Biochemistry

Article

Cite This: *Biochemistry* 2018, 57, 4469−4477

pubs.acs.org/biochemistry

Functional Evaluation of the π-Helix in the NAD(P)H:FMN Reductase of the Alkanesulfonate Monooxygenase System

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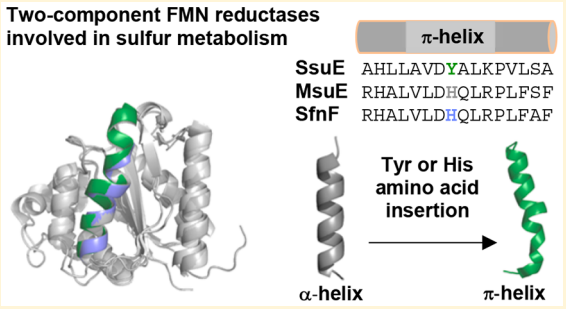
Supporting Information

ABSTRACT: A subgroup of enzymes in the NAD(P)H:FMN reductase family is comprised of flavin reductases from two-component monooxygenase systems. The diverging structural feature in these FMN reductases is a π-helix centrally located at the tetramer interface that is generated by the insertion of an amino acid in a conserved α4 helix. The Tyr insertional residue of SsuE makes specific contacts across the dimer interface that may assist in the altered mechanistic properties of this enzyme. The Y118F SsuE variant maintained the π−π stacking interactions at the tetramer interface and had kinetic parameters similar to those of wild-type SsuE. Substitution of the π-helical residue (Tyr118) to Ala or Ser transformed the enzymes into flavin-bound SsuE variants that could no longer support flavin reductase and desulfonation activities. These variants existed as dimers and could form protein−protein interactions with SsuD even though flavin transfer was not sustained. The ΔY118 SsuE variant was flavin-free as purified and did not undergo the tetramer to dimer oligomeric shift with the addition of flavin. The absence of desulfonation activity can be attributed to the inability of ΔY118 SsuE to promote flavin transfer and undergo the requisite oligomeric changes to support desulfonation. Results from these studies provide insights into the role of the SsuE π-helix in promoting flavin transfer and oligomeric changes that support protein−protein interactions with SsuD.



Bacterial two-component FMN-dependent systems are composed of a flavin reductase and a monooxygenase enzyme that catalyze the oxidation of diverse sulfonated substrates. The role of the flavin reductase is to supply reduced flavin to the monooxygenase enzyme, while the monooxygenase enzyme utilizes the reduced flavin to activate molecular oxygen. The two-component alkanesulfonate monooxygenase system is upregulated in bacteria during sulfur starvation to cleave the C−S bonds of various alkanesulfonates generating sulfite and the corresponding aldehyde.[1] The sulfite generated is incorporated into various sulfur-containing biomolecules. Interestingly, some bacteria express additional two-component systems under sulfur limitation to provide these organisms with a more complex mechanism for sulfur acquisition.[2−5] The utilization of two-component systems for sulfur acquisition suggests a similar mechanism is utilized to ensure that cellular sulfur concentrations are maintained. Conserved structural features may optimize the catalytic properties of two-component FMN-dependent systems. The structural and conformational changes of two-component monooxygenases must be synchronized to simultaneously support flavin reduction, reduced flavin transfer, and the oxidation of substrates. However, the structural properties that enable these flavin reductases to transfer the labile reduced flavin product to their monooxygenase partner have not been fully evaluated.

The SsuE FMN reductase of the alkanesulfonate monooxygenase system belongs to the NAD(P)H:FMN reductase family based on a conserved flavodoxin fold.[6] Enzymes in this family are comprised primarily of FMN-bound flavin reductases, but a subgroup of FMN reductases within the family are two-component monooxygenase systems that catalyze flavin transfer. These two-component FMN reductases have a π-helical structure located at the tetramer interface (Figure 1A).[6] This SsuE π-helix is generated by the insertion of a tyrosine residue (Tyr118) in the conserved α4 helix that generates a change in intrastrand hydrogen bonding resulting in wide turns.[6] Identification of π-helices as discrete secondary structures is often overlooked when characterizing three-dimensional structures of proteins.[7−10] Extensive structural analyses have determined that π-helices in protein structures are often misannotated and provide a distinct function in the majority of enzymes that contain this structure.[7−10] The π-helical structures can modify the conformation of an enzyme to provide alternative noncovalent contacts and/or destabilize the region to provide a gain of function. The conserved π-helices in structurally related flavin reductases of two-component systems suggest this secondary structural element provides these enzymes with a common function.

In the three-dimensional structure of SsuE with bound flavin, hydrogen bonding occurs between the hydrogen of the hydroxyl group of Tyr118 and the oxygen atom backbone carbonyl of

