Probing the flavin transfer mechanism in alkanesulfonate monooxygenase system
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Abstract

Bacteria acquire sulfur through the sulfur assimilation pathway, but under sulfur limiting conditions bacteria must acquire sulfur from alternative sources. The alkanesulfonate monooxygenase enzymes are expressed under sulfur-limiting conditions, and catalyze the desulfonation of wide-range of alkanesulfonate substrates. The SsuE enzyme is an NADPH-dependent FMN reductase that provides reduced flavin to the SsuD monooxygenase. The mechanism for the transfer of reduced flavin in flavin dependent two-component systems occurs either by free-diffusion or channeling. Previous studies have shown the presence of protein-protein interactions between SsuE and SsuD, but the identification of putative interaction sights have not been investigated. Current studies utilized HDX-MS to identify protective sites on SsuE and SsuD. A conserved α-helix on SsuD showed a decrease in percent deuteration when SsuE was included in the reaction. This suggests the role of α-helix in promoting protein-protein interactions. Specific SsuD variants were generated in order to investigate the role of these residues in protein-protein interactions and catalysis. Variant containing substitutions at the charged residues showed a six-fold decrease in the activity, while a deletion variant of SsuD lacking the α-helix showed no activity when compared to wild-type SsuD. In addition, there was no protein-protein interactions identified between SsuE and his-tagged SsuD variants in pull-down assays, which correlated with an increase in the $K_d$ value. The α-helix is located right next to a dynamic loop region, positioned at the entrance of the active site. The putative interaction site and dynamic loop region located so close to the active site of SsuD suggests the importance of this region in the SsuD catalysis. Stopped-flow studies were performed to analyze the lag-phase which signifies the stabilization and transfer of reduced flavin from SsuE to SsuD. The SsuD variants showed a decrease in lag-phase, which could be because of a downturn in flavin transfer. A competitive assay was devised to evaluate the mechanism of flavin transfer in the alkanesulfonate monooxygenase system. A variant of SsuE was generated which interacted with SsuD, but was not able to reduce FMN. Assays that included varying concentrations of Y118A SsuE and wild-type SsuE in the coupled assays showed a decrease in the desulfonation activity of SsuD. The decrease in activity could be by virtue of Y118A SsuE competing with the wild-type SsuE for the putative docking site on SsuD. These studies define the importance of protein-protein interactions for the efficient transfer of reduced flavin from SsuE to SsuD leading to the desulfonation of alkanesulfonates.

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 hard 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
alkanesulfonate (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 have reported the presence of physical interactions between SsuE and SsuD leading to the formation of a transient complex (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. Substitutions of these protected regions had a direct impact on SsuD activity, highlighting the role of protein-protein interactions in the transfer of reduced flavin. 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. The studies described will help to enhance knowledge regarding reduced flavin transfer in two-component systems. Previous studies have already highlighted the regions involved in protein-protein interactions between SsuE and SsuD. To identify the role of protein-protein interactions an inactive SsuE variant was used in a competitive assay with wild-type SsuD and SsuE. Also the rate of production of reactive oxygen species, due to the autoxidation of reduced flavin, was calculated using resazurin dye. Single turnover stopped-flow kinetics was performed to determine the stability of reduced flavin upon substitutions at the interaction sites. The reported results will enable us to understand the mechanism of reduced flavin transfer in the two-component alkanesulfonate monooxygenase system.
Materials and methods

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. 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 ∆D251-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 described (7). The expression and purification of all SsuD variants was performed as previously reported (12).

3. Evaluation of FMNH₂ transfer between SsuE and SsuD variants.

Rapid reaction kinetic analyses monitoring both flavin reduction and oxidation was performed with the D251A/D252A/E253A, ∆D251-A261 or wild-type SsuD (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 mM Tris-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)
without SsuD in order to monitor flavin reduction and subsequent non-enzymatic flavin oxidation.

