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Functional Role of a Conserved Arginine Residue Located on a Mobile Loop of Alkanesulfonate Monooxygenase

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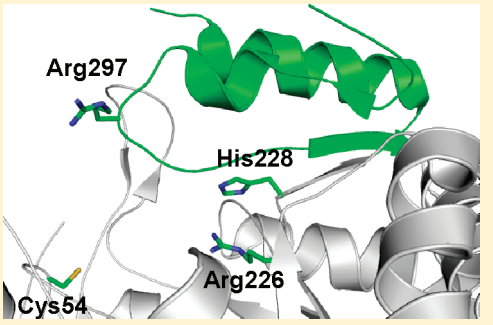
Supporting Information

ABSTRACT: The structure of the flavin-dependent alkanesulfonate monooxygenase (SsuD) exists as a TIM-barrel structure with an insertion region located over the active site that contains a conserved arginine (Arg297) residue present in all SsuD homologues. Substitution of Arg297 with alanine (R297A SsuD) or lysine (R297K SsuD) was performed to determine the functional role of this conserved residue in SsuD catalysis. While the more conservative R297K SsuD possessed a lower k_{cat}/K_m value ($0.04 \pm 0.01\ \mu M^{-1}\ min^{-1}$) relative to wild-type ($1.17 \pm 0.22\ \mu M^{-1}\ min^{-1}$), there was no activity observed with the R297A SsuD variant. Each of the arginine variants had similar K_d values for flavin binding as wild-type SsuD ($0.32 \pm 0.15\ \mu M$), but there was no measurable binding of octanesulfonate. The low levels of activity for the R297A and R297K SsuD variants correlated with the absence of any detectable C4a-(peroxy)flavin formation in stopped-flow kinetic studies. Single-turnover experiments were performed in the presence of SsuE to evaluate both the reductive and oxidative half-reaction. With wild-type SsuD a lag phase is observed following the reductive half-reaction by SsuE that represents flavin transfer or conformational changes associated with the binding of substrates. Evaluation of the Arg297 SsuD variants in the presence of SsuE showed no lag phase following reduction by SsuE, and the flavin was oxidized immediately following the reductive half-reaction. These results corresponded with a lack of detectable changes in the proteolytic susceptibility of R297A and R297K SsuD in the presence of reduced flavin and/or octanesulfonate, signifying the absence of a conformational change in these variants with the substitution of Arg297.



Bacterial organisms have specific sulfur requirements that must be met to synthesize essential sulfur-containing cofactors and metabolites. When bacteria are under limiting sulfur conditions, a distinct set of proteins are expressed that allow the organism to obtain sulfur from alternative sources.[1,2] The two-component alkanesulfonate monooxygenase system expressed under sulfur limitation allows bacterial organisms to use alkanesulfonates as a source of sulfur. The system utilizes an NAD(P)H[1]-dependent flavin reductase to catalyze the reduction of FMN. The flavin reductase (SsuE) transfers the reduced flavin to the alkanesulfonate monooxygenase (SsuD), which catalyzes the liberation of sulfite from 1-substituted alkanesulfonates (Scheme 1).[3] The sulfite is subsequently reduced and incorporated into sulfur-containing compounds. This enzyme system is found in a diverse group of bacterial organisms, indicating the importance of this system in maintaining appropriate sulfur levels for biological processes.

The SsuD enzyme exists as a homotetramer and is structurally related to the bacterial luciferase family, which includes bacterial luciferase and LadA.[4−7] While these enzymes share relatively low amino acid sequence identity, three-dimensional structures of the enzymes in this family show common overall structural features. All members of this family form a triosephosphate isomerase (TIM)-barrel fold, with the active site located at the C-terminal end of the β barrel.[4−7] The LadA enzyme is a homodimer of identical subunits, and the structure of bacterial luciferase is a dimer of homologous α and β subunits.[5−8] The primary structural difference between the two subunits in bacterial luciferase is the presence of a loop region that is part of an insertion sequence in the α subunit.[5,6] This loop region was also identified in SsuD and is largely unresolved in the original three-dimensional structure, suggesting conformational mobility in this region.[4] In several TIM-barrel proteins, these flexible loops contribute to the structure of the enzyme active site or play a functional role both in the binding of substrates and in enzyme catalysis.[9−11] In these lid-containing TIM-barrel proteins, loop closure protects the substrate and catalytic intermediates from bulk solvent.[12−18] This lid-gating mechanism would be catalytically important for the two-component monooxygenase enzymes to sequester reduced flavin and protect flavin intermediates generated during catalysis. Studies have been performed to evaluate the role of this flexible loop in bacterial luciferase. The loop was protected from proteolysis in the presence of phosphate and reduced flavin.[19,20] A variant of bacterial luciferase containing a loop deletion in the α subunit was still able to bind substrates and generate the carboxylic acid product; however, the bioluminescence was

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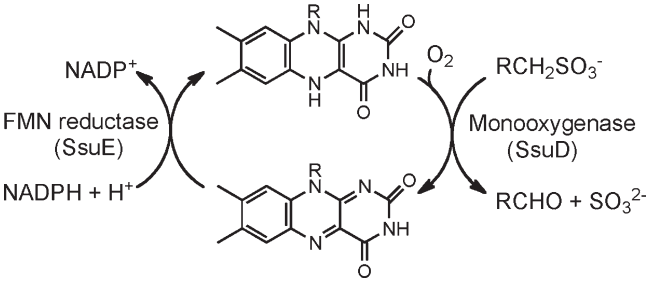
Scheme 1



