EXPOSING THE PROTEIN-PROTEIN INTERACTION SITES AND PROBING THE FLAVIN TRANSFER MECHANISM IN THE ALKANESULFONATE MONOOXYGENASE SYSTEM FROM ESCHERICHIA COLI

by

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Except where reference is made to the work of others, the work described in this thesis is my own or was done in collaboration with my advisory committee.

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ABSTRACT

Sulfur is an essential element for all living organisms. Bacteria acquire sulfur through a sulfur assimilation pathway, but under sulfur limiting conditions bacteria must acquire sulfur from alternative sources. These alternative sources of sulfur are sulfonates and sulfonate esters, which are naturally occurring compounds. The *ssu* operon is induced under sulfur limiting conditions and is comprised of genes that encode for a flavin reductase (SsuE), alkanesulfonate monooxygenase (SsuD), and alkanesulfonate transporter proteins (SsuA, SsuB and SsuC). The SsuE enzyme reduces FMN with NADPH and reduced flavin is then transferred to SsuD. The SsuD uses the reduced flavin and dioxygen to catalyze the desulfonation of diverse alkanesulfonates producing the corresponding aldehyde and sulfite. The reduced flavin is a highly unstable molecule and it readily reacts with dioxygen to form hydrogen peroxide and superoxide radicals. The two mechanisms for the transfer of reduced flavin in two-component system are free diffusion and direct channeling. Previous studies have identified stable protein-protein interactions between SsuE and SsuD that are thought to play a role in flavin transfer events. Protein-protein interactions may also influence the oligomeric state of SsuE and SsuD. Evaluating the influence of protein-protein interactions over the dynamic changes in oligomeric states of proteins and its role in transfer of reduced flavin molecule from SsuE to SsuD will help us in understanding the desulfonation mechanism carried out by this system.

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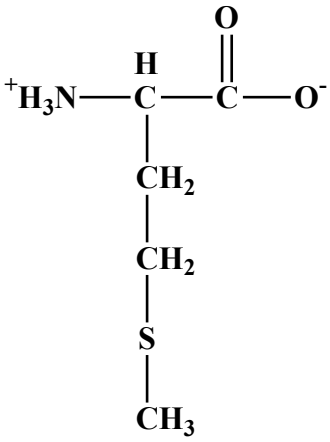
CHAPTER ONE

LITERATURE REVIEW

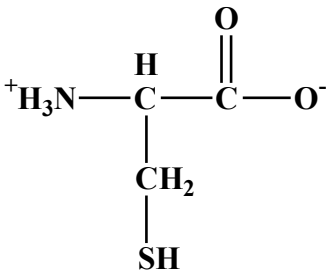
1.1 Sulfur assimilation in bacteria

Bacteria require sulfur for cell growth. Sulfur exists primarily as a component of cysteine and methionine incorporated into proteins Figure 1.1). In addition to proteins, sulfur is an important part of many cofactors like biotin, coenzyme A, coenzyme M, thiamine, and lipoic acid. Sulfur is important for the synthesis of naturally occurring amino acids and in the redox-active component of disulfide bonds and iron-sulfur centers. In microbes, cysteine biosynthesis by sulfate assimilation represents the pathway by which inorganic sulfur is reduced and incorporated into organic compounds (8, 9).

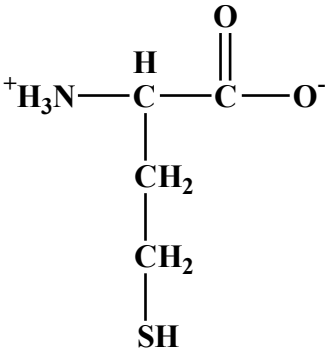
In the sulfur assimilation pathway the sulfate and thiosulfate are transported into the cell by the ATP binding-cassette (ABC)-type transporter proteins (Figure 1.2). This transport occurs with the help of sulfate permease (CysU, CysW, CysA, and sulfur binding proteins) (10). Sulfate permease is a membrane bound protein that assists in the transport of sulfate inside the cell. For sulfur assimilation sulfate must be activated to adenosine 5'-phosphosulfate (APS). In this step sulfate is linked to a phosphate residue by an anhydride bond by consumption of ATP and release of pyrophosphate. This reaction is catalyzed by ATP sulfurylase, which is a bifunctional enzyme comprising of two-subunits (CysN and CysD). The CysN has GTPase activity whereas



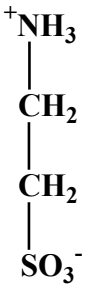
Methionine



Cysteine



Homocysteine



Taurine

Figure 1.1 Naturally occurring sulfur-containing amino acids

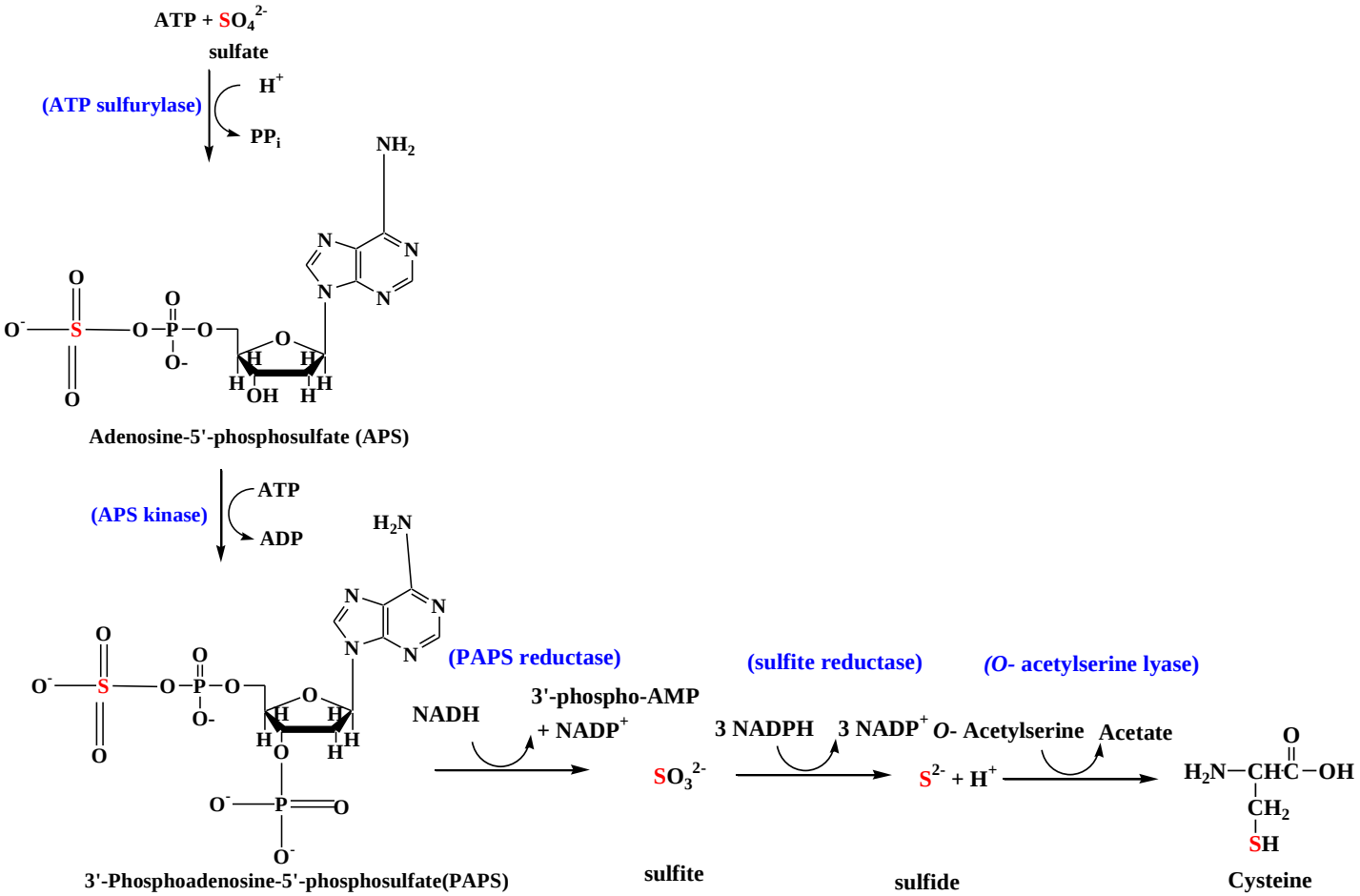


Figure 1.2 Sulfate assimilation pathway and cysteine biosynthesis in *E. coli* (Adapted from (118)).

CysD is sulfate adenylyltransferase subunit 2 (10). APS is further phosphorylated by APS kinase which catalyzes the formation of phosphoadenosine5'-phosphosulfate (PAPS). PAPS serves as a source of activated sulfate for sulfotransferases and other enzymes that catalyzes the formation of many sulfur containing co-factors, amino acids and other biological compounds. Since the reaction equilibrium of ATP-sulfurylase favors the reverse direction i.e., formation of ATP and sulfate, the products of forward reaction (APS and pyrophosphate) must be further metabolized by the enzyme APS kinase (9, 11).The PAPS is reduced to sulfite by thioredoxin dependent PAPS reductase (CysH). This reaction occurs in the presence of NADPH and adenosine 3'-5' diphosphate (PAP) is released as a by-product. It has been reported that PAP is also produced in fatty-acid metabolism from coenzyme A, it is thus suggested that PAP may play a role in coupling sulfur metabolism with lipid metabolism (12). The sulfite produced is then reduced by NADPH-sulfite reductase (CysJ, CysI and, CysG) to produce sulfide (9). In the sulfur-assimilation pathway the sulfide is converted to cysteine upon its reaction with *O*-acetylserine in the presence of *O*-acetylserine lyase (10). Cysteine biosynthesis is catalyzed by serine transacetylase (CysE) and *O*-acetylserine sulfhydrylase A (CysK). CysE and CysK are two subunits of the enzyme cysteine synthase (13). The *O*-acetylserine produces *N*-acetylserine as an intermediary metabolite due to the spontaneous cyclization of *O*-acetylserine (9). The reaction is irreversible which means only *O*-acetylserine acts as a sulfur acceptor. The *N*-acetylserine is shown to act as an inducer for the *cys* regulon (9).

The *E. coli* chromosome has eighteen genes responsible for sulfur assimilation. The genes involved in sulfate activation and reduction (*cysD, cysN, cysC, cysH, cysI and, cysJ)* are located on the same operon (14). The operons of all cysteine biosynthetic genes are grouped as the cysteine regulon (15, 16). The transcription of cysteine biosynthetic genes is regulated by the

binding regulator CysB upstream of the promoter region (17, 18). The CysB regulator is also capable of down-regulating transcription (19). The binding of CysB to the positive regulation sites of the *cys*operon is stimulated by an inducer *O*-acetylserine. This leads to the formation of a transcription initiation complex (17). Along with the central transcription activator CysB, the proteins of the biosynthetic pathway are also regulated by sulfate, sulfur intermediates in the pathway, and cysteine (14).

The feedback inhibition of serine transacetylase for controlling the *cys* genes is dependent on the cysteine concentration inside the cell (16). It has also been reported that cysteine regulates the expression of *cys*genes (16).In many microbes the synthesis of methionine is linked to cysteine biosynthesis through alternate metabolic pathways. In *Escherichia coli* the origin of methionine synthesis originated from the homoserine pathway, which starts with the synthesis of an activated *O*-succinyl homoserine derivative (20). The condensation of the activated homoserine derivative with cysteine leads to the formation of cystathione. The cystathione β-lyase enzyme catalyzes the release of homocysteine and serine. In *E. coli* two isoforms of methionine synthase namely MetE and MetH can assist in the conversion of homocysteine to methionine by transferring a methyl group (Figure 1.3) (20). Methionine can act as a universal methylating agent by producing *S*-adenosylmethionine (SAM). Many of these methylations are essential for cellular functions. SAM synthetase is an enzyme that combines methionine with ATP to produce*S*-adenosylmethionine. Cysteine is essential for the biosynthesis of thiocysteine. Thiocysteine provides the sulfur for iron-sulfur clusters. The thiocysteine is generated by the lysis of cystine by the oxidation of two cysteine residues (21). The biosynthesis of thiamine requires cysteine to produce a thiazole group, which combines with pyrimidine to form thiamine

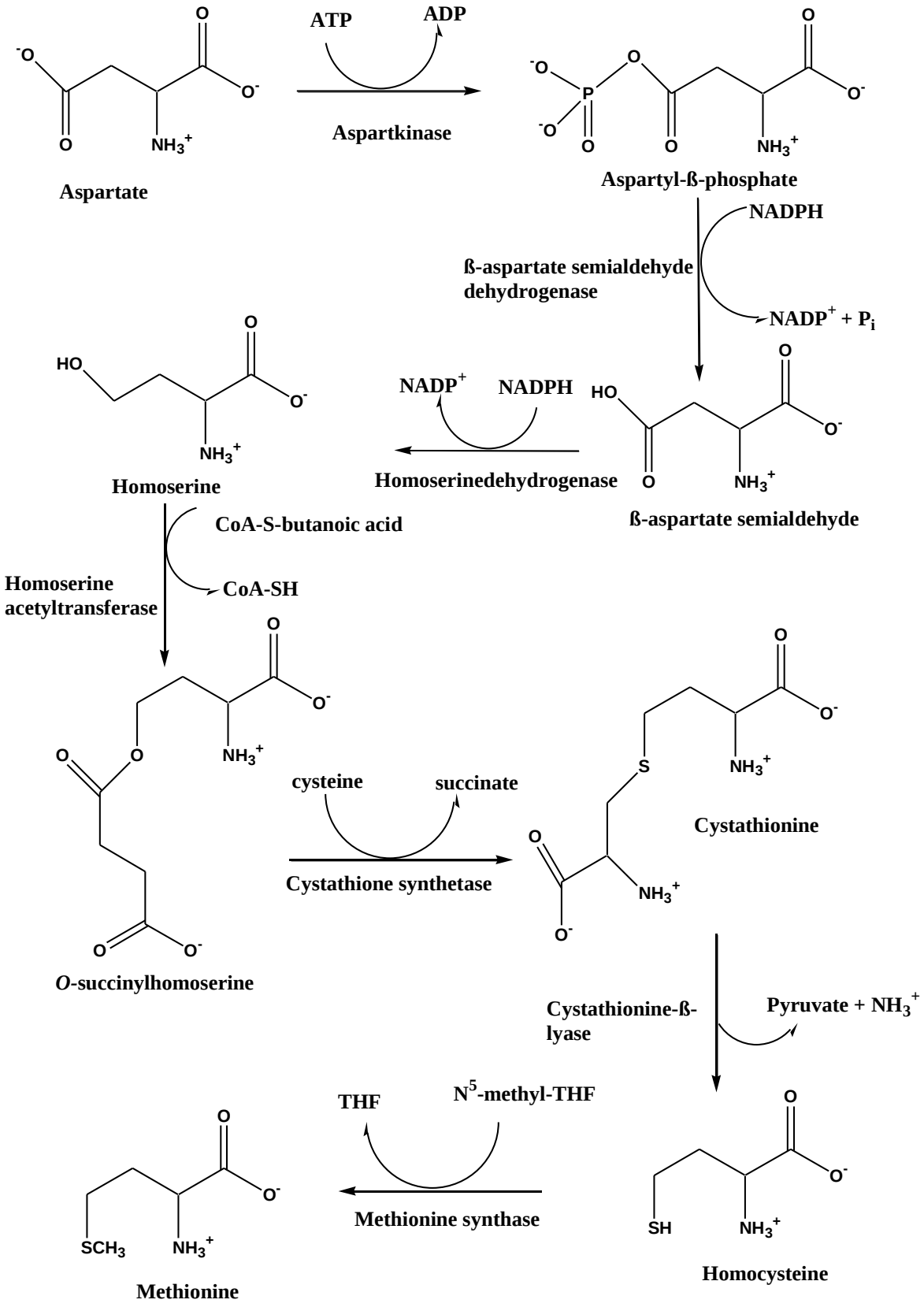


Figure 1.3 Methionine biosynthesis in *Escherichia coli.* (Adapted from (20))

monophosphate (22). Thus under normal circumstances the *cys*regulon controls the production of cysteine and various other sulfur-containing compounds.

1.2 Regulation of sulfur starvation induced enzyme systems in bacteria

Sulfate forms an important component of sulfur-containing biomolecules and is vital for the survival of bacterial cells. Sulfate starvation triggers the expression of sulfate-starvation induced proteins or Ssi proteins in *E. coli*. The proteins expressed by genes on the *ssuEADCB* and *tauABCD* operons are examples of Ssi proteins (Figure 1.4) (8). The *tau* operon expresses genes for taurine dioxygenase (TauD) which utilizes taurine as its sulfur source, whereas the *ssu* operon expresses genes for a flavin reductase (SsuE) and alkanesulfonate monooxygenase (SsuD) which utilizes a wide-range of alkanesulfonates as alternate sulfur sources.

1.2.1 Taurine / α-ketoglutarate dioxygenase

Taurine dioxygenase (TauD) is a member of the non-heme Fe(II) oxygenase family, which converts taurine (2-aminoethanesulfonate) to sulfite and aminoacetaldehyde (8).TauD is an α-ketoglutarate dependent enzyme that converts α-ketoglutarate to succinate and CO_2 (Figure 1.5) (8). Other members of this family include enzymes that modify protein side-chains, repair alkylated-damaged DNA, and degrade compounds that are found in the environment (sulfonates). All members of this family form a high-valent iron-oxo intermediate that participates in catalysis. TauD shares a high sequence similarity with 2,4-dichlorophenoxyacetic acid (2,4-D)/α-ketoglutarate-dioxygenase (TfdA). Both of these enzymes couple the oxidative decarboxylation of α-ketoglutarate to succinate and CO_2. Despite the lack of sequence similarity members of this superfamily utilize similar mechanisms to form reactive oxygen intermediates. Enzymes in the α-ketoglutarate-dependent dioxygenase superfamily catalyze a wide variety of biological oxidation reactions.

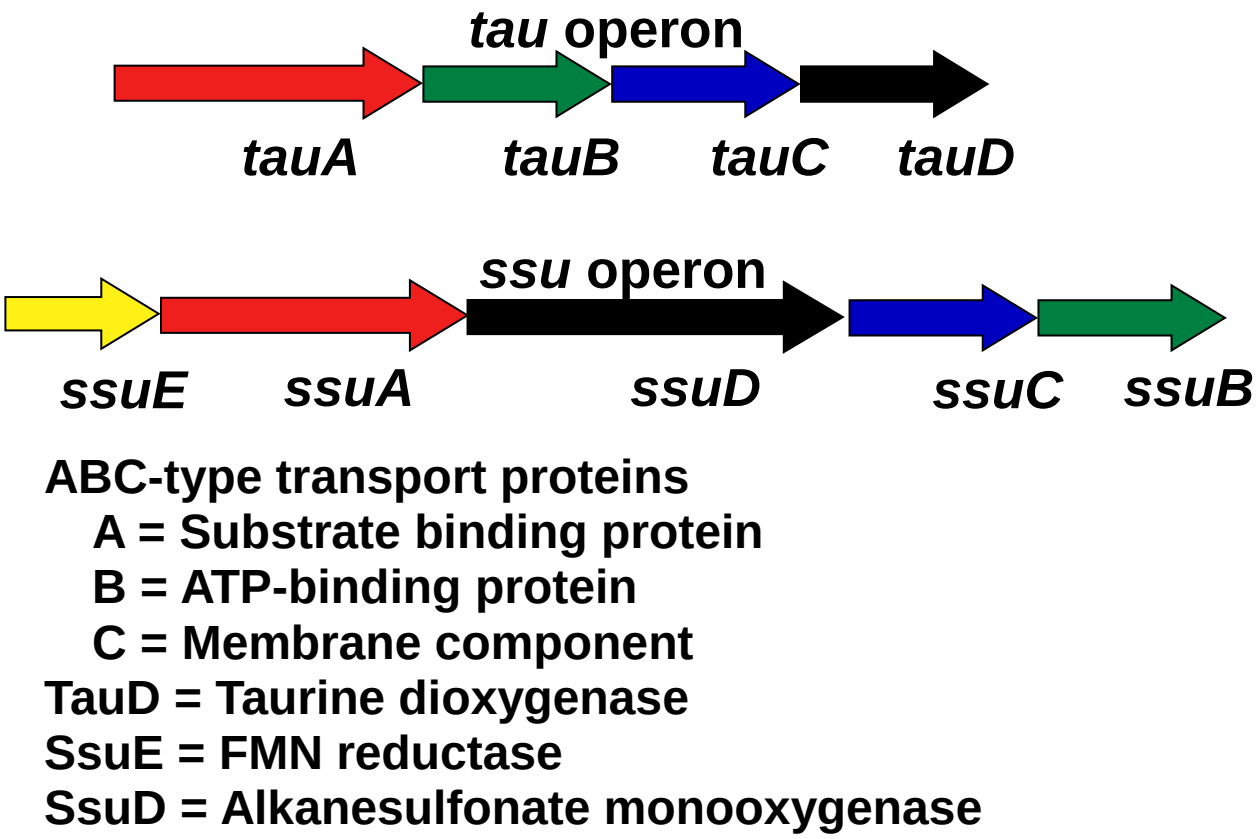


Figure 1.4 The *tau* and *ssu* sulfur starvation induced operons.(Adapted from (8)).

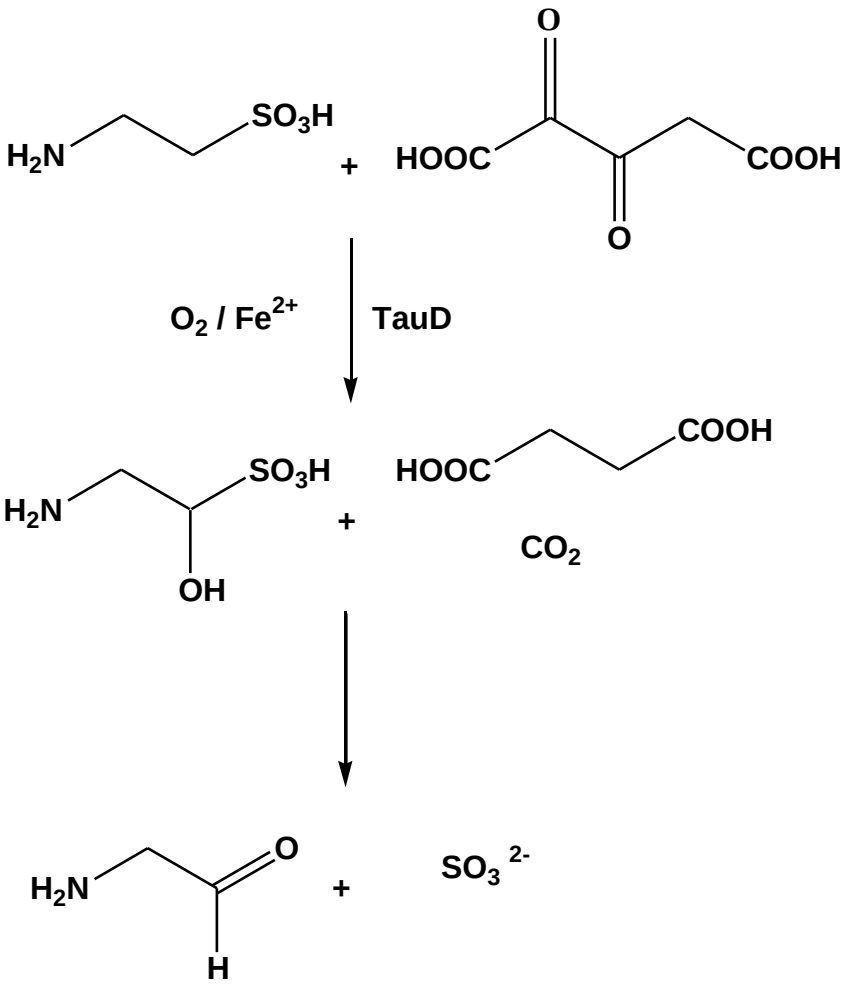


Figure 1.5 Catalytic reaction of the sulfur starvation induced TauD enzyme (Adapted from (8)).