4. Kinetics of resazurin reduction

Stopped-flow kinetic analyses were performed to evaluate reactive oxygen species upon autoxidation of FMNH$_2$ due to unsuccessful transfer to SsuD variants DDE(251/252/253)AAA and ∆D251-A261 SsuD. 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, ∆D251-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 mM Tris-HCl, pH 7.5, and 100 mM NaCl. The resazurin exhibit 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 100 s. The traces were best fit to an integrated first-order reaction using KaleidaGraph software (Abelbeck Software, Reading, PA):

$$[A]_t = [A]_0 e^{-kt}$$

Where, $[A]_0$ is the initial concentration of Resazurin dye, $[A]$ is the final concentration of Resazurin dye, and $k$ represents the rate of reduction of Resazurin at 570 nm ($k_{570}$).

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 at 524 nm. Aliquots of SsuD (0.02 – 0.95 µM) were titrated against FMN-bound SsuE. Similar experiments were performed for wild-type SsuE. The equation used to determine the concentration of SsuD bound to SsuE was (7):
\[
[SsuD]_{\text{bound}} = [SsuE] \left( \frac{I_0 - I_c}{I_0 - I_f} \right) 
\]  
(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 \([SsuD]_{\text{bound}}\) \((y)\) were then separately plotted against \([\text{FMN}]_{\text{total}}\) and \([\text{SsuD}]_{\text{total}}\) \((x)\) respectively, to obtain the dissociation constant \((K_D)\) for SsuE and SsuD binding by using following equation:

\[
y = \frac{K_D + x + n}{2} - \sqrt{\left(\frac{K_D + x + n}{2}\right)^2 - 4xn} 
\]  
(3)

The linear dependence of SsuD activity on the concentration of SsuE was first established. The activity of SsuD (0.06 µM) was measured 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 mM Tris-HCl pH 7.5 and 100 mM NaCl. 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 mM Tris-HCl, pH 7.5 and 100 mM NaCl 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 without Y118A SsuE.

RESULTS

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 studies have highlighted that in the stopped-flow kinetic traces at 450 nm there is a 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 and ΔD251-A261 SsuD variant. Flavin oxidation occurred immediately following flavin reduction. The kinetic traces for D251A/D252A/E253A and ΔD251-A261 SsuD were 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 variant 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.

2. Quantifying the production of reactive oxygen species

The reduced flavin can undergo autoxidation in the presence of dioxygen to produce reactive oxygen 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 kinetic traces obtained at 570 nm for each variant represents the production of resorufin which can be correlated with autoxidation of FMNH$_2$ producing reactive oxygen species (Figure 2). The kinetic traces were best fit to an integrated first-order rate equation, and the rate constants obtained were then compared to rate constants with SsuE with and without wild-type SsuD. From Table the rate constants ($k$) for SsuD variants DDE(251/252/253)AAA and ΔD251-A261 at 0.5 μM FMN were 0.0020 ± 0.0001 s$^{-1}$ and 0.0022 ± 0.0002 s$^{-1}$. At similar concentration of FMN the rate constants ($k$) for SsuE without and with SsuD were 0.0031 ± 0.0005 s$^{-1}$ and 0.0012 ± 0.0007 s$^{-1}$. Unexpectedly the reaction rates and kinetic traces for ΔD251-A261 SsuD and SsuE were different signifying that the reduced flavin was still getting productively transferred from SsuE to ΔD251-A261 SsuD although at a lower rate than for SsuE and wild-type SsuD. These results suggest that the transfer of reduced flavin is not entirely dependent on protein-protein interactions between SsuE and SsuD. But the protein-protein interactions generally increase the rate of reduced flavin transfer and make it more effective.
Figure 1 Kinetics of flavin reduction and oxidation by DDE(251/252/253)AAA (●), ∆D251-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, ∆D251-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.
Table 1: The rate of reduction of resazurin when SsuE is alone or coupled with wild-type, DDE(251/252/253)AAA, and ΔD251-A261 SsuD.

|                      | $k_{570}$ (s$^{-1}$) |
|----------------------|----------------------|
| Wild-type SsuE       | 0.0031 ± 0.0005      |
| SsuE and SsuD        | 0.0012 ± 0.0007      |
| SsuE and ΔD251-A261  | 0.0020 ± 0.0001      |
| SsuE and ΔD251-A261  | 0.0022 ± 0.0002      |