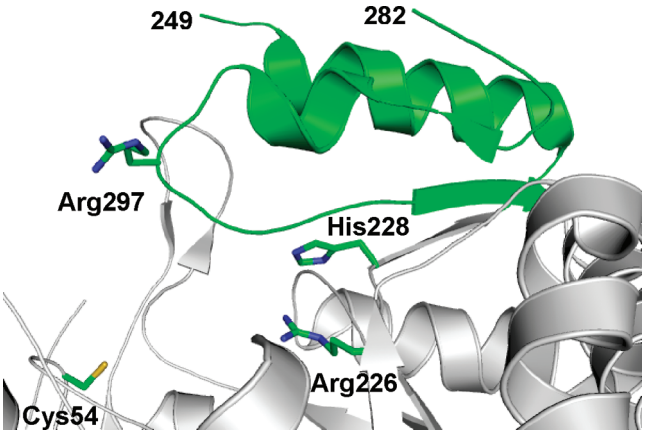


Figure 1. Putative active site of SsuD. The conserved SsuD active site residues are His228, His226, and Cys54. The insertion region (highlighted in green) contains the mobile loop that is proposed to close over the active site. The electron density from amino acid residue 250 to 282 was poorly defined. The Arg297 residue is located on this insertion region. The active site was generated and rendered with PyMOL (PDB ID: 1M41).[4]

decreased by 2 orders of magnitude.[21] Substitution of conserved loop amino acid residues Gly275 and Phe261 also resulted in a decrease in bioluminescence.[22] Both variants exhibited increased rates for the nonproductive dark decay of the C4a-hydroperoxyflavin and decreased rates for decanal oxidation. These studies demonstrated the importance of the flexibility of Gly275 and hydrophobic properties of Phe261 in catalysis and established the importance of the loop in contributing to the active site architecture.[22] Alanine substitutions at 15 amino acids within the mobile loop were also generated to determine if specific amino acid residues were responsible for the decrease in bioluminescence with the loop deletion.[23] Alanine substitutions at Lys283 and Lys286 in bacterial luciferase led to a decrease in bioluminescence and were thought to play a role in excluding bulk solvent from the active site. While a portion of this loop region was unstructured in initial crystal structures of bacterial luciferase in the absence of substrates, a structuring of the loop in the α subunit was observed in a recent three-dimensional structure of bacterial luciferase with bound oxidized FMN.[24]

A conserved arginine residue (Arg297) in the insertion sequence that contains the putative mobile loop is found in all bacterial SsuD enzymes. On the basis of the three-dimensional structure of *E. coli* SsuD, this arginine residue is located on the loop region that lies over the putative active site of the enzyme (Figure 1). Sequence-based alignments with SsuD demonstrated that this arginine residue is conserved and similarly positioned in other two-component flavin-dependent enzymes including nitrilotriacetate monooxygenase (NtaA), pristinamycin synthase subunit A (SnaA), and dibenzothiophene desulfurization enzyme (SoxA).[4] When SsuD was initially characterized, it contained an aberrant Arg297 to Cys mutation that eliminated enzymatic activity.[4] In the three-dimensional structure, the arginine residue is positioned away from the active site and would have to undergo a conformational change in order to be catalytically relevant. Evidence for a conformational change with the binding of substrates has been demonstrated through kinetic studies with SsuD and may be associated with the closing of the loop region.[25] Two variants of SsuD were constructed in which Arg297 was replaced by alanine or lysine. Kinetic and structural analyses were performed to evaluate the function of this residue in the SsuD-catalyzed desulfonation reaction. These studies define a mechanistic role for Arg297 and suggest a similar function for the conserved arginine residue in other flavin-dependent two-component monooxygenases.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotide primers were from Invitrogen (Carlsbad, CA). Flavin mononucleotide phosphate (FMN), nicotinamide adenine dinucleotide phosphate (NADPH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), dimethyl sulfoxide (DMSO), guanidine·HCl, EDTA, sodium chloride, urea, D-glucose, glucose oxidase, potassium phosphate (monobasic and dibasic), ammonium bicarbonate, calcium chloride, phenylmethylsulfonyl fluoride (PMSF), and TPCK-treated trypsin were purchased from Sigma (St. Louis, MO). Glycerol was obtained from Fisher (Pittsburgh, PA). Octanesulfonate was from Fluka (Milwaukee, WI).

Site-Directed Mutagenesis, Expression, and Purification. Mutagenesis of the *ssuD* gene was performed with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The CGT codon for Arg297 was replaced with GCG and AAA for Ala and Lys, respectively. The pET21a plasmid containing the *ssuD* gene was used as the template for PCR-amplification. The entire *ssuD* coding region containing the appropriate mutations was confirmed by sequence analysis at Davis Sequencing (Davis, CA). Each plasmid containing the substituted *ssuD* gene was transformed into *E. coli* BL21(DE3) supercompetent cells (Invitrogen, Carlsbad, CA). The variants generated are referred to as R297A (Arg297 to Ala) and R297K (Arg297 to Lys) SsuD.

The wild-type and variant SsuD proteins were expressed from a pET21a expression vector in *E. coli* strain BL21(DE3), and the purification of each protein was performed as previously described.[26] Following purification, stocks of the variant and wild-type SsuD enzymes were stored in 25 mM potassium phosphate buffer (pH 7.5), 100 mM NaCl, and 10% glycerol at −80 °C. The SsuD concentration was determined spectrophotometrically using a molar absorption coefficient of 47 900 M^{-1} cm^{-1} at 280 nm. Wild-type SsuE was also expressed and purified as previously described, and the concentration was determined spectrophotometrically using a molar absorption coefficient of 20 340 M^{-1} cm^{-1} at 280 nm.[26]

Substrate Binding and Steady-State Kinetic Analyses. A coupled assay measuring the sulfite product was used to determine the steady-state kinetic parameters of the variant and wild-type SsuD enzymes as described previously with slight modifications.[25] For wild-type SsuD the reaction was initiated

Table 1. Dissociation Constants and Steady-State Kinetic Parameters for R297K, R297A, and Wild-Type SsuD