Received: May 14, 2018
Revised: July 2, 2018
Published: July 6, 2018

DOI: 10.1021/acs.biochem.8b00544
Biochemistry 2018, 57, 4469−4477

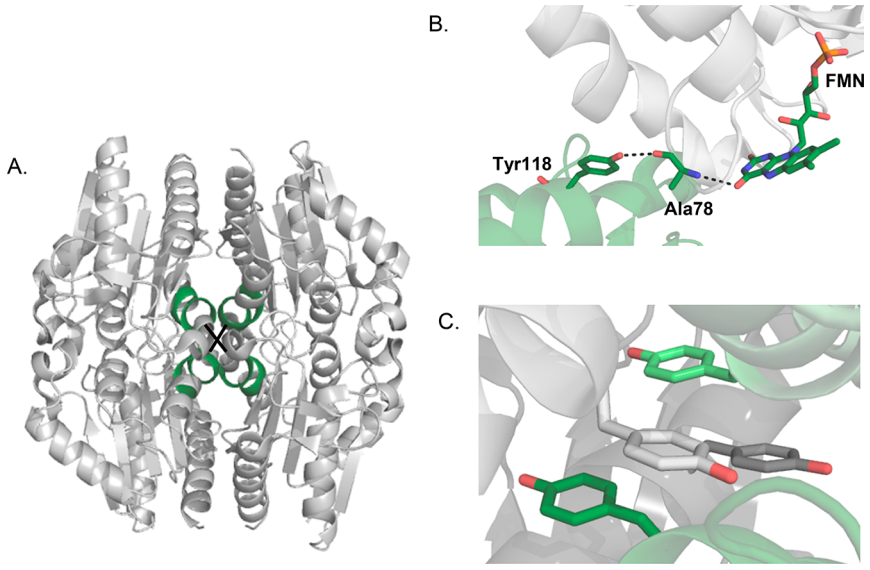


Figure 1. Structural analysis of the π-helix in SsuE. (A) Location of the π-helix (green) at the tetramer interface of SsuE. The black lines at the interface represent the contacts made between the π-helix of each monomer and the opposite monomer in the dimer pair. (B) Hydrogen bonding interactions between Tyr118 and the oxygen of the carbonyl group of Ala78 across the tetramer interface of SsuE. The amide group of Ala78 forms hydrogen bonding interactions with the C4 carbonyl oxygen of the bound flavin. (C) Observed π-stacking interactions between Tyr118 residues at the tetramer interface of SsuE. The Tyr118 π-stacking interactions occur at a diagonal between monomers of the dimer pair. The structures were rendered with Pymol using Protein Data Bank entry 4PTZ (SsuE).[6]

Ala78 across the tetramer interface (Figure 1B).[6,11] The amide nitrogen of Ala78 forms a hydrogen bond to the O4 atom of FMN bound to the active site. Although not noted in the original three-dimensional structure, Tyr118 also forms π-stacking interactions with the Tyr118 residues across the tetramer interface (Figure 1C). The SsuE tetramer interface region includes a region of the π-helix and comprises the protein−protein interaction site with SsuD that facilitates flavin transfer. Therefore, the tetramer of SsuE would need to undergo an oligomeric shift to a dimer for this region of SsuE to effectively form protein−protein interactions with SsuD. In preliminary investigations, substitution of Tyr118 with Ala resulted in a FMN-bound SsuE variant.[11] Although the Y118A SsuE variant retained flavin specificity for FMN, the flavin remained bound even when reduced. The reduced Y118A SsuE variant showed slow reactivity in NADPH oxidase assays, but supported electron transfer to ferricyanide. In addition, there was no measurable sulfite production by SsuD when it was coupled with Y118A SsuE, which correlated with the inability of Y118A SsuE to support multiple flavin reduction turnovers.[11] Preliminary investigations of Y118A SsuE were important in providing a basic foundation regarding the general properties of the π-helix. Additional Tyr118 SsuE variants were generated to further evaluate the role of the π-helix in promoting the oligomeric changes in SsuE that promote flavin transfer.

EXPERIMENTAL PROCEDURES

Materials. *Escherichia coli* strains [XL-1 Blue and BL21(DE3)] were purchased from Stratagene (La Jolla, CA). Plasmid vectors and pET21a were obtained from Novagen (Madison, WI). DNA primers were synthesized by Invitrogen (Carlsbad, CA). Ampicillin, streptomycin sulfate, lysozyme, potassium bromide, ammonium sulfate, reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), bovine serum albumin (BSA), glycine, lysozyme, ethylenediaminetetraacetic acid (EDTA), potassium phosphate (monobasic anhydrous and dibasic anhydrous), dimethyl sulfoxide (DMSO), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), lysozyme, and urea were from Sigma (St. Louis, MO). Isopropyl β-D-1-thiogalactopyranoside (IPTG), sodium chloride, and glycerol were obtained from Macron Fine Chemicals (Center Valley, PA). 1-Octanesulfonate was purchased from Fluka (Milwaukee, WI).

Site-Directed Mutagenesis and Purification of the Y118 SsuE Variants. Variants of Tyr118 in SsuE were generated to investigate the roles of the π-helix in flavin reduction and transfer to SsuD and in the regulation of oligomeric-state changes. The SsuE primers were designed as 27-base oligonucleotides for the Y118S, Y118F, and ΔY118 SsuE variants. The primers were ordered from Life Technologies by substituting TAT for Tyr with TCT or TTT or deleting the TAT codon for the Y118S, Y118F, or ΔY118 SsuE variant, respectively. The Qiagen kit plasmid purification protocol was utilized to prepare the SsuE plasmid used for site-directed mutagenesis. Following site-directed mutagenesis, the SsuE variants were confirmed through DNA sequencing analysis (Eurofins/Genomics, Louisville, KY). The previously generated Y118A SsuE variant was also utilized in these studies.[11] The SsuD and SsuE enzymes were expressed and purified from a pET21a expression vector in *E. coli* strain BL21(DE3) as previously described.[12] The protein fractions for the flavin-free SsuE enzymes and SsuD were collected based on the ultraviolet−visible (UV−vis) absorbance at 280 nm. For the flavin-containing SsuE variants, the fractions were collected based on the UV−vis absorbance at 457 nm. Circular dichroism spectroscopy to evaluate the overall gross secondary structure of the variants was performed as previously outlined.[13]

Kinetic Analysis of the Y118 SsuE Variants. NADPH oxidase activity was assessed with the Y118 SsuE variants as previously described.[11,14,15] The flavin reductase assays were performed with the Y118 SsuE variants (0.04 μM) by varying FMN concentrations (0.25−1.5 μM) at a fixed NADPH

DOI: 10.1021/acs.biochem.8b00544
Biochemistry 2018, 57, 4469−4477

concentration (200 μM) and monitoring NADPH oxidation through the decrease in the UV−vis absorbance at 340 nm. Electron transfer to ferricyanide was performed with FMN-bound wild-type and ΔY118 SsuE (0.04 μM), varying concentrations of NADPH (5−50 μM), and 1 mM ferricyanide in 25 mM Tris-HCl (pH 7.5) and 100 mM NaCl at 25 °C. The initial rates for both experiments were obtained by monitoring the decrease in absorbance at 340 nm with the oxidation of the reduced pyridine nucleotide.