TauD is expressed in *E. coli* in response to sulfur starvation. The TauD enzyme enables the bacterial cell to utilize taurine as a sulfur source during sulfur starvation. Taurine is present in the tissues of humans and other animal species as an intracellular amino acid. These sulfur starvation induced enzyme systems can utilize taurine and other sulfonates from soil. The operon in which TauD is encoded is *tauABCD,* which also expresses genes for TauABC (125). TauABC are ABC-type transporter proteins in which TauA is a substrate-binding protein, TauB is a ATP-binding protein, and TauC is an intracellular membrane component protein.

The expression of *tauD* is dependent on the presence of a functional CysB. The *tau* operon is repressed in the presence of cysteine and sulfate (8). The sulfate repression of *tauABCD*is different from the regulation of other CysB-dependent genes on the *cys*regulon. The *cbl*gene product LysR-type transcriptional regulator that is related to CysB is also required for the expression of the *tau* operon (126). The Cbl protein is a general transcription factor for the sulfonate-sulfur utilization gene or operon. An interrelationship occurs between CysB and Cbl for *tauABCD*operon activation. The expression of this operon is also dependent on the presence of an inducer *N*-acetyl–L-serine. Transcription from promoters is initiated by CysB protein binding to activation sites just upstream of the -35 region of the promoter. The inducer facilitates the interaction of CysB with these sites (17, 127, 128). The single binding site for Cbl protein is located between bp -68 and -112 of the *tau* control region. The Cbl protein is a primary positive regulator of the *tau* promoter. On the other hand CysB is involved in controlling the *tau* operon region (125).

1.2.2 Alkanesulfonate monooxygenase system in E. coli

The *ssu* operon expresses the proteins involved in replenishing the sulfur reserve under sulfur limiting conditions (8) (23). SsuE and SsuD comprise the two-component alkanesulfonate

monooxygenase system in *E. coli* and a wide range of other bacteria (Figure 1.6) (24). The SsuE enzyme reduces FMN utilizing NADPH (8). The SsuD in turn accepts the reduced flavin from SsuE and catalyzes the oxygenolytic cleavage of C-S bonds in alkanesulfonates (24, 25). It is a co-dependent system in which SsuD shows a linear dependence on SsuE for its catalytic activity. The SsuABC are transporter proteins which serve a similar purpose as the TauABC proteins.

The *cys* genes that control the *ssu* operon are positively regulated by the LysR type transcriptional activators CysB and inducer *N*-acetylserine (129). The bacterial cells with *cbl* mutations were not able to use taurine or aliphatic sulfonates as sulfur source. Also, many *Ssi* proteins were either absent or present in very low concentrations in these *cbl* mutant cells which were grown under sulfur-starvation conditions (125). Thus, the Cbl protein might be an activator for genes that are expressed under sulfur-starvation conditions (Figure 1.7). The expression of *ssu* genes requires the presence of Cbl which binds to the -35 region upstream of the *ssu* operon. The binding of Cbl in close proximity of the -35 region may assist in bringing Cbl in direct contact with RNA polymerase (131). The role of CysB for the expression of the *ssu* operon has not been fully elucidated. But it has been shown that CysB is required for the expression of Cbl and maybe an indirect activator of *ssu* (132). Thus, differences exist between the expression of *cys* genes necessary for the reduction of sulfate, biosynthesis of cysteine, and *tau* genes. The alkanesulfonate monooxygenase system requires flavin to maintain their functionalities hence this system is classified as a flavin-dependent two-component alkanesulfonate monooxygenase system. Unlike many other flavin-dependent systems, alkanesulfonate monooxygenase utilizes flavin as a substrate and not as a cofactor. Although this

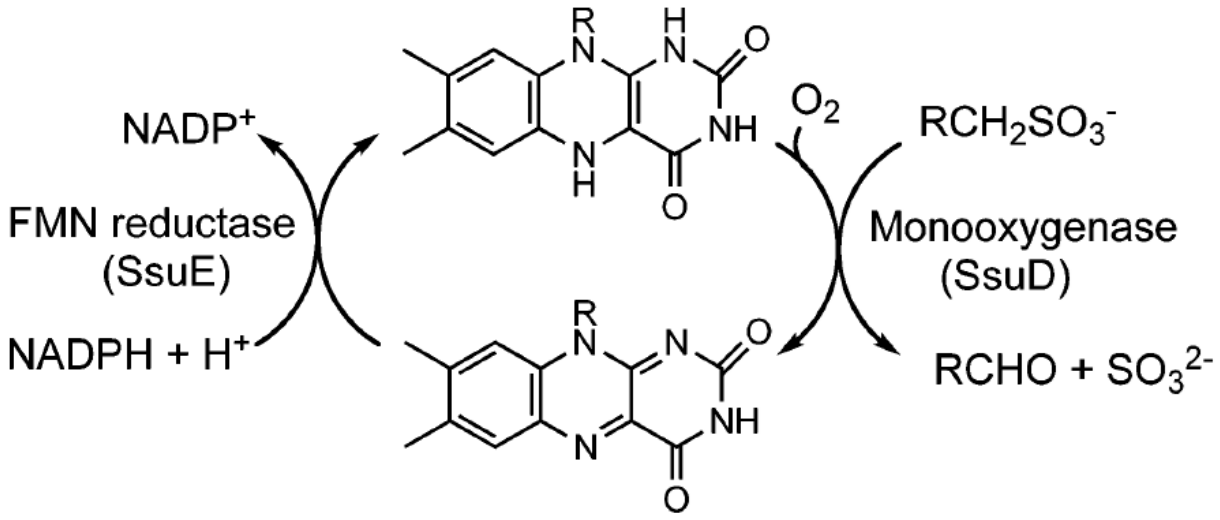


Figure 1.6 Catalytic reaction of the alkanesulfonate monooxygenase system. (Adapted from (8)).

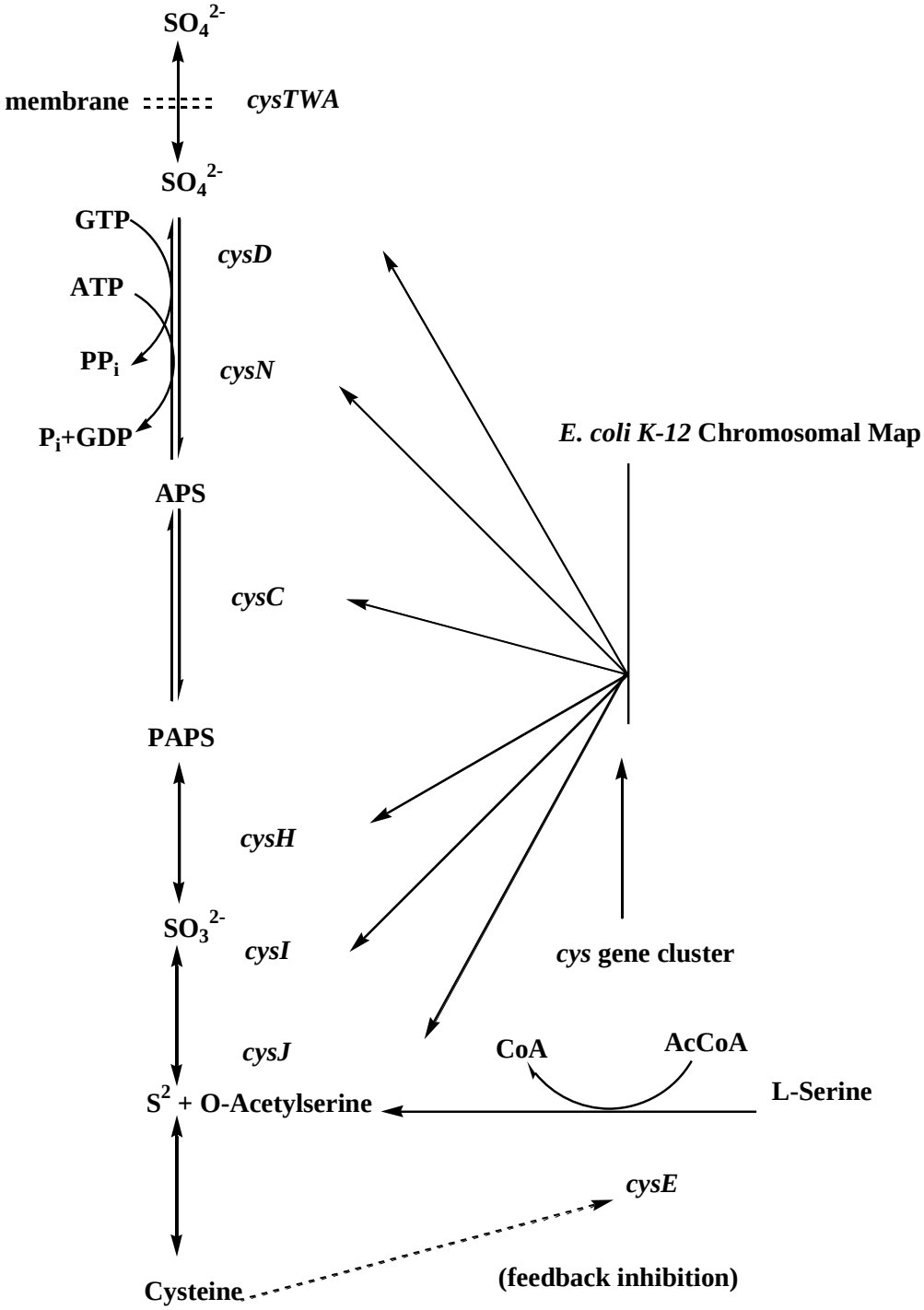


Figure 1.7 Regulation of the *cys* regulon for sulfur assimilation in *E. coli.* (Adapted from 8)

system is expressed under sulfur-limiting stress conditions, efforts are underway to link the expression of the *ssu* operon with oxidative stress conditions.

1.3 The mechanism of the alkanesulfonate monooxygenase system

The monooxygenase enzymes utilize reduced flavin to activate dioxygen generating C4a-(hydro)peroxyflavin reactive intermediates. The catalytic ability of this intermediate is dependent on the active site environment of SsuD which stabilizes this reactive intermediate (133-135). This C4a-(hydro)peroxyflavin intermediate can act as either a nucleophile or electrophile depending on the reaction being catalyzed (136-140). The alkanesulfonate monooxygenase system carries out an oxygenolytic cleavage of the C-S bond of alkanesulfonates.

1.3.1 FMN reductase (SsuE)

For most of the identified FMN reductases properties that are associated with two-component systems, the three-dimensional structures are still unknown. The only reported FMN reductases structures are of EmoB, Frase1, FRP, and SsuE (37, 38, 39, 40). The crystal structure of SsuE shows a homotetrameric structure (40). The SsuE enzyme has the tertiary and quaternary structural properties that are highly similar to EmoB. All members of the flavodoxin-like superfamily bind FMN at a common site, but in case of SsuE the FMN binding site is deeply nestled into the structure. The oligomeric state of members of the NADPH-dependent FMN reductase family has been shown to vary upon FMN binding. A dimer-tetramer equilibrium is believed to exist in this family which is facilitated by flavin binding. With the exception of SsuE, the members of NADPH-dependent FMN reductase family form a tetramer upon flavin binding.

In case of SsuE the FMN binding weakens the tetramer and shifts the equilibrium towards dimer formation (120). This unique behavior can be explained with the presence of π-helix near the FMN binding site. A π-helix is a secondary structure found in over 15% of the known proteins. It is believed to be an evolutionary adaptation formed by the insertion of single amino acid in the α-helix(). In case of SsuE the π-helix may assist in the FMN binding by weakening the tetrameric structure. The other two enzymes with in this family with a π-helix in there structure are EmoB and an uncharacterized oxidoreductase 3K1Y. The 3K1Y is believed to be a reductase which is part of a two-component system as its gene is in a gene cluster encoding an ABC-transporter homologue and an SsuD homologue. In all these three enzymes the π-helix is centered on Tyr118 residue.

The reductase enzymes involved in two-component systems catalyze the reduction of oxidized flavin with reducing equivalents provided by NAD(P)H. Typically the reductase enzymes have a specificity for either FMN or FAD. The SsuE enzyme shows a higher specificity towards FMN with greater k_{cat}/K_mvalues (24). The binding affinity of SsuE is 1000-fold higher towards oxidized FMN compared to reduced flavin (29, 141). Apart from SsuE all the other members of NADPH-dependent reductase family utilize FMN as a prosthetic group and employ a ping-pong mechanism for its reduction. The SsuE enzyme uses flavin as a substrate and was shown to exist as a dimer in solution (24, 28). The kinetic mechanism of SsuE follows an ordered sequential mechanism (Figure 1.8) (28). A recent crystal structure of FMN-soaked SsuE highlighted FMN as a cofactor (40). SsuE have also been suggested to use a ping-pong mechanism for its catalytic cycle as shown for EmoB (40). The purified recombinant SsuE protein is flavin free and further studies are under way to evaluate the substrate binding mechanism. It has been reported that the kinetic mechanism of SsuE was altered to an equilibrium ordered mechanism in the presence of

SsuD and octanesulfonate. There was also a 10-fold decrease in the K_m value for FMN in the presence of SsuD and octanesulfonate (28). The NADPH and FMN substrates bind to SsuE to form a ternary complex (MC-1). Following the formation of MC-1 the first phase (k_1) is the formation of an initial charge transfer complex between NADPH and FMN (CT-1). The second phase (k_2) leads to the formation of the second charge transfer complex involving $FMNH_2$ and $NADP^+$(CT-2). The change of CT-1 to CT-2 is the hydride transfer step from NADPH to FMN. The final step is the decay of CT-2 of the Michaelis complex with bound products (28). The hydride transfer is thus the rate-limiting step in the flavin reduction by SsuE. The charge transfer complex formed during the flavin reduction by SsuE is relatively weak compared to flavin-bound reductase enzymes. Since the role of SsuE is to reduce FMN and transfer it to SsuD, a mechanism involving a tightly bound FMN would not be useful for promoting flavin transfer.

1.3.2 The monooxygenase (SsuD)

The members of the bacterial luciferase family share low sequence identity; however, the three-dimensional structures of these enzymes have common structural features (85). The enzymes in this family are TIM-barrel structures and their active sites are located on the C-terminal ends of the β-barrel. The TIM-barrel is a conserved protein fold consisting of eight α-helices and eight parallel β-strands. The structure is named after the glycolytic enzyme triosephosphate isomerase (TIM). The structure of SsuD deviates from a typical TIM barrel fold as it has a dynamic loop region located in an insertion sequence. For TIM-barrel proteins the reactive intermediates in the active site are protected by a mobile loop. Amino acid sequence alignment of *E. coli* SsuD with homologues from different organisms show that the dynamic loop region is highly conserved. Previous studies have suggested that a conformational change of the SsuD dynamic loop is

initiated by binding of the substrate and protects the peroxyflavin intermediate generated in the desulfonation mechanism. The disordered loop of SsuD closes over the active site after substrate binding and is responsible for conformational changes observed in kinetic studies (Figure 1.11) (143). The SsuD enzyme belongs to the bacterial luciferase family. The SsuD enzyme catalyzes the desulfonation of a wide-range of sulfonated compounds to generate aldehyde and sulfites (24). The desulfonation is catalyzed by the formation of a highly reactive C4a-(hydro)peroxyflavin intermediate generated by the reaction of reduced flavin with dioxygen (Figure 1.9) (25).The SsuD enzyme has a 100-fold lower K_D value for reduced flavin as compared to SsuE. The specificity of SsuE and SsuD for different redox forms of flavin may also play a role in the effective transfer of reduced flavin. This important feature of the two-component alkanesulfonate monooxygenase system could prevent uncoupling of the desulfonation reaction.

Flavin monooxygenase enzymes catalyze the oxidations of substrates through electrophilic or nucleophilic substitution reactions (Figure1.12) (28). The C4a-(hydro)peroxyflavin intermediate can act either as an electrophile or nucleophile in the reaction depending on the mechanism employed by the enzyme. A C4a-peroxyflavin (FL-OO-) intermediate would be involved in electrophilic substitution and C4a-hydroperoxyflavin (FL-OOH) would be more useful in a nucleophilic addition reactions. In SsuD enzyme rapid reaction kinetic evaluation supports a mechanism involving the formation of C4a-peroxyflavin intermediate, but this flavin intermediate is not stable due to the presence of higher amount of polar amino acid residues in the active site of SsuD (25). In case of SsuD the organosulfonated substrate requires a more electrostatic environment for its stability. Due to this increased electrostatic environment the flavin intermediate cannot be adequately stabilized for isolation.

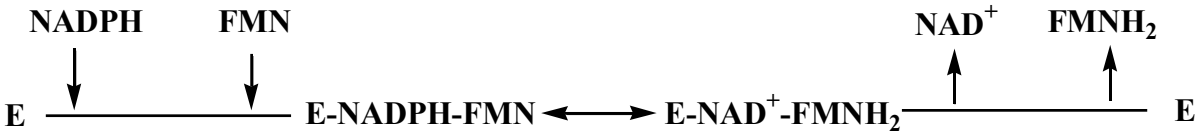


Figure 1.8 Reaction schemes for NADPH and FMN binding to SsuE. (Adapted from (44)).

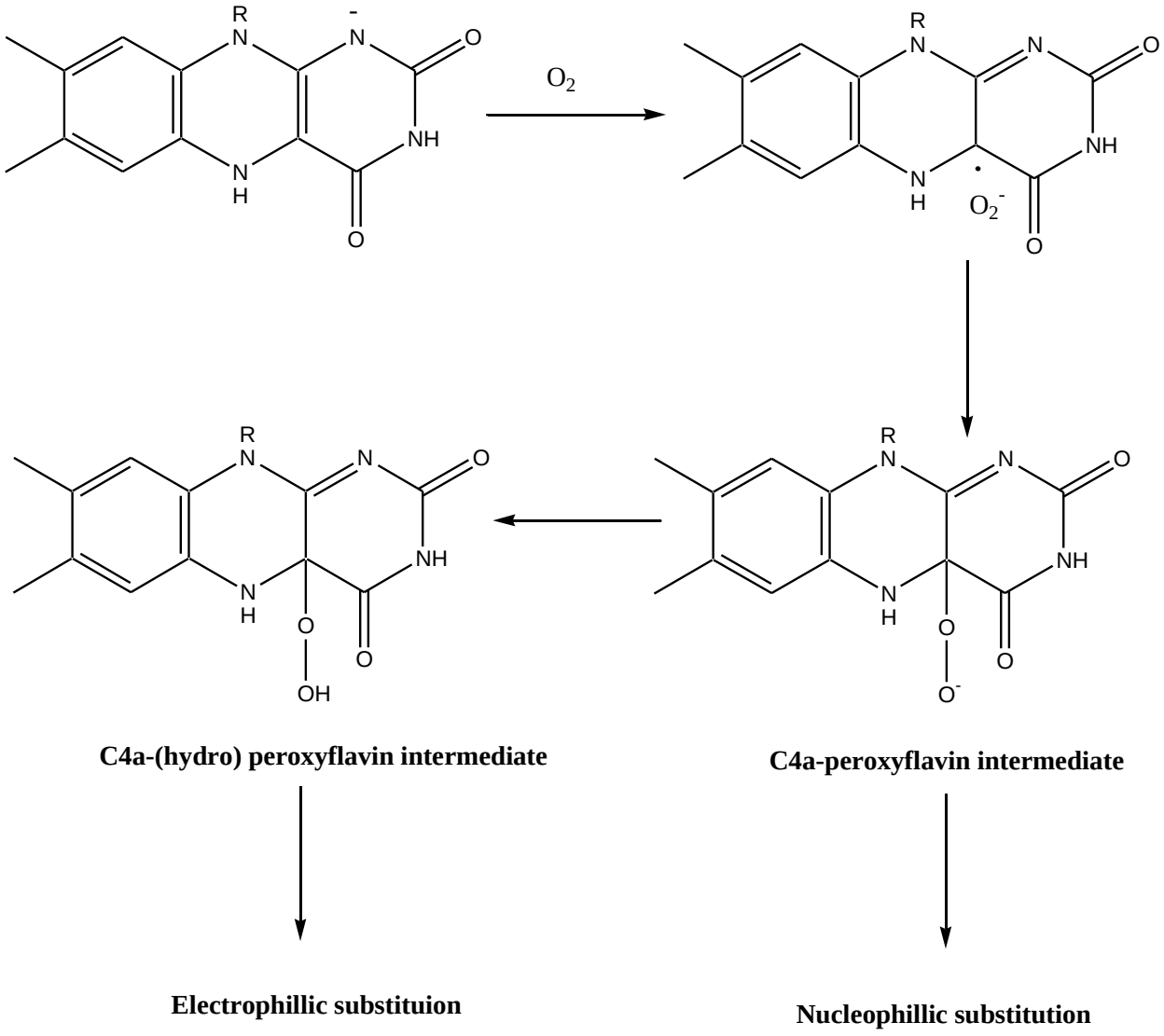


Figure 1.9 The formation of reactive flavin intermediates C4a-peroxyflavin intermediate and C4a-(hydro) peroxyflavin intermediates. (Adapted from 44)

Figure 1.10 Three-dimensional structure of NADPH-dependent FMN reductase (SsuE) from *Escherichia coli* with FMN bound at the interface of monomer. The structure was rendered with PyMOL after PDB:4PTZ (120).

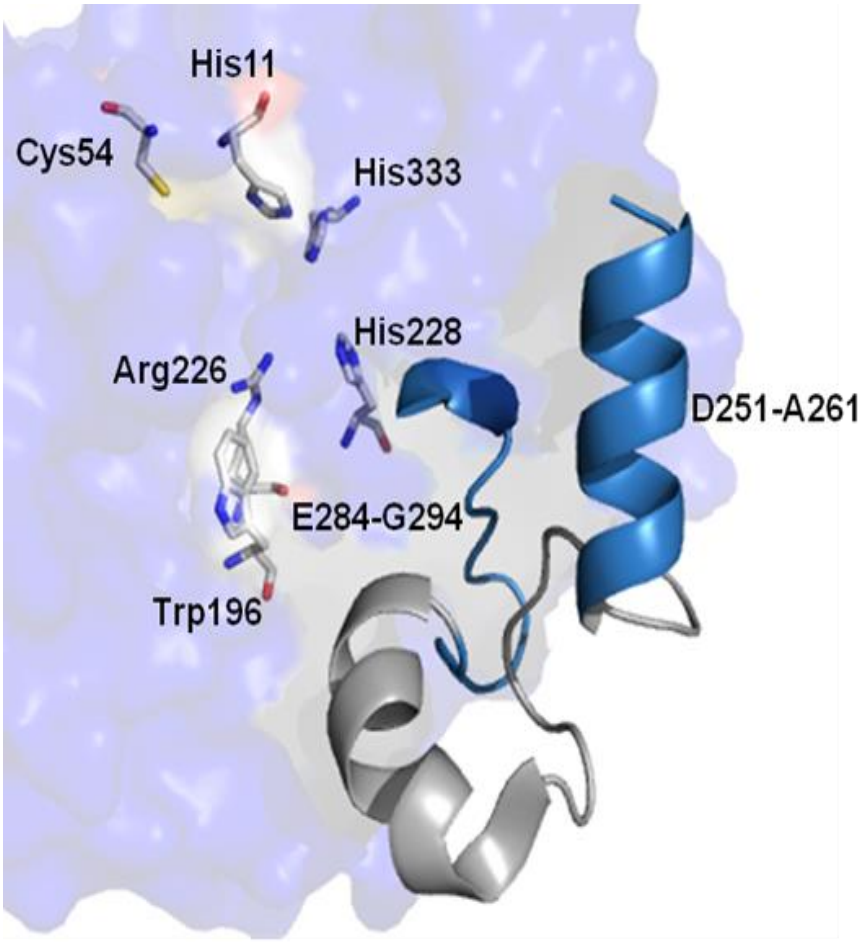


Figure 1.11 The SsuD monomer with the active site highlighted relative to the solvent protected region and dynamic loop region. The structure is rendered with PyMOL PDB:1M41 (120).

Several amino acid residues (His228, His11, His333, Cys54, and Arg226) located in the active site of SsuD have similar arrangement as catalytically relevant amino acids in the bacterial luciferase family. But similar active site environment often promote diverse functionalities despite having almost identical structural motifs. The role of these conserved active site residues of SsuD in catalysis were determined by pH dependence on k_{cat} and k_{cat}/K_m in the pH range of 5.8-10.0. Experiments were performed in this pH range because SsuD reacts with many buffers as aminosulfonic acids are substrates of SsuD. The k_{cat}/K_mpH profile indicates the optimal protonation state of ionizable groups located on the free enzyme and/or substrate in order for the reaction to commit to the first irreversible step of catalysis while the pH dependence of k_{cat} reflects ionizable groups on the enzyme-substrate complex required for catalysis to occur including product release. The k_{cat}/K_m profile for SsuD reaction revealed a single titratable group with pKa value of 6.9 ± 0.1. Also the pH of k_{cat} for SsuD revealed a similar titratable group of pKa value of 6.6 ± 0.1. Despite the small difference in these values they likely represent the same group. Previous studies have highlighted that the desulfonation reaction by SsuD occurs through an ordered binding mechanism where $FMNH_2$ binds to the enzyme first followed by the binding of either 1-octanesulfonate or O_2. If the lower pK_a value represents the same group in both k_{cat} and k_{cat}/K_mprofiles, then it can be concluded that the deprotonated form of this group is necessary both for the formation of final enzyme-substrate complex and for catalysis. Histidine is an amino acid whose pKa is consistent with the values identified by pH profile assays. But steady-state kinetic assay failed to establish His228 or any other active site histidine residue to be essential for catalysis. Moreover this pKa value was present in the pH profile of all His and Cys variants with minor variations. These minor variations were attributed to the presence of a sticky substrate. The presence of these sticky substrates had a negligible effect on the overall k_{cat} and

k_{cat}/K_m pH-independent values which means that while some of these active site amino acids may be involved in maintaining the active-site environment via substrate binding interactions but they are not critical for catalysis. Hence, the pH profiles for H228A, H11A, H333A, and C54A SsuD confirm that neither of these amino acids are responsible for the lower pKa value. In bacterial luciferase deuterium isotope studies have suggested that N1 position of $FMNH_2$ is contributing to the lower pKa value of 6.9. It is thus possible that N1 position of FMNH2 is contributing to the lower pKa value in SsuD as well. A hollow was observed from the fits of the C54A SsuD k_{cat} and k_{cat}/K_mprofiles which indicated the presence of a sticky proton in the enzyme-substrate complex. The observed hollow indicated that the octanesulfonate dissociates faster than the ionizable proton in the C54A SsuD variant. It is believed that Cys54 would be required for therapid dissociation of the N1 proton from $FMNH_2$ in the presence of octanesulfonate. This rapid dissociation has been shown to be responsible for the formation of a reactive flavin intermediate at a significant rate. The Arg226 residue at the active site of SsuD has also been shown to serve a crucial role in catalysis. The R226A and R226K variants were completely inactive. Thus the pH dependence on the kinetic parameters of R226A and R226K was not determined as neither variant could catalyze detectable levels of sulfite production. Although the substrate binding was not affected for these variants no observable formation of C4a-(hydro)peroxyflavin intermediate was observed in rapid reaction kinetic studies. It has been indicated that Arg226 could possess multiple roles. The positively charged groups such as arginine are highly conserved near the active site of Baeyer-Villiger flavin monooxygenases in order to stabilize peroxyflavin intermediates. The absence of such positively charged stabilizing group near the active site can lead to inactivation of enzyme due to destabilization of flavin intermediate. Also results from the chemical rescue experimenst with guanidine have suggested

that the orientation of and chemical properties associated with guanido group of Arg226 is crucial in catalysis.

1.4 Reactions catalyzed by two component systems

The oxygenases of two-component enzyme system catalyze a wide range of reactions (Figure 1.13) (119). Many of the FMN-dependent monooxygenase enzymes in the family share mechanistic similarities with each other. These enzymes are dependent on an FMN reductase to provide them with $FMNH_2$, which they then utilize to activate molecular oxygen.

The bacterial luciferase enzyme is found in several aquatic and terrestrial organisms including *Vibrio harveyi*, *Vibrio fischer* and, *Photobacteriumphosphorium* (67). Bacterial luciferase utilizes long-chain aliphatic aldehydes, reduced flavin, and dioxygen to produce an aliphatic carboxylic acid and bioluminescence (68,69,70). LadA isolated from *Geobacillusthermodenitrificans* converts long chain alkanes to the corresponding alcohol (71). LadA can utilize a wide range of alkanes ranging from C15-C36 chain lengths (8). Recently identified FMN-dependent monooxygenase enzymes in some bacterial species like *Rhodococcus* sp. and *Paenibacillus* sp. utilize dibenzothiophene (DBT) for sulfur acquisition (72, 73). The dibenzothiophene desulfurization enzyme C (DszC) FMN-dependent monooxygenase catalyzes two initial steps in the conversion of DBT to DBT sulfone (Figure 1.12). A second FMN-dependent monooxygenase DszA converts DBT sulfone to 2-hydroxybiphenyl-2-sulfinite. In this pathway the single FMN reductase provides reduced flavin to two separate monooxygenases. Two monooxygenases have been identified that catalyze the degradation of the chelating agent nitrilotriacetic acid (NTA) and

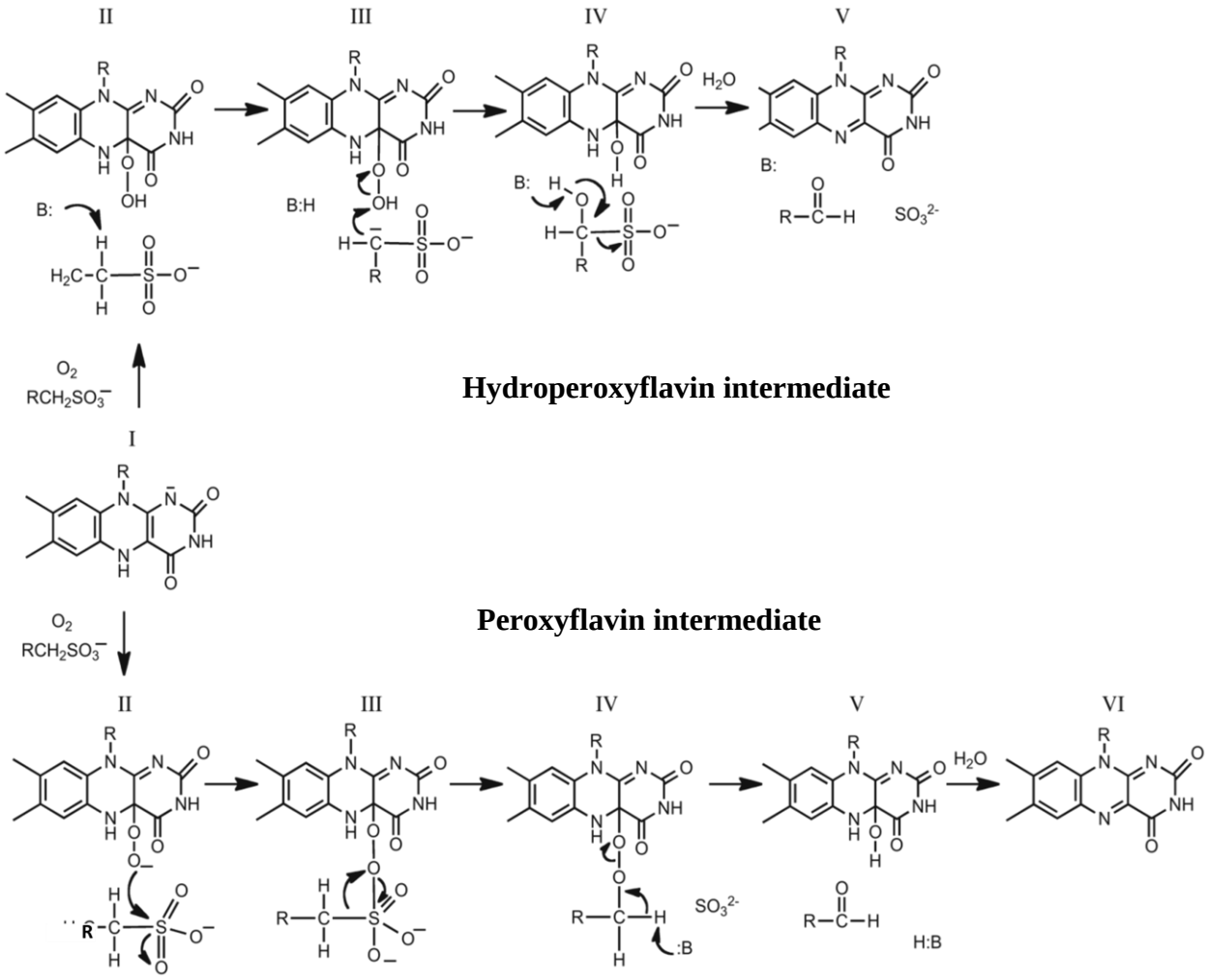
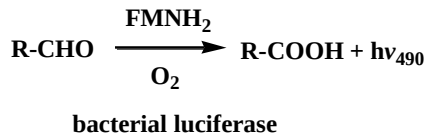
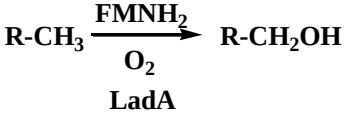
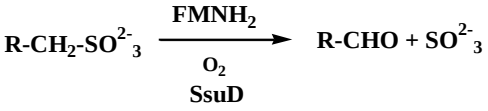


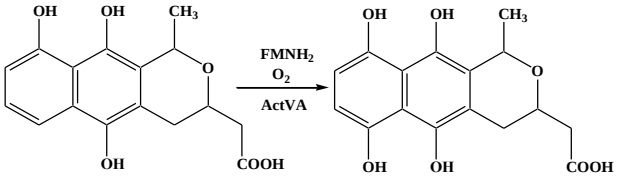
Figure 1.12 Proposed mechanism of SsuD desulfonation. (Adapted from (44))

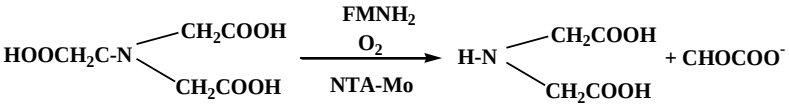
ethylenediaminetetracetic acid (EDTA)as a carbon, and nitrogen source. The enzyme FMN-dependent monooxygenase NtaA converts NTA to iminodiacetate and glyoxylate and utilizes $Mg^{2+}$to coordinate the substrate (74,75,76). The monooxygenase EmoA catalyzes the oxidation of EDTA to ethylenediaminetriacetate and glyoxylate and EmoA enzyme can degrade a wide-range of complex substrates including EDTA, NTA, and diethylene triaminepentacetic acid (DTPA) (77). The C2 subunit of HPA hydroxylase from *Acinetobacter baumannii* can catalyze the hydroxylation of p-hydroxyphenylacetate (HPA) to 3,4-dihydroxyphenylacetate (DHPA) (78). The monooxygenase from this system can utilize either FMN or FAD in the hydroxylation reaction (79). The FMN-dependent monooxygenases are also involved in antibiotic biosynthesis. The ActVA monooxygenase catalyzes the final step in actinorhodin biosynthesis and SnaA is involved in the synthesis of polyunsaturated cyclic peptolidepristinamycin. Pristinamycin acts as an inhibitor of protein synthesis (80, 81, 82, 83). The reaction for this enzyme involves oxidation in place of oxygenation. All the enzymes discussed play a key role in bacterial cells and are dependent on the reductase counterparts for the transfer of reduced flavin. For enzymes that use flavin as a substrate the transfer of reduced flavin occurs by a direct transfer mechanism. Flavin transfer by a direct transfer mechanism would rely on protein-protein interactions between the reductase and oxygenase.











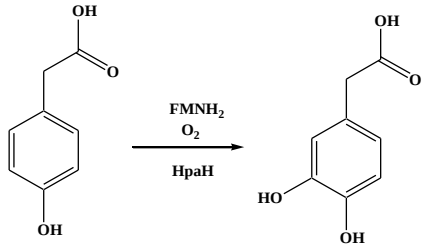


Figure 1.13 Reactions catalyzed by FMN-dependent monooxygenase enzymes (Adapted from (119)).

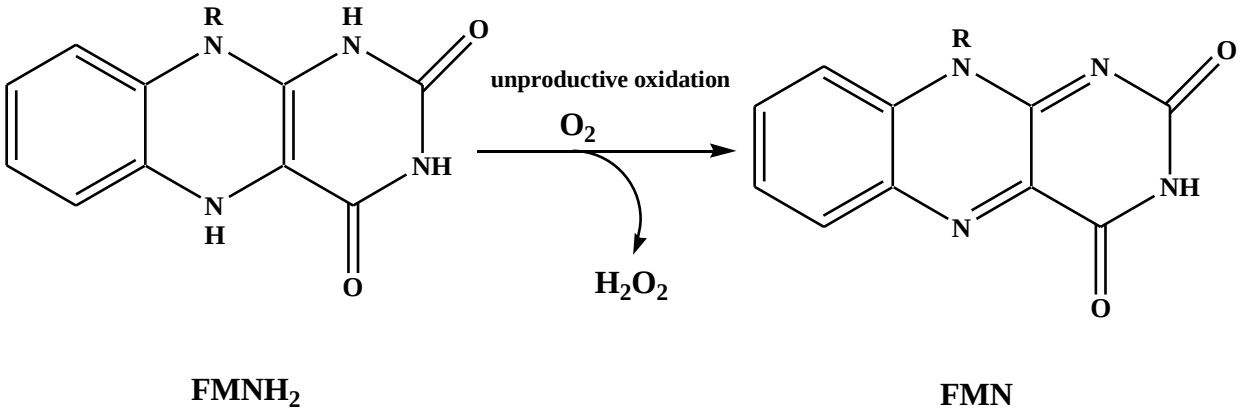


Figure 1.14 Autoxidation of reduced FMN to yield reactive oxygen species (ROS) like H_2O_2 (Adapted from (124)).

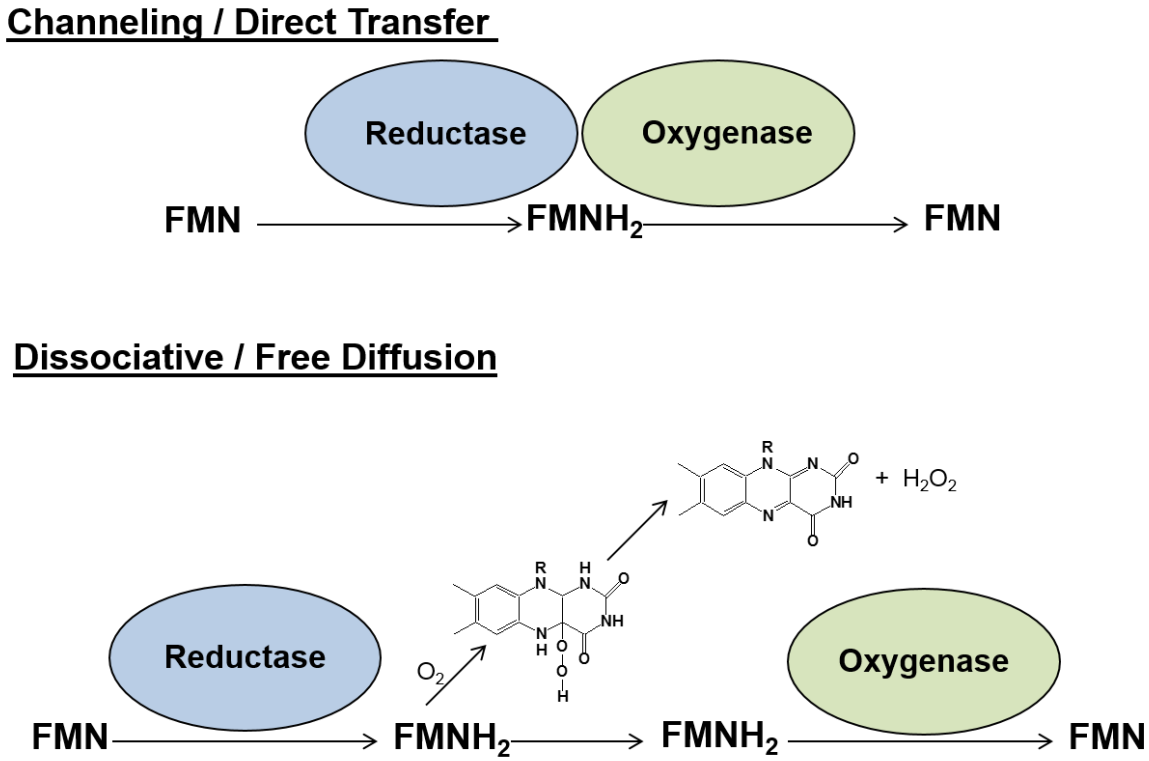


Figure 1.15 Two general mechanisms for reduced flavin transfer in the two-component enzyme systems (Adapted from (122)).

1.5 Mechanism of reduced flavin transfer in two-component systems

Reduced flavin can react with dioxygen non-enzymatically to form reactive oxygen species (Figure 1.14) (26). The reduced flavin and oxygen forms an initial complex in which an electron is transferred from a singlet reduced flavin to triplet O_2. This leads to the formation of a radical pair which collapses due to spin inversion to form flavin peroxide (27). The flavin (hydro)peroxide is unstable in aqueous solution and dissociates heterolytically to H_2O_2 and oxidized flavin (124). Due to the high lability of reduced flavin, the efficient transfer of reduced flavin is of paramount importance in two-component enzyme systems. Depending on the system under investigation the transfer of reduced flavin can occur either by a free-diffusion or channeling mechanism (Figure 1.15) (122). Some monooxygenase enzymes that have been characterized have shown to transfer the reduced flavin from FMN reductases by a free-diffusion mechanism. In other two-component systems the genes required for the expression of the reductase and monooxygenase are located on the same operon. Thus their genetic locations should provide some exclusivity and similar structural motifs which may promote protein-protein interactions.

1.5.1 Free diffusion mechanism for $FMNH_2$ transfer in two-component systems

The transfer of reactive metabolites efficiently between proteins is important for all biological systems. Efficient transfer is also important to avoid side reactions. In aerobic systems the transfer of reduced flavin can have negative effects on the cell if not properly maintained. The autoxidation of reduced flavin can generate hydrogen peroxide and reactive oxygen species. In many systems like bacterial luciferase the transfer of reduced flavin is pivotal for activity (42,43). Bacterial luciferase (LuxAB) is a flavin dependent monooxygenase that catalyzes the

transfer of long-chain aldehydes to generate carboxylic acid and visible light. Bacterial luciferase can accept reduced flavin from a variety of flavin oxidoreductases like FRE, and FRP (44). There was no stable complex formation between bacterial luciferase and FRPin pull-down assays (45,46). LuxG was identified as the reductase for bacterial luminescence. Also, no stable protein-protein interactions were identified between LuxG from *Photobacteriumleiognathi* and *Pl*LuxAB by gel filtration experiments (44). The genes for LuxG and LuxAB are located on the same operon. The kinetic data for reduced flavin transfer between LuxG and LuxAB supports free-diffusion. The transient kinetic methods were employed in this study to measure the individual rate constant and analyze quantitatively to distinguish between the two modes of transfer for reduced flavin. The kinetics of release of reduced flavin from LuxG was not influenced by the presence of LuxAB, even though the concentration of LuxAB was much higher than that of LuxG (53).The binding of reduced flavin to LuxAB and the subsequent reaction carried out were also shown not to be dependent on the presence of LuxG. The double-mixing experiments were performed by using LuxG and a variety of reduced flavin acceptors to show that the reaction kinetic is consistent with the free-diffusion model. Similar techniques were also previously employed to show the presence of free-diffusion mechanism for reduced flavin transfer in HPAH (C1-C2) from *A. baumannii* and ActVA-ActVB system from *S. coelicolor*. It has also been demonstrated that LuxAB can accept reduced flavin from flavin oxidoreductases that are not from luminous bacteria (43, 46, 47). The flavin-dependent monooxygenase system p-hydroxyphenylacetate hydroxylase (HPAH) from *Acinetobacter baumanni* is a two-component system in which C1 is an oxygenase and C2 is a reductase. Recent studies have shown that complex formation between C1 and C2 is not required for reduced flavin transfer(48, 49, 50). Similar results were seen in the ActVAB system where protein-protein interactions were not

observedby pull-down assays and the transfer of reduced flavin was not dependent on complex formation (24). Also the oxygenase component of HAPH and ActVAB system was able to accept reduced flavin from cytochrome c at an identical rate constant. The rate constants for these systems were dependent on the dissociation of reduced flavin from the reductase (49, 50). Therefore, protein-protein interactions were not required and the flavin transfer is dependent on product dissociation for these systems. The ActVB and C2 from HPAH did not show any physical interactions with their respective reductase components in affinity chromatography assays (51). Although the mechanism for the transfer of reduced flavin varies among different enzyme systems they must all contribute towards minimizing the wasteful autoxidation of reduced flavin. Most of the studies supporting the free-diffusion of reduced flavin have ultimately based their discussions around the release of reduced flavin by the reductase or the binding capacity of the oxygenase towards reduced flavin. It should be noted that all the reduced flavin dependent oxygenases are inherently designed to utilize reduced flavin as a substrate to catalyze their reactions. The situation becomes complicated when consider that multiple enzymes within the bacterial cell accept reduced flavin. The FMN cofactor is a substrate or medium for electron transfer in various redox reactions. This raises a distinct possibility that reduced flavin will never reach the desired destination if free-diffusion is the only means of flavin transfer.

1.5.2 Direct transfer of $FMNH_2$ in two-component systems

An alternate mechanism for reduced flavin transfer in two-component systems is through protein-protein interactions. Flavin reductase P is an important enzyme in the reductase-luciferase system from *Vibrio harveyi.* A direct interaction of reduced flavin between two enzymes has been shown to exist. The kinetic analyses of flavin reductase FRP catalysis have

subsequently provided evidence for protein-protein interactions and have offered a kinetic mechanism for direct transfer of reduced flavin (55). The FRP kinetic mechanism of FMN shifted from a ping-pong to a sequential mechanism in the presence of luciferase. This meant that the protein-protein interactions or the conformational change associated with it plays role in the mechanism of FRP (56). Evidence to support protein-protein interactions was further strengthened by fluorescence anisotropy experiments that provided evidence for the FRP-luciferase complex (56). Fluorescence anisotropy experiments were performed by labeling FRP with a fluorophore eosin. The fluorescence anisotropy signals are sensitive to molecular aggregation states. The three cysteines that are located away from the active site of FRP were used for labeling. The fluorescence intensity decreased as the luciferase concentration increased in the sample mixture. Further evidence for the presence of direct transfer channeling mechanism for transfer of reduced flavin between FRP reductase and LuxAB was provided by BRET experiments in which the transfer of light emitted by the LuxAB reaction via BRET to the fluorescent YFP-FRP implied that LuxAB and FRP are located close to each other. Further evidence that FRP and LuxAB physically interact were provided steady-state experiments which showed an alteration in FRP kinetics in the presence and absence of LuxAB. These studies provide an evidence for the formation of a transient complex between FRP and luciferase.

1.5.3 Mixed transfer mechanism for reduced flavin transfer

Another, possible mechanism for reduced flavin or reactive intermediate transfer can be a combination of free-diffusion and direct transfer. Flavin dependent oxygenases have heme, non-heme iron, copper, or flavin-dependent active sites involved in their oxygenation reactions (102-106). These enzymes also differ structurally ranging from single-components to complex

systems. The two-component styrene monooxygenase (SMO) from *Pseudomonas* is a typical flavin-dependent system with a highly conserved reductase and monooxygenase component. Previous rapid reaction kinetic studies and numerical simulation models have shown the presence of complex formation between SMOA (reductase) and SMOB (oxygenase) (108). A real-time assay was employed to evaluate that NADH and styrene were oxidized in 1:1 ratio when SMOA:SMOB were in high concentration. But upon lowering the SMOA:SMOB ratio and increasing FAD concentration the efficiency of reaction decreased. Based on the comparison of experimental data with numerical simulations it was stated that steady-state coupling of NADH and styrene oxidations were in agreement with reduced flavin transfer upon transient complex formation between SMOA and SMOB. Conversely SMOB was able to carry out epoxidation of substrate in the absence of its reductase component, by using alternative source for reduced FAD (117). Thus these studies suggest that styrene monooxygenase (SMO) demonstrate both a dissociative and direct transfer mechanism for the transfer of reduced flavin (31).

1.6 Role of Reactive Oxygen species in promoting oxidative stress and protein damage

The flavin containing monooxygenase enzymes can inadvertently play a key role in promoting oxidative stress in bacterial cells. As mentioned the reduced flavin is labile and canundergo autoxidation to produce hydrogen peroxide and other reactive oxygen species. Reactive by-products of these futile reactions include superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radicals ($^{\cdot}OH$). In aerobic organisms such products can also be derived from sequential univalent reductions of molecular oxygen catalyzed by electron transfer in the respiratory chain. In *E. coli* the respiratory chain can account for over 80% of H_2O_2 production (92). In the environment, ionizing, near-UV radiation, or numerous compounds

that generate intracellular cellular O_2^- can also cause oxidative stress. The reactive oxygen species generally target DNA, RNA, proteins, and lipids. The hydroxyl radicals generated from H_2O_2 through Fenton reactions leads to modifications of macromolecules. The free radicals can also directly react with polyunsaturated fatty acids in membranes and can initiate lipid peroxidation. Lipid peroxidation decreases membrane fluidity and can alter membrane properties by disrupting membrane-bound proteins. Due to disruption of membrane-bound proteins more radicals are formed and polyunsaturated fatty acids are broken down to multiple unwanted products such as aldehyde derivatives (93). These aldehydes can diffuse from their site of origin and can react with other biomolecules inside the cell. The aldehyde compounds that are produced as a by-product of oxidative stress are also called second toxic messengers. The aldehyde species reactwith both the base and sugar moieties of DNA leading to a single and double strand break in the backbone. These alterations on DNA strands lead to lesions that block replication (94). In proteins the reactive oxygen species can lead to oxidation of sulfhydryl groups, reduction of disulfides, oxidative adduction of amino acids residues near the metal-binding sites, reaction with aldehydes, protein-protein cross linking, and peptide fragmentation leading to loss of protein activity.

1.7 Defense mechanisms against reactive oxygen species

Cells have evolved and developed defense mechanisms that either keep the concentration of O^{2-} derived radicals at appropriate levels or repair oxidative damages. Iron plays a key role in cellular systems and Fe^{2+} is necessary for the growth of all living organisms. Iron solubilization and metabolism are strictly regulated at two levels : (1) the entrance to the cell by specific membrane bound receptors, and (2) inside the cell, by two proteins, bacterioferritin and ferritin. The nonenzymatic antioxidants such as NADPH, β-carotene, ascorbic acid, α-tocopherol, and

glutathione (GSH) are molecules that present and assist in maintaining the intracellular reducing environment and scavenge reactive oxygen species. GSH assist in maintaining a strong reducing environment inside the cell and its reduced form is maintained by glutathione reductase using NADP. There are also certain specific enzymes that help in decreasing the reactive oxygen species in the cell. Superoxide dismutase (SOD) is an iron-containing enzyme that converts O^{2-} to H_2O_2 and O_2 (95). In *E. coli* H_2O_2 is removed by two catalases hydroperoxidase I (HPI) and hydroperoxidase II (HPII) (96). Glutathione peroxidase and DT-diaphorase are also scavenging enzymes. Under stress conditions DNA repairing enzymes like endonuclease IV and exonuclease III are induced (97). In prokaryotic cells there are certain enzymes that help in repairing the damages on primary structure of proteins. Thioredoxin reductase is one such repair and modification agent. Methionine sulfoxide reductase helps in repairing the oxidation of methionine to methionine sulfoxide.

1.8 Genetic responses to oxidative stress

All aerobic organisms show genetic responses towards oxidative stress. Transcriptional activators OxyR and SoxRS in *E. coli* provides defense against peroxides and superoxides respectively (Figure 1.16) (98, 124). The SoxRS operon is comprised of 15 genes that encode enzymes like Mn-SOD, endonuclease IV, glucose-6- phosphate dehydrogenase, a fumarase, aconitase, and ferredoxin reductase. The *oxyR* gene controls the genes encoding HPI catalase, glutaredoxin, glutathione reductase, and NADPH-dependent alkylhydoperoxide reductase. The activation of these proteins greatly increases the bacterial cells resistance towards oxidative stress. More recent studies have suggested that OxyR and SoxR are always present in *E. coli* cells, but are generally inactive in unstressed cells (99). When the redox state of the cytosol is

altered due to oxidative stress OxyR is reversibly activated by formation of intramolecular disulfide bonds (100, 101). The glutaredoxin which controls the cellular disulfide machinery is responsible for reversing the activation of OxyR. But the gene responsible for encoding glutaredoxin is regulated by OxyR. This provides a mechanism of autoregulation, which uses the formation and reduction of disulfide bonds as a switch allowing a rapid response against oxidative stress.

1.9 $FMNH_2$ transfer mechanism in the alkanesulfonate monooxygenase system

The flavin-dependent two-component alkanesulfonate monooxygenase system is considered an atypical flavoprotein because the flavin isnot tightly bound through covalent or non-covalent interactions as a cofactor. Therefore an important step for this flavin-dependent two-component system 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 for many of these systems.

1.9.1 Evaluation of static interactions between SsuE and SsuD

The genes for the SsuE and SsuD enzymes in *E. coli* are located on the same operon which suggests that these two proteins may work in close contact with each other to carry out a desulfonation reaction. A stable complex was identified in affinity chromatography experiments using 6X his-tagged SsuD as bait for SsuE. The coelution and intermediary structure formation between SsuE and SsuD was not due to nonspecific protein-protein interactions because no such interactions were seen between SsuD and an unrelated protein. A trifunctional cross-linker was also used to probe protein-protein interactions between SsuE and SsuD. This reagent contains an amine reactive site, a photoreactivearylazide, a biotin label and a cleavable disulfide bond. After reacting this reagent with a mixture of SsuE and SsuD the silver-stained gel showed a protein

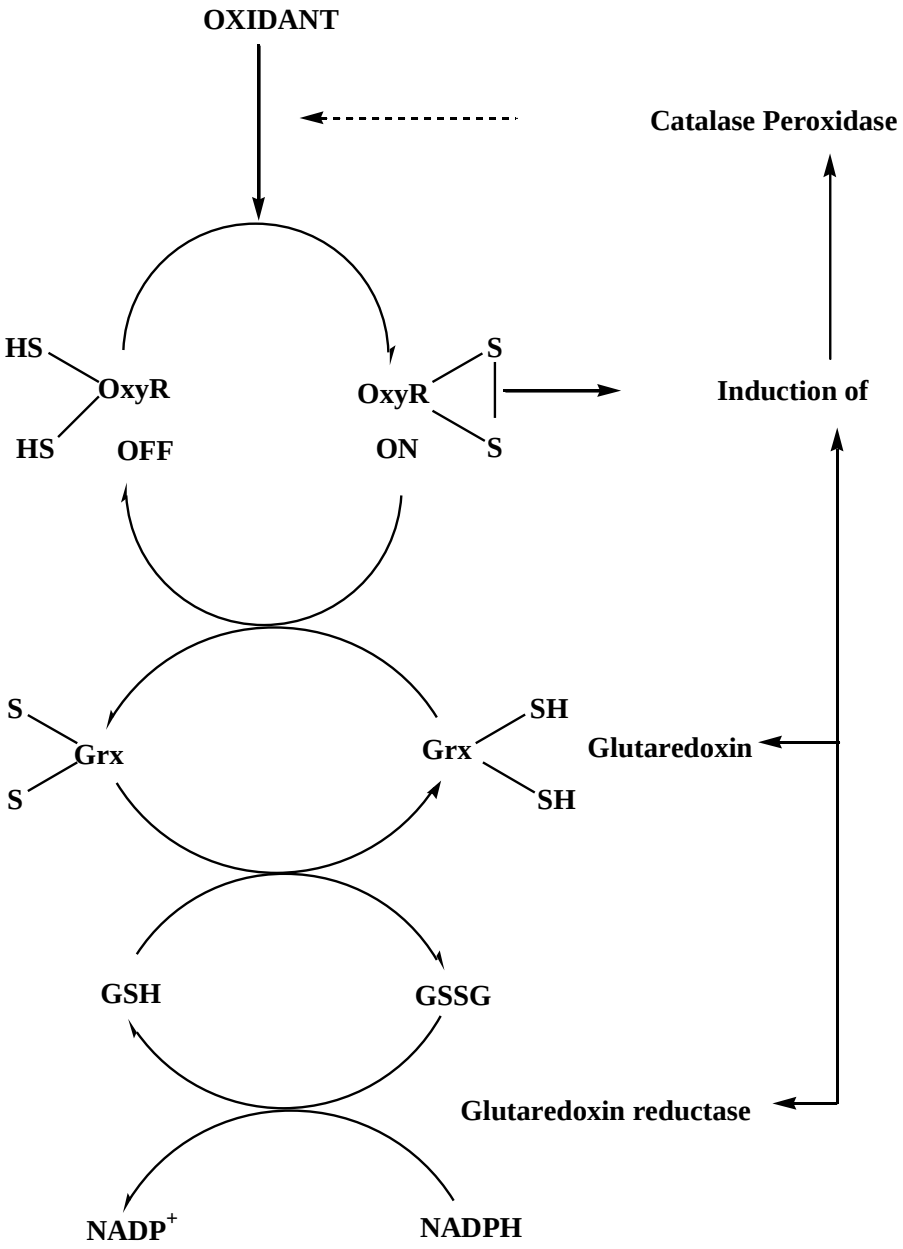


Figure 1.16 Oxidative stress activates OxyR by formation of a disulfide bond. OxyR triggers the expression of reductive activities such as enzymes that degrade the oxidant and reduce disulfide bonds. Reduction of disulfide bonds provides negative feedback (Adapted from (124)).

band at around 63 kDa that correlated with the monomer of SsuE and SsuD covalently bound with the cross-linking reagent. The control experiment with only SsuE or SsuD with the reagent showed no such high-molecular band (32). The results from these studies clearly demonstrated that static protein-protein interactions do exist between SsuE and SsuD.

1.9.2 Kinetic studies for the protein-protein interactions between SsuE and SsuD.

Kinetic studies were performed to evaluate protein-protein interactions between SsuE and SsuD. The kinetic mechanism of SsuE was altered from an ordered sequential mechanism to an equilibrium ordered mechanism in the presence of SsuD and octanesulfonate (28). This change in mechanism was also correlated with a 10-fold increase in the K_m value of FMN for SsuE. The far-UV circular dichroism spectra highlighted that any change that occur due to protein-protein interactions between SsuE and SsuD may be only due to minor perturbations in the protein structure that would not be translated to the overall secondary structure. These studies were performed in the presence of FMN with SsuE and SsuD. There was a decrease in molar ellipticity observed by visible CD spectra which was attributed due to a change in the flavin environment. This change in flavin environment was then further analyzed by using fluorescence spectroscopy. A decrease in fluorescence was observed when flavin binds to SsuE, but when the FMN-bound SsuE was titrated against SsuD an increase in fluorescence was observed that reached a saturation point. This increase in fluorescence was associated with a change in the flavin environment due to protein-protein interactions and the conformational changes associated with those interactions.

1.10 Summary

Flavin is an important cofactor for the transfer of electrons between different pathways in the cell. In the bacterial cell flavin can function as a cofactor or substrate. The reduced flavin is highly labile and can easily react with dioxygen molecule to produce reactive oxygen species like H_2O_2, O_2^- and other oxygen radicals. These reactive species are harmful to the cell and can lead to cell death. The bacterial cell has developed specific mechanisms to carefully monitor the flavin concentration in the cell. There has been considerable development in understanding the mechanism of reduced flavin transfer in two-component enzyme systems. But there is no clear consensus on one transfer mechanism. Based on various results that have been reported the transfer mechanism for reduced flavin is system dependent. In the alkanesulfonate monooxygenase system SsuE reduces oxidized FMN in the presence of NADPH to produce $FMNH_2$. This reduced flavin is then transferred to SsuD where it activates a dioxygen molecule to form C-4a (hydro)peroxyflavin intermediate which is the reactive oxygen intermediate involved in the breakdown of the C-S bond in alkanesulfonate. FMN acts a substrate in the alkanesulfonate monooxygenase system, hence the transfer of reduced flavin from SsuE to SsuD is a key step.

In this study experiments have been performed to establish the protein-protein interactions sites between SsuE and SsuD. How the protein-protein interactions can lead to more effective transfer of reduced flavin is an interesting question. Even though the previous studies have established the presence of physical interactions between SsuE and SsuD, the finding of specificamino acid residues involved in such interactions further promote the mechanistic studies of the alkanesulfonate monooxygenase system. The alkanesulfonate monooxygenase system is expressed during sulfur-starvation conditions. The alkanesulfonate monooxygenase enzyme

comprise an interesting two-component enzyme system because of their role in providing sulfate to the bacterial cell during sulfur-starvation conditions and its presence in a large group of bacteria. Therefore, the studies described here will help us understand how the enzyme system prevents the two-component system from uncoupling by efficiently transferring reduced flavin.

CHAPTER TWO

Exposing the Alkanesulfonate Monooxygenase Protein-Protein Interaction Sites

2.1 Introduction

The alkanesulfonate monooxygenase system is involved in replenishing sulfur resources during sulfur-limitation in various bacteria (2, 3). An interesting mechanistic feature of the alkanesulfonate monooxygenase system is the transfer of reduced flavin from the NADPH[1]-dependent flavin mononucleotide reductase (SsuE) to the alkanesulfonate monooxygenase (SsuD). Unlike canonical flavoproteins, SsuE utilizes FMN as a substrate rather than a cofactor.[3] The transfer of reduced flavin in two-component systems could occur through a diffusion or direct transfer mechanism involving protein-protein interactions. There are a large number of flavin-dependent two-component systems identified in bacteria, but there is no clear consensus on the mechanism of reduced flavin transfer. Different mechanisms for the transfer of reduced flavin have been reported depending on the system studied (1, 4). Reduced flavin is a labile molecule and can undergo autoxidation to form reactive oxygen species (15, 16). Therefore, flavin transfer between SsuE and SsuD in the alkanesulfonate monooxygenase system could be facilitated by protein-protein interactions to prevent the generation of reactive oxygen species. Protein-protein interactions have been identified between SsuE and SsuD, but the location of these interaction sites

has not previously been determined (1). The protein-protein interactions may assist in the hand-off of reduced flavin from SsuE to SsuD, providing a protected passage for effective transfer of reduced flavin.

The three-dimensional structure of the SsuD enzyme is composed of a triosephosphate isomerase (TIM)-barrel fold, but contains several insertion sequences that deviate from the $(\beta/\alpha)_8$ TIM-barrel core (17). An insertion sequence positioned over the active site contained a loop region with poorly defined electron density.[17] Protein dynamics appear to play a critical role in the alkanesulfonate monooxygenase system, and may assist in flavin transfer. The loop region undergoes a conformational change with the binding of substrates, and was shown to protect reduced flavin from non enzymatic oxidation (18). Closure of the dynamic loop over the SsuD active site would be dependent on reduced flavin transfer from SsuE to SsuD. Putative protein-protein interaction regions would likely be located near the active site opening under the dynamic loop. Although structural dynamics of SsuD have been investigated, the oligomeric state and dynamic changes of SsuE during flavin reduction and transfer is currently not known (18, 19). Recent studies suggest a change in the oligomeric state occurs with the binding of flavin (20). The SsuE enzyme existed as a tetramer in the three-dimensional structure of SsuE, but in the presence of oxidized flavin the oligomeric state of SsuE was shifted to a dimer. Changes in the oligomeric state of SsuE with the binding of substrates could also directly impact protein-protein interactions with SsuD. Due to the lability of $FMNH_2$, the transfer of reduced flavin from SsuE to SsuD would need to be rapid and efficient. Thus, protein-protein interactions would bring the active sites of SsuE and SsuD in close proximity to each other, resulting in efficient $FMNH_2$ transfer. These protein-protein interactions may trigger conformational changes across the SsuD structure to enhance the desulfonation reaction. If protein-protein interactions play a

role in flavin transfer, then the protein dynamics of SsuE and SsuD would need to be finely synchronized events. The studies described herein were performed to determine if specific interaction sites are responsible for promoting protein-protein interactions between SsuE and SsuD. In order to better understand the mechanistic details of reduced flavin transfer between SsuE and SsuD, the protein-protein interaction sites were identified by amide hydrogen/deuterium exchange mass spectrometry (HDX-MS). Substitutions at the interaction region on SsuD were generated, and the ability of the variants to promote flavin transfer was evaluated. The reported results identify the interaction sites on SsuE and SsuD, while establishing a solid foundation for defining the mechanism behind reduced flavin transfer in the two-component alkanesulfonate monooxygenase system.

2.2 Experimental procedures

2.2.1 Materials

All chemicals were purchased from Sigma-Aldrich, Bio-Rad, or Fisher. *Escherichia coli* strain BL21(DE3) was purchased from Stratagene (La Jolla, CA). DNA primers were synthesized by Invitrogen (Carlsbad, CA). The expression and purification of wild-type SsuE and SsuD variants was performed as previously reported (10).

2.2.2 Hydrogen-Deuterium Exchange Mass Spectrometry

Pepsin-digestion of SsuE and SsuD was initially performed to identify peptide fragments through collision-induced dissociation.The SsuE or SsuD enzymes (60 μM) were digested with 5 mg/mL pepsin for 5 min, and the digested peptides were separated on a Phenomenex 50 × 2–mm microbore C18 HPLC column over 15 min at 0.1 mL/min using a linear gradient of 0-50%

solvent B (98% acetonitrile, 2% H_2O, 0.4% formic acid). Peptides were sequenced with a BrükerHCTUltra PTM Discovery mass spectrometer in positive ion mode by data dependent MS/MS,and identified by PEAKS Client 6 (Bioinformatics Solutions Inc.). Individual HDX-MS experiments were performed with 60 µM (2 µL) of SsuE or SsuD in 25 mM potassium phosphate, pH 7.5, and 5 mM DTT. The SsuE-SsuD complex was generated by combining equal volumes (1 µL each) of 120 µM SsuE and SsuD. The HDX reaction was initiated with the addition of 23 µL of D_2O (99.9%) (21). All the samples were incubated at 25 ˚C for 30 s, after which the reaction was stopped with 25 µL of quench buffer (pH 2.3, 0 °C). The samples were then immediately digested on ice with 2 µL of 5 mg/mL pepsin for 5 min. The digested peptides were separated on a Phenomenex 50 × 2–mm microbore C18 HPLC column over 15 min at 0.1 mL/min using a linear gradient of 0-50% solvent B (98% acetonitrile, 2% H_2O, 0.4% formic acid) (22). All samples for HDX were prepared individually and evaluated on the same day as the corresponding water and 100 % deuteration controls. The separated peptides were sequenced with a BrükerHCTUltra PTM Discovery mass spectrometer in positive ion mode by data dependent MS/MS. The spectra from each HDX-MS sample were analyzed using HDExaminer (Sierra Analytics). The percentage of deuterium incorporated for each peptide was determined with eq 1 (21).

$$\%D = \frac{(m_t - m_0)}{(m_{100} - m_0)} \times 100 \qquad (1)$$

wherem_t, m_0, m_{100} are the average molecular masses of the same peptide in the partially deuterated at 30 s, non-deuterated, and the fully-deuterated control samples, respectively.

2.2.3 Construction of the SsuD variants

The protected regions identified on SsuE and SsuD by HDX-MS contain polar amino acid residues that may be involved in non covalent interactions. Charged amino acids located on the

SsuD α helix at positions 251, 252 and, 253 were substituted with alanine to generate the DDE(251/252/253)AAA SsuD variant. The variants of SsuD were generated using the pET21a plasmid containing the *ssud* gene, as previously described (23). The primers were designed to replace the nucleotide base for Asp251, Asp252, and Glu253 with the alanine codon GCG, to generate DDE(251/252/253)AAA SsuD. The primers used to construct the SsuD variant containing a deletion of the α-helix from positions D251-A261 was generated by PCR-amplification using the primers 5′ TATCGCGAGGAGACAGGCCGATTTCGCCC 3′ and 5′ GCTTTGGAGGAGGTTTCATCATCAAGATGCG 3′ that includes a *BseR1* recognition sequence. The purified PCR product was digested with *BseR1* for one hour at 37 ˚C. After digestion, the truncated vector was ligated using T4 DNA ligase at 16 ˚C overnight. The C-terminal His-tagged ΔD251-A261 SsuD variant was generated by replacing the TAA stop codon of ΔD251-A261 *ssuD*with the alanine codon GCG. All the variant constructs were confirmed by DNA sequence analysis at Davis Sequencing (University of California, Davis). The SsuD variants containing the desired substitution or deletion were transformed into chemically competent *E. coli* BL21(DE3) cells for protein expression. The expression and purification of all SsuD variants was performed as previously reported (10).

2.2.4 Kinetic analyses

The assays to determine the kinetic parameters for the SsuD variants were performed as previously described (24). The reactions were initiated by the addition of NADPH (500 µM) into a reaction mixture containing wild-type SsuD or the SsuD variants (0.2 µM), SsuE (0.6 µM), FMN (2.0 µM), and varying concentrations of octanesulfonate (5-1000 µM) in 25 mMTris-HCl, pH 7.5, and 100 mMNaCl at 25 ˚C. Because of diminished activity of ΔD251-A261 SsuD, a final protein concentration of 2 µM was used with 6 µM SsuE and 20 µM FMN.

Fluorimetric titrations were used to determine the K_d values of wild-type SsuD and the SsuD variants for reduced flavin (24).Anaerobic SsuD solutions 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 ultra high purity argon gas for 15 min. Both protein and flavin solutions 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). Aliquots of reduced flavin (0.2 - 9.1 µM) were added to a 1 mL solution of wild-type SsuD or the SsuD variants (0.5 µM) in 25 mM potassium phosphate, pH 7.5, and 10% glycerol and the fluorescence spectra were recorded following a one min incubation after each addition. Protein samples were excited at 280 nm, and the fluorescence emission intensity was recorded at 344 nm following each addition of $FMNH_2$. The K_d value was determined as previously described (9, 24).

$$[S]_{bound} = [E]\frac{I_0 - I_c}{I_0 - I_f} \qquad (2)$$

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

$$y = \frac{(K_d + X + n) - \sqrt{(K_d + X + n)^2 - 4xn}}{2} \qquad (3)$$

Y and *X* represent the concentration of bound and free substrate, respectively, following each addition. K_D is the maximum binding at equilibrium with the maximum concentration of substrate.

2.2.5 Affinity chromatography binding assays

The assay to determine the physical interactions between 6X His-tagged ΔD251-A261 SsuD and native SsuE was performed using a Ni-NTA column with His-tagged protein as a receptor and native protein as a ligand. The His-tagged ΔD251-A261 SsuD variant and native SsuE were expressed by inoculating with the strain of bacteria containing the desired recombinant gene. A liter of LB-ampicillin medium containing expressed His-tagged ΔD251-A261 SsuD was harvested by centrifugation at 5000 g for 20 min. The harvested cells were then resuspended with 100 mL of 25 mM potassium phosphate, pH 7.5, and 10% glycerol containing 20 mg/mL of lysozyme. The resuspended cell pellet was sonicated followed by centrifugation (10,000 g, 20 min) and the lysate containing His-tagged ΔD251-A261 SsuD was loaded on a Ni-NTA column. The column was washed with 100 mL of 25 mM potassium phosphate, pH 7.5, and 10% glycerol to remove adventitiously bound protein. A liter of LB-Amp medium containing native SsuE was then treated similarly to ΔD251-A261 SsuD and the resulting lysate was loaded on the Ni-NTA column followed by an additional wash with 100 mL of 25 mM potassium phosphate, pH 7.5, and 10% glycerol. The column was washed with 200 mL of 25 mM potassium phosphate, pH 7.5, 10% glycerol, and 125 mM imidazole to remove all unbound proteins followed by 25 mM potassium phosphate, pH 7.5, 10% glycerol, and 300 mM imidazole to elute His-tagged ΔD251-A261 SsuD and any interacting proteins. Fractions from both imidazole washes were collected and analyzed by 12% SDS-PAGE.

2.2.6 Fluorimetric titration of FMN-bound SsuE with SsuD

Fluorimetric analyses were performed to determine the effects of substitutions and deletions of SsuD on the binding affinity with SsuE (1). The FMN-bound SsuE protein was prepared by titrating 0.4 µM FMN with 1 µL aliquots of SsuE for a total of 10-15 additions (0.04 to 0.4 µM). The protein samples were excited at 450 nm and fluorescence emission intensity measured at 524 nm. The FMN-bound SsuE solution was titrated with aliquots of D251A, DDE(251/252/253)AAA, Δ251-261 or wild-type SsuD (0.02 to 0.95 µM). The concentration of SsuD bound to SsuE was determined with eq 4.

$$[SsuD]_{bound} = [SsuE]\left[\frac{I_0 - I_c}{I_0 - I_f}\right] \qquad (4)$$

where [SsuE] represents the initial concentration of enzyme, I_0 is the initial fluorescence intensity of FMN prior to the addition of SsuD, I_c is the fluorescence intensity of FMN following each addition of SsuD, and I_f is the final fluorescence intensity. The concentration of SsuD bound (y) was then plotted against the total SsuD concentration (x) to obtain the dissociation constant (K_d) for binding between SsuE and SsuD fitting the plot to eq 5.

$$y = \frac{(K_d + X + n) - \sqrt{(K_d + X + n)^2 - 4xn}}{2} \qquad (5)$$

2.3 Results

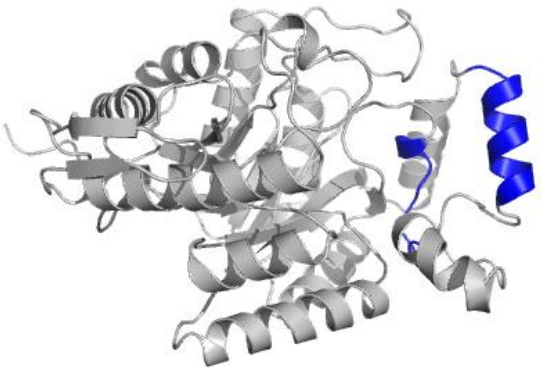
2.3.1 Hydrogen-deuterium Isotope Exchange Mass Spectrometry

Stable protein-protein interactions have been shown to exist between SsuE and SsuD, but the specific regions of each enzyme involved in these interactions have not been identified (1). Results from previous studies suggest that the protein-protein interactions between SsuE and SsuD are essential to promote flavin transfer (18). In order to evaluate the protein-protein

interaction sites, HDX-MS was utilized to identify regions of SsuE and SsuD that may be protected in a stable complex. The amount of deuterium exchange into backbone amides for a specified time for SsuE and SsuD separately were compared to that for the corresponding protein in the SsuE-SsuD complex. Differences in solvent accessibility for specific regions were observed for SsuE and SsuD upon complex formation (Figure 2.1). For SsuE, regions 78-89 (KAAYSGALKTLL) and 119-125 (YALKPVL) showed a >20% decrease in deuterium incorporation in the SsuE-SsuD complex (Figure 2.2). There was a comparable decrease in deuterium incorporation upon complex formation for SsuD regions 251-261 (DDETIAKAQAA) and 285-295 (EISPNLWAGVG) (Figure 2.3). Protected peptides on SsuE are located on two distinct α-helical regions (Figure 2.1 B). The protected SsuD D251-A261 peptide is located on an α-helix, and the E285-G295 peptide comprised a region of an α-helix and a short β–strand.Protected regions identified on SsuD are positioned at the active site opening, and are connected to each other by a dynamic loop (Figure 2.4). The dynamic loop was previously shown to close over the active site with the binding of substrates (18, 19). The location of the protected regions adjacent to the active site suggests that protein-protein interactions between SsuE and SsuD align the active sites to assist in flavin transfer.

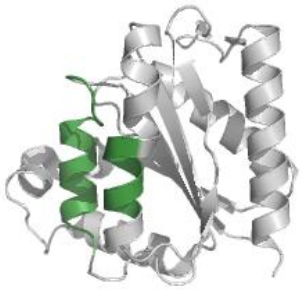
There are numerous charged amino acids located on the α-helical protected region of SsuD (Asp251, Asp252 and Glu253, and Lys257) and SsuE (Lys77, Lys86 and Lys121). These distinct positively and negatively charged amino acids of SsuE and SsuD may form salt bridges to promote protein-protein interactions. Substitutions were made at the protected sites in order to evaluate the role of these charged amino acids in protein-protein interactions. The substitution of charged residues were performed with SsuD due to the decreased structural stability of SsuE with even minor amino acid alterations, and were focused on the region comprising 251-261

(A)



SsuD
251-DDETIAKAQAA-261
285-EISPNLWAGVG-295

(B)

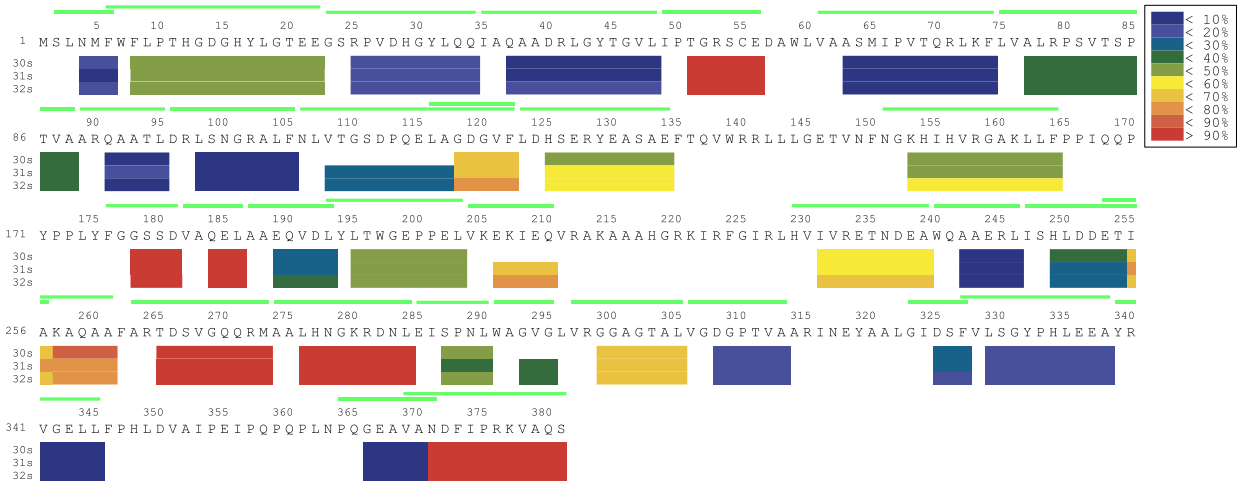


SsuE
78-KAAYSGALKTLL-89
118-YALKPVL-124

Figure 2.1 Solvent protected regions in the SsuE-SsuD complex identified by HDX-MS. (A)SsuD monomer highlighting regions 251-261 (DDETIAKAQAA) and 285-295 (EISPNLWAGVG)protected in the SsuE-SsuD complex. (B) SsuE monomer highlighting regions protected in the SsuE-SsuD complex.The structures were rendered with PyMOL. SsuE, PDB: 4PTY; SsuD, PDB: 1M41 with residues 250-282 constructed with PDB entry1NQK and 3RAO) (Adapted from (7)).

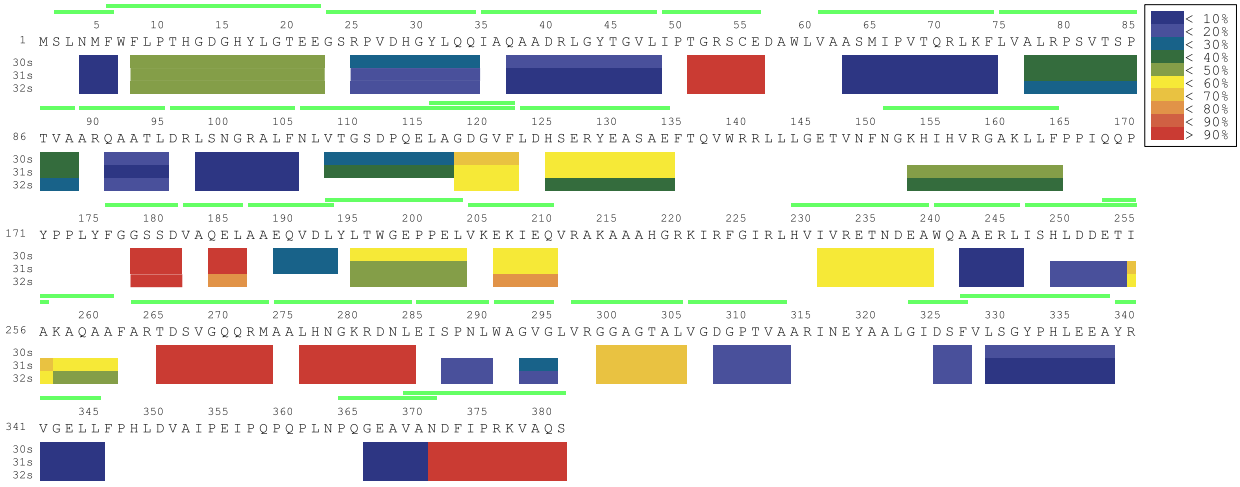
(A)

SsuD alone



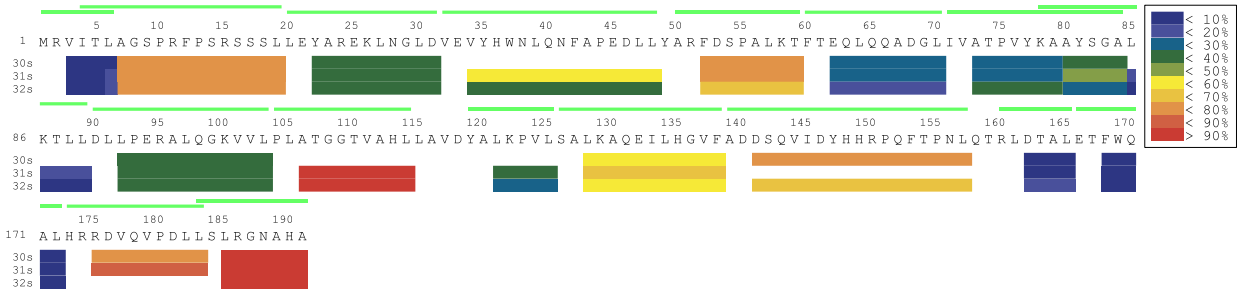
(B)

SsuD in complex with SsuE



(C)

SsuE alone



(D)

SsuE in complex with SsuD

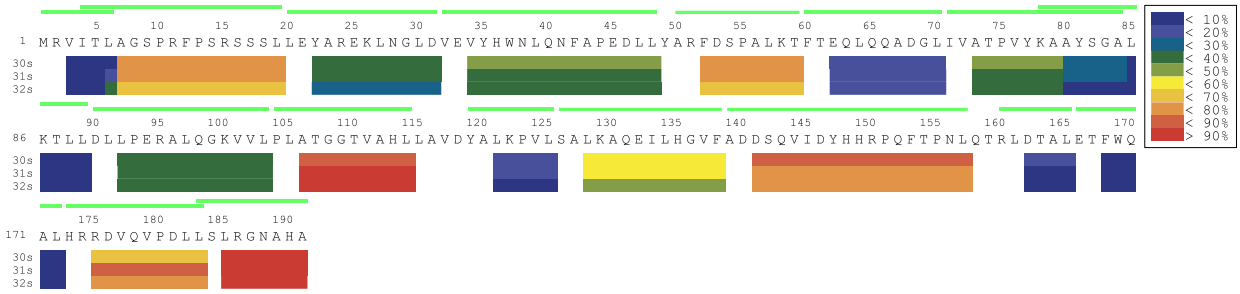


Figure 2.2 (A) The peptide map sequences for SsuD (251-262) (285-295) and SsuE (78-89) (118-124) with or without complex. Peptides were sequenced with Bruker HCT Ultra PTM discovery mass spectrometry in positive in mode by data dependent MS/MS. (Adapted from (41)).

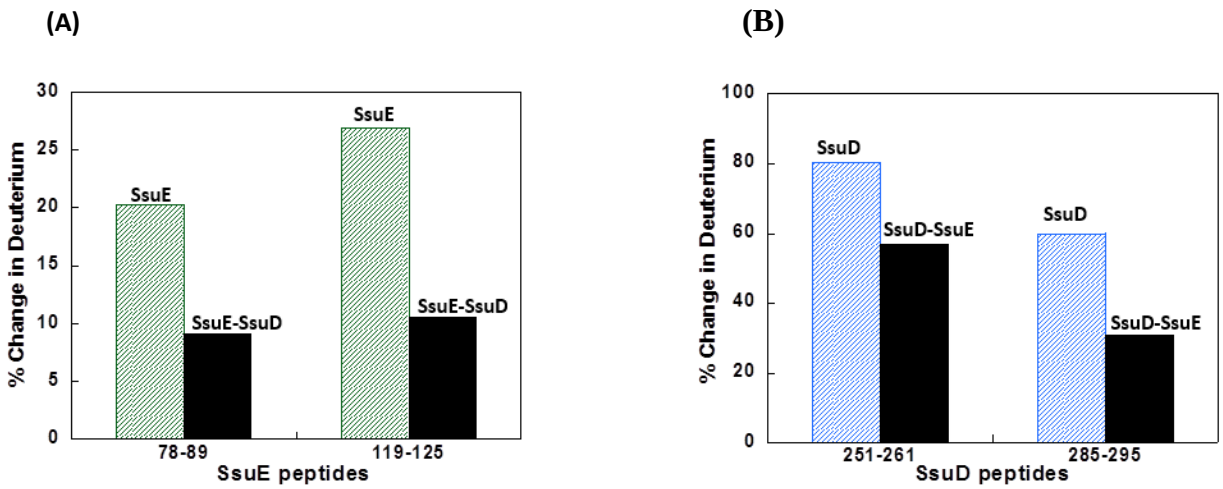


Figure 2.3 Change in deuterium incorporations at regions (A) 78-89 and 119-125 on SsuE and (B) 251-261 and 285-295 on SsuD upon SsuE-SsuD complex formation. (Adapted from (41)).

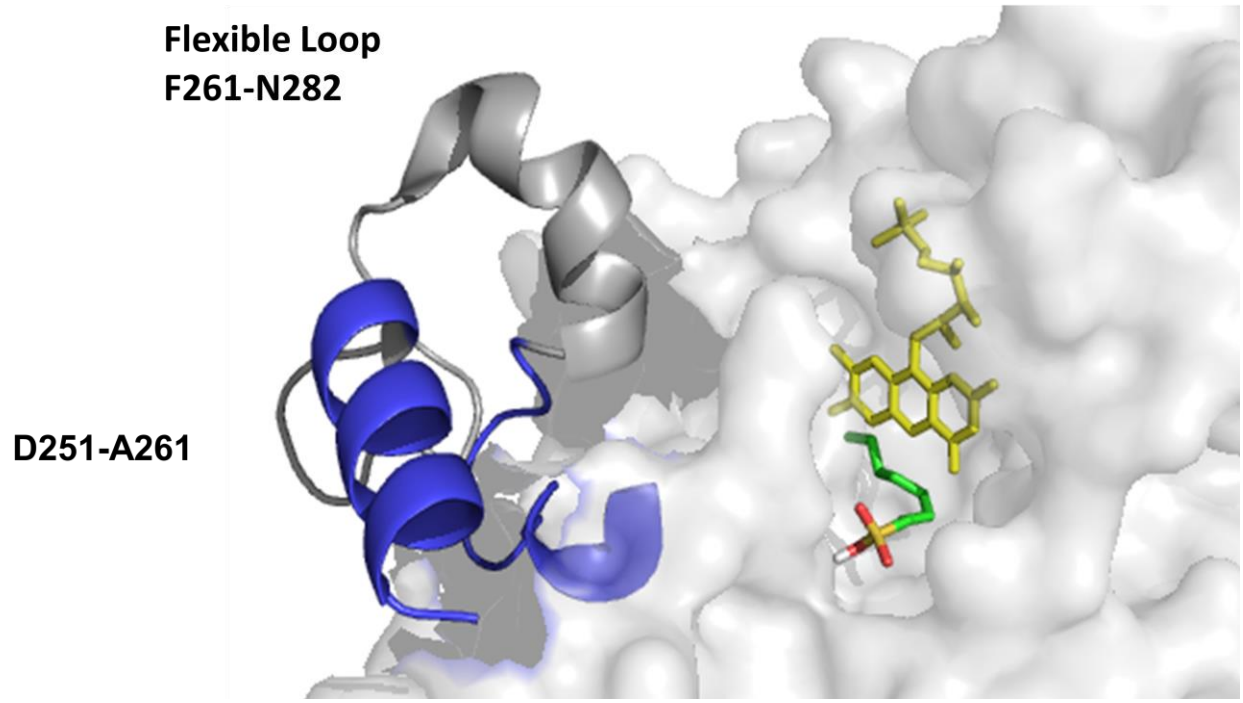


Figure 2.4 The SsuD monomer with the active site highlighted relative to the solvent protected region. Protected SsuD peptides D251-A261 and E284-G294 (blue)are located on an insertion region adjacent to the active site. The active site is shown with the flavin (yellow) and octanesulfonate bound.The structure was rendered with PyMOL (PDB:1M41), and the contiguous loop region was constructed with PDB entry 1NQK and 3RAO (Adapted from (8)).

(DDETIAKAQAA). This region comprises an insertion region leading to a divergence from the canonical TIM-barrel structure. Variants of SsuD containing single and double substitutions of negatively charged amino acid residues located on region 251-261 had similar catalytic properties as wild-type SsuD. Variants containing multiple substitutions of the charged amino acids (DDE(251/252/253)AAA SsuD) and a deletion (ΔD251-A261 SsuD) of the α-helical region of SsuD were generated in order to better assess the functional role of this site.

2.3.2 Steady-state kinetic analyses of the SsuD variants

The steady-state kinetic activity of SsuD is routinely evaluated in a coupled assay with SsuE supplying reduced flavin. The effects of the SsuD substitutions on reduced flavin transfer from SsuE to SsuD were assessed in coupled assays monitoring the release of sulfite from the desulfonation of octanesulfonate. Single and double variants of the charged residues in theprotected region from 251 to 261 had similar steady-state kinetic properties as wild-type (data not shown). The k_{cat}/K_mvaluefor DDE(251/252/253)AAA SsuD with octanesulfonate showed a ~4-fold decrease compared to wild-type SsuD (Table 2.1).Deletion of the α-helix from D251-A261 showed no defects in the overall gross secondary structures, but exhibited no detectable activity under standard assay conditions. Increasing the concentration of ΔD251-A261 SsuD, SsuE, and FMN resulted in partial recovery of the activity for the deletion variant, but the k_{cat}/K_m value was still decreased 10-fold compared to wild-type SsuD (Table 2.1). These results show that the catalytic activity of SsuD was affected upon substitution and deletion at the solvent protected sites identified in HDX-MS.

Table 2.1: Kinetic parameters for wild-type, DDE(251/252/253)AAA, and Δ251-261 SsuD.

	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}/min^{-1}$)	K_d[a] (μM)
wild-type SsuD	53 ± 4	43 ± 5	1.2 ± 0.1	1.31 ± 0.05
DDE(251/252/253)AAA SsuD	51 ± 1	157 ± 6	0.30 ± 0.01	1.24 ± 0.04
ΔD251-A261 SsuD	-[b]	-	-	1.27 ± 0.06

[a]Determined under anaerobic conditions as described in Experimental Procedures.

[b]No activity detected under standard steady-state conditions.

2.3.3 Fluorimetric titration of anaerobic $FMNH_2$ against SsuD variants

Fluorimetric titrations were performed to determine if the decrease in enzymatic activity for the SsuD variants was due to a disruption in reduced flavin binding. To evaluate flavin binding with the SsuD variants, $FMNH_2$ was titrated into a sample of DDE(251/252/253)AAA or ΔD251-A261A SsuD. The spectra were recorded with an excitation wavelength at 280 nm and emission intensity measurements at 344 nm. The K_d values for $FMNH_2$ binding to DDE(251/252/253)AAA and ΔD251-A261A SsuD were comparable to wild-type SsuD (1.31 ± 0.05) at 1.24 ± 0.04 and 1.27 ± 0.06 μM, respectively (Table 2.1). These results suggest thatsubstitutions and deletions in the protected α-helical region did not alter the binding affinity of $FMNH_2$. Therefore, the change in activity of DDE(251/252/253)AAA and ΔD251-A261A SsuD was not due to a decrease in the the binding affinity of $FMNH_2$.

2.3.4 Pull-down Assays between SsuE and 6X his-tagged SsuD variants

An uncoupling of reduced flavin transfer from SsuE to ΔD251-A261 SsuD due to disruption of protein-protein interactions could lead to the absence of activity observed in steady-state kinetic assays. In order to examine if the protected α-helical region on SsuD plays an important role in protein-protein interactions that promote reduced flavin transfer, pull-down assays with His-tagged ΔD251-A261 SsuD and native SsuE were performed. Static protein-protein interactions between SsuE and His-tagged SsuD were previously identified through pull-downassays (32). Expressed His-tagged ΔD251-A261 SsuD from a cell lysate was loaded onto a Ni-NTA column.Following a phosphate buffer wash to remove unbound protein, wild-type SsuE from a cell lysate was loaded onto the Ni-NTA column containing bound His-tagged ΔD251-A261 SsuD. The column was first washed with 125 mM imidazole buffer to remove any unbound

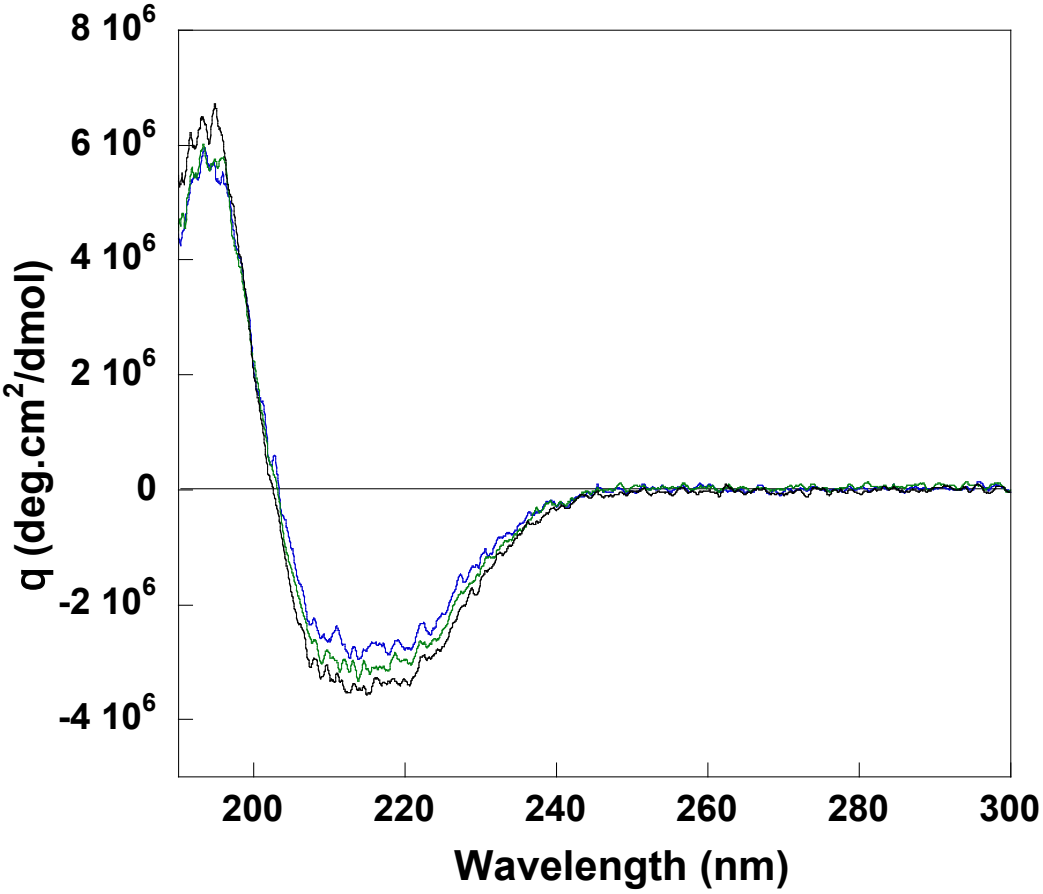


Figure 2.5 A CD-graph for secondary structures for wild-type, DDE(251/252/253)AAA, and ΔD251-A261 SsuD. (Adapted from (41)).

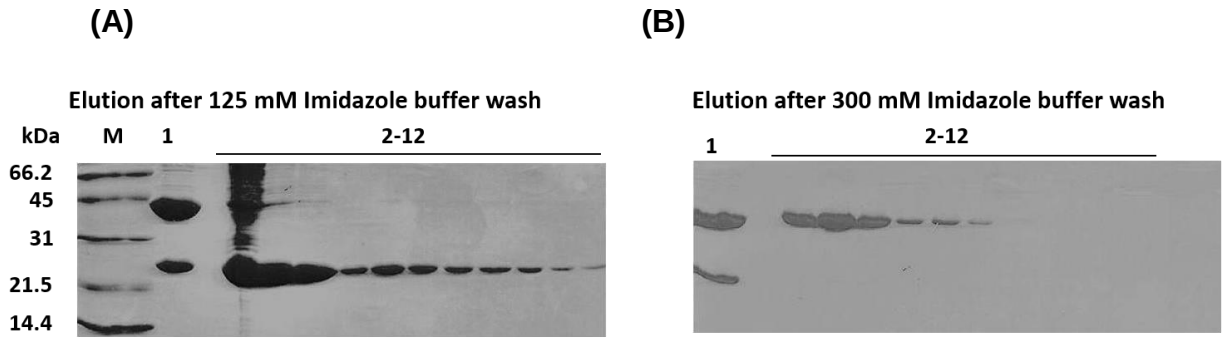


Figure 2.6 SDS-PAGE (12% acrylamide) from affinity chromatography experiments with SsuE and the 6×His-tagged ΔD251-A261 SsuD. Clarified cell lysate containing expressed 6×His-tagged ΔD251-A261 SsuD was loaded onto a Ni-NTA column. Followed a column wash, clarified cell lysate containing expressed native SsuE was loaded on the column. (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), lanes 4-15 represent fractions after the 125 mM imidazole wash. (B) SDS-PAGE of fractions after the 300 mM imidazole wash, lane 1 is protein standard, lane 2 is wild-type SsuD (41.7 kDa) and SsuE (21.3 kDa), and lanes 4-15 represent fractions after the 300 mM imidazole wash over time. (Adapted from (41)).

protein followed by a second wash with 300 mM imidazole buffer to remove bound His-tagged ΔD251-A261 SsuD. Analysis of the eluted protein fractions by SDS-PAGE showed that unbound wild-type SsuE eluted with the 125 mM imidazole wash, and His-tagged ΔD251-A261 SsuD eluted from the column with the 300 mM imidazole wash (Figure 2.3). In previous studies, SsuE coeluted with His-tagged SsuD in the 300 mM imidazole buffer wash due to protein-protein interactions. The separate elution of wild-type SsuE and ΔD251-A261 SsuD indicates that protein-protein interactions were disrupted with the deletion of the α-helical region. Although DDE(251/252/253)AAA SsuD had only a 4-fold decrease in activity, wild-type SsuE did not coelute with the His-tagged variant in comparable experiments. The absence of protein-protein interaction observed in the pull-down assays were likely due to a decrease in the binding affinity between SsuE and the SsuD variants.

2.3.5 Fluorimetric titration of FMN bound SsuE against SsuD variants

Fluorimetric titrations were performed to determine if the absence of protein-protein interactions in the pull down assays were due to a decrease in the binding affinity between SsuE and the SsuD variants. It was previously demonstrated that the emission spectra from 470-650 nm shows a decrease in flavin fluorescence when SsuE binds FMN (32). Titration of the SsuE-FMNcomplex with SsuD results in an increase in flavin fluorescence due to protein-protein interactions causing a change in the local flavin environment of SsuE (Figure 2.5). The FMN-bound SsuE complex was titrated with wild-type, DDE(251/252/253)AAA, or ΔD251-A261SsuD, and the spectrarecorded at an excitation of 450 nm with emission intensity measurements from 470-650 nm. As previously described, the wild-type SsuD enzyme has a high affinity for SsuE (32). A comparable K_d value of 0.040 ± 0.004 μM was determined in these studies for the interaction of wild-type SsuD and SsuE (Table 2.2). Titrations performed with the

FMN-bound SsuE complex and DDE(251/252/253)AAA SsuD variant demonstrated saturation behavior with a K_d value of 0.21 ± 0.03 μM even though there were no observable interactions in pull-down assays. It was not possible to obtain an accurate K_d value for ΔD251-A261 SsuD with SsuE because there was no increase in the fluorescence intensity with the addition of SsuD (Figure 2.5B). The relatively small change in fluorescence could be due to nonspecific interactions of the variants with SsuE. The titration experiments corroborated the results observed in the pull-down assays that protein-protein interactions with SsuE were weakened with the DDE(251/252/253)AAA and ΔD251-A261 SsuD variants.

2.4 Discussion

Organisms utilize reduced flavin in diverse oxidative reactions. For many flavoproteins the flavin is tightly or covalently bound to the enzyme, and the reductive and oxidative half-reaction flavin is transferred between the reductase and oxygenase enzymes, and efficient transfer of reduced flavin is a priority to prevent the desulfonation reaction from decoupling. The mechanism of transfer of the reduced flavin in two-component enzyme systems is poorly understood, but could either occur through a diffusion or direct transfer mechanism. Free reduced flavin is highly labile and can undergo non enzymatic oxidation to produce reactive oxygen species (15, 16). Therefore, inefficient flavin transfer could increase oxidative stress in bacteria leading to cellular damage. A direct transfer mechanism would provide a protective transfer mechanism for reduced flavin without the risk of non enzymatic oxidation. Flavin transfer by a direct transfer mechanism would rely on protein-protein interactions between the reductase and oxygenase. Previous studies have identified protein-protein interactions between SsuE and SsuD. The SsuE-SsuD complex binds in a 1:1 stoichiometric ratio with a dissociation

Table 2.2: Binding affinity of SsuE with wild-type, D251A SsuD, DDE(251/252/253)AAA, and ΔD251-A261 SsuD.

	K_d (μM)
Wild-type SsuD	0.039 ± 0.004
D251A SsuD	0.064 ± 0.015
DDE(251/252/253)AAA SsuD	–[a]
ΔD251-A261 SsuD	–

[a] The K_d could not be determined because titration of this variant did not reach saturation.

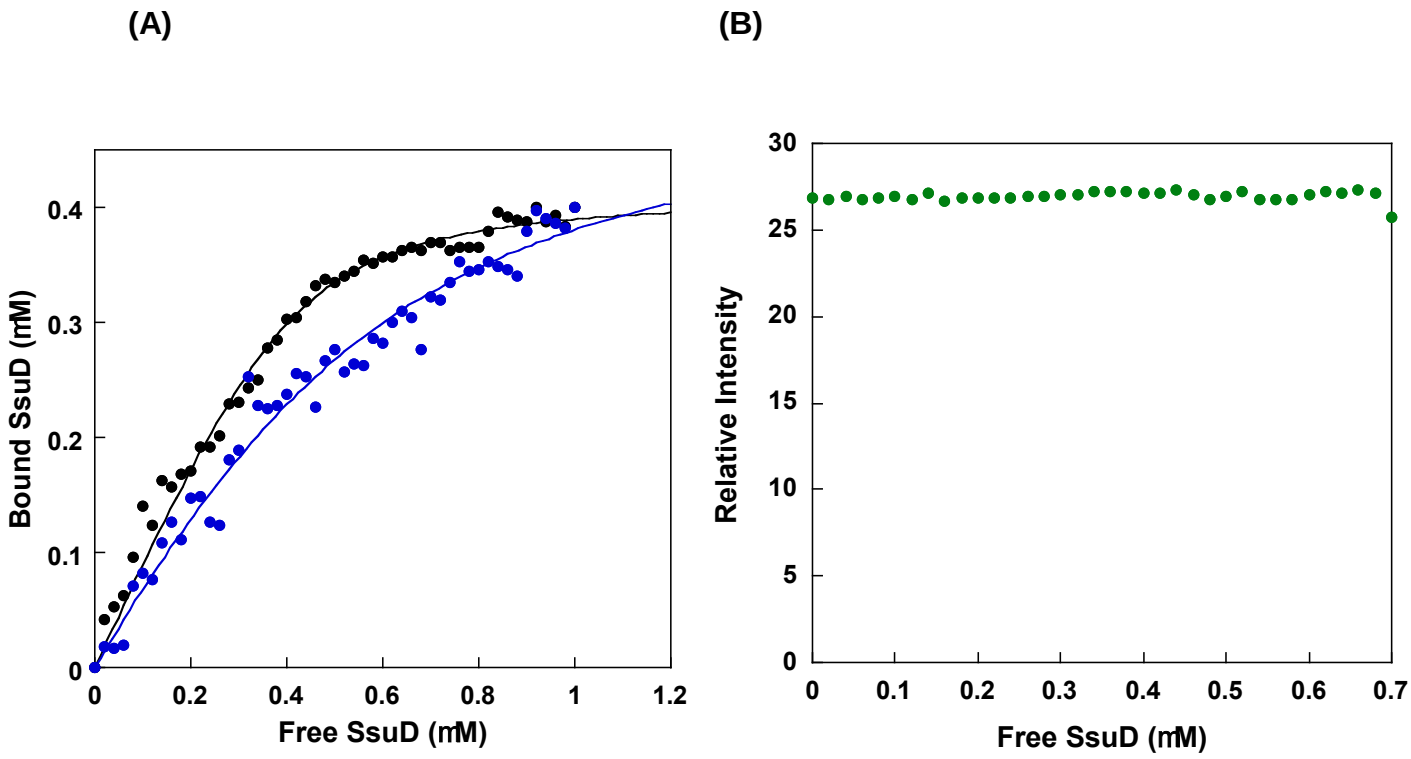


Figure 2.7 Fluorometric titration of wild-type or SsuD variants to FMN-bound SsuE. (A) Plot of the data for wild-type SsuD (black), andDDE(251/252/253)AAA (blue) determined with eq 1 from the change in fluorescent intensities. (B) Relative intensity ofΔD251-A261SsuD (green). 0.40 µM concentration of FMN-bound SsuE was titrated with wild-type or the SsuD variants (0.02-0.95 µM). (Adapted from (41)).

constant of 0.022 ± 0.001 μM. In the presence of SsuD and octanesulfonate the kinetic mechanism of SsuE was modified from a sequential to a rapid equilibrium ordered mechanism, suggesting that protein-protein interactions between SsuE and SsuD altered the kinetic properties of SsuE (10). Protein-protein interactions would bring the two active sites in close proximity to each other to assist in the efficient transfer of reduced flavin. The higher reduced flavin affinity observed for SsuD relative to SsuE ensures that once SsuE release reduced flavin it will readily bind to SsuD and be protected from nonenzymatic oxidation (24). Results from HDX-MS suggested that specific regions on SsuE and SsuD were protected from hydrogen deuterium exchange when both proteins were included in the reaction in the absence of the flavin substrate. The rate of deuterium incorporation decreased at specific sites on both proteins at a similar percent. For SsuD, the percent deuterium incorporation decreased for peptides D251-A261 and E285-G295. A similar decrease in deuterium incorporation was observed for SsuE peptides K78-L89 and Y119-L125 SsuE. These results support and extend our previous findings that protein-protein interactions occur between SsuE and SsuD, and identified where these putative contact sites were located. The SsuD D251-A261 α-helix contains polar residues, and is located adjacent to the previously characterized dynamic loop region (17). The polar residues located on the SsuD D251-A261 α-helix are conserved across SsuD enzymes from numerous bacterial sources.

Variants with multiple substitutions of the charged residues and a deletion of the α-helix were generated to assess how the SsuD α-helical region on the insertion region may assist in protein-protein interactions with SsuD. The steady-state kinetic assays monitoring desulfonation by SsuD are performed with SsuE supplying reduced flavin to the reaction. The SsuE enzyme is in excess over SsuD so that the reduced flavin is not limiting in the reaction. Any decrease in the observed activity with the SsuD variants could be attributed to an inability of SsuE to effectively

transfer reduced flavin. Single variant substitutions in the SsuD D251-A261 region did not result in a significant change in the steady-state kinetic parameters of SsuD. However, multiple substitutions of the charged residues to generate the DDE(251/252/253)AAA SsuD variant resulted in a 4-fold decrease in the k_{cat}/K_m value. Although the DDE(251/252/253)AAA SsuD showed only a modest decrease in activity, substitutions on the α-helix may have weakened the protein-protein interactions with SsuE leading to ineffective transfer of reduced FMN to SsuD. There was no measurable activity with ΔD251-A261 SsuD under standard reaction conditions. Relative concentrations of the reaction components were increased 10-fold in order to obtain measurable activity for the ΔD251-A261 SsuD variant. Results from fluorescence titration experiments with the SsuD variants gave similar K_d values for reduced flavin binding as wild-type SsuD. Therefore, the altered desulfonation activity of the SsuD variants was not due to a decrease in the flavin affinity.

The absence of activity in the steady-state kinetic assays for ΔD251-A261 SsuD was attributed to a disruption in protein-protein interactions. The inability of the deletion variant to form protein-protein interactions was corroborated in pull-down assays with His-tagged ΔD251-A261 SsuD and wild-type SsuE. Comparable control experiments demonstrated the coelution of His-tagged wild-type SsuD and SsuE from the Ni-NTA column with a higher concentration of imidazole as was previously observed. The absence of protein-protein interactions between SsuE and ΔD251-A261 SsuD in the fluorimetric titration studies supported the findings from pull-down experiments. Desulfonation activity was observed with a 10-fold increase in flavin and enzyme concentration, suggesting the overall structural properties of the variant were still viable as supported by circular dichroism spectroscopy. Although the DDE(251/252/253)AAA SsuD did not interact with SsuE in pull-down assays, there was only a 5-fold decrease in the affinity

between FMN-bound SsuE with DDE(251/252/253)AAA SsuD compared to wild-type SsuD with SsuE in fluorimetric titrations. The observed discrepancies can be attributed to the enhanced sensitivity of detection in the fluorimetric titrations, and the overall decrease in affinity can be attributed to a decrease in protein-protein interactions between SsuE and the SsuD variant due to alterations on the α-helix. It is significant that the activity and interactions of DDE(251/252/253)AAA SsuD with SsuE were not extensively disrupted. The deletion of the negatively charged residues may have led to a slight decrease in the sensitivity of the interactions, but the presence of the helical region may still maintain some limited interactions. In steady-state kinetic assays, SsuE is typically in a 3-fold excess over SsuD so reduced flavin is not limiting. While protein-protein interaction sites are present on SsuE and SsuD, disruption of these sites may not affect the ability of reduced flavin to be transferred if a flavin supply is in excess. Therefore, flavin transfer could be successfully transferred by diffusion under specified conditions.

The α-helix of SsuD appears to play a key role in dynamic and conformational changes required for interaction with SsuE (18, 19). The SsuD enzyme is a TIM-barrel protein expanded by insertion regions that deviate from the $(\beta/\alpha)_8$ barrel core. In the three-dimensional structure of SsuD, there was an area of poorly defined electron density located in an insertion sequence (17). The insertion sequence was later shown to contain a dynamic loop region (18, 19). Further studies suggested a conformational change of the SsuD dynamic loop is initiated by the binding of substrates and protects the peroxyflavin intermediate generated in the desulfonation mechanism. Deletion of the dynamic loop of SsuD resulted in the loss of activity due to the altered active site environment that was not able to protect reactive reduced flavin from bulk solvent (18). Variants of SsuD containing deletions of the dynamic loop region were inactive, but

were still able to effectively bind to SsuE. It was postulated that an additional role of the dynamic loop would be to assist with flavin binding following the flavin transfer event. The HDX-MS protected sites of SsuD were located on either side of the dynamic loop region at the active site opening. The decreased deuterium exchange in this region would not be caused by loop closure on these sites, because the loop only closes over the active site with the binding of substrates. The inability of the SsuD deletion variant to bind to SsuE was likely caused by disruption of the protein-protein interaction sites. These studies also support a model where SsuE and SsuD can interact even in the absence of substrates, and corroborates previous observations.

The results from these studies provide an interesting perspective on the mechanism of flavin transfer. 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. A diffusion and/or a direct transfer mechanism has been observed in other two-component systems depending on the enzyme system under investigation and the mechanism employed (8, 9). The formation of stable protein-protein interactions between SsuE and SsuD suggest a direct flavin transfer mechanism is preferred in the alkanesulfonate monooxygenase system. Disruption of the protein-protein interaction sites does not completely eliminate the desulfonation activity of SsuD under specific reaction conditions. Unlike many two-component flavin-dependent systems, the alkanesulfonate monooxygenase system is found in a broad range of bacteria. The alkanesulfonate monooxygenase enzymes have only been shown to be expressed when sulfur is limiting to the bacterial cell, and is therefore under tight metabolic regulation (2, 28). In addition to SsuE and SsuD, the *ssu* operon expresses an ABC-type transporter for the transport of alkanesulfonate substrates. The highly regulated coexpression of the alkanesulfonate monooxygenase enzymes and the substrate-specific transporter during sulfur starvation ensures

that the system is readily available for the desulfonation event. While most bacteria that utilize the alkanesulfonate monooxygenase system express both SsuE and SsuD, SsuE in *Bacillus subtilis* has not been identified (29). This suggests that another FMN reductase is able to substitute for SsuE during sulfur limitation, and protein-protein interactions may not be mandatory for desulfonation to occur. Based on the reported studies, reduced flavin transfer can occur without protein-protein interactions in *E. coli* when reduced flavin is supplied in excess over SsuD. However, efficient transfer of reduced flavin is only observed with specific interactions between SsuE and SsuD. Flavin transfer between the FRP reductase and bacterial luciferase was also shown to occur by a diffusion or a direct transfer mechanism (25). Results from steady-state coupling experiments with styrene monooxygenase suggested that flavin transfer occurs by both a direct transfer and diffusion mechanism. In numerous two-component systems there have been no measurable protein-protein interactions, but alternative mechanisms to regulate flavin transfer have been identified (8,26,27). The 4-hydroxyphenylacetate 3-monooxygenase enzyme in *E. coli* is also coexpressed with the corresponding reductase, but there were no protein-protein interactions observed in this system (9). Efficient reduced flavin transfer is thought to be dependent on the higher affinity of the monooxygenase for reduced flavin, and increased cellular concentrations of the monooxygenase relative to the reductase. While a higher affinity for reduced flavin has also been observed for SsuD compared to SsuE, the higher affinity would not exclude the existence of protein-protein interactions. The concentrations of SsuE and SsuD have not been determined in *E. coli*; therefore, it is not readily apparent how protein-protein interactions play a role under cellular conditions. A protein of similar molecular weight as SsuE is often observed on SDS-PAGE following purification of the recombinant SsuD enzyme suggesting that protein-protein interactions do indeed occur under

cellular conditions, and these proteins may not just be expressed during sulfur limitation. The results provide a foothold to further evaluate other structural properties of the alkanesulfonate monooxygenase system that define the mechanism of desulfonation.

CHAPTER THREE

Probing the Flavin Transfer Mechanism in the Alkanesulfonate Monooxygenase System

3.1 Introduction

Sulfur is essential for the survival and growth of all living organisms. Bacteria utilize inorganic sulfur for the biosynthesis of sulfur-containing amino acids and cofactors. Sulfur exists as sulfonate and sulfonate esters in the soil. These xenobiotic compounds are difficult to break down and require enzymatic activity for their catalysis. Therefore bacteria must have an alternative mechanism to acquire sulfur when sulfur is limiting (1). Bacteria express a specific set of proteins under sulfur-limitation conditions (2). The sulfonate-sulfur utilization (*ssu*) proteins enable bacteria to utilize xenobiotic compounds like alkanesulfonates as a sulfur source. The *ssu* operon is induced when sulfur is limiting and encodes a NADPH-dependent FMN-reductase (SsuE) and $FMNH_2$-dependent alkanesulfonate monooxygenase (SsuD) (2,3). SsuE reduces FMN to $FMNH_2$ which is then transferred to SsuD. The SsuD enzyme utilizes the reduced flavin to catalyze the oxygenolytic cleavage of the C-S bond in alkanesulfonates (4). Depending on the system being studied, the transfer of reduced flavin can occur either by a channeling or dissociative mechanism. For some flavin dependent two-component systems the transfer of reduced flavin can be best explained as a combination of the two mechanisms (5). The flavin transfer mechanism in the

flavin-dependent alkanesulfonate monooxygenase system is important to understand the catalytic mechanism of this two-component system.

Previous studies evaluating protein-protein interactions and substrate binding suggest that a cooperative mechanism is involved in the transfer of reduced flavin between SsuE to SsuD (6). Recent studies have utilized HDX-MS to highlight specific regions on SsuE and SsuD involved in protein-protein interactions (7). These regions were protected from hydrogen-deuterium exchange due to protein-protein interactions between SsuE and SsuD. Notable charged amino acid residues were reported on the protected regions of SsuD that were shown to be involved in protein-protein interactions. To determine the role of protein-protein interactions in the flavin transfer substitutions were performed at these protected regions. Single turnover stopped-flow kinetics was performed to determine the effect on reduction and oxidation of flavin upon substitutions at the interaction sites. To identify the role of protein-protein interactions in the reduced flavin transfer an inactive SsuE variant was used in a competitive assay with wild-type SsuD and SsuE. The rate of production of reactive oxygen species, due to the autoxidation of reduced flavin, was calculated using resazurin dye. The reported results enable us to better determine the importance of protein-protein interactions to promote reduced flavin transfer in the two-component alkanesulfonate monooxygenase system.

3.2 Experimental procedures

3.2.1 Materials

All chemicals were purchased from Sigma-Aldrich, Bio-Rad, or Fisher. DNA primers were synthesized by Invitrogen (Carlsbad, CA). The expression and purification of wild-type SsuE and SsuD variants was performed as previously reported (10).

3.2.2 Construction, expression and purification of recombinant proteins

Previous studies have identified protected peptides on SsuE and SsuD by HDX-MS (7). The charged amino acids were substituted and deleted to generate DDE (251/252/253) AAA and ΔD251-A261 SsuD variants. The Tyr118 of SsuE was also substituted to generate an inactive variant Y118A SsuE. The substitutions and deletions of amino acids were performed as previously described (7). Expression and purification of all SsuD variants was performed as previously reported (12).

3.2.3 Evaluation of $FMNH_2$ transfer between SsuE and SsuD variants.

Rapid reaction kinetic analyses monitoring both flavin reduction and oxidation was performed with the D251A/D252A/E253A, ΔD251-A261, or wild-typeSsuD (35 μM), SsuE (35 μM), and FMN (25 μM)in 50 mM potassium phosphate, pH 7.5, and 10 % glycerol mixed against NADPH (250 μM) and octanesulfonate (250 μM) in air-equilibrated 10 mMTris-HCl, pH 8.5 and 10 % glycerol at 4 ˚C. All the experiments were carried out in a single mixing mode by mixing equal volumes of solutions and monitoring the reaction by single wavelength analysis at 450 nm. A control experiment was performed with SsuE (35 μM) in the absence of SsuD to monitor flavin reduction and subsequent non-enzymatic flavin oxidation.

3.2.4 Kinetics of resazurin reduction

Stopped-flow kinetic analyses were performed to evaluateif reactive oxygen species are generated due to ineffective transfer of reduced flavin to the SsuD variants. The SsuE:SsuD:FMN ratio was maintained at 1:1:1 to avoid unnecessary flavin oxidation which may lead to exaggerated results. The reactions were performed by mixing DDE(251/252/253)AAA,

ΔD251-A261 or wild-type SsuD (0.5 µM), SsuE (0.5 µM), octanesulfonate (100 µM), and FMN (0.5 µM) in one syringe against NADPH (200 µM) and resazurin dye (50 µM) in air-saturated 50 mMTris-HCl. pH 7.5, and 100 mMNaCl. The resazurin exhibits a peak-shift from 600 nm to 570 nm upon reduction. All reactions were performed in photodiode array mode and traces were evaluated at 570 nm over 100s. The initial traces were best fit to a linear equation using KaleidaGraph software (Abelbeck Software, Reading, PA) :

$$a = b - kx \qquad (3.1)$$

3.2.5 Competition assay

Spectrofluorimetric analyses were performed to determine the binding affinity of Y118A SsuE with SsuD(6). The protein samples were excited at 450 nm and emission intensity measurements were monitored at 524 nm. Aliquots of SsuD (0.02 – 0.95 µM) were titrated against FMN-bound Y118A SsuE. Similar experiments were performed for wild-type SsuE. The equation used to determine the concentration of SsuD bound to SsuE was (7) :

$$[SsuD]_{bound} = [SsuE]\left[\frac{I_0 - I_c}{I_0 - I_f}\right] \qquad (3.2)$$

where [SsuE] represents the initial concentration of enzyme, $I_0$is the initial fluorescence intensity of FMN prior to addition of SsuD, $I_c$is the fluorescence intensity of FMN following each addition of SsuD, and $I_f$is the final fluorescent intensity. The concentration of $[SsuD]_{bound}$ (y) was plotted against $[FMN]_{total}$ or $[SsuD]_{total}$ (x) respectively, to obtain the dissociation constant (K_D) for SsuE and SsuD binding by using following equation :

$$y = \frac{(K_D + x + n) - \sqrt{(K_D + x + n)^2 - 4xn}}{2} \qquad (3.3)$$

The linear dependence of SsuD activity on the concentration of SsuE was established by measuring the activity of SsuD (0.06 μM) against varying concentration of SsuE (0 – 0.12 μM). The reaction mixture contained FMN (2 μM), NADPH (250 μM), and octanesulfonate (1000 μM) in 25 mMTris-HCl, pH 7.5, and 100 mMNaCl. The quantification of final product and determination of rate was performed as previously described (10).

The competitive assay between Y118A SsuE and SsuE/SsuD was modeled on a previously described procedure (14). Reaction mixtures containing SsuE/SsuD (0.06 μM), FMN (2 μM), NADPH (250 μM), and octanesulfonate (1000 μM) in 25 mMTris-HCl, pH 7.5 and 100 mMNaCl were mixed with varying concentration of Y118A SsuE (0.02 – 3.0 μM). The quantification of final product and determination of rate was performed as previously described (10). The control experiment was performed for the reaction mixture in the absence of Y118A SsuE.

3.3 Results

3.3.1 Rapid Reaction Kinetic studies of Flavin Transfer

The role of protein-protein interaction in the transfer of reduced flavin from ssue to the SsuD variants was probed through rapid reaction kinetic analyses. This experiment was performed to evaluate if reduced flavin has decreased stability due to a decrease in protein-protein interactions. Stopped-flow analyses were performed by mixing NADPH and 1-octanesulfonate with D251A/D252A/E253A, ΔD251-A261 or wild-type SsuD and SsuE. The absorbance for oxidized flavin is at 450 nm. As the reaction progresses the absorbance at 450 nm decreases, signifying the reduction of flavin. Prior stopped-flow kinetic studies identified the presence of an apparent lag phase between the reductive (SsuE) and oxidative (SsuD) half-reaction (12). This lag phase

was related to the transfer of reduced flavin and/or the conformational changes that occur with the binding of substrate to SsuD. This lag phase was not observed in kinetic traces obtained with the D251A/D252A/E253A or ΔD251-A261 SsuD variant. Flavin oxidation occur immediately following flavin reduction. The kinetic traces for D251A/D252A/E253A and ΔD251-A261 SsuDwere similar to the kinetic trace obtained for SsuE and FMN in the absence of SsuD suggesting that the flavin is not effectively oxidized by the SsuD variants following flavin reduction. Even though D251A/D252A/E253A and ΔD251-A261 SsuD have a similar affinity for reduced flavin as wild-type SsuD in fluorescent titrations, the majority of reduced flavin was not protected from the air-saturated solvent and was readily oxidized.

3.3.2 Quantifying the production of reactive oxygen species

The reduced flavin can undergo autoxidation in the presence of dioxygen to produce reactiveoxygen species. The role of protein-protein interactions in the transfer of reduced flavin was further evaluated through rapid reaction kinetic analyses to determine if variations in the protected regions of SsuD are responsible for an increase in $FMNH_2$ oxidation. Resazurin (blue and non fluorescent) is reduced to resorufin (pink and highly fluorescent) by reactive oxygen species like superoxide ions. The autoxidation of $FMNH_2$ produces reactive oxygen species like hydrogen peroxide, and superoxide radicals which would provide electrons to reduce the resazurin dye producing resorufin. Rapid reaction kinetic analysis monitoring autoxidation of $FMNH_2$ was monitored with DDE(251/252/253)AAA, ΔD251-A261, or wild-type SsuD with SsuE in the presence of FMN. The absorbances obtained at 570 nm for each variant were converted to concentrations of resorufin formed by using molar extinction coefficient of 117216 $Lmol^{-1}cm^{-1}$ (Figure 3.2). The values were best fit to a linear rate equation, and the rate

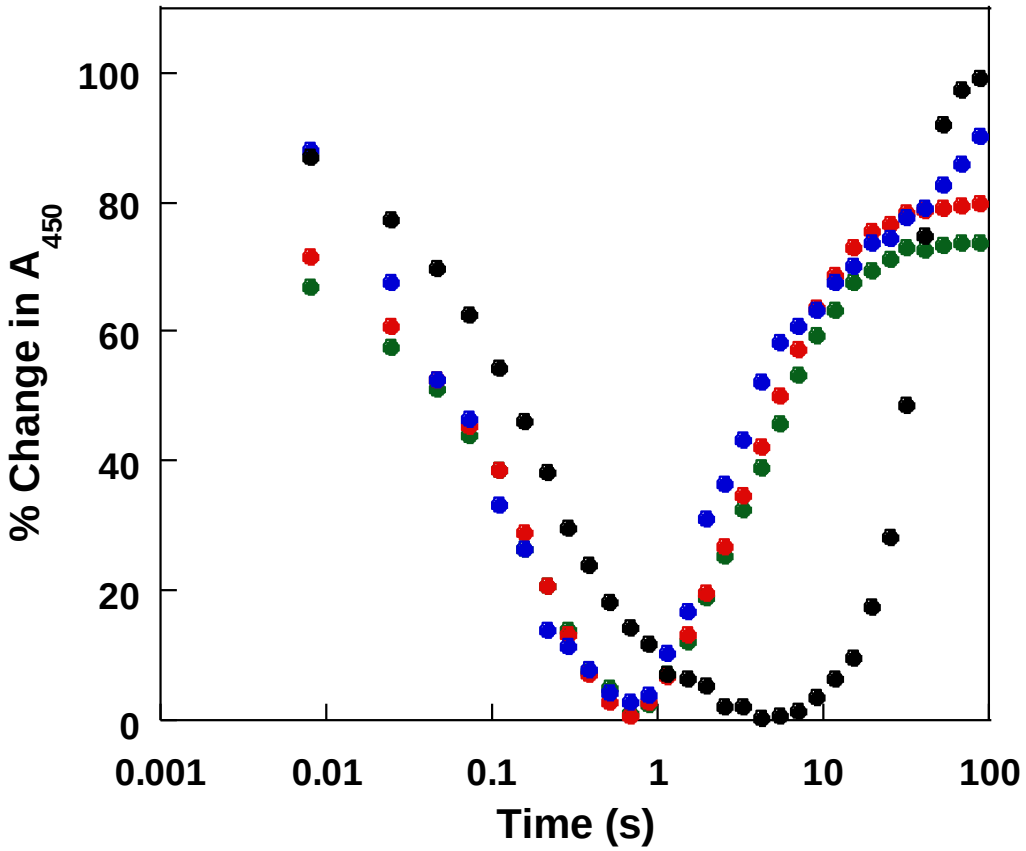


Figure 3.1 Kinetics of flavin reduction and oxidation by DDE(251/252/253)AAA (●), ΔD251-A261 (●), or wild-type SsuD (●) and SsuE or SsuE only (●). Stopped-flow kinetic traces were obtained at 4˚C by mixing DDE(251/252/253)AAA, ΔD251-A261 SsuD, or wild-type SsuD (35 μM when included in the reaction), SsuE (35 μM), and FMN (25 μM), against NADPH (250 μM) and octanesulfonate (250 μM) monitored at 450 nm.

constants obtained were then converted to initial rates and compared to rates of SsuE with and without wild-type SsuD. As shown in Table3.1 the initial rates for SsuD variants DDE(251/252/253)AAA and ΔD251-A261 at 0.5 μM FMN were 3.427 ± 0.050 and 4.428 ± 0.020 $\mu M^{-1}min^{-1}$. At similar concentration of FMN the initial rates for SsuE without and with SsuD were 5.432 ± 0.050 and 1.570 ± 0.070 $\mu M^{-1}min^{-1}$. The initial rates for ΔD251-A261SsuD and SsuE were almost similar signifying the transfer of reduced was affected upon deletion ofthe SsuD α-helix.

3.3.3 Binding and Competitive analyses of Y118A SsuE

If the protein-protein interactions between SsuE and SsuD are important for reduced flavin transfer then the disruption of these interactions by using an inactive SsuE variant should result in a decrease in SsuD activity. The Y118A SsuE variant has flavin bound as a cofactor. The Y118A SsuE can interact with SsuD without transferring reduced flavin. Fluorescence spectroscopy experiments were performed to determine the K_d value for protein-protein interactions between Y118A SsuE and SsuD. The FMN-bound Y118A SsuE variant was titrated against SsuD. There was an increase in flavin fluorescence after each addition of SsuD at the emission wavelength of 524 nm. The concentration of SsuD bound was plotted against total SsuD to obtain a K_d value of 0.040 ± 0.004 and 0.122 ± 0.014 μM for wild-type and Y118A SsuE, respectively. Although, there is a 3-fold decrease in binding affinity for Y118A SsuE for SsuD as compared to wild-type SsuE, the Y118A SsuE variant is still able to interact with SsuD. The competitive assay required that the Y118A SsuE variant competes with wild-type SsuE for interaction sites on SsuD. For these experiments to be valid the observed rate of sulfite production by SsuD must be dependent on the SsuE concentration. Sulfite production by SsuD

Table 3.1: The initial rate of reduction of resazurin when SsuE is alone or coupled with wild-type, DDE(251/252/253)AAA, and ΔD251-A261 SsuD.

	v_o/E_t ($\mu M^{-1}min^{-1}$)
Wild-type SsuE	5.43 ± 0.05
SsuE and SsuD	1.57 ± 0.07
SsuE and DDE(251/252/253) AAA SsuD	3.42 ± 0.01
SsuE and ΔD251-A261 SsuD	4.42 ± 0.02

The absorbances were converted to concentration by using the molar extinction coefficient for resazurin of 117216 $\mu L \mu mol^{-1} cm^{-1}$

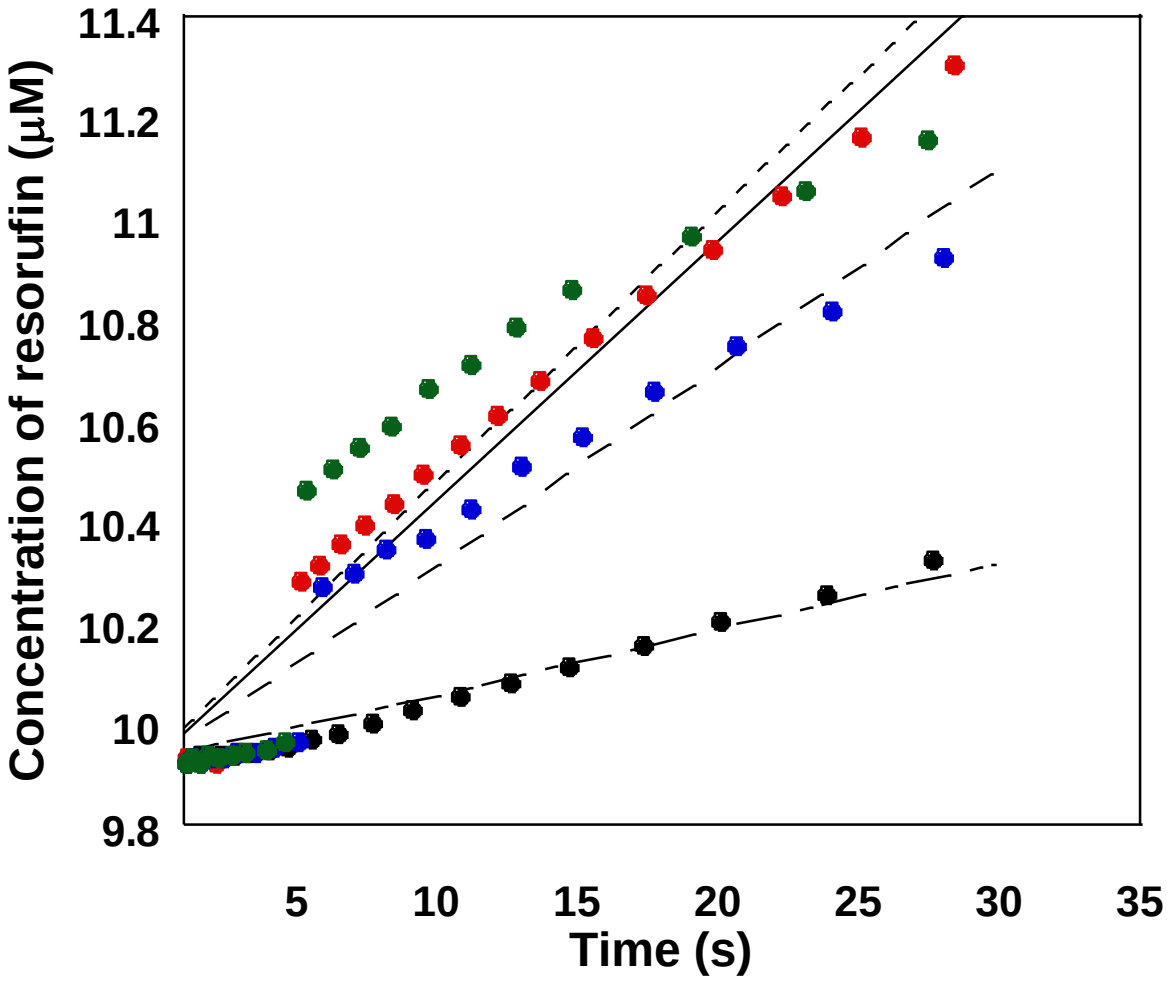


Figure 3.2 Stopped-flow assay to determine the rate of resazurin reduction by DDE(251/252/253)AAA (●), ΔD251-A261 (●), or wild-type SsuD (●) and SsuE or SsuE only (●). Stopped-flow kinetic traces were obtained at 4˚C by mixing DDE(251/252/253)AAA, ΔD251-A261, or wild-type SsuD (0.5 μM when included in the reaction), SsuE (0.5 μM), and FMN (0.5 μM) against NADPH (200 μM), resazurin (15 μM), and octanesulfonate (100 μM) monitored at 570 nm for 100 s. The traces were then fitted to linear equation 3.1.

increased with increasing concentrations of SsuE between 0 – 0.1 μM SsuE with the concentrations of SsuD held constant (0.06 μM). Therefore, a concentrations of 0.06 μM SsuE, which falls within the linear dependence range, was used in the competitive assays. The net rate of sulfite production was decreased upon addition of increasing concentrations of Y118A SsuE. At a 5:1 ratio of Y118A SsuE to wild-type SsuE, the sulfite production decreased by almost 4-folds.

3.4 Discussion

The flavin-dependent alkanesulfonate monooxygenase system is a two-component enzyme system that utilizes flavin as a substrate instead of a tightly bound cofactor. The flavin reductase (SsuE) provides reduced flavin to the monooxygenase enzyme (SsuD). SsuD uses reduced flavin to activate a dioxygen molecule forming a C4a-(hydro)peroxyflavin intermediate involved in the oxygenolytic cleavage of C-S bonds in alkanesulfonate. This reaction replenishes the sulfite concentration in bacteria when sulfur is limiting. The genes for the SsuE and SsuD enzymes are expressed from the same operon which suggests a close relationship between the two enzymes (15-22). Previously studied flavin-dependent two-component systems propose a direct or dissociative mechanism for the transfer of reduced flavin. A dissociative mechanism for the transfer of reduced flavin is prevalent in many two-component systems (23, 24, 25). The dissociative mechanism depends on passive diffusion for the transfer of reduced flavin to the monooxygenase. In the flavin-dependent two-component enzyme systems the flavin reductases have a higher affinity for oxidized flavin and the monooxygenases have a higher affinity for

reduced flavin (8, 25, 26, 27, 28). The dissociative mechanism is based on the rapid transfer of flavin between the reductase and monooxygenase half of enzymes. Conversely, a direct transfer

Table 3.2: The dissociation constant for wild-type and Y118A SsuE for FMN and SsuD

	K_d^{FMN}	K_d^{SsuD}
wild-type SsuE	0.008 ± 0.003	0.040 ± 0.004
Y118A SsuE	0.044 ± 0.005	0.124 ± 0.014

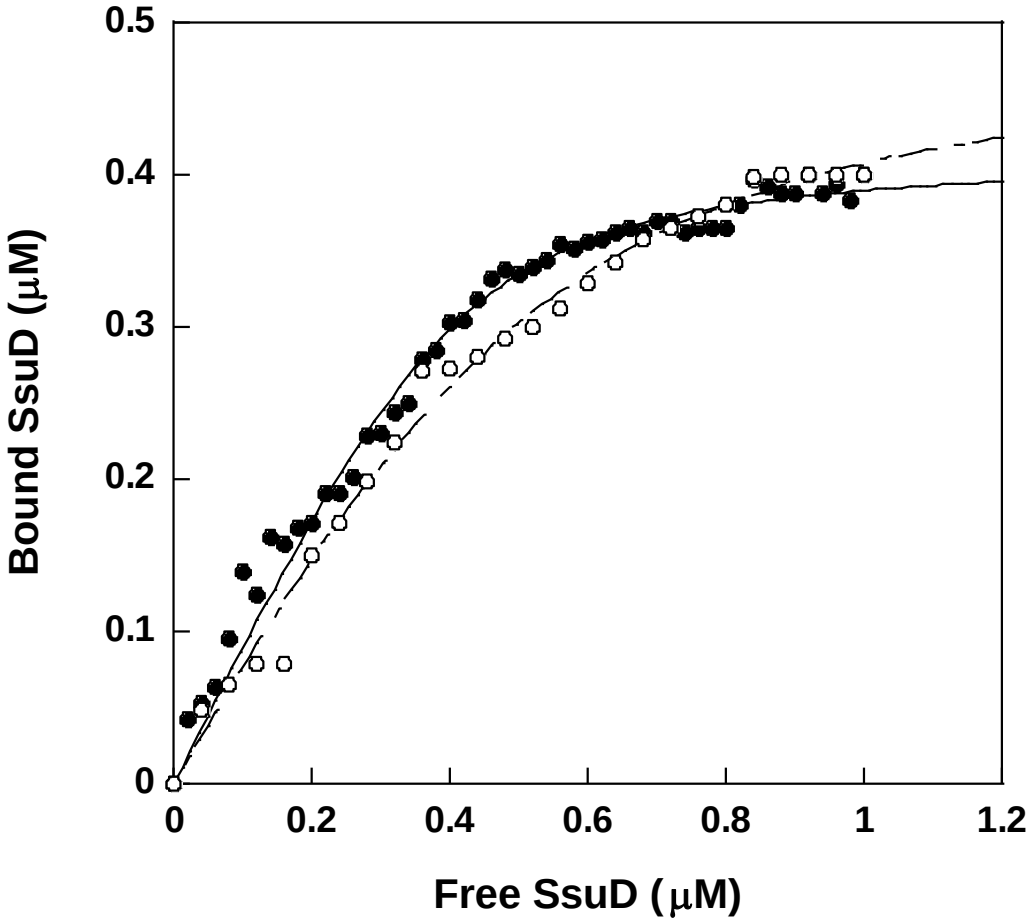


Figure 3.3 Fluorimetric titration of Y118A (○) and wild-type (●) SsuE with FMN. A 0.1 µM concentrations of SsuE enzyme was titrated against 0.022 – 0.44 µM of FMN. Emission intensity measurements of 344 nm were measured using an excitation wavelength at 280 nm.

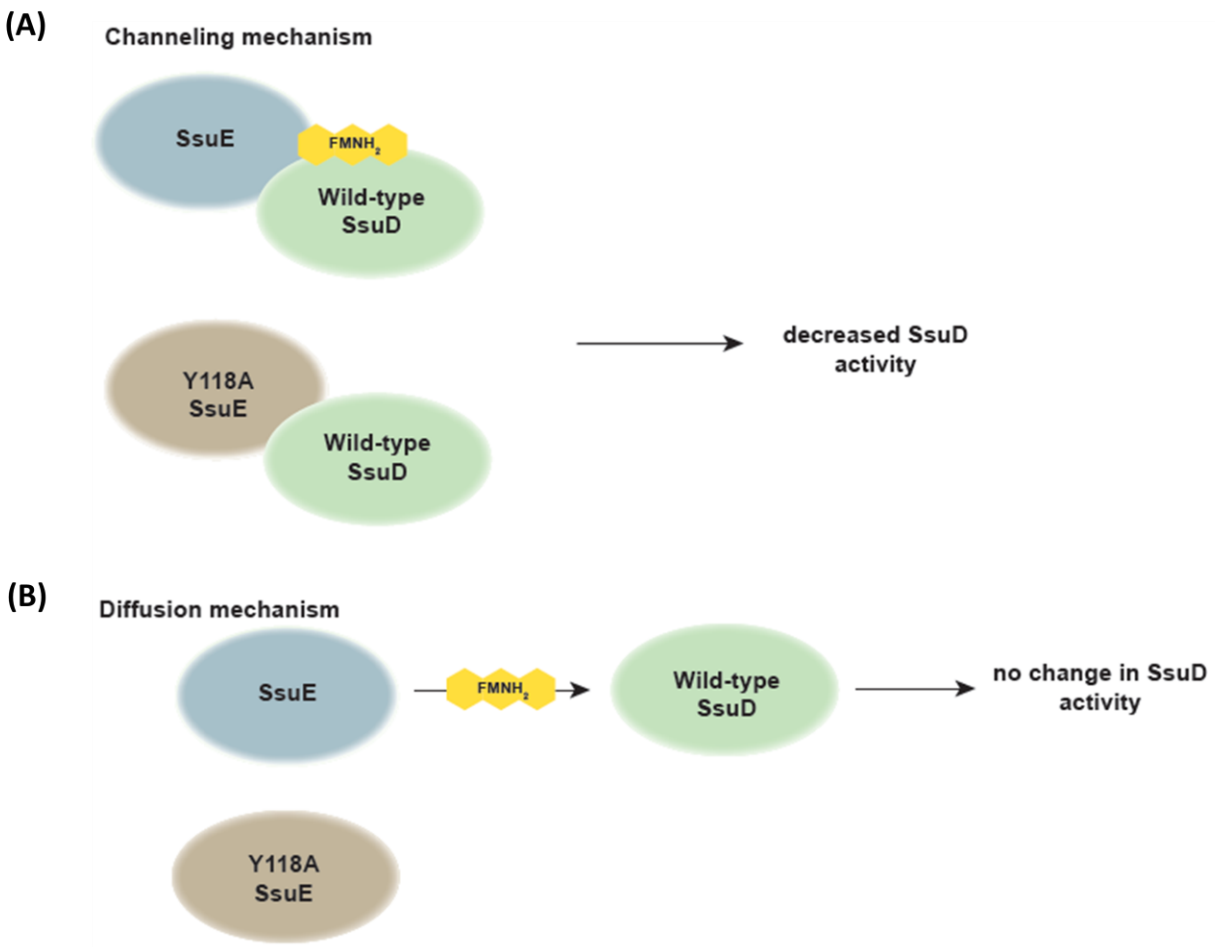


Figure 3.4 A model describing possible channeling and diffusion mechanisms exhibited by SsuE/SsuD system. (A) If channeling mechanism is essential for the transfer of reduced flavin than the presence of Y118A SsuE should hinder the transfer process thereby decreasing the SsuD activity. (B) But if diffusion mechanism is prevalent than the presence of Y118A SsuE should have no effect on reduced flavin transfer efficiency thus no change in SsuD activity should be observed.

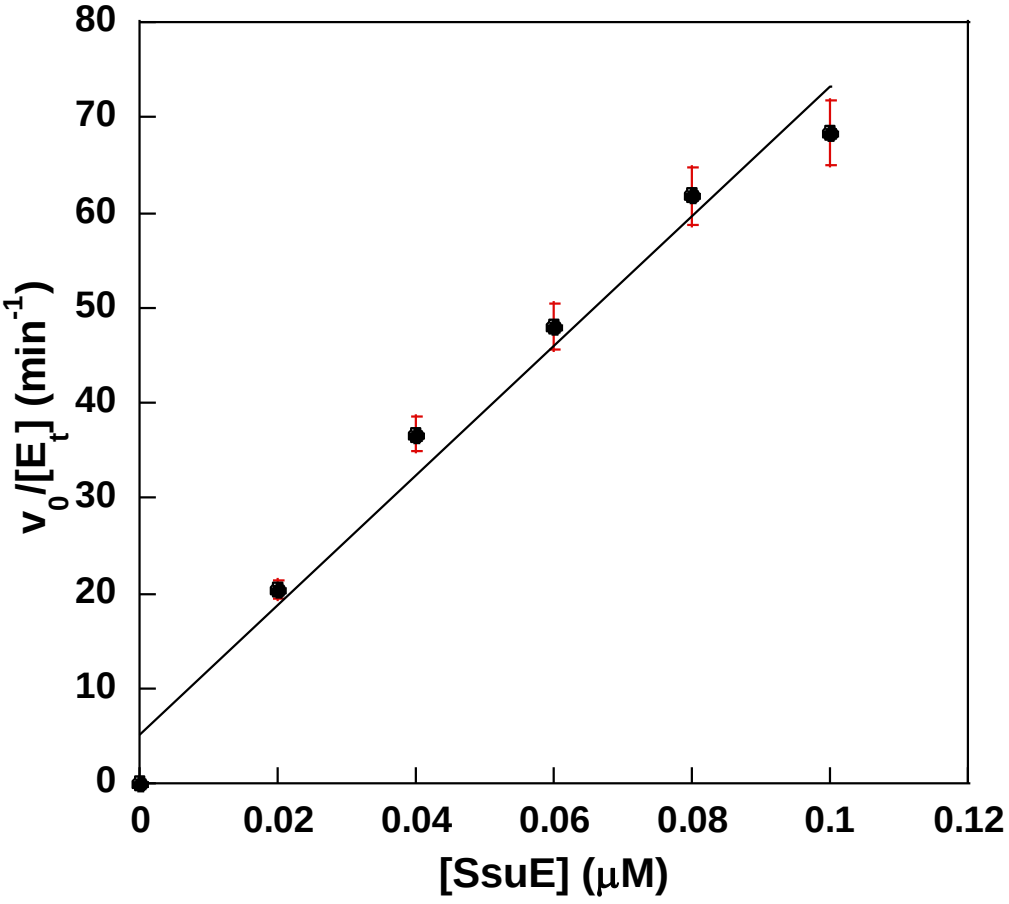


Figure 3.5 Approximation of linear dependence of the initial rate of sulfite production by SsuD (0.06 μM) with varying concentrations of SsuE (0 – 0.1 μM). The assays were performed in 25 mMTris-HCl at pH 7.5 using FMN (2 μM), octanesulfonate (100 μM), and NADPH (250 μM).

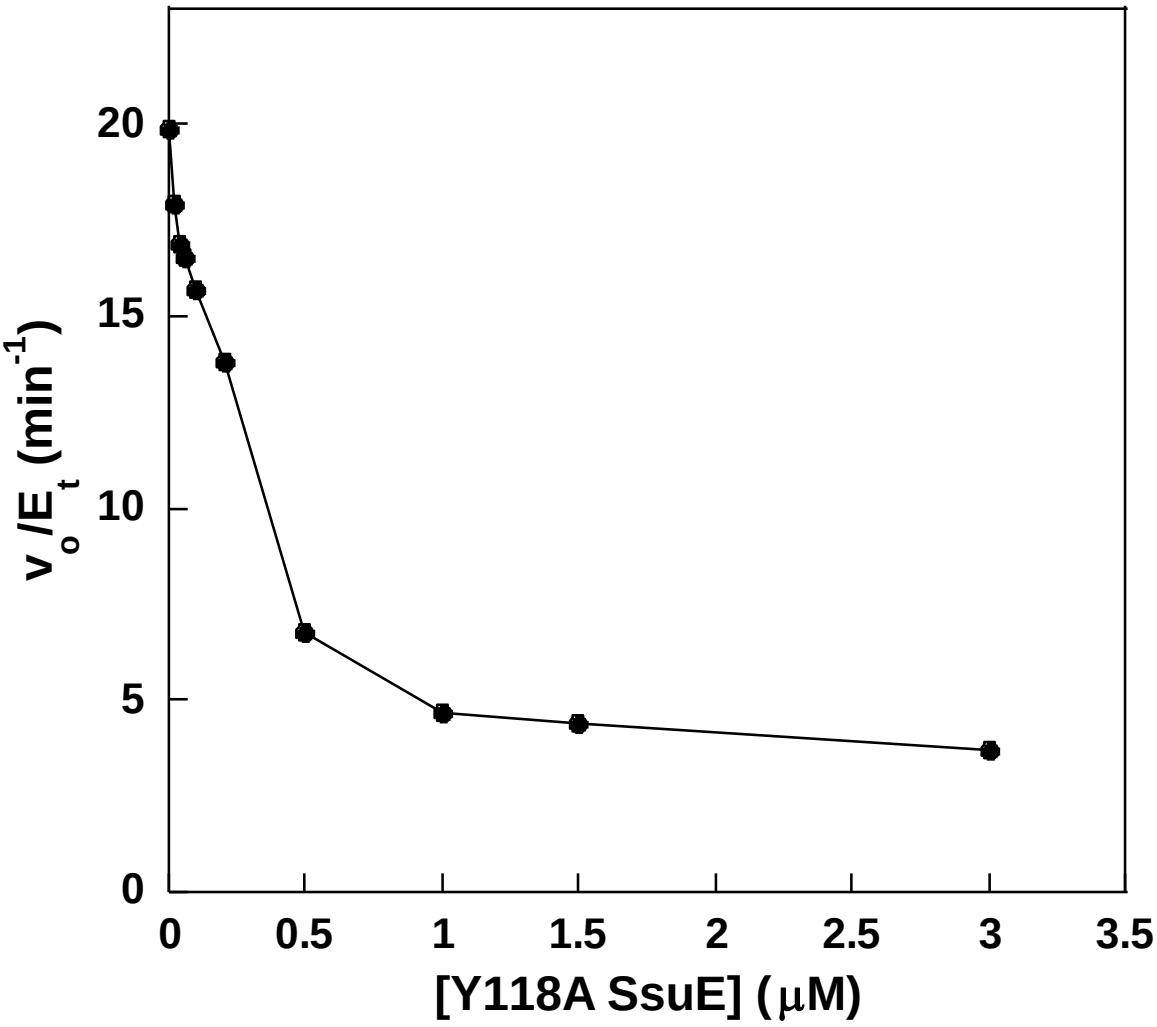


Figure 3.6 Competition assays between SsuD/SsuE and Y118A SsuE. The assays were performed with FMN (2 µM), NADPH (250 µM), SsuE and SsuD (0.06 µm) with varying concentrations of Y118A SsuE (0 – 3 µM) in 25 mMTris-HCl, pH 7.5 at 25 ˚C.

mechanism minimizes the contact between reduced flavin and the external environment (6, 29, 30). The direct transfer mechanism enhances the transfer of reduced flavin by either forming a molecular channel or by bridging the distance between two-active sites by bringing them in close proximity to each other through protein-protein interactions. Various kinetic and biophysical studies have provided support for a channeling mechanism for the transfer of reduced flavin between FRP and bacterial luciferase (29, 30). Steady-state kinetic studies on styrene monooxygenase (SMO) have shown the presence of both a dissociative and direct transfer mechanism for reduced flavin transfer (31). The presence of site-specific protein-protein interactions between SsuE and SsuD have been suggested in the alkanesulfonate monooxygenase system (6, 7). The HDX-MS experiment identified protected regions on SsuE and SsuD, which were shown to play a key role in protein-protein interactions (7). The protected region of SsuD had a highly conserved α-helix, which was located adjacent to the active site. The α-helix of SsuD appeared to play a key role in dynamic and conformational changes required for interaction with SsuE. The protein-protein interactions promoted by the movement of the conserved α-helix will bring the two active sites in close proximity and increase the effectiveness of reduced flavin transfer between SsuE and SsuD.

In the two-component flavin-dependent systems the oxidative and reductive reactions occur on two separate enzymes providing independent analyses of each reaction. Single rapid reaction kinetic experiments were performed to monitor the reduction and oxidation of FMN. The kinetic traces obtained at 450 nm following flavin oxidation in the presence of both SsuE and SsuD showed a lag phase possibly due to reduced flavin transfer and conformational changes associated with the transfer. Comparable experiments with DDE(251/252/253)AAA and ΔD251-

A261 SsuD gave kinetic traces lacking a significant lag phase. The kinetic traces for DDE(251/252/253)AAA and ΔD251-A261 SsuD were similar to traces obtained in the absence of SsuD with SsuE alone suggesting that autoxidation of reduced flavin occurred in these variants. The DDE(251/252/253)AAA SsuD showed partial activity as compared to wild-type SsuD under standard steady-state conditions (7). Thus it was interesting that kinetic traces for DDE(251/252/253)AAA SsuD were similar to kinetic traces obtained in the presence of SsuE alone. The ΔD251-A261 SsuD is deprived of an entire α-helix region, thus explaining a complete loss of activity under standard steady-state conditions (7). The reaction mixture containing SsuE and SsuD showed a slower reduction of free flavin, suggesting the presence of SsuD has some effect on the active site of SsuE. Although the eventual slower oxidation of reduced flavin for SsuE and SsuD corroborates with the conformational changes associated with protein-protein interactions. The absence of lag phase for both the SsuD variants correlates with decreased or the complete absence of activity for DDE(251/252/253)AAA and ΔD251-A261 SsuD,respectively.

The mechanism of direct transfer of reactive metabolite has often been difficult to show, but a few examples do exist. The channeling transfer mechanisms are mostly found in multifunctional enzymes with two distinct active sites located on one peptide or different subunits. For example in case of tryptophan synthase the two active sites on different subunits are connected to each other via a hydrophobic tunnel (32). This tunnel helps in the transfer of an indole intermediate between two active sites. A similar intermediate transfer is observed in glutamine phosphoribosyl pyrophosphate amidotransferase for the transfer of ammonia (33). When the two active sites are located on separate enzymes, channeling or direct transfer can be demonstrated using a competition assay. In the alkanesulfonate monooxygenase system the activity of SsuD was dependent on concentrations of SsuE as shown by the linear dependent assay. The SsuE

variant is capable of binding SsuD, and thus acts as a competitor for docking sites on SsuD. The Y118A SsuE enzyme at increasing concentrations binds to SsuD preventing SsuE from docking with SsuD. Previous studies have highlighted the role of protein-protein interactions and the conformational changes that ensue with the transfer of reduced flavin from SsuE to SsuD (7). The Y118A SsuE variant shows a lower binding affinity towards SsuD but was able to compete with wild-type SsuE. The Y118A SsuE variant cannot transfer reduced flavin to SsuD and thus forms an inactive complex with SsuD. The SsuD utilizes reduced flavin in the presence of dioxygen to catalyze C-S bonds in alkanesulfonates, but the reduced transfer of $FMNH_2$ to SsuD leads to a decrease in SsuD activity. Thus the protein-protein interactions must play a key role for the effective transfer of reduced flavin from SsuE to SsuD.

CHAPTER FOUR

SUMMARY

The flavin-dependent alkanesulfonate monooxygenase system from *Escherichia coli* is composed of NAD(P)H-dependent FMN reductase (SsuE) and an $FMNH_2$ –dependent alkanesulfonate monooxygenase (SsuD) (8). The SsuE enzyme reduces an oxidized FMN to $FMNH_2$ in the presence of NAD(P)H. The SsuD is capable of cleaving the C-S bond of a wide range of 1-substituted alkanesulfonate in the presence of O_2 and $FMNH_2$ (8, 32). The SsuD enzyme is proposed to catalyze the desulfonation reaction through a C4a-(hydro)peroxyflavin reactive intermediate (59). Although it has been shown that physical interactions do exist between SsuE and SsuD, the exact peptides involved in such interactions were not known. In addition, how the reduced flavin gets effectively transferred from SsuE to SsuD has not been fully addressed. An important feature for the two-component enzyme systems that use flavin as a substrate is the transfer of reduced flavin from one component to another. Based on the static interactions between SsuE and SsuD direct transfer of reduced flavin was proposed but enough evidence was put forward to support the hypothesis. This research has focused on exposing the protein-protein interaction sites on SsuE and SsuD, as well as probing the role of these interactions for the effective transfer of reduced flavin in the alkanesulfonate monooxygenase system.

4.1 Catalytic mechanism of the alkanesulfonate monooxygenase system

The flavin-dependent monooxygenase enzymes are capable of catalyzing a wide range of biochemical reactions. The flavin-dependent monooxygenase of the two-component enzyme systems catalyzes a reaction in three important steps: 1) accepting a reduced flavin from the reductase component 2) activating a dioxygen molecule to form a reactive oxygenating intermediate C4a-(hydro)peroxyflavin 3) utilizing the reactive intermediate to cleave the substrate (28). In many two-component enzymes the flavin is a substrate instead of a covalently bound cofactor. In such systems the reductive and oxidative half-reactions occur on separate enzymes. The SsuE enzyme follows an ordered sequential mechanism for the reduction of flavin in which either FMN or NADPH binds first to the enzyme (28). The characterization of SsuD suggested that it also follows an ordered sequential mechanism in which reduced flavin binds first and octanesulfonate binds last. Also it has been shown that each substrate binding is associated with a conformational change. The SsuD can employ two possible mechanisms for the desulfonation of alkanesulfonates. It can perform a nucleophilic attack by a C4a-peroxyflavin intermediate or an electrophilic attack with C4a-(hydro)peroxyflavin reactive intermediate (153). The alkanesulfonate peroxyflavin adduct can then undergo a Baeyer-Villiger rearrangement followed by proton abstraction from an active site base. This leads to the production of oxidized flavin, sulfite and a corresponding aldehyde. The identity of the active site base is still not clear. The results from isotope effect have indicated that Arg226 stabilizes the C4a-(hydro)peroxyflavin intermediate.

4.2 Protein-protein interaction sites on SsuE and SsuD

Many biophysical experiments have been performed to detect the protein-protein interactions between SsuE and SsuD. Results from affinity chromatography showed the formation of a transient complex between (32). Spectrofluorimetry studies suggested the presence of a tight binding affinity between SsuE and SsuD with 1:1 stoichiometric ratio, but the location of these interaction regions was still unknown. HDX-MS studies were employed to identify the the protected peptides on SsuE and SsuD during complex formation. Regions D251-A261 and E284-G294 on SsuD and K78-L89 and Y118-L124 on SsuE showed maximum change in deuterium incorporations upon complex formation. The peptide regions D251-A261 and E284-G294 on SsuD are located on each side of the previously diagnosed dynamic loop region which plays a key role in reduced flavin binding.

4.3 α-helix adjacent to the active site of SsuD

The peptide D251-A261 on SsuD is a complete α-helix and is adjacent to the active site. Also this peptide is directly connected to the dynamic loop region which helps in reduced flavin binding (90). Substitutions were performed on this α-helix to determine the change in protein-protein interactions and overall activity of SsuD. The D251A SsuD variant did not show any significant change in the activity or binding affinity towards SsuE. The DDE(251/252/253)AAA SsuD variant showed a 10-fold increase in K_d value and a 6-fold decrease in k_{cat}/K_m value. These changes are significant and highlight a disruption in protein-protein interactions upon substitutions. The deletion variant ΔD251-A261 SsuD was inactive under standard steady-state conditions and showed no biding affinity towards SsuE in spectrofluorimetric assays. Also no complex formation was noticed between native SsuE and his-tagged ΔD251-A261 SsuD by

affinity chromatography experiments. These results further indicated the importance of protected peptides of SsuD in promoting protein-protein interactions with SsuE.

4.4 Reduced flavin transfer between SsuE and SsuD

The transfer of reduced flavin is an important step for the transfer of reduced flavin in two-component enzyme systems. The main requirement for the direct transfer of reduced flavin is protein-protein interaction between two enzymes. But disruption of such interactions should lead a decrease in the efficiency of flavin transfer. Stopped-flow assays monitoring the reductive and oxidative half reactions of flavin showed a decrease in the stability of proteins upon substitutions at the interaction sites. For this assay presence of a lag phase signifies the transfer of reduced flavin or the conformational changes associated with it. But for DDE(251/252/253)AAA and ΔD251-A261 SsuD variants no such lag phase was observed. The kinetic traces for SsuD variants resembled with the traces for SsuE alone. Competition assays with Y118A SsuE variants also demonstrated a decrease in flavin transfer efficiency and lower SsuD activity upon disruption of protein-protein interactions. Y118A SsuE variant can interact with wild-type SsuD but is unable to transfer flavin. Thus the mutant SsuE competes with wild-type SsuE for interaction sites on SsuD and hence disrupts the flow of reduced flavin. In summary, the protein-protein interaction triggers a wide-range of conformational changes which assists in an efficient transfer of reduced flavin from SsuE to SsuD.

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