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Steady-State Kinetic Isotope Effects Support