The steady-state coupled assay with SsuE and SsuD monitoring sulfite production was performed as previously described. The reactions were initiated with the addition of 500 μM NADPH to a reaction mixture containing wild-type SsuE or its variants (0.6 μM), FMN (2 μM), SsuD (0.2 μM), and octanesulfonate (5−2000 μM) in 25 mM Tris-HCl (pH 7.5) and 0.1 M NaCl at 25 °C. The reaction was quenched after 3 min with 8 M urea, and the sulfite product was quantified as previously described.[15] All assays were performed in triplicate, and steady-state kinetic parameters were determined by fitting the data to the Michaelis−Menten equation.

Rapid-reaction kinetic analysis with the Y118 SsuE variants was performed using an Applied Photophysics SX.18 MV stopped-flow spectrophotometer as previously outlined.[16] The single-turnover experiment was performed by mixing wild-type SsuE or its Tyr118 variants (35 μM) with wild-type SsuD (35 μM) and FMN (30 μM) in 50 mM potassium phosphate (pH 7.5), 0.2 M NaCl, and 10% glycerol in one syringe against 250 μM NADPH and 50 μM octanesulfonate in 10 mM Tris-HCl (pH 8.5), 0.1 M NaCl, and 10% glycerol in the second syringe. Similar rapid kinetic experiments were set up without SsuD and octanesulfonate in the assay to monitor the flavin reduction half-reaction and subsequent non-enzymatic flavin oxidation.[16] The experiments were performed in single-mixing mode by reacting equal volumes of solutions from each syringe. The change in absorbance at 450 nm was monitored over 100 s. The kinetic trace was standardized and plotted in Kaleidagraph (Synergy).

Fluorimetric Titrations of the Y118 SsuE Variants with SsuD. Binding of flavin to wild-type SsuE and its variants was monitored on a Cary Eclipse Agilent (Santa Clara, CA) fluorescence spectrophotometer with an excitation wavelength at 280 nm and emission measurements at 344 nm. A 1.0 mL solution of flavin-free variant or wild-type SsuE (0.1 μM) in 25 mM potassium phosphate (pH 7.5) and 0.1 M NaCl was titrated with FMN (from 0.022 to 0.44 μM), and the fluorescence spectrum was recorded following a 2 min incubation after each addition. All assays were performed in triplicate, and the K_d value was determined as previously described.[12] Bound FMN was determined with eq 1.

$$[\mathrm{FMN}]_{\mathrm{bound}} = [\mathrm{SsuE}]\frac{I_o - I_c}{I_o - I_f} \quad (1)$$

where $[\mathrm{FMN}]_{\mathrm{bound}}$ represents the concentration of FMN-bound SsuE, [E] represents the initial concentration of the enzyme, I_o is the initial fluorescence intensity of the enzyme prior to the addition of the substrate, I_c is the fluorescence intensity of the enzyme following each addition, and I_f is the final fluorescence intensity. The concentration of FMN bound was plotted against the free substrate concentration to obtain the dissociation constant (K_d) according to eq 2.

$$y = \frac{K_d + x + n - \sqrt{(K_d + x + n)^2 - 4xn}}{2} \quad (2)$$

where y and x represent the concentration of the bound and free substrate, respectively, following each addition and K_d is the maximum binding at equilibrium with the maximum concentration of the substrate.

Protein−protein interactions between the FMN-bound Y118 SsuE variants and SsuD were evaluated by fluorescence spectroscopy. The FMN-bound Y118 SsuE variants (0.4 μM) in 25 mM potassium phosphate (pH 7.5) were titrated with 0.04−1.1 μM aliquots of SsuD as previously described.[13] The fluorescence emission at 525 nm was measured using an excitation wavelength of 450 nm on a Cary Eclipse Agilent (Santa Clara, CA) fluorescence spectrophotometer with slit widths set at 5 nm. The samples were prepared, and experiments were performed in triplicate for wild-type SsuE and each variant. The concentration of SsuD bound to SsuE was determined with eq 1, replacing $[\mathrm{FMN}]_{\mathrm{bound}}$ with [SsuD] in the equation. 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 wild-type SsuE or the variants and SsuD fitting the plot to eq 2.

Competition Assays with Y118A and Wild-Type SsuE. The role of the π-helix in flavin transfer was evaluated in a competition assay.[17] The reaction mixture contained SsuE (0.06 μM), FMN (2 μM), SsuD (0.06 μM), octanesulfonate (1 mM), NADPH (0.25 mM), and varying concentrations of Y118A SsuE (0.01−0.3 μM) in 25 mM Tris (pH 7.5) and 0.1 M NaCl in a total volume of 0.5 mL. The end point assay was initiated by the addition of NADPH, the reaction mixture incubated for 3 min at 25 °C, and the reaction quenched with the addition of 8 M urea (167 μL). The sulfite product was measured as previously described.[15] The rates obtained were plotted against the concentration of Y118A included in the reaction mixture. An experiment to evaluate the dependence of desulfonation on the concentration of wild-type SsuE was performed in the absence of Y118A SsuE by varying the concentration of wild-type SsuE (0.01−0.12 μM) at constant concentrations of substrates and SsuD as outlined above.

Evaluating the Oligomeric States of the Y118 SsuE Variants. Analytical size-exclusion chromatography of the Y118 SsuE variants was performed on an Agilent 1260 Infinity GPC/SEC chromatograph. The molecular weights of the Y118 SsuE variants (50 μM) were determined using an Agilent Bio SEC-3, 4.6 mm × 300 mm, 100 Å column. The variants were eluted from the column with 150 mM sodium phosphate (pH 7.0) and 100 mM NaCl with a flow rate of 0.2 mL/min monitored at 280 nm. A Bio-Rad gel filtration standard with a molecular weight range from 1.35 to 670 kDa was used to generate a curve to fit the molecular weights based on retention times. The standard curve (log of the molecular weight vs retention time) was generated on the basis of the elution time monitored at 280 nm.