	K_m, octanesulfonate (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})	K_d, $FMNH_2$[a] (μM)
WT SsuD[b]	44.0 ± 8.3	51.7 ± 2.1	1.17 ± 0.22	0.32 ± 0.15
R297K SsuD	35.2 ± 4.6	1.5 ± 0.1	0.04 ± 0.01	1.09 ± 0.16
R297A SsuD	ND[c]	ND	ND	1.46 ± 0.17

[a] Determined under anaerobic conditions as described in Experimental Procedures. [b] Previously reported.[25] [c] No activity detected.

with the addition of NADPH (500 μM) into a reaction mixture containing SsuD (0.2 μM), SsuE (0.6 μM), FMN (2 μM), and varying concentrations of octanesulfonate (5−1500 μM) in 25 mM potassium phosphate (pH 7.5) and 10% glycerol. Because of decreased enzymatic activity with the variants, assays contained a final concentration of 3.0 μM R297A or R297K SsuD, 9.0 μM SsuE, and 5 μM FMN. The sulfite product was quantified as previously described.[25]

Binding of reduced flavin to the variant SsuD enzymes was monitored by spectrofluorimetric titration. A solution of R297A or R297K SsuD (0.5 μM) in 25 mM potassium phosphate (pH 7.5), 10% glycerol, and 100 mM NaCl (1.0 mL total volume) was titrated with a solution of $FMNH_2$ (0.26−9.42 μM $FMNH_2$ for R297A SsuD and 0.26−9.19 μM $FMNH_2$ for R297K) under anaerobic conditions, and the fluorescence was recorded after each addition.[25] Octanesulfonate binding to the SsuD variants was investigated by similar fluorimetric titration methods employed for flavin binding. Aliquots of an anaerobic solution of octanesulfonate in an airtight titrating syringe were added to an anaerobic solution of either R297A or R297K SsuD (1 μM), with $FMNH_2$ (2 μM) in 25 mM potassium phosphate (pH 7.5), 10% glycerol, and 100 mM NaCl (1.0 mL total volume). The fluorescence spectrum for each titration were recorded with an excitation wavelength at 280 nm and emission intensity measurements at 344 nm.

Rapid-Reaction Kinetics Analyses. All stopped-flow experiments were carried out using an Applied Photophysics SX.18 MV stopped-flow spectrophotometer. Reduced flavin solutions were prepared as previously described.[25] Experiments to evaluate the oxidation of reduced flavin by the Arg297 variants were performed by mixing $FMNH_2$ (15 μM) in one drive syringe against R297A or R297K SsuD (45 μM) and octanesulfonate (50−2000 μM before mixing) in air-equilibrated 25 mM potassium phosphate (pH 7.5) and 10% glycerol in the other drive syringe. All experiments were carried out in single-mixing mode at 4 °C by mixing equal volumes of the solutions and monitoring the reactions by single wavelength analyses at 370 and 450 nm. Kinetic traces were fit to the following rate equation with KaleidaGraph software (Abelbeck Software, Reading, PA).

$$A = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + C \quad (1)$$

where k_1, k_2, and k_3 are the apparent rate constant for the different phases, A is the absorbance at time t, A_1 and A_2 are amplitudes of each phase, and C is the absorbance at the end of the reaction.

Stopped-flow kinetic experiments monitoring both the reductive and oxidative half-reactions were performed with aerobic solutions of R297A, R297K, or wild-type SsuD (35 μM), SsuE (35 μM), and FMN (25 μM) mixed against NADPH (250 μM) in air-saturated 25 mM potassium phosphate (pH 7.5) and 10% glycerol at 4 °C. When included in the reaction, octanesulfonate (250 μM) was present in the drive syringe with NADPH. All experiments were carried out in single-mixing mode by mixing equal volumes of the solutions and monitoring the reactions by single wavelength analyses at 450 nm.

Proteolytic and Mass Spectrometric Analyses. The susceptibility of the variants and wild-type SsuD to proteolysis was investigated with trypsin in the absence and presence of $FMNH_2$ and octanesulfonate. Samples of R297A, R297K, or wild-type SsuD enzymes (24 μM) were treated with 10 μg/mL TPCK-treated trypsin in 200 mM ammonium bicarbonate (pH 8.4), 1 mM $CaCl_2$, and 1 mM HCl. An anaerobic solution of flavin (100 μM) was made in 25 mM phosphate buffer (pH 7.5), 10 mM EDTA, and 10% glycerol. The flavin solution was bubbled with argon gas for 20 min before being transferred to an anaerobic chamber and reduced inside a gastight Hamilton syringe with a long wavelength UV lamp. The concentration of $FMNH_2$ in each reaction mixture was 29 μM, and the concentration of octanesulfonate was 250 μM when included in the reaction. Samples (10 μL) were taken at various times (15−300 s) and added to 2 μL of PMSF (6 mg/mL) in 100% isopropanol to quench the reaction. The samples were separated by SDS-PAGE and quantified using the ImageJ software (NIH, Bethesda, MD) to determine the percent digestion. For analysis of the tryptic digests by mass spectrometry, 2% formic acid (final concentration) was added to the sample prior to direct injection into a Waters Q-TOF Premier mass spectrometer. The multiply charged protein was deconvoluted using MaxEnt 1 to obtain the protein molecular mass. Peptide mapping was performed through database analysis with GPMAW software (Lighthouse, Odense, Denmark).

RESULTS

Steady-State Kinetic Analysis and Substrate Affinity of the Arg297 SsuD Variants. Steady-state kinetic analyses were performed to determine if substitution of the conserved arginine residue resulted in any modifications in the kinetic parameters of SsuD. While the K_m value for octanesulfonate with R297K SsuD was comparable to wild-type, the k_{cat} value was decreased ~35-fold (Table 1), resulting in a 30-fold decrease in the k_{cat}/K_m value for the R297K SsuD variant compared to wild-type. The R297A SsuD variant exhibited no detectible production of sulfite, even at increased protein concentrations. The absorbance values at 412 nm for 2-nitro-5-benzoic acid production were similar to the absorbance values obtained in control experiments in the absence of enzyme. The absorbance values were below the detectable limit for sulfite of 4 μM. These results suggest that Arg297 located on the proposed mobile loop region is essential for desulfonation by SsuD, and the charge on the amino acid may play a role in catalytic function.

