine and the closure of a flexible loop over the active site (124, 155). The sulfite product of the alkanesulfonate monooxygenase system is then assimilated into various sulfur-containing compounds for the survival and growth of the organism (36). Sulfonate buffers and alkanesulfonates with varying carbon lengths can be utilized by the alkanesulfonate monooxygenase system as substrates (36). The ability of SsuD to utilize a broad range of substrates ensures bacteria enough alternate sulfur sources during sulfur limitation.

![Scheme 3.1. Proposed SsuD mechanism involving a C4a-peroxyflavin intermediate (37).](image)
The flavin-dependent two-component systems are considered atypical flavoproteins because the flavin cofactors are not tightly bound to proteins through covalent or non-covalent interactions. Therefore, a distinctive feature of these flavin-dependent two-component systems is the transfer of the reduced flavin from one enzyme component to the other. Although an increasing number of bacterial flavin-dependent two-component systems have been identified, the mechanism of flavin transfer has not been fully determined (60, 75, 78, 82, 87, 88, 94, 156-159, 160-164). Reduced flavin transfer could either occur through a dissociative mechanism from the reductase to the monooxygenase, or by a coupled channeling mechanism involving protein-protein interactions between the enzyme components.

It is postulated that flavin transfer occurs through a channeling mechanism in the alkanesulfonate monooxygenase system. Previous studies reported an altered kinetic mechanism for SsuE in the presence and absence of SsuD and the alkanesulfonate substrate, which was attributed to the interactions between SsuE and SsuD (81). Direct protein-protein interaction between SsuE and SsuD with the formation of a stable complex has been identified, suggesting flavin transfer occurs through a channeling mechanism (167). In order for the reduced flavin to access the active site of SsuD without undergoing non-enzymatic oxidation, the SsuD flexible loop region was thought to participate in conformational changes that assist in flavin transfer events (179). Nonetheless, there has been no direct kinetic evidence to support the channeling flavin transfer mechanism in the alkanesulfonate monooxygenase system. A method has been described to directly evaluate substrate transfer mechanism utilizing an inactive donor enzyme variant (180). Because the donor enzyme SsuE is highly unstable in the
alkanesulfonate monooxygenase system, a modified method was designed using an SsuD variant with reduced flavin affinity to kinetically discern between a dissociative or channeling mechanism for reduced flavin transfer. In addition, rapid kinetic studies were performed to investigate if the kinetics of each enzyme is affected in the presence of the other enzyme component. The reported results provide insights into the flavin transfer mechanism in the two-component alkanesulfonate monooxygenase system.
3.2 Material and Methods

3.2.1 Materials

Potassium phosphate (monobasic and dibasic), flavin mononucleotide phosphate (FMN), nicotinamide adenine dinucleotide phosphate (NADPH), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), glucose, glucose oxidase, ammonium sulfate, ampicillin, streptomycin sulfate, lysozyme, ethylenediaminetetraacetic acid (EDTA) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). Glycerol, isopropyl-β-D-thiogalactoside (IPTG) and sodium chloride were obtained from Fisher (Pittsburgh, PA). Octanesulfonate was from Fluka (Milwaukee, WI). QIAprep Spin Miniprep Kit and QIAquick PCR Purification Kit were from Qiagen (Valencia, CA). Super-competent cells *E. coli* BL21 (DE3) were from Stratagene (La Jolla, CA). Oligonucleotide primers were from Invitrogen (Carlsbad, CA).

3.2.2 Construction, expression, and purification of recombinant proteins

The Y128F/S179A SsuD variant was generated using the pET21a plasmid containing the *ssuD* gene as the template for PCR-amplification. The TAC codon for Tyr128 was replaced with TTC for Phe, and the TCA codon for Ser179 was replaced with GCA for Ala. The PCR products were purified with the QIAquick PCR Purification Kit (Stratagene, La Jolla, CA), and digested with *Dpn*I to remove parental plasmids. The SsuD variant was confirmed by sequence analysis at Davis Sequencing (Davis, CA). The vector containing the correct gene sequence for the SsuD variant was transformed into
super-competent cells *E. coli* BL21 (DE3) for protein expression. The expression and purification of the variant and wild-type SsuD proteins were performed as previously described (81).

### 3.2.3 Steady-state activity assays

The steady-state kinetic parameters for wild-type and Y128F/S179A SsuD were determined based on previously reported methods (47). Reactions were initiated by the addition of NADPH (500 μM) into a solution containing SsuD (0.2 μM), SsuE (0.6 μM), FMN (2.0 μM), and varying concentrations of octanesulfonate (5-1000 μM) in 25 mM potassium phosphate buffer, pH 7.5, 10% glycerol at 25 °C. Because of decreased enzymatic activity with the SsuD variant, assays contained a final concentration of 3.0 μM Y128F/S179A SsuD, 9.0 μM SsuE, 500 μM NADPH and 5.0 μM FMN. The sulfite product was quantified as previously described (47).

### 3.2.4 Reduced flavin affinity

A previously described spectrofluorimetric titration method was used to determine the $K_d$ values of wild-type and Y128F/S179A SsuD for reduced flavin with slight modifications (47, 81). Protein solutions were prepared in a glass titration cuvette by at least 15 cycles of evacuation followed by equilibration with ultrahigh purity argon gas. Flavin was prepared in 25 mM potassium phosphate, pH 7.5, and 10% glycerol was bubbled with ultrahigh purity argon gas for 15 min. Both protein and flavin solution were then incubated in an anaerobic glovebox for 20 min after adding glucose (20 mM) and glucose oxidase (10 Units) to remove trace amounts of dioxygen. The anaerobic FMN solution was photoreduced by two consecutive 15 min irradiations in the presence of EDTA (10 mM). For the titration of Y128F/S179A SsuD with reduced flavin, aliquots of
reduced flavin (2.63-56.42 µM) were added to a 1 mL solution of Y128F/S179A SsuD (0.5 µM) in 25 mM potassium phosphate, pH 7.5, and 10% glycerol and the fluorescence spectra were recorded following a 5-minute-incubation after each addition. The addition of reduced flavin was performed in an anerobic glovebox to ensure anaerobiosis. Protein samples were exited at 280 nm and the emission intensity recorded at 344 nm on a Perkin-Elmer LS 55 luminescence spectrometer (Palo Alto, CA). The $K_d$ value was determined as previously described (47).