RESULTS

General Properties of the Purified Y118 SsuE Variants. The π-helix centrally located at the tetramer interface in SsuE is derived from an insertion of a tyrosine residue in conserved helix α4 of FMN-bound reductases belonging to the NAD(P)H:FMN reductase family. To investigate the functional and structural roles of the π-helix in SsuE, several variants of Tyr118 were generated. The Y118S SsuE variant was generated to conserve the hydroxyl group of Tyr118, which is within hydrogen bonding distance to Ala78 across the tetramer interface. Although these hydrogen-bonding interactions were

DOI: 10.1021/acs.biochem.8b00544
Biochemistry 2018, 57, 4469−4477

previously noted, π-stacking interactions have also been observed between the Tyr118 residues of each monomer. These π-stacking interactions occur between monomers of the dimer pair at the tetramer interface. A Y118F SsuE variant was generated to conserve the phenyl group associated with the tyrosine residue. In addition, a deletion of Tyr118 was generated because the known π-helices in flavin reductases of two-component monooxygenase systems are maintained by the insertion of a tyrosine in conserved helix α4. All the variants, including the previously generated Y118A SsuE variant, were expressed and purified as previously described.[12] The purified Y118S SsuE variant was FMN-bound as was observed with Y118A SsuE, but the Y118F and ΔY118 SsuE variants were flavin-free when purified similar to that of wild-type SsuE.[12] There were no significant perturbations in the gross secondary structure of the Y118 SsuE variants compared to that of wild-type SsuE based on circular dichroism spectroscopy (Figure S1).

Steady-State Kinetics Properties of the Y118 SsuE Variants. The Y118A SsuE variant had no NADPH oxidase activity because of the slow reactivity of reduced flavin with dioxygen but could transfer electrons to ferricyanide.[11] NADPH oxidase assays were also performed with additional Y118 SsuE variants to determine if electron transfer to dioxygen was affected by the π-helix substitutions. The flavin reductase activity was monitored under steady-state kinetic conditions for the Y118S, Y118F, and ΔY118 SsuE variants by monitoring the oxidation of NADPH. The Y118S and ΔY118 SsuE variants could not sustain flavin reductase activity, similar to results obtained with the Y118A SsuE variant. However, the Y118F SsuE variant had a k_{cat}/K_m value of 4000 ± 30 μM^{-1} min^{-1}, comparable to the wild-type value of 2300 ± 200 μM^{-1} min^{-1} (Table 1). The affinities of the Y118 SsuE variants for FMN were evaluated to determine if flavin binding was affected. The Y118 SsuE variants containing a single substitution had K_d values for FMN binding comparable to that of wild-type SsuE (Table 1).[12] A 10-fold lower binding affinity for flavin binding was observed with the ΔY118 SsuE deletion variant than with wild-type SsuE. Although the ΔY118 SsuE variant was unable to support NADPH oxidase activity, the 10-fold decrease in flavin affinity would not account for the absence of activity because the flavin should still bind at the saturating concentrations of FMN used in the flavin reductase assays. As previously observed, each variant had approximately one FMN bound per monomer.[12] While NADPH oxidase activity was not supported, the deletion variant could transfer electrons to ferricyanide giving a k_{cat}/K_m value of 6.2 ± 1 μM^{-1} min^{-1} compared to the wild-type SsuE value of 3.4 ± 0.4 μM^{-1} min^{-1}, and a previously reported k_{cat}/K_m value of 10 ± 1 μM^{-1} min^{-1} with Y118A SsuE.[11]

Table 1. Steady-State Kinetic Parameters for the Y118 SsuE Variants Measuring NADPH-Dependent FMN Reductase Activity[a]

	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} min^{-1})	$K_{d(FMN)}$ (nM)
wild-type SsuE	255 ± 27	0.11 ± 0.02	2300 ± 200	17 ± 1
Y118A SsuE[b]	–	–	–	8 ± 1
Y118S SsuE	–	–	–	7 ± 1
Y118F SsuE	316 ± 2	0.08 ± 0.02	4000 ± 30	50 ± 10
ΔY118 SsuE	–	–	–	160 ± 10

[a]All assays were performed in triplicate, and the kinetic parameters were determined as described in Experimental Procedures. [b]Value could not be determined under the experimental conditions.

Flavin reductases of two-component systems must transfer reduced flavin successfully to the monooxygenase enzymes for the insertion of single oxygen atom(s) into their respective substrates. Coupled assays were performed to evaluate the ability of the Y118 SsuE variants to support desulfonation. There was no measurable desulfonation activity observed in coupled assays monitoring sulfite production with the Y118S SsuE and ΔY118 SsuE variants as was previously observed for Y118A SsuE (Table 2).[11] However, desulfonation activity was observed with the Y118F SsuE variant with a k_{cat}/K_m value of 2.0 ± 0.5 μM^{-1} min^{-1} compared to the wild-type SsuE value of 2.2 ± 0.3 μM^{-1} min^{-1} (Table 2). The absence of desulfonation activity with the Y118S and ΔY118 SsuE variants correlated with the absence of NADPH oxidase activity and suggests the flavin is not transferred to SsuD following flavin reduction.

Table 2. Desulfonation Activity of Wild-Type SsuD and the Y118 SsuE Variants[a]

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
wild-type SsuE	93 ± 7	2.2 ± 0.3	43 ± 6
Y118A SsuE[b]	–	–	–
Y118S SsuE	–	–	–
Y118F SsuE	16 ± 4	2.0 ± 0.5	8 ± 1
ΔY118 SsuE	–	–	–

[a]All assays were performed in triplicate, and the steady-state kinetic parameters were determined as described in Experimental Procedures. [b]Value could not be determined under the experimental conditions.