Spectrofluorimetric titrations were performed to determine whether the Arg297 substitutions had an effect on the binding of $FMNH_2$ and octanesulfonate. The dissociation constants (K_d) for the binding of reduced flavin to the SsuD variants were determined by titrating $FMNH_2$ into a sample of R297A or R297K SsuD. The K_d value for $FMNH_2$ binding to each SsuD variant was 1.09 ± 0.16 and 1.46 ± 0.17 μM for R297K or R297A SsuD, respectively (Table 1). These K_d values are comparable to the values obtained for wild-type SsuD (0.32 ± 0.15 μM) and suggest that substitution of the arginine residue to either lysine or alanine does not significantly alter the binding of $FMNH_2$ in fluorescent titrations.[25] Similar titration experiments were performed to

Table 2. Rate Constants for the Oxidation of Reduced Flavin by R297A, R297K, and Wild-Type SsuD in the Absence and Presence of Octanesulfonate

	370 nm		
	k_1 (s^{-1})	k_2 (s^{-1})	k_3 (s^{-1})
−octanesulfonate			
WT SsuD[a]	12.9 ± 0.3	1.80 ± 0.03	0.08 ± 0.01
R297A SsuD	3.32 ± 0.07	0.81 ± 0.02	
R297K SsuD	1.39 ± 0.01	0.24 ± 0.02	
+octanesulfonate			
WT SsuD[a]	28.9 ± 1.1	1.50 ± 0.02	0.19 ± 0.03
R297A SsuD	3.18 ± 0.07	0.91 ± 0.04	
R297K SsuD	1.97 ± 0.01	0.44 ± 0.02	

[a] Previously reported.[25]

determine if the binding of octanesulfonate to the R297K or R297A SsuD/$FMNH_2$ complex was affected by the arginine substitutions. There was no apparent quenching of the intrinsic fluorescence with the addition of octanesulfonate, and a K_d for octanesulfonate could not be obtained even with octanesulfonate additions over 300 μM. These results were somewhat unexpected, as the R297K SsuD variant still possessed some activity. However, altered binding of octanesulfonate by R297A SsuD may correlate with the absence of fluorescent quenching during fluorescent titrations.

Rapid Reaction Kinetics of Flavin Oxidation. The role of Arg297 in the desulfonation reaction was further probed through rapid reaction kinetic analyses to evaluate if flavin oxidation was affected in the absence and presence of octanesulfonate. Stopped-flow kinetic analyses was performed with each variant at 370 and 450 nm mixing free $FMNH_2$ with R297A or R297K SsuD, in the absence and presence of octanesulfonate. The C4a-(hydro)peroxyflavin is proposed to be the flavin intermediate responsible for the desulfonation reaction and has a spectral signature observable at 370 nm. Previous studies with SsuD identified three phases at 370 nm in the absence and at low concentrations (≤100 μM) of octanesulfonate. This initial phase (k_1) following mixing of $FMNH_2$ with SsuD in the absence or with low octanesulfonate concentrations (≤100 μM) likely represents a flavin adduct that forms prior to regeneration of FMN and was attributed to the C4a-(hydro)peroxyflavin reactive intermediate[25] (Table 2). The rates obtained for the last two phases were assigned to the decay of the flavin intermediate back to the oxidized form (k_2 and k_3) (Table 2). The kinetic traces obtained at 450 nm with wild-type SsuD represents oxidation of the reduced flavin, and the C4a-(hydro)peroxyflavin reactive intermediate is not observed at this absorbance. The kinetic traces at 370 nm for R297A SsuD were best fit to a double-exponential equation with rate constants of 3.32 ± 0.07 s^{-1} (k_1) and 0.81 ± 0.02 s^{-1} (k_2) in the absence of octanesulfonate (Figure 2A, ●), and 3.18 ± 0.07 s^{-1} (k_1) and 0.91 ± 0.04 s^{-1} (k_2) with the addition of octanesulfonate (Figure 2A, ○) (Table 2). The initial phase previously attributed to the formation of the C4a-(hydro)peroxyflavin intermediate with wild-type SsuD at 370 nm in the absence or with low octanesulfonate concentrations was not observed in any of the kinetic traces of flavin oxidation with R297A SsuD.[25] The inability of R297A SsuD to generate the C4a-(hydro)peroxyflavin intermediate correlates with the absence of activity in steady-state kinetic