3.2.5 Rapid reaction kinetic analysis

Stopped-flow kinetic analyses were carried out using an Applied Photophysics SX. 18 MV stopped-flow spectrophotometer (47). All stopped-flow experiments were carried out in single-mixing mode at 4 °C by mixing equal volumes of the solutions, and monitoring the reactions by single wavelength analyses at 450 nm.

To investigate if the kinetics of each enzyme is affected in the presence of the other enzyme component, stopped-flow experiments with varying concentrations of substrates were performed. For the evaluation of the effect of SsuD and octanesulfonate on SsuE catalysis, wild-type SsuD (35 µM), SsuE (35 µM), and FMN (25 µM) in 25 mM potassium phosphate, pH 7.5, 10% glycerol were mixed against NADPH (250 µM) and octanesulfonate (0-1000 µM) in air-equilibrated 10 mM Tris-HCl buffer, pH 8.5, 10% glycerol. For the evaluation of the effect of SsuD and dioxygen on SsuE catalysis, wild-type SsuD (35 µM), SsuE (35 µM), and FMN (25 µM) in anaerobic 25 mM potassium phosphate, pH 7.5, 10% glycerol were mixed against NADPH (250 µM), octanesulfonate (250 µM) and dioxygen (0-60%) in anaerobic 10 mM Tris-HCl buffer, pH 8.5, 10% glycerol buffer. Control experiments were performed by mixing SsuE (35
μM) and FMN (25 μM) in 25 mM potassium phosphate, pH 7.5, 10% glycerol with NADPH (60-250 μM) and octanesulfonate (250 μM) in air-equilibrated 10 mM Tris-HCl buffer, pH 8.5, 10% glycerol. The single wavelength traces of flavin reduction by SsuE at 450 nm were fitted to the following equations using KaleidaGraph software (Abelbeck Software, Reading, PA):

\[ A = A_1 e^{(-k_1 t)} + A_2 e^{(-k_2 t)} + C \]

where \( k_1 \) and \( k_2 \) are the apparent rate constants for the different phases, \( A \) is the absorbance at time \( t \), \( A_1 \) and \( A_2 \) are amplitudes of each phases, and \( C \) is the absorbance at the end of the reaction.

Stopped-flow experiments were performed to investigate the rapid reaction kinetics of Y128F/S179A SsuD. SsuE (30 μM), Y128F/S179A SsuD (30 μM), and FMN (25 μM) in 25 mM potassium phosphate, pH 7.5, 10% glycerol were mixed against NADPH (250 μM) and octanesulfonate (250 μM) in air-equilibrated 10 mM Tris-HCl buffer, pH 8.5, 10% glycerol. In order to evaluate the flavin transfer mechanism between SsuE and SsuD, stopped-flow experiments were performed by mixing SsuE (30 μM), wild-type SsuD (30 μM), Y128F/S179A SsuD (0-30 μM) and FMN (25 μM) in 25 mM potassium phosphate, pH 7.5, 10% glycerol against NADPH (250 μM) and octanesulfonate (250 μM) in air-equilibrated 10 mM Tris-HCl buffer, pH 8.5, 10% glycerol. The kinetic traces of flavin oxidation in the presence of both wild-type and varying concentration of variant SsuD were compared to the kinetic trace of flavin oxidation in the presence of wild-type SsuD alone.
3.3 Results

3.3.1 Rapid reaction kinetics of flavin reduction and oxidation

In the alkanesulfonate monooxygenase system, the flavin reductive and oxidative half-reactions occur on separate enzymes, and there is a defined separation between these two events (155). In steady-state kinetic analysis, the SsuE mechanism was altered in the presence of SsuD and octanesulfonate, suggesting that SsuD affects flavin reduction possibly by forming protein-protein interactions with SsuE (81). Although reductive half-reaction is linked with the oxidative half-reaction under steady-state conditions, whether the flavin reduction by SsuE and oxidation by SsuD affect each other under pre-steady-state conditions have never been investigated.

In order to evaluate if SsuE is affected by SsuD in catalysis under single-turnover conditions, stopped-flow kinetic analyses were performed monitoring flavin reduction by SsuE in the presence of SsuD with varying octanesulfonate and dioxygen substrates. In the presence of SsuD and octanesulfonate, the onset of flavin reduction by SsuE was initiated earlier with increasing concentrations of octanesulfonate (Figure 3.1). This change was shown to be dependent on the octanesulfonate concentration. When these kinetic traces of flavin reduction were fitted, the rates were not significantly altered. The rate of flavin reduction was 8.8 s\(^{-1}\) for the kinetic trace without octanesulfonate, while the rate of flavin reduction was 12.1 s\(^{-1}\) for the kinetic trace with 1000 μM octanesulfonate. A control experiment in the absence of SsuD showed that kinetic traces monitoring flavin
reduction were unchanged with varying concentrations of octanesulfonate, confirming that octanesulfonate alone has no influence on SsuE catalysis (data not shown). These results suggest that the initiation of flavin reduction by SsuE starts faster in the presence SsuD and octanesulfonate. Interestingly, in the presence of SsuD, dioxygen had similar effect on flavin reduction by SsuE compared to octanesulfonate, further suggesting that the oxidative half-reaction by SsuD has an impact on the reductive half-reaction by SsuE (Figure 3.2). The ability of the SsuD enzyme to affect flavin reduction by SsuE under single turnover conditions suggests physical contacts may exist between the two components during catalysis.