Rapid-Reaction Kinetic Studies of the Y118 SsuE Variants. The inability of Y118A, Y118S, and ΔY118 SsuE to support desulfonation in the coupled assay suggested that alteration of the π-helix could disrupt flavin transfer. Rapid-reaction kinetic experiments were performed to monitor the flavin transfer event in the SsuE variants. The reductive and oxidative half-reactions of each variant were monitored at 450 nm under single-turnover conditions. From previous studies, the reductive half-reaction catalyzed by wild-type SsuE is followed by a lag phase in the presence of SsuD (Figure 2A,B, black filled circles). The lag phase has been proposed to represent the transfer of reduced flavin from SsuE to SsuD, which involves protein−protein interactions and conformational changes associated with the binding of reduced flavin by SsuD. This lag phase between flavin reduction and oxidation is not observed with wild-type SsuE when SsuD and octanesulfonate are not included in the reaction because the oxidation of reduced flavin occurs immediately following the reduction phase (Figure 2A,B, black empty circles). The kinetic traces of Y118A SsuE in the absence of SsuD showed a decrease in absorbance at 450 nm representing flavin reduction (Figure 2A, red empty circles). Flavin reduction occurred in two phases with an initial fast phase followed by a slower reduction phase. Full oxidation of the flavin that occurs with wild-type SsuE following the reductive half-reaction was not observed with the Y118A SsuE variant even when SsuD was included in the reaction. There was a slow increase in absorbance that plateaued at an amplitude comparable to that of the start of the slow reductive half-reaction (Figure 2A, red empty and filled circles). Similar reductive kinetic traces were obtained with the ΔY118 SsuE variant in the absence or presence of SsuD; however, the slow increase in

DOI: 10.1021/acs.biochem.8b00544
Biochemistry 2018, 57, 4469−4477

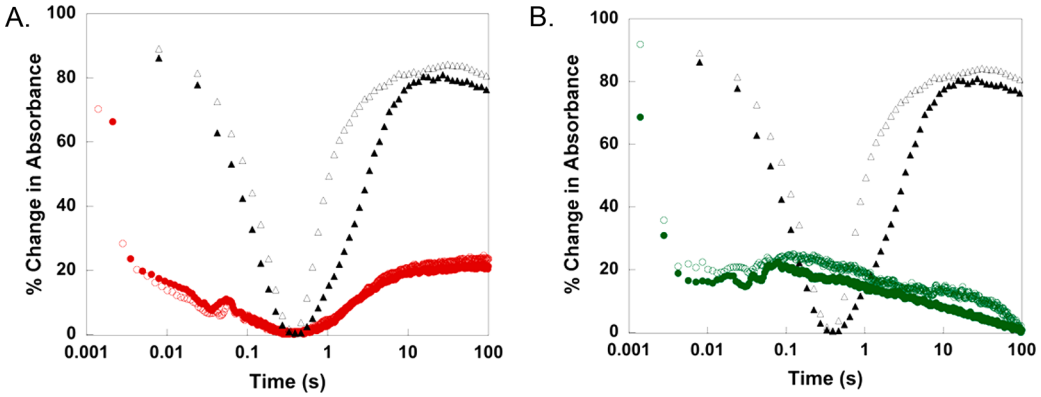


Figure 2. Rapid-reaction kinetic analyses of Y118A, ΔY118 SsuE, and wild-type SsuE. (A) Kinetic traces of Y118A SsuE (red, empty circles), wild-type SsuE (△), Y118A SsuE and SsuD (red, filled circles), and wild-type SsuE and SsuD (▲). (B) Kinetic traces of ΔY118 SsuE (green, empty circles), wild-type SsuE (△), ΔY118 SsuE and SsuD (green filled circles), and wild-type SsuE and SsuD (▲). Kinetic traces were obtained by following flavin reduction and oxidation at 450 nm. Each reaction was performed in triplicate. Stopped-flow kinetic traces were obtained at 4 °C by mixing wild-type SsuE or the Y118 variants (35 μM) and FMN (30 μM) with NADPH (250 μM) monitored at 450 nm. Additional traces were obtained with SsuD (35 μM) and octanesulfonate (50 μM) included in the reaction.

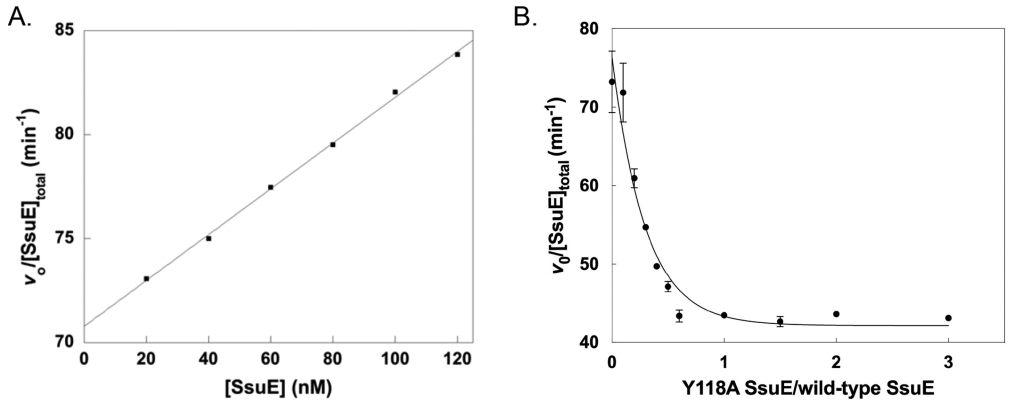


Figure 3. Competition studies for evaluating the role of the π-helix in reduced flavin transfer and protein−protein interactions between SsuE and SsuD. (A) Linear dependence of the desulfonation activity of SsuD on the concentration of SsuE. (B) Desulfonation activity of SsuD in coupled assays with SsuE and increasing concentrations of Y118A SsuE. Each reaction was performed in triplicate, and the rates obtained were plotted against the concentration of Y118A included in the reaction.

absorbance following full flavin reduction was not observed (Figure 2B, green empty and filled circles).

Evaluation of Protein−Protein Interactions between the Variants of Tyr118 in SsuE and Wild-Type SsuD. Results from hydrogen−deuterium exchange mass spectrometry studies identified protein−protein interaction sites in SsuE and SsuD.[18] In the SsuE enzyme, the interacting regions include the π-helix located at the tetramer interface that is associated with Tyr118 inserted at helix α4 (Figure 1A).[6,18] Fluorimetric titrations were performed to evaluate the effect of perturbing the π-helix in SsuE on protein−protein interactions with SsuD. The Y118A and Y118F SsuE variants interacted with SsuD, giving K_d values of 5.9 ± 0.6 and 10 ± 1 nM, respectively, compared to a K_d value of 18 ± 1 nM for wild-type SsuE. The ΔY118 SsuE variant showed no change in flavin fluorescence with the addition of SsuD, suggesting that the SsuE deletion variant could not form protein−protein interactions with SsuD.

Although Y118A SsuE could form static protein−protein with SsuD, competition assays were performed to further evaluate the ability of Y118A SsuE to interact with SsuD during catalysis.[17] Initial studies were performed to determine the concentrations at which the initial velocity is dependent on wild-type SsuD (Figure 3A). The competitive assay contained fixed amounts of SsuD and wild-type SsuE (in a 1:1 ratio) and varying concentrations of Y118A SsuE. Unlike wild-type SsuE, the Y118A variant could not support desulfonation activity by SsuD.[11] Because both Y118A and wild-type SsuE enzymes were shown to interact with SsuD, the two should compete for the interaction sites in SsuD, resulting in a decline in catalytic activity when both enzymes are included in the assay. The observed desulfonation activity would be dependent on only the SsuD interaction sites occupied by wild-type SsuE because the Y118A SsuE cannot transfer reduced flavin. If the transfer of reduced flavin between SsuE and SsuD occurs through diffusion, the catalytic activity would not change with increasing amounts of Y118A SsuE because the reduced flavin would diffuse through the bulk solution to SsuD. Conversely, if the ΔY118 SsuE variant cannot interact with SsuD, then there should be no change in kinetic activity when the SsuE deletion variant is included in the reaction with wild-type SsuE and SsuD. There was a decrease in desulfonation activity observed with increasing concentrations of Y118A SsuE, suggesting that Y118A SsuE was competing with wild-type SsuE for the interaction sites in SsuD (Figure 3B). The decrease in desulfonation activity reached a plateau after 1 equiv of Y118A SsuE was added. The observed desulfonation activity by SsuD was dependent only

DOI: 10.1021/acs.biochem.8b00544
Biochemistry 2018, 57, 4469−4477

on the interaction with wild-type SsuE because Y118A SsuE cannot transfer reduced flavin to SsuD. The ability of wild-type SsuE and the Y118A SsuE variant to compete for the protein interaction sites in SsuD during desulfonation under steady-state conditions supports a channeling mechanism for the transfer of reduced flavin between the alkanesulfonate monooxygenase system enzymes.

Evaluation of the Oligomeric States of Y118 SsuE Variants. Wild-type SsuE has been observed in different oligomeric states that appears to be dependent on the addition of flavin. In the presence of flavin, the SsuE enzyme shifts from a tetramer to a dimer with the addition of flavin. This oligomeric shift may regulate protein−protein interactions and the flavin transfer event. The π-helix located at the tetramer interface may play a role in regulating these potential oligomeric-state changes.[6,11] The oligomeric states of the Y118 SsuE variants were evaluated to determine the effect of substituting or deleting Tyr118 on the quaternary structure of SsuE. The Y118F SsuE variant existed as a tetramer in the absence of flavin with a molecular weight of 84 ± 11 kDa based on the monomeric molecular weight of wild-type SsuE (21.3 kDa). The flavin-bound Y118A and Y118S SsuE variants were dimers with molecular weights of approximately 42 kDa (Table 3). Deflavination of Y118A and Y118S SsuE did not convert the dimeric states of the variants into tetramers, implying the oligomeric states of Y118A and Y118S were independent of the presence of flavin (Table 3). The ΔY118 SsuE variant had a molecular weight of 79 ± 6 kDa, which correlated to the approximate tetrameric structure given.

Table 3. Evaluating the Quaternary Structure of the Y118 SsuE Variants

	molecular weight (kDa)[a]	oligomeric structure[b]
	Flavin-Bound	
wild-type SsuE	73 ± 5	tetramer
Y118A SsuE	42 ± 1	dimer
Y118S SsuE	42 ± 1	dimer
	Flavin-Free	
Y118A SsuE	39 ± 1	dimer
Y118S SsuE	40 ± 1	dimer
Y118F SsuE	79 ± 6	tetramer
ΔY118 SsuE	84 ± 11	tetramer

[a]The molecular weight was obtained as described in Experimental Procedures. The molecular weight values reflect the average from three separate experiments. [b]The oligomeric states were determined using a monomeric molecular weight of 21 kDa for wild-type SsuE.

DISCUSSION

Identifying the unique structural properties of enzymes is pivotal for understanding their defined functions. The π-helices are characterized by a single-residue insertion into α-helices that generates a wide turn caused by the alteration of intrastrand hydrogen bonding and augments existing protein functions.[9] The π-helix previously identified in SsuE was generated through the insertion of a tyrosine residue (Tyr118) in helix α4 of this flavodoxin-folded protein.[6] It was initially observed that the π-helix is conserved in flavin reductases that belong to two-component monooxygenase systems with known structures that included EmoB from *Mesorhizobium* sp. BNC1 and an SsuE homologue from *Corynebacterium diptheriae*.[19] In preliminary investigations, substitution of Tyr118 in SsuE with Ala generated a flavin-bound variant that displayed kinetic properties that were different from those of wild-type SsuE.[11] The Y118A SsuE variant was purified with FMN bound, but the enzyme could not support continuous turnover in NADPH oxidase assays. In addition, there was no desulfonation activity in coupled assays with SsuD monitoring sulfite production. Although the reduced Y118A SsuE variant had slow NADPH oxidase activity, the enzyme supported the transfer of electrons to ferricyanide. Interestingly, a histidine residue appears to mimic the role of tyrosine in generating π-helices for flavin reductases of two-component monooxygenase systems involved in sulfur metabolism (Figure 4). The SfnF enzyme supplies reduced flavin to dimethylsulfone monooxygenase (SfnG), and the MsuE enzyme supplies reduced flavin to methanesulfonate monooxygenase (MsuD).[3,5,20]