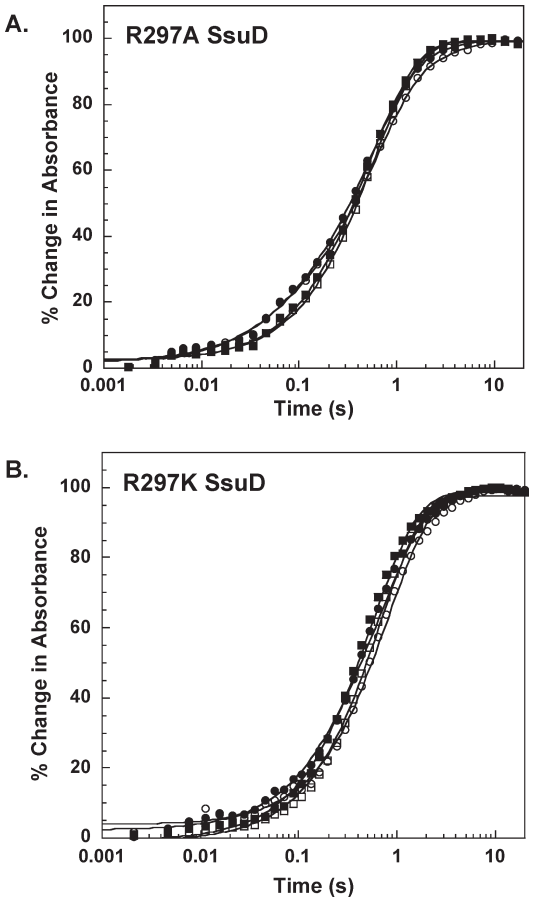


Figure 2. Kinetics of flavin oxidation by R297A, R297K, or wild-type SsuD. (A) Stopped-flow kinetic traces were obtained at 4 °C mixing free $FMNH_2$ (15 μM) with R297A (45 μM) in air-saturated buffer at 370 nm (●) and 450 nm (■) and free $FMNH_2$ (15 μM) mixed with R297A SsuD (45 μM) and 1-octanesulfonate (100 μM) in air-saturated buffer at 370 nm (○) and 450 nm (□). (B) Stopped-flow kinetic traces for R297K SsuD were performed as described for R297A SsuD. The labeling is the same as described for R297A SsuD. The kinetic traces shown represent an average of three separate experiments. The solid lines are the fits of the kinetic traces to eq 1.

experiments and suggests that the peroxyflavin is not stabilized. Although R297K SsuD still possessed slight activity in steady-state kinetic assays, there was no observable accumulation of the C4a-(hydro)peroxyflavin. Kinetic traces at 370 nm for R297K SsuD were best fit to a double-exponential equation with similar rate constants within error as R297A SsuD, 1.39 ± 0.01 s^{-1} (k_1) and 0.24 ± 0.02 s^{-1} (k_2) in the absence of octanesulfonate (Figure 2B, ●), and 1.97 ± 0.01 s^{-1} (k_1) and 0.44 ± 0.02 s^{-1} (k_2) with octanesulfonate included in the reaction (Figure 2B, ○). The rapid reaction kinetic traces for R297A and R297K SsuD at 450 nm with and without octanesulfonate were fit to a double-exponential equation and essentially overlapped (Figure 2A,B, ■ and □). There was no clear hyperbolic dependence on k_1 with increasing octanesulfonate concentration as had previously observed with wild-type SsuD.[25] The hyperbolic dependence was associated with the two-step process for the binding of octanesulfonate. Octanesulfonate was proposed to bind in rapid equilibrium to SsuD followed by an isomerization or chemical step.[25] If the octanesulfonate-dependent step is attributed to a conformational change, the absence of a dependence on the k_{obs} with

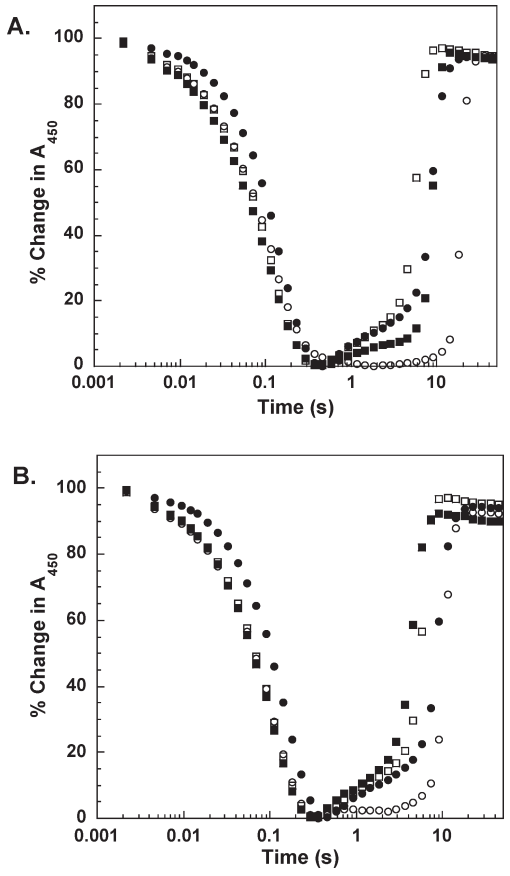


Figure 3. Kinetics of flavin reduction and oxidation by R297A, R297K, or wild-type SsuD and SsuE or with SsuE only. Stopped-flow kinetic traces were obtained at 4 °C mixing R297A, R297K, or wild-type SsuD (35 μM when included in the reaction), SsuE (35 μM), and FMN (25 μM) against NADPH (250 μM) monitored at 450 nm. (A) Kinetic traces of R297A (□), R297K (■), and wild-type (○) SsuD in the presence of SsuE or with SsuE only (●). (B) Kinetic traces of R297A (□), R297K (■), and wild-type (○) SsuD in the presence of SsuE or with SsuE only (●) with the addition of octanesulfonate (250 μM). The kinetic traces shown represent an average of three separate experiments.

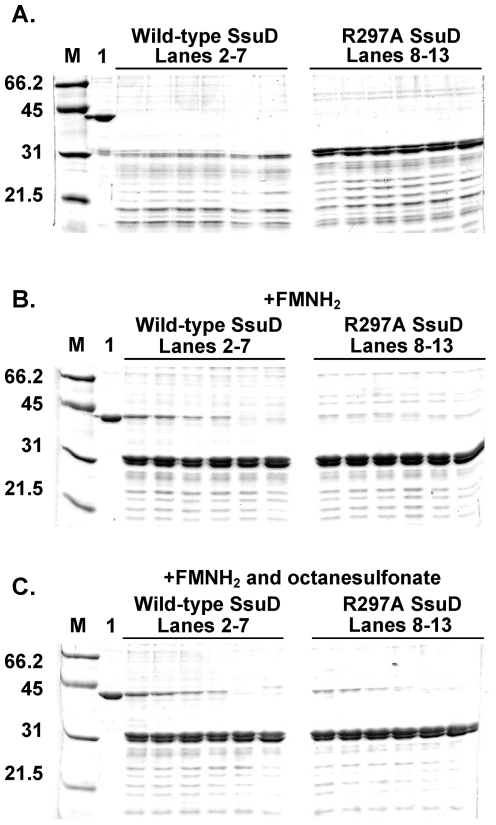


Figure 4. Effect of substrates on the proteolytic susceptibility of wild-type and R297A SsuD by limited tryptic digestion: (A) wild-type and R297A SsuD in the absence of substrates; (B) wild-type and R297A SsuD in the presence of $FMNH_2$; (C) wild-type and R297A SsuD in the presence of $FMNH_2$ and octanesulfonate. Gels A−C: molecular weight marker (M), wild-type SsuD (lane 1), aliquots were removed and quenched with PMSF after 15 s (lanes 2 and 8), 30 s (lanes 3 and 9), 45 s (lanes 4 and 10), 1 min (lanes 5 and 11), 2 min (lanes 6 and 12), or 5 min (lanes 7 and 13).

increasing octanesulfonate concentration further supports the inability of this substrate to productively bind to the R297 SsuD variants.

The rapid reaction kinetic studies for R297A and R297K SsuD indicate that there is no accumulation of the C4a-(hydro)peroxyflavin intermediate. The location of this residue on the putative mobile loop suggests that Arg297 may be important in stabilizing loop closure. Closure of the mobile loop may be essential in protecting the reactive flavin intermediates from bulk solvent and to ensure productive substrate binding. Single-turnover experiments were performed in the presence of SsuE to evaluate the reduced flavin transfer step from SsuE to SsuD. By including SsuE in the reaction, the flavin transfer step prior to the oxidative half-reaction catalyzed by SsuD could be monitored. In stopped-flow kinetic traces at 450 nm in the absence of octanesulfonate, there was an apparent lag phase between the reductive (SsuE) and oxidative (SsuD) half-reaction (Figure 3A, ○). This lag phase was somewhat abbreviated in the kinetic trace of wild-type SsuD in the presence of both flavin and octanesulfonate (Figure 3B, ○). This lag phase may represent the transfer step and/or conformational changes that occur with the binding of substrates to SsuD. This lag phase was not observed in kinetic traces obtained with the R297A SsuD variant with or without octanesulfonate included in the reaction (Figure 3A,B, □). Instead, flavin oxidation occurred immediately after flavin reduction. The kinetic traces obtained for R297A SsuD were similar to the trace obtained with SsuE and flavin in the absence of SsuD (Figure 3A, ●). The kinetic trace of R297K SsuD in the absence of octanesulfonate initially showed immediate oxidation of the flavin similar to the kinetic trace of R297A SsuD (Figure 3A, ■); however, the oxidation of the flavin then slowed and was intermediary between the kinetic trace of R297A and wild-type SsuD. When octanesulfonate was included in the reaction, the kinetic trace of R297A and R297K SsuD (Figure 3B, □ and ■, respectively) were both similar to the kinetic trace obtained with SsuE and flavin in the absence of SsuD (Figure 3B, ●). Even though R297A and R297K SsuD have a similar affinity for reduced flavin as wild-type in fluorescent titrations, the majority of the reduced flavin was not protected from bulk solvent and was readily oxidized in the presence of octanesulfonate under catalytic conditions.

Proteolytic Susceptibility of Wild-Type and Arg297 SsuD Variants. Kinetic studies with wild-type SsuD suggest that conformational changes occur with the binding of substrates. This conformational change may be associated with loop closure as

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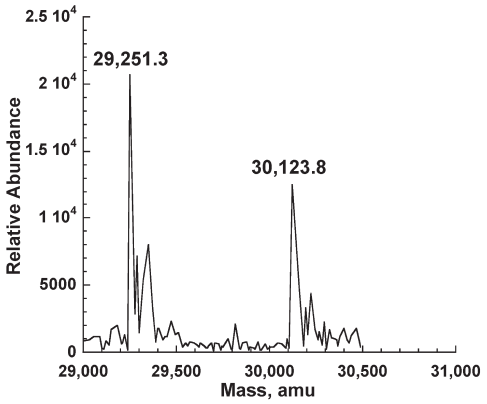
A.



B.

Mass		Peptide	
29251.3	1	SLNMFWFLPTHG....ETIAKAQAAFAR	263
30123.8	1	SLNMFWFLPTHG....AFARTDSVGQQR	271

Figure 5. Mass spectrometric analysis of wild-type SsuD following limited tryptic digestion. (A) Peptide fragments with the highest relative abundance from limited trypsin proteolysis. (B) Peptide mapping of the peptide peaks identified by mass spectrometry with the highest relative abundance using the known SsuD amino acid sequence.

observed with other TIM-barrel proteins, and Arg297 may play a role in securing the closed conformation of SsuD. The susceptibility of R297A, R297K, and wild-type SsuD to limited tryptic digestion was investigated to determine if Arg297 is involved in the binding of substrates and/or assisting in loop closure. Aliquots of the reaction mixtures of R297A or wild-type SsuD enzyme digested with trypsin were quenched at specified times over 5 min. The wild-type and R297A SsuD enzymes in the absence of substrates appeared to be rapidly digested, with no intact protein left after 15 s (Figure 4A). Wild-type SsuD was digested appreciably slower in the presence of $FMNH_2$, with 20% of the intact protein remaining after 30 s (Figure 4B). Similar protection was observed in the presence of both $FMNH_2$ and octanesulfonate (Figure 4C). Conversely, the R297A SsuD variant was nearly completely digested after 30 s in the presence of $FMNH_2$ only and $FMNH_2$ with octanesulfonate (Figure 4B,C). The R297K SsuD variant exhibited comparable proteolytic susceptibility as R297A SsuD (Figure S1). The results with wild-type SsuD suggest that the binding of substrates protects the enzyme from proteolytic digestion, and the majority of the protection was observed with the binding of reduced flavin. The similarity between the time-dependent digestion of R297A and R297K SsuD with either $FMNH_2$ only or both $FMNH_2$ and octanesulfonate suggests that the arginine located at position 297 on the mobile loop in SsuD may be important in stabilizing a conformation change initiated with the binding of reduced flavin. Digestion of wild-type SsuD (42 kDa) resulted in the appearance of two bands with nearly equal intensity resolving at ~30 kDa. Results from mass spectrometric analysis of partially digested wild-type SsuD identified two peptide fragments with a high relative abundance at 29.3 and 30.1 kDa (Figure 5A). These peptide fragments contained the tryptic digestion site on the loop region following Arg263 (29.3 kDa peptide fragment) and Arg271 (30.1 kDa peptide fragment) (Figure 5B). The presence of a tryptic target on the loop region indicates the loop region is the accessible proteolytic site. The tryptic sites become partially protected during substrate binding, suggesting conformational changes associated with loop movement are important in catalysis.

DISCUSSION

The binding of substrates by SsuD involves conformational changes that are critical to catalysis. Results from previous studies demonstrated that SsuD was unable to bind octanesulfonate unless flavin was initially bound, suggesting that flavin induces a conformational change that promotes catalysis.[25] A second conformational change likely occurs with the binding of octanesulfonate in rapid equilibrium to the SsuD/$FMNH_2$ complex. The three-dimensional structure of SsuD has a disordered region in an insertion sequence, suggesting that this region may be partially mobile.[4] This disordered region lies near the putative active site of SsuD and is highly conserved in all SsuD homologues. It is postulated that the disordered loop in SsuD closes over the active site following the binding of substrate(s) and may be responsible for the conformational changes observed in kinetic studies.[4] Loop closure would be a valuable mechanistic strategy for enzymes that utilize flavin as a substrate. Because the flavin must move in and out of the active site, the active site of these enzymes may be more accessible compared to enzymes that utilize flavin as a bound prosthetic group. The formation of reactive flavin intermediates during catalytic turnover would be protected with loop closure over the active site.

The Arg297 residue located on the insertion sequence of SsuD is conserved among the SsuD amino acid sequences from various bacterial organisms. The SsuD recombinant enzyme initially characterized contained an Arg297 to Cys substitution that inactivated the enzyme, but the role of Arg297 in catalysis was never evaluated.[3] The conserved nature and location of this residue on the insertion sequence near the disordered loop region suggest that Arg297 is directly contributing to catalytic function. The arginine residue may interact with another amino acid or substrate functional group to stabilize the requisite conformational changes necessary for catalysis. Results from steady-state kinetic analysis demonstrated that substitution of Arg297 with either Lys or Ala altered the kinetic parameters compared to wild-type SsuD. The R297K SsuD variant had a 30-fold decrease in the k_{cat}/K_m value, while the R297A SsuD variant had no detectible activity. In order to obtain measurable activity, the concentration of R297K SsuD had to be increased. The decrease in the catalytic efficiency of the R297K SsuD variant is primarily due to a decrease in the k_{cat} value. Even though the R297K SsuD variant had partial activity, the lysine substitution would not be properly positioned to make the necessary contacts essential for full catalytic activity. Because of the significant reduction in activity for both SsuD variants, the affinity of each Arg variant for $FMNH_2$ and octanesulfonate was investigated. There was a 3-fold increase in the K_d value determined for $FMNH_2$ with R297K and a 5-fold increase in the K_d value for R297A SsuD compared to wild-type SsuD, indicating that substitution of Arg297 with Lys or Ala caused little change in flavin binding. When the SsuD/$FMNH_2$ complex was titrated with octanesulfonate, there was no observable quenching of the intrinsic fluorescence signal. Although the flavin is still able to bind to the arginine variants in fluorescent titrations, it may no longer trigger the conformational change needed to allow the binding of octanesulfonate because of

alternate binding. The arginine residue could stabilize a conformational change induced by reduced flavin binding that correlates with loop closure.

A C4a-(hydro)peroxyflavin intermediate is proposed to be involved in the desulfonation mechanism by SsuD. Evidence for this flavin intermediate was previously obtained through rapid reaction kinetic analyses in the absence and at low concentrations of octanesulfonate.[25] The C4a-(hydro)peroxyflavin has a signature absorbance spectrum at ~370 nm. The generation of the C4a-(hydro)peroxyflavin intermediate was observed as an initial fast phase in kinetic traces obtained at 370 nm but were absent from kinetic traces monitoring flavin oxidation at 450 nm. The absence of loop closure with the substitution of Arg297 would likely lead to the accessibility of the flavin to bulk solvent causing the destabilization of flavin intermediates. Kinetic traces at 370 nm obtained for the oxidation of $FMNH_2$ with R297A and R297K SsuD in the absence of octanesulfonate substrate were best fit to a double-exponential equation. There was no initial phase (k_1) correlating with the formation of the C4a-(hydro) peroxyflavin intermediate as was previously observed with wild-type SsuD.[25] Evidence for the formation of the C4a-(hydro) peroxyflavin intermediate was only observed at low concentrations of octanesulfonate ($\leq$ 100 μM) in stopped-flow kinetic studies performed with wild-type SsuD due to the reaction proceeding at increased rates at higher substrate concentrations.[25] Even at lower octanesulfonate concentrations, there was no initial phase at 370 nm associated with the generation of the C4a-(hydro)peroxyflavin for R297A and R297K SsuD. In addition, there was no dependence on the k_{obs} with increasing octanesulfonate concentrations as previously observed with wild-type SsuD.[25] The inability of the C4a-(hydro)peroxyflavin intermediate to be generated correlates with the lack of activity for the R297A SsuD variant. If conformational changes are indeed disrupted in R297A SsuD, then the flavin is likely accessible to solvent and the C4a-(hydro)peroxyflavin would not be stabilized leading to nonenzymatic oxidation of the flavin with no detectable activity. The observed modest activity of R297K SsuD does not translate to observed conformational changes, leading to the stabilization of the C4a-(hydro)peroxyflavin.

In the two-component flavin-dependent enzymes the reduced flavin must be transferred from the reductase to the oxygenase enzyme. Therefore, the oxidative and reductive half-reactions occur on separate enzymes, resulting in a lag time between the two events. This distinct separation allows one to observe the partitioning of flavin reduction, the transfer of flavin from SsuE to SsuD including conformational changes associated with substrate binding, and the subsequent oxidation by SsuD under single turnover conditions. 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 the transfer of reduced flavin from SsuE to SsuD and/or a conformational change associated with the binding of $FMNH_2$, while similar experiments with R297A SsuD gave kinetic traces devoid of a significant lag phase. The kinetic trace for R297A SsuD was comparable to the kinetic trace obtained in the absence of SsuD, suggesting that the flavin was oxidized nonenzymatically. Although there was no lag phase observed with R297K SsuD, flavin oxidation was slower than R297A SsuD or free flavin. The kinetic traces of R297A and R297K SsuD obtained in the presence of octanesulfonate were both similar to SsuE alone. It is unclear why the rate of flavin oxidation of R297K SsuD was similar to the R297A SsuD and SsuE only kinetic trace in the presence of octanesulfonate, but slower in the absence of octanesulfonate. The absence of a lag phase with both FMN and octanesulfonate that likely correlates with a conformational change agrees with the absence of C4a-(hydro)peroxyflavin accumulation.

If Arg297 facilitates conformational changes associated with loop movement, substitution of Arg297 would impair the ability of SsuD to protect reduced flavin from nonenzymatic oxidation. The inability of the R297A and R297K SsuD variants to undergo the necessary conformational changes to promote catalysis was supported through studies evaluating proteolytic susceptibility. In the absence of substrates, both R297A, R297K, and wild-type SsuD enzymes were rapidly digested (<15 s). With the addition of $FMNH_2$ there was 20% of wild-type SsuD remaining after 30 s, indicating some level of protection with flavin binding. The susceptible proteolytic sites were located on the insertion region containing the putative mobile loop. The mobile loop in bacterial luciferase showed similar protection from limited proteolysis in the presence of reduced flavin and was thought to become more ordered and less accessible with the binding of reduced flavin.[20] The same level of protection was not observed with R297A or R297K SsuD in the presence of reduced flavin. The R297A and R297K SsuD variants exhibited a similar susceptibility to proteolysis in the absence or presence of both $FMNH_2$ and octanesulfonate. Therefore, the arginine variants were not able to undergo the conformation changes observed with wild-type SsuD. These results suggest that Arg297 helps stabilize the conformational change associated with loop movement. The detrimental effect of the arginine substitution on catalysis resulted in a more accessible active site to bulk solvent due to the inability of R297A and R297K SsuD to undergo the conformational change associated with loop closure. The accessible active site resulted in the inability of the flavin intermediate to be adequately stabilized.

Loop closure in TIM-barrel enzymes plays an important role as a lid gating mechanism to protect reaction intermediates from bulk solvent.[9−18] Studies have also shown that loop closure in TIM excludes water from the enzyme active site resulting in a hydrophobic environment that is essential in the stabilization of transition state intermediates and alters the pK_a of catalytically relevant amino acid side chains.[11,12,27−30] In bacterial luciferase phenylalanine residues that create a hydrophobic active site environment were shown to play a critical role in substrate binding and in the stabilization of flavin intermediates.[31] Several enzymes that rely on loop closure for catalytic activity contain conserved basic amino acid side chains near the loop region. A lysine residue directly interacts with the substrate phosphate group in TIM, while an arginine residue plays a similar role in orotidine 5′-monophosphate decarboxylase (OMPDC).[17,32−36] Mutation of these amino acid residues leads to a substantial decrease in activity that can be partially recovered by the addition of guanidinium and alkylammonium ions in OMPDC and TIM, respectively.[36,37] In addition, truncated substrates lacking the phosphate group are activated with the addition of phosphate in studies with TIM.[38−40] A change in the conformation of the disordered loop region of bacterial luciferase was also observed with the addition of phosphate.[19] These studies suggest that although the phosphate is not directly involved in catalysis, the interaction between the loop region and substrate phosphate group is necessary for the isomerization reaction. The protection of reactive flavin intermediates in the two-component flavin-dependent oxygenase enzymes is essential for catalysis. The oxygenase enzyme is likely in an open conformation with the

binding of reduced flavin but must undergo a conformational change to protect the reactive C4a-(hydro)peroxyflavin intermediate. The Arg297 residue located near the putative mobile loop region in SsuD may interact with the FMN phosphate group. The position of Arg297 in the three-dimensional structure of SsuD is pointing away from the proposed active site.[4] If the arginine is critical for catalysis, then there is likely movement of the amino acid side chain into the active site pocket, making it available for substrate interactions. Although reduced FMN was able to bind in equilibrium titration experiments to the enzyme, there are additional active site residues proposed to play a role in flavin binding. The interaction of the arginine residue with the substrate may not be important in the actual binding of the flavin but may assist in stabilizing a conformational change that favors loop closure. An added effect of these conformational changes would be protection of catalytic intermediates through the exclusion of bulk solvent. Further studies to evaluate the structural and functional role of the dynamic loop region of SsuD are currently being performed to broaden our understanding of these complex systems.

■ ASSOCIATED CONTENT

Supporting Information. Figure S1 demonstrating the proteolytic susceptibility of the R297K SsuD variant. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

DMSO, dimethyl sulfoxide; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; FMN, flavin mononucleotide; $FMNH_2$, reduced flavin mononucleotide; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); PMSF, phenylmethylsulfonyl fluoride; SsuE, alkanesulfonate flavin reductase; SsuD, alkanesulfonate monooxygenase; TIM, triosephosphate isomerase.

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