In order to evaluate if SsuD is affected by SsuE in catalysis under single-turnover conditions, stopped-flow kinetic analyses were performed monitoring flavin oxidation by SsuD in the presence of SsuE with varying concentrations of NADPH. The flavin oxidation started earlier with lower concentrations of NADPH, and the flavin stayed reduced for a longer period of time with the increasing concentration of NADPH (Figure 3.3). The lag phase separating the reductive and oxidative half-reactions was not apparent with low concentration of NADPH. This lag phase was previously thought to represent flavin transfer/conformational changes events where the flavin remains reduced (155). The requirement of high NADPH concentration for the apparent lag phase suggests that flavin is being constantly reduced by SsuE and equilibrium between flavin reduction and flavin oxidation is reached in the lag phase with an excess of NADPH in the reaction. Due to the absence of the apparent lag phase, it is hard to fit the kinetic traces and difficult to distinguish flavin oxidation by SsuD and flavin transfer/conformational
changes events. As a result, it is unclear whether the oxidative half-reaction catalyzed by SsuD catalysis is affected by the catalysis of SsuE or not.
Figure 3.1. Rapid reaction kinetics of flavin reduction by SsuE in the presence of SsuD and varying octanesulfonate concentration. Stopped-flow kinetic traces monitoring reductive half reaction were followed at 450 nm and 4 °C after mixing wild-type SsuD (35 μM), SsuE (35 μM), and FMN (25 μM) against NADPH (250 μM) and octanesulfonate (0 μM ●, 50 μM ■, 200 μM ◆ and 1000 μM ▲) in aerobic buffer.
Figure 3.2. Rapid reaction kinetics of flavin reduction by SsuE in the presence of SsuD and varying dioxygen concentration. Stopped-flow kinetic traces were followed at 450 nm and 4 °C after mixing wild-type SsuD (35 μM), SsuE (35 μM), and FMN (25 μM) against NADPH (250 μM), octanesulfonate (250 μM) and dioxygen (0% ●, 30% ■ and 60% ▲) in anaerobic buffer.
Figure 3.3. Rapid reaction kinetics of flavin reduction by SsuE and flavin oxidation by SsuD with varying NADPH concentration. Stopped-flow kinetic traces were followed at 450 nm and 4 °C after mixing wild-type SsuD (35 μM), SsuE (35 μM), and FMN (25 μM) against NADPH (60 μM ●, 90 μM ○, 120 μM ■, 150 μM □, 180 μM ◆, 210 μM ◊ and 250 μM ▲) and octanesulfonate (250 μM) in aerobic buffer.
3.3.2 Kinetic parameters of Y128F/S179A SsuD

In order to discern between a dissociative and a channeling flavin transfer mechanism in the alkanesulfonate monooxygenase system, a method was developed requiring an SsuD variant with decreased activity and reduced flavin affinity. If the flavin transfer occurs through a dissociative mechanism, the SsuD variant will not compete with wild-type enzyme for reduced flavin. Due to the reduced FMNH$_2$ affinity of the SsuD variant, the FMNH$_2$ will only diffuse into wild-type SsuD when both wild-type and variant SsuD are present. When comparing flavin oxidation in the presence of wild-type SsuD with or without the variant SsuD, the kinetic trace representing flavin oxidation would not change if the flavin is transferred dissociatively. On the other hand, if the flavin transfer occurs through a channeling mechanism, the reduced flavin will be actively transferred within a transient SsuE-SsuD complex. The SsuD variant is still able to compete with wild-type SsuD for the interaction site(s) on SsuE despite its low affinity for reduced flavin. Therefore, a portion of reduced flavin will not be directly transferred to wild-type enzyme in the presence of both wild-type and variant SsuD. A channeling flavin transfer mechanism would result in an altered flavin oxidation kinetic trace when the SsuD variant is included in the reaction mixture containing wild-type SsuD. In conclusion, this variant competition method is able to demonstrate the flavin transfer mechanism by comparison of the flavin oxidation in the presence of wild-type SsuD with or without the variant.

The crystal structures of bacterial luciferase and LadA with FMN bound revealed a tyrosine and serine residue within hydrogen bonding distance with the FMN phosphate oxygens (116, 123). Based on the three dimensional structure, the SsuD active site
architecture is similar to the active site of bacterial luciferase and LadA (121). The phosphate oxygens of LadA FMN modeled in the SsuD active site were shown to be within hydrogen bonding distance to Tyr128 and Ser179 (Figure 3.4). Therefore active site residues Tyr128 and Ser179 from SsuD are speculated to be involved in flavin binding. The ability of these amino acid residues to coordinate the reduced flavin substrate was examined by substituting Tyr128 with phenylalanine and Ser179 with alanine by site-directed mutagenesis. The dissociation constant ($K_d$) for the binding of reduced flavin to Y128F/S179A SsuD was determined by fluorimetric titrations (Table 3.1). Compared to wild-type SsuD, the $K_d$ value for reduced flavin binding to Y128F/S179A SsuD was found to be approximately 20-fold higher (Table 3.1), suggesting that Tyr128 and Ser179 plays a role in coordinating the reduced flavin substrate. The steady-state kinetic parameters of Y128F/S179A SsuD showed a 5-fold decrease $k_{cat}$ value. The $K_m$ value for octanesulfonate was increased nearly 7-fold. These changes in the kinetic parameters resulted in a 40-fold decrease in the $k_{cat}/K_m$ value for the Y128F/S179A SsuD variant compared to wild-type (Table 3.1). These combined results suggest that the Y128F/S179A SsuD variant cannot catalyze the desulfonation reaction efficiently due to its reduced FMNH$_2$ affinity.

In order to evaluate ability of Y128F/S179A SsuD to oxidize reduced flavin under pre-steady-state conditions in the presence of SsuE, stopped-flow analyses monitoring flavin reduction and oxidation at 450 nm were performed. Distinctive differences were observed between kinetic traces representing flavin oxidation by Y128F/S179A and wild-type SsuD (Figure 3.5). In the kinetic trace obtained with wild-type SsuD, the apparent lag phase was observed, suggesting the rate of flavin reduction and oxidation
Figure 3.4. Three-dimensional structures of SsuD active site with FMN modeled in. The tyrosine 128 and serine 179 are shown in hydrogen bonding distance with the phosphate group of FMN. The structure was generated and rendered with PyMOL (PDB ID:1M41) (121).
### Table 3.1. Kinetic parameters for Y128F/S179A and wild-type SsuD.