Additional variants of Tyr118 in SsuE were generated to further assess the functional effects of mutating the π-helix in SsuE. Substitution of Tyr118 with serine eliminated the aromatic group but maintained a hydroxyl group that could participate in hydrogen bonding across the tetramer interface with Ala78. The Y118S SsuE variant was purified with FMN bound and displayed no measurable reductive or desulfonation kinetic activity similar to Y118A SsuE.[11] Even though FMN was bound as purified, Y118S SsuE had an affinity for flavin similar to that of wild-type SsuE. Previously noted noncovalent interactions may not be optimized with the less bulky and shorter serine residue, resulting in kinetic properties similar to those of Y118A SsuE. While the Ser substitution adversely affected flavin transfer, the Y118F SsuE enzyme was flavin-free and had flavin reductase and desulfonation activities comparable to those of wild-type SsuE. In addition, the binding affinity of Y118F SsuE for FMN and the oligomeric structure was comparable to that of flavin-free wild-type SsuE. These kinetic similarities between Y118F and wild-type SsuE could be attributed to π-stacking interactions maintained at the tetramer interface by the phenyl groups of phenylalanine. Aromatic amino acids are often involved in π-stacking interactions if the distance between them is <7 Å.[21] The Tyr118 π-stacking interactions occur at a diagonal between monomers of the dimer pair at a π-stacking distance of ∼5.3 Å, which confers the tetrameric core with parallel-displaced π-stacking interactions (Figure 1B).[22] Insertional residues that generate the π-helix often display aromatic properties.[7,9] Substitution of Tyr with Phe may be able to maintain the overall structure of the π-helix and support π-stacking interactions. Therefore, the formation of π-stacking interactions may play a role that is more relevant than that of H-bonding interactions in maintaining the overall structure and function of SsuE. Interestingly, a histidine residue appears to mimic the role of tyrosine in generating π-helices in recently determined structures of flavin reductases of two-component monooxygenase systems involved in sulfur metabolism. Because the π-helices in flavin reductases of two-component monooxygenase systems evolved through a tyrosine or histidine insertion, the Tyr118 residue was deleted (generating ΔY118 SsuE) to reverse the residue insertion in SsuE. The ΔY118 SsuE variant had no measurable flavin reductase or desulfonation activity similar to Y118A SsuE, but there was a 10-fold decrease in flavin affinity compared to that of wild-type SsuE.

In previous studies, the Y118A SsuE variant showed slow oxygen reactivity under steady-state kinetic conditions.[11] The reactivities of flavoproteins with dioxygen are quite varied and depend on different structural factors.[23−26] The trafficking of reduced flavin in two-component flavin-dependent systems remains an enigma because of the unstable nature of free

DOI: 10.1021/acs.biochem.8b00544
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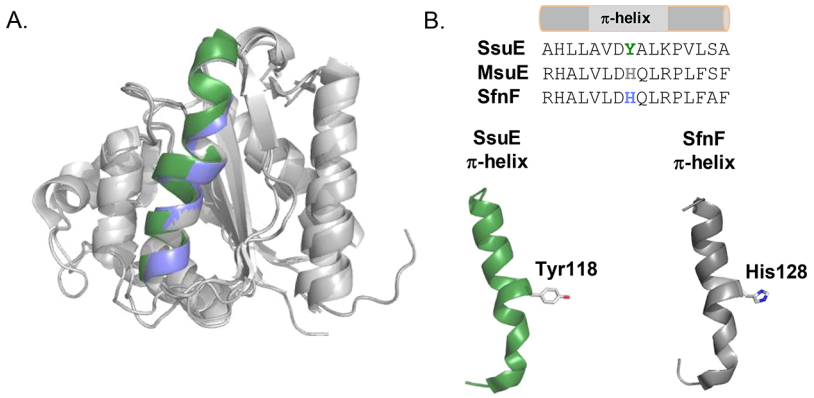

Figure 4. Evaluation of the π-helix in the two-component NAD(P)H:FMN reductases involved in desulfonation. (A) Overlay of the NAD(P)H:FMN reductases involved in sulfur acquisition. (B) Highlighted π-helices of SsuE (green), MsuE (blue), and SfnG (gray). The structures for SsuE (Protein Data Bank entry 4PTZ) and SfnF (Protein Data Bank entry 4c76, chain a) were rendered with Pymol. The structure of MsuE was modeled with I-Tasser using Protein Data Bank entry 4c76, chain a, as the threading template.[6,31,32] (B) Sequence alignment of the π-helical region of the NAD(P)H:FMN reductases involved in sulfur metabolism. The π-helical region of the NAD(P)H:FMN reductases is generated by the insertion of Tyr or His into a conserved α-helix. Alignments were performed with the Clustal Omega database maintained by EMBL-EBI.[33,34]

reduced flavin. If not successfully delivered to the dedicated monooxygenase enzyme, reduced flavin could undergo nonenzymatic oxidation producing reactive oxygen intermediates. Flavin reduction in two-component flavin-dependent systems must be tightly controlled to facilitate flavin transfer. Protein−protein interactions were previously observed between SsuE and SsuD, and these interaction sites are located at the tetramerization region that includes the π-helix.[18] In addition, the active site is located near the tetramer interface, suggesting that oligomeric changes may expose the active site of SsuE for catalysis and this is a mechanism for initiating reduced flavin transfer to SsuD (Figure 5). The Y118A and Y118S SsuE

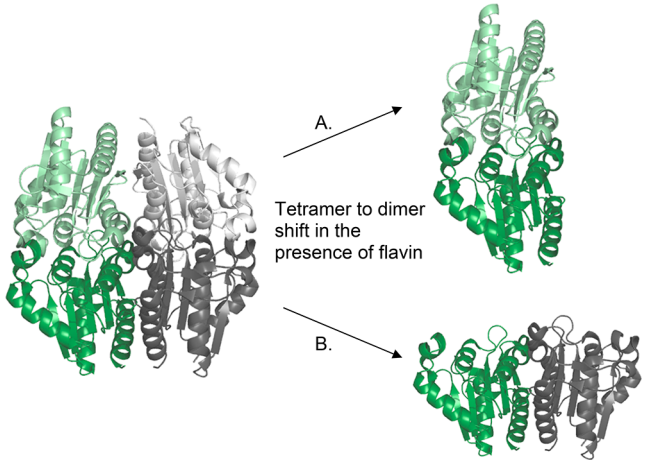

Figure 5. Oligomeric properties of SsuE. The SsuE enzyme exists as a tetramer (85.2 kDa) in the absence of flavin but dissociates into a dimer with FMN bound (42.6 kDa). The tetramer is shown with each dimer pair colored green and gray. Generation of the interacting dimer could occur along the interface formed from the dimer of dimers (A) or could also result from the disruption of the dimer pairs (B).[6]

variants were FMN-bound as purified and existed as dimers. However, the dimeric structures were maintained even when the Y118A and Y118S SsuE enzymes were deflavinated. Substitution of Y118 with Ala did not adversely affect the interaction of the SsuE variants with SsuD even though desulfonation activity was not observed. The dimeric structure of these variants would expose the protein−protein interaction sites, and a tetramer−dimer shift with the binding of flavin would not be necessary to promote protein−protein interactions. Although protein−protein interactions were maintained, the Y118A SsuE variant could not transfer reduced flavin to SsuD. The ability of Y118A SsuE to form stable protein−protein interactions with SsuD was also observed in competition assays with wild-type SsuE and SsuD. The variant could effectively compete with wild-type SsuE for SsuD binding sites, resulting in an overall decrease in desulfonation activity because the flavin was not transferred. The observed rate decrease for SsuD supports our previous findings that flavin transfer occurs through a channeling mechanism. There would be no change in the rate of desulfonation if the flavin were transferred to SsuD through a diffusion mechanism, because the reduced flavin would be released into the bulk solvent and bound by SsuD. Conversely, the ΔY118 SsuE variant was predominantly tetrameric even in the presence of flavin and could not undergo the oligomeric shift necessary for protein−protein interactions with the monooxygenase enzyme. Deletion of Tyr118 adversely affected the ability of SsuE to interact with SsuD, suggesting that the deletion of this residue shifted the enzyme to behave as canonical flavoproteins within the family. However, ΔY118 SsuE appears to be able to protect reduced flavin from reaction with dioxygen like Y118A SsuE does.

Distinct functional properties separate SsuE from other canonical flavoproteins within the family, which include changes in the oligomeric state to facilitate protein−protein interactions with SsuD that initiate flavin transfer. The primary structural difference within this family of flavin reductases that would account for this enhanced function is the presence of the π-helix. The interactions of the π-helix across the tetramer interface assist in stabilizing the adjacent active site through defined noncovalent interactions, and these interactions are promoted by a wider turn of the π-helix.[9] Substitution of Tyr118 with alanine or serine would essentially serve as an amino acid place holder for Tyr118, but the resulting substitution would likely disrupt the π-helical structure. The majority of enzymes belonging to the NAD(P)H:FMN reductase family lack the insertional residue that promotes generation of the π-helix and are canonical flavin reductases that favor a tetrameric quaternary

DOI: 10.1021/acs.biochem.8b00544
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structure.[9] Enzymes within this family lack a monooxygenase partner and instead catalyze the direct reduction of compounds such as quinones, chromates, and arsenate.[27,28] Deletion of Tyr118 may transform SsuE to more closely resemble the canonical flavin reductases within the family, leading to the formation of a stable tetrameric structure and eliminating protein−protein interactions.

Specific structural features are required to mediate oligomerization of SsuE. The π-helix appears to be a key structural feature that leads to changes in the oligomeric state, which enhances protein−protein interactions for efficient flavin transfer in FMN reductases of two-component systems. The generation of π-helices often leads to the energetic destabilization of enzymes, resulting in an increase in dynamics that can enhance enzyme function.[9] One of the two π-helical regions in lipoxygenase was shown to provide an access point for fatty acid binding because of the increased flexibility of this region.[29] The π-helix in methane monooxygenase was shown to provide substrate flexibility for the enzyme.[30] Further evaluation of the three-dimensional structure identified two overlapping π-helices, with an observed peristaltic-type shift of one of the π-helices when the substrate binds.[9] Observed shifts and increased flexibility essential for substrate binding would not occur with a more stable α-helix. Insertion of Tyr118 in SsuE crafts a π-helix at the tetramer interface essential for regulation of oligomeric-state changes and could be involved in promoting flavin transfer. The presence of the π-helix at the tetramer interface of NAD(P)H:FMN reductases may lead to a more dynamic region that promotes the oligomeric changes needed for protein−protein interactions with the monooxygenase partner. The structural and kinetic studies reported herein provide critical insight into the functional role of π-helices in flavin reductases of two-component monooxygenase systems.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b00544.

Circular dichroism of the Y118 SsuE variants (PDF)

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Funding

This work was supported by Grant 1244320 (to H.R.E.) from the National Science Foundation.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

CD, circular dichroism; FAD, oxidized flavin adenine dinucleotide; FMN, oxidized flavin mononucleotide; $FMNH_2$, reduced flavin mononucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; SsuE, alkanesulfonate monooxygenase flavin reductase; SsuD, alkanesulfonate monooxygenase; SfnF, dimethylsulfone monooxygenase flavin reductase; SfnG, dimethylsulfone monooxygenase; MsuE, methanesulfonate monooxygenase flavin reductase; MsuD, methanesulfonate monooxygenase.

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