The absorbance were converted to concentration by using the molar extinction coefficient for resazurin of 117216 µLµmol$^{-1}$cm$^{-1}$.
Figure 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 change in absorbance observed was converted to concentrations by using resazurin molar extinction coefficient of 117216 µLµmol⁻¹cm⁻¹ to calculate the rate of reduction of resazurin. The traces were then fitted to integrated first-order equation 1.
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 see a decrease in SsuD activity. The Y118A is an inactive variant with FMN bound to it. The Y118A SsuE variant cannot transfer flavin to SsuD. 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 $0.040 \pm 0.004$ and $0.122 \pm 0.014$ 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 increased with increasing concentrations of SsuE between 0 – 0.1 µM SsuE with the concentrations of SsuD held constant (0.06 µM). Therefore, a concentration 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. Similar results were obtained for wild-type SsuE pre-incubated with SsuD for 15 minutes prior to the assay.
Table 2 The dissociation constant for wild-type and Y118A SsuE for FMN and SsuD

|                  | $K_d^{FMN}$ (µM) | $K_d^{SsuD}$ (µM) |
|------------------|------------------|-------------------|
| wild-type SsuE   | 0.008±0.003      | 0.040±0.004       |
| Y118A SsuE       | 0.044±0.005      | 0.124±0.014       |
Figure 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 4 Model describing channeling and diffusion mechanisms exhibited by SsuE/SsuD system.
Figure 5 Linear dependence of the initial rate of sulfite production by SsuD (0.06 µM) on varying concentrations of SsuE (0 – 0.1 µM). The assays were performed in 25 mM Tris-HCl at pH 7.5 using FMN (2 µM), octanesulfonate (100 µM), and NADPH (250 µM).
Figure 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 mM Tris-HCl, pH 7.5 at 25 °C.
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 (SsuD), which activates a dioxygen molecule to form a C4a-(hydro)peroxyflavin intermediate that performs the oxygenolytic cleavage of C-S bonds in alkanesulfonate. This reaction replenishes the sulfite concentration in the bacteria when sulfur is limiting. The genes for SsuE and SsuD are expressed from the same operon which indicates the presence of 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 of reduced flavin for its transfer from the reductase 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 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 turnover experiments were performed on the stopped-flow 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 ∆D251-A261 SsuD gave kinetic traces lacking a significant lag phase. The kinetic traces for DDE(251/252/253)AAA and ∆D251-A261 SsuD were similar to traces obtained in the absence of SsuD with SsuE alone, which suggests the autoxidation of reduced flavin. The DDE(251/252/253)AAA SsuD showed partial activity as compared to wild-type SsuD under standard steady-state conditions (7). Thus its surprising to see the kinetic traces for DDE(251/252/253)AAA SsuD similar to kinetic traces obtained in the presence of SsuE alone. The ∆D251-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 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 shown 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 utilized reduced flavin in the presence of dioxygen to break down C-S bond in alkanesulfonates. But with 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.
These studies provide an intriguing viewpoint for the transfer of reactive metabolites in the two-component systems. In the alkanesulfonate monooxygenase system the transfer of reduced flavin is a key step leading to desulfonation by SsuD. The genes for SsuE and SsuD are located on same operons but upon expression functions as separate entities, albeit towards a common goal. The transfer of reactive metabolites in the two-component systems can occur either by direct transfer or a free-diffusion mechanism depending on the system being studied. Previous studies have shown the formation of a stable intermediary structure between SsuE and SsuD (6,7). The formation of this stable structure might suggest the presence of direct transfer mechanism for the transfer of reduced flavin, but the high binding affinity of each enzyme towards a particular oxidation state of FMN might contradict that suggestion. Previous studies have shown the importance of α-helix (D251-A261) of SsuD in promoting the protein-protein interactions with SsuE (7). The disruption at this helical region affected not only the binding affinity of SsuD towards SsuE but also the efficiency of reduced flavin transfer. The alkanesulfonate monooxygenase system is a stress-response protein that gets activated during sulfate-limiting conditions. Thus the regulation of these enzymes is tightly regulated with specific functions in order to replenish bacteria with sulfate.

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