<table>
<thead>
<tr>
<th></th>
<th>$k_{\text{cat}}$ (min^{-1})</th>
<th>$K_{m}$ (μM)</th>
<th>$k_{\text{cat}}/k_{\text{cat}}$ (μM min^{-1})</th>
<th>$k_d(\text{FMNH}_2)$ (^a) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type SsuD (^b)</td>
<td>51.7 ± 2.1</td>
<td>44.0 ± 8.3</td>
<td>1.2 ± 0.2</td>
<td>0.30 ± 0.15</td>
</tr>
<tr>
<td>Y128F/S179A SsuD</td>
<td>8.6 ± 1.6</td>
<td>294.8 ± 55.9</td>
<td>0.03 ± 0.006</td>
<td>5.43 ± 0.60</td>
</tr>
</tbody>
</table>

\(^a\) Determined under anaerobic conditions as described in Material and Methods

\(^b\) Previously reported (47)
reached equilibrium with excess NADPH. In the control experiment without SsuD, the flavin reduced by SsuE was auto-oxidized by the dioxygen in solution immediately following flavin reduction (Figure 3.5, ◆). It is reported that the rate of flavin auto-oxidation is $1.43 \text{ S}^{-1}$ while the rate of enzymatic flavin oxidation by SsuD is $0.16 \text{ S}^{-1}$ (47). Therefore, no apparent lag phase was observed in the control experiment without SsuD because the rate of non-enzymatic flavin auto-oxidation is faster than the rate of flavin reduction by SsuE and the equilibrium cannot be reached. Similar to the control experiment in the absence of SsuD, the kinetic trace obtained with Y128F/S179A SsuD did not contain the apparent lag phase (Figure 3.5, ○). The kinetic trace of flavin oxidation by Y128F/S179A SsuD lies in between the kinetic trace obtained with wild-type SsuD and the kinetic trace without SsuD, resembling a mixture of enzymatic flavin oxidation and non-enzymatic flavin oxidation. These results suggest that Y128F/S179A SsuD cannot utilize the reduced flavin efficiently due to decreased affinity, which interrupts the flavin transfer event to SsuD.

3.3.3 Evaluation of flavin transfer mechanism

Flavin transfer can occur either through a dissociation mechanism or a channeling mechanism in the alkanesulfonate monooxygenase system. In order to kinetically discern between the two mechanisms, a variant competition method was developed by including Y128F/S179A SsuD in single turnover reactions monitoring flavin reduction by SsuE and the subsequent oxidation of the flavin by wild-type SsuD. When varying concentrations of Y128F/S179A SsuD (10-30 μM) were added in the reaction mixture of SsuE (30 μM), wild-type SsuD (30 μM), and FMN (25 μM) under single turnover condition, altered flavin oxidation traces were observed (Figure 3.6 A). The flavin oxidation was delayed in
the presence of both wild-type and Y128F/S179A SsuD compared to flavin oxidation by wild-type SsuD alone. The observed delay in flavin oxidation corresponds to a longer lag phase between flavin reduction and oxidation, which increased with increasing concentration of Y128F/S179A SsuD (Figure 3.6 B). Because this lag phase is thought to represent the length of time in which the rate of flavin reduction is in equilibrium with the rate of flavin oxidation during flavin transfer, a longer lag phase would suggest that the flavin transfer event is prolonged with the addition of Y128F/S179A SsuD. Based on the results from the completion method, the Y128F/S179A SsuD is able to effectively compete with wild-type SsuD during catalysis, strongly supporting a channeling flavin transfer mechanism in alkanesulfonate monooxygenase system.
Figure 3.5. Comparison of enzymatic flavin oxidation by wild-type SsuD, Y128F/S179A SsuD, and non-enzymatic flavin oxidation. Kinetics traces following the reduced flavin oxidation at 450 nm and 4 °C after mixing SsuE (30 μM), FMN (25 μM), and wild-type SsuD (◆ 30 μM) or Y128F/S179A SsuD (■ 30 μM) or buffer (●) against octanesulfonate (250 μM) and NADPH (250 μM). The kinetic traces shown represent an average of three separate experiments.
Figure 3.6. The effect of varying concentration of Y128F/S179A SsuD on the flavin oxidation by wild-type SsuD. A: Kinetics traces following the flavin reduction and oxidation at 450 nm and 4 °C after mixing SsuE (30 μM), FMN (25 μM), wild-type SsuD (30 μM) and Y128F/S179A SsuD (0 μM ●, 10 μM ■, 20 μM ◆ and 30 μM ▲) against octanesulfonate (250 μM) and NADPH (250 μM). The kinetic traces shown represent an average of three separate experiments. B: Kinetic traces of flavin oxidation from A.
3.4 Discussion

The alkanesulfonate monooxygenase system is a two-component flavin-dependent enzyme system that utilizes the flavin cofactor as a substrate instead of a covalently or tightly bound prosthetic group. The two-component flavin-dependent systems rely on a flavin reductase to provide reduced flavin to the monooxygenase enzyme for activity. In addition, the genes expressing the two enzyme components are often located in the same operon, indicating a close cooperative relationship between the two enzymes (42). A mechanistic challenge for these two-component monooxygenase systems is the transfer of reduced flavin from the flavin reductase to the monooxygenase (94, 165).

In the flavin-dependent two-component systems that have been studied, the flavin transfer event can either occur through a dissociative mechanism or a channeling mechanism. In the dissociative mechanism, the reduced flavin is released from the reductase and passively diffuses into the cellular environment, followed by binding to the monooxygenase. This entire free diffusion flavin transfer process is performed without protein-protein interaction between the two components. Typically, the flavin reductases have a higher affinity for the oxidized flavin, while the monooxygenases have a higher affinity for the reduced flavin (47, 94). The preference of the reduced flavin binding to the monooxygenase ensures that the flavin once reduced will be rapidly transferred. The dissociative flavin transfer mechanism has been reported in several two-component systems (156-159, 160, 163). In these systems, direct protein-protein interactions were
not observed by kinetic, fluorescence and affinity chromatography studies (156-159, 160, 161). Free diffusion has been proposed as the mechanism of flavin transfer in bacterial luciferase system because in vitro analyses have demonstrated that bacterial luciferase can utilize reduced flavin supplied by different flavin reductases (78, 82, 87, 88, 160). However, this argument is based on the hypothesis that these flavin reductases are not structurally related. The possibility of these flavin reductases containing similar common structural motifs cannot be eliminated without evaluating the three dimensional structures of different flavin reductases. If these flavin reductases contain similar structural motifs that are able to interact with bacterial luciferase, a channeling flavin transfer mechanism becomes possible between bacterial luciferase and these flavin reductases. Even though the reduced flavin is able to diffuse rapidly from the reductases to the monooxygenases, it can also be oxidized rapidly in solution generating reactive dioxygen species leading to the damage of cellular components. Therefore, it is speculated by a few groups that the channeling flavin transfer mechanism would be favored in cellular systems (75, 81, 162-164, 167). The channeling mechanism is defined as the reduced flavin being actively transferred through a molecular channel within a protein complex formed between the reductase and monooxygenase. A substrate channeling mechanism for flavin transfer has also been reported in bacterial luciferase system through biochemical and biophysical analysis (75, 162-164). Stable protein-protein complex formation was manifested between monomeric bacterial luciferase and FRP through fluorescence anisotropy studies in Vibrio harveyi (164). Results from kinetic analyses also demonstrated that FRP exhibited a ping-pong kinetic pattern in a single-enzyme spectrophotometric assay monitoring the NADPH oxidation, while the kinetic mechanism changed to a sequential
pattern in a luciferase-coupled assay measuring light emission (162). As a result, a channeling flavin transfer mechanism between bacterial luciferase and FRP was proposed in *Vibrio harveyi*. In addition, a novel fusion enzyme of FRP and bacterial luciferase was generated and characterized (165). This fusion enzyme closely mimics a single-component bifunctional monooxygenase. In the coupled bioluminescence reaction, the total quantum outputs observed with the fusion enzyme without exogenously added FMN was higher than the case with 25 μM FMN added, suggesting that the bioluminescence activity of the fusion enzyme relies on a shuffling of the FRP-bound-FMN between the reductase and luciferase within the fusion protein. Therefore, it is speculated that a channeling flavin transfer mechanism would be favored *in vivo* in order for the two-component bacterial luciferase systems to achieve maximum efficiency.

A channeling flavin transfer mechanism has also been proposed in the two-component alkanesulfonate monooxygenase system. Flavin reduction by SsuE has been reported to switch from an ordered sequential mechanism to a rapid equilibrium ordered mechanism in the presence of SsuD and octanesulfonate (81). In addition, SsuE also showed a 10-fold increase in $K_m$ value for FMN with the addition of SsuD and octanesulfonate. The altered mechanism for SsuE suggested that the SsuE catalysis is influenced by the SsuD under steady-state conditions. The increased $K_m$ value for FMN may indicate that the flavin reduction by SsuE is synchronized with SsuD catalysis to avoid the production of excess reduced flavin. Static protein-protein interaction was also identified between SsuE and SsuD by chemical cross-linking, visible CD spectroscopy and affinity chromatography (167). In addition, the dissociation constant for SsuD
binding to SsuE was determined to be 0.0022 ± 0.0010 μM by fluorescence spectroscopy (167). If these physical contacts between SsuE and SsuD exist during catalysis, it is consistent with the observation of an altered SsuE mechanism in the presence of SsuD and a proposed channeling flavin transfer mechanism in the two-component alkanesulfonate monooxygenase system.

Even though the flavin transfer mechanism for bacterial luciferase system is still under debate and a channeling mechanism may occur in both bacterial luciferase system and alkanesulfonate monooxygenase system, how the flavin is actively delivered within the protein complex may differ in these two-component systems. An unresolved mobile loop was observed above the active site of both bacterial luciferase and SsuD (121, 122). Deletion of the bacterial luciferase loop region resulted in the flavin being effectively transferred in in vivo studies, suggesting the mobile loop of bacterial luciferase does not participate in flavin transfer (119). However, the SsuD mobile loop of bacterial luciferase was suggested to not only induce essential conformational changes important for catalysis but also play a dual role in assisting in flavin transfer (179).

In order to further evaluate the flavin transfer mechanism in alkanesulfonate monooxygenase system, rapid reaction kinetic studies were performed to investigate if the kinetics of each enzyme was affected in the presence of the other enzyme under pre-steady-state conditions. In the presence of SsuD and octanesulfonate substrate, the onset of flavin reduction by SsuE was initiated earlier with higher octanesulfonate concentration. It was previously established that octanesulfonate is not a substrate of SsuE and does not form any interactions with SsuE (81). It is speculated that higher octanesulfonate concentrations increase the rate of conformational changes of SsuD and
promotes a more rapid and effective communication between SsuE and SsuD, which enhances the onset flavin reduction by SsuE. This change was shown to be dependent on octanesulfonate concentration. A control experiment without SsuD showed that the flavin reduction was not affected by octanesulfonate alone. These results suggest that the dynamics of SsuD affect the catalysis of SsuE. Rapid reaction kinetic analyses evaluating the effect of dioxygen on the catalysis of SsuE and SsuD also suggested that the onset of flavin reduction by SsuE starts earlier with higher concentration of dioxygen, further suggesting that dioxygen-induced conformational changes of SsuD has an impact on the reductive half-reaction by SsuE. Interestingly, this impact by dioxygen was not as significant as octanesulfonate, indicating the SsuD conformational change induced by dioxygen may not be as substantial as the one induced by octanesulfonate. When the effect of SsuE catalysis on flavin oxidation by SsuD was evaluated, flavin oxidation was initiated earlier with lower concentration of NADPH. Because the lag phase between the reductive and oxidative half-reactions was not apparent in the kinetic traces with lower concentrations of NADPH, it is hard to distinguish and describe the flavin transfer and oxidation. Therefore, it is uncertain if oxidative half reaction by SsuD is influenced by SsuE. Nevertheless, the overall results of these studies were sufficient to conclude that the conformational changes of SsuD induced by its substrate influence the flavin reduction by SsuE, indicating protein-protein interaction and communication exist between SsuE and SsuD during catalysis. The structural evidences of static protein-protein interactions and the kinetic evidences under both steady-state and pre-steady-state conditions strongly suggest a channelling flavin transfer mechanism in the alkanesulfonate monooxygenase system.
A novel method has been described to directly evaluate the substrate transfer mechanism between the donor enzyme and the acceptor enzyme (180). In the original method, an inactive variant of the donor enzyme was generated to compete with the wild-type donor enzyme in coupled activity assays. If the activity of the acceptor enzyme is decreased with increasing concentrations of the inactive donor variant, less substrate is transferred from the active donor enzyme to the acceptor enzyme because more complex is formed between the inactive donor variant and the acceptor enzyme, indicating a channeling substrate transfer mechanism. If the activity of the acceptor enzyme is not affected by the addition of the inactive donor variant, all substrate produced by the active donor enzyme can efficiently diffuse into the acceptor enzyme, indicating a dissociative substrate transfer mechanism.

Even though a channeling flavin transfer mechanism in the alkanesulfonate monooxygenase system is indicated by analyzing flavin reduction and static protein-protein interaction, a direct evaluation of the flavin transfer event during catalysis has not been performed. Therefore, a variant completion method was utilized to directly study the flavin transfer during catalysis. Because the donor SsuE enzyme in the alkanesulfonate monooxygenase system is highly unstable, the inactive SsuE variants generated were susceptible to aggregation and precipitation during the purification process (data not shown). Thus, a modified method was developed by using an SsuD variant with a decreased affinity for reduced flavin. Modeling the flavin coordinates in the flavin-bound LadA three-dimensional structure into SsuD places Tyr128 and Ser179 in a position that coordinates the phosphoryl group of flavin. The Y128F/S179A SsuD variant was generated as the candidate with decreased flavin affinity for the evaluation of
the flavin transfer mechanism. The perturbed flavin binding ability and the decreased catalytic efficiency of Y128F/S179A SsuD suggested the ability of this variant to catalyze the desulfonation reaction was greatly weakened due to its decreased reduced flavin affinity. In the rapid reaction kinetic analysis, distinct differences were observed between the flavin oxidation by Y128F/S179A or wild-type SsuD. Similar to the control of non-enzymatic flavin oxidation, the kinetic trace obtained with Y128F/S179A SsuD did not show an apparent lag phase. The kinetic trace of flavin oxidation by Y128F/S179A SsuD resembled a mixture of enzymatic flavin oxidation and non-enzymatic flavin oxidation, suggesting that some reduced flavin is oxidized non-enzymatically. This non-enzymatic flavin oxidation could be due to the reduced flavin not being properly transferred between the SsuE and Y128F/S179A SsuD variant. The flavin reduced by SsuE is likely to be oxidized by dioxygen in SsuE or in solution because of the impaired flavin binding ability of Y128F/S179A SsuD. Therefore, Y128F/S179A SsuD could serve as a proper inactive variant to investigate flavin transfer through designed competition methods.

In order to kinetically discern between the two mechanisms, reduced flavin oxidation by wild-type SsuD was monitored under single turnover conditions in the presence of different concentrations of Y128F/S179A SsuD. If the flavin transfer occurs through a dissociation mechanism, the flavin reduced by SsuE should dissociate into solution. Because wild-type SsuD has a 20-fold higher reduced flavin affinity than Y128F/S179A SsuD, the reduced flavin in solution should only be diffused into wild-type SsuD when both enzymes are present. As a result, flavin oxidation by wild-type SsuD should not be affected in the presence of Y128F/S179A SsuD. If flavin transfer occurs through a
channeling mechanism, the reduced flavin should be actively transferred within a transient SsuE-SsuD complex. Because Y128F/S179A SsuD will compete with wild-type SsuD for the interaction site(s) on SsuE, a portion of the reduced flavin will not be directly transferred to wild-type SsuD. It was demonstrated that the kinetic trace monitoring reduced flavin oxidation by Y128F/S179A SsuD does not contain the lag phase observed in flavin oxidation by wild-type SsuD. Therefore, the overall kinetic trace of flavin oxidation should be altered with both wild-type and Y128F/S179A SsuD included in the reaction if the flavin transfer occurs through a channeling mechanism.

When varying concentrations of Y128F/S179A SsuD were included in the reaction mixture of SsuE, wild-type SsuD, and FMN under single turnover condition, the flavin oxidation trace was altered. The change in flavin oxidation induced by Y128F/S179A SsuD was also shown to be dependent on the variant concentration. Interestingly, a longer lag phase was observed for the altered flavin oxidation in the presence of Y128F/S179A SsuD. When the transient SsuE-Y128F/S179A SsuD complex forms, the reduced flavin may not be transferred properly and is retained in SsuE after the dissociation of the SsuE-Y128F/S179A SsuD complex due to the decreased flavin affinity of the variant. Because there was unoccupied free wild-type SsuD in solution, the SsuE-reduced flavin could reassociates with wild-type SsuD to transfer reduced flavin to wild-type SsuD for oxidation (Figure 3.7). The longer lag phase is believed to be caused by this protein-protein equilibrium complex. These results suggest that a transient SsuE-SsuD complex is formed during catalysis, strongly supporting a channeling mechanism for flavin transfer.
Figure 3.7. Schematic representation of flavin reduction and oxidation process with the presence of SsuE, wild-type SsuD and Y128F/S179A SsuD. In the presence of both wild-type (green circle) and Y128F/S179A SsuD (grey circle), the SsuE (blue square) is able to form protein-protein complex with both SsuD after reducing FMN (yellow triangle). In the SsuE/wild-type SsuD complex, the FMNH$_2$ is transferred directly to wild-type SsuD for enzymatic oxidation. In the SsuE/Y128F/S179A SsuD complex, the FMNH$_2$ is not transferred and retained in SsuE due to the reduced affinity of Y128F/S179A SsuD for FMNH$_2$. The FMNH$_2$ bound SsuE then dissociates Y128F/S179A SsuD and re-associates with unoccupied wild-type SsuD in solution, followed by enzymatic oxidation. The resulted flavin oxidation in the presence of both wild-type and Y128F/S179A SsuD is a mixture of a normal and a slowed enzymatic flavin oxidation.
The flavin cofactors play an important role in biological systems due to their ability to assume versatile redox states. A large number of flavoproteins fulfill their functions by relying on the flavin cofactor to transfer electrons. The reduced flavin is extremely reactive toward molecular dioxygen, generating H$_2$O$_2$ and reactive oxygen species that are damaging to cellular components. In order to eliminate the production of these unwanted toxic compounds, the intracellular concentration of free reduced flavin should be cautiously controlled. In the flavin-dependent two-component systems, the reduced flavin is transferred from the reductase to the monooxygenase. Alternating flavin transfer mechanisms have been reported depending on the specific system. For the two-component systems that utilize a dissociative flavin transfer mechanism, the reduced flavin diffusion occurs faster than the reaction with free dioxygen in solution. Therefore, the production of damaging reactive oxygen species can be avoided. For the two-component systems that utilizing a channeling flavin transfer mechanism, transient complex is formed between the reductase and the monooxygenase. As a result, the reduced flavin is protected from the bulk solvent in the complex. The evidences provided in this study thoroughly supported a channeling flavin transfer mechanism in the alkanesulfonate monooxygenase system, and explains how the reduced flavin is protected from non-enzymatic oxidation during the desulfonation reaction.
Chapter 4
Summary

The flavin-dependent two-component alkanesulfonate monooxygenase system from *E. coli* is composed of a NAD(P)H-dependent FMN reductase (SsuE) and an FMNH$_2$-dependent alkanesulfonate monooxygenase (SsuD). In the presence of NAD(P)H, the SsuE enzyme catalyzes the reduction of FMN to FMNH$_2$. In the presence of O$_2$ and FMNH$_2$, the SsuD enzyme is capable of cleaving the C-S bond of a wide range of 1-substituted alkanesulfonates, resulting in the liberation of sulfite and production of a corresponding aldehyde (36, 47). The SsuD enzyme is proposed to catalyze the desulfonation reaction through a C4a-(hydro)peroxyflavin intermediate. Although an unresolved loop region above the SsuD active site was suggested to protect the flavin peroxide intermediate, direct characterization of this unresolved loop region has not been performed (155). In addition, how SsuD mechanistically protect and stabilize the reactive intermediate has not been fully addressed. A distinct feature of the two-component alkanesulfonate monooxygenase system is that the reduced flavin supplied by SsuE is transferred to SsuD for the desulfonation reaction. Although a channeling flavin transfer mechanism has been suggested with the identification of static SsuE-SsuD interaction, the evidence was not enough to conclude that the flavin transfer occurs though a channeling mechanism during catalysis. This research has focused on elucidating the functional role of the SsuD unresolved loop region, as well as the flavin
transfer mechanism during catalysis with a combination of biophysical, biochemical and kinetic approaches.

4.1 Catalytic mechanism of the alkanesulfonate monooxygenase

In the two-component systems, the flavin-dependent monooxygenase usually catalyze a variety of reactions in three steps: 1) utilizing reduced flavin supplied by a flavin reductase; 2) activating dioxygen to form a C4a-(hydro)peroxyflavin intermediate; 3) oxygenating the substrate. The initial characterization of SsuD has suggested an ordered substrate binding mechanism with the reduced flavin binding first and octanesulfonate binding last (47). In addition, each substrate binding event is likely to be associated with conformational changes (47, 155). In the proposed mechanism of SsuD, a C4a-peroxyflavin intermediate performs a nucleophilic attack on the sulfonate functional group. The alkanesulfonate peroxyflavin adduct then undergoes Baeyer-Villiger rearrangement and proton abstraction from an active site base, leading to the generation of hydroxyflavin, alkanealdehyde and sulfite. This mechanism is supported by the identification of a C4a-(hydro)peroxyflavin intermediate through rapid reaction kinetic analysis. The active site Cys54 residue was suggested to directly or indirectly stabilize the flavin peroxide intermediate by hydrogen bonding (124). Based on site-directed mutagenesis studies and kinetic characterization, the active site Cys54 was considered to not directly catalyze the desulfonation reaction as an active site base. In addition, the evaluation of active site His228 residue also suggested that it is not the catalytic base (data not shown). Although pH profile of SsuD suggest that an amino acid must be deprotonated in order to achieve the optimal activity, the identity of the catalytic base still remains unclear (data not shown).
4.2 Functional role of the SsuD unresolved loop region in catalysis

In the three-dimensional structure of SsuD, a conserved loop region located above the putative active site was unresolved in an insertion region that deviates from TIM-barrel core, suggesting mobility of this loop region (121). In the initial characterization, a highly conserved Arg297 on this insertion region was speculated to involve in catalysis due to the diminishing enzymatic activity with an aberrant mutation of Arg297→Cys (121). It was speculated that the Cys mutation at position 279 would form a disulfide bond with the Cys54 located in the active site, sacrificing the mobility of the unresolved loop region and resulting in the abolished activity (121). Further site-directed mutagenesis studies, biochemical and kinetic analysis of the Arg297 residue suggested that this conserved residue plays an important role in the desulfonation reaction, and confirmed that the mobility of the unresolved loop is crucial to the catalysis (155). The substitution of Arg297 with Lys or Ala did not significantly alter the secondary structural components or the substrate affinity. The R297A and R297K SsuD showed impaired catalytic activity and were unable to accumulate critical C4a-(hydro)peroxyflavin intermediate, indicating that Arg297 is required for the protection and stabilization of the reactive flavin peroxide intermediate (155). Studies evaluating proteolytic susceptibility of the unresolved loop region of wild-type SsuD and the Arg297 variants suggested that the SsuD Arg297 facilitates necessary conformational changes induced by reduced flavin binding (155). These conformational changes correlate with loop closure and are suspected to protect the intermediate and promote catalysis.
Three variants containing a partial deletion of the unresolved loop region were constructed to directly evaluate the functional properties of this region. Although the secondary structures and the substrate affinity of all three loop-deleted SsuD variants were not significantly altered, the catalytic activity toward sulfite production was completely abolished, suggesting an absolute requirement of this loop region for catalysis. Further near-UV CD spectroscopic studies evaluating the reduced flavin binding environment in the active site of loop-deleted SsuD variants demonstrated that the SsuD unresolved loop region is important in stabilizing the flavin environment of SsuD in order to facilitate catalysis. In addition, proteolytic susceptibility experiments on the loop-deleted SsuD variants suggested that the loop closure requires the full-length loop region, and a shortened loop region would not induce the requisite conformational changes necessary for catalysis. The rapid reaction kinetic analysis in the presence of both SsuE and loop-deleted SsuD variants suggested that the reduced flavin was oxidized non-enzymatically due the lack of protection from the unresolved loop. Even though the static protein-protein interactions were not compromised between SsuE and loop-deleted SsuD variants, the slower onset of flavin reduction and immediate flavin oxidation in the presence of loop-deleted SsuD variants implied that the unresolved loop region may be involved in promoting flavin reduction by SsuE and flavin transfer from SsuE to SsuD during catalysis. Therefore, dual roles of inducing essential conformational changes and assisting flavin transfer were proposed for the SsuD unresolved loop region. These studies provided better understanding of the mechanistic roles of the SsuD unresolved loop region in the desulfonation reaction.
4.3 Flavin transfer mechanism in the alkanesulfonate monooxygenase system

The flavin transfer event for two-component alkanesulfonate monooxygenase system in *E. coli* has been speculated to occur through a substrate channeling mechanism. The altered reaction mechanism of SsuE in the presence of SsuD and octanesulfonate suggested that protein-protein interactions exist between the two enzyme components of alkanesulfonate monooxygenase system (81, 167). The static SsuE-SsuD interactions were later identified through several approaches including pull-down assays, chemical cross-linking and near-UV CD spectroscopy (167). Although a channeling flavin transfer mechanism has been proposed for two-component the alkanesulfonate monooxygenase system based on the static protein-protein interactions, there has been no direct kinetic evidence to support the channeling flavin transfer mechanism during the desulfonation reaction.

Therefore, rapid reaction kinetic studies were performed to investigate if the kinetics of each enzyme is affected in the presence of the other enzyme component. The onset of the flavin reduction by SsuE was initiated earlier in the presence of SsuD and substrates, suggesting that the dynamics of SsuD affect the catalysis of SsuE. It is also implied that the higher substrate concentration increases the rate of conformational changes of SsuD and promotes a more rapid and effective communication between SsuE and SsuD, resulting in an earlier onset of flavin reduction by SsuE. In order to kinetically discern between the channeling and dissociative flavin transfer in the alkanesulfonate monooxygenase system, a competition method was developed by utilizing an SsuD variant with decreased reduced flavin affinity. Based on the thee-dimensional structure similarities with related protein, SsuD active site residues Tyr128 and Ser179 were
speculated to be involved in reduced flavin binding. In order to generate an SsuD variant with reduced flavin affinity, the Y128F/S179A SsuD was generated via site-directed mutagenesis. Steady-state kinetic analysis confirms that ability of Y128F/S179A SsuD to catalyze the desulfonation reaction was weakened due to its decreased reduced flavin affinity. Based on the results from rapid reaction kinetic analysis, it is concluded that the reduced flavin can be transferred from SsuE to Y128F/S179A SsuD, while a portion of the transferred flavin is oxidized non-enzymatically due to the impaired flavin binding ability of Y128F/S179A SsuD. Therefore, Y128F/S179A SsuD could serve as a proper implement to investigate flavin transfer mechanism through the designed competition method. To directly evaluate the flavin transfer mechanism, the flavin reduction and oxidation was monitored when varying concentrations of Y128F/S179A SsuD were added to a reaction mixture containing SsuE and wild-type SsuD. The kinetic trace representing flavin oxidation was significantly altered when Y128F/S179A SsuD was added, suggesting that a transient SsuE-SsuD complex was formed and Y128F/S179A SsuD actively competed with wild-type SsuD for the interaction site(s) on SsuE. As a result, a portion of the reduced flavin was not directly transferred from SsuE to wild-type SsuD, resulting in altered flavin oxidation traces. This change in flavin oxidation induced by Y128F/S179A SsuD was also shown to be dependent on the variant concentrations, highly supporting a channeling flavin transfer mechanism. Based the altered kinetic trace of flavin oxidation trace with the addition of Y128F/S179A SsuD, a complex dissociation/re-association model was proposed. When the transient SsuE-Y128F/S179A SsuD complex forms, the reduced flavin may not be transferred properly and is retained in SsuE after the dissociation of the SsuE-Y128F/S179A SsuD complex due to the
decreased flavin affinity of the variant. Because there was unoccupied free wild-type SsuD in solution, the reduced flavin bound SsuE re-associates with wild-type SsuD and transfers the reduced flavin to wild-type SsuD for enzymatic oxidation, resulting an altered flavin oxidation trace with a prolonged lag phase. This model explains the observation of a prolonged lag phase in the flavin oxidation kinetic trace when Y128F/S179A SsuD was added to compete with wild-type SsuD, supporting complex formation and a channeling flavin transfer mechanism in the two-component alkanesulfonate monooxygenase system.

In summary, the functional role of the SsuD unresolved loop region and the flavin transfer mechanism of the two-component alkanesulfonate monooxygenase system has been evaluated in depth. Conformational changes involving the closure of the unresolved loop region play critical roles to ensure catalysis: controlling substrate binding order, protecting the reduced flavin from bulk solvent, stabilizing the reactive C4a-(hydro)peroxyflavin intermediate, and facilitating flavin transfer from SsuE to SsuD. In the two-component alkanesulfonate monooxygenase system, the flavin transfer occurs through a channeling mechanism, where the reduced flavin is actively shuffled within a transient SsuE-SsuD complex. Therefore, the system is able to strictly control the oxidation of the reactive reduced flavin and avoid production of damaging reactive oxygen species. These studies on the structural dynamics of SsuD and flavin transfer mechanism are essential to understanding the mechanism of the desulfonation reaction catalyzed by the two-component alkanesulfonate monooxygenase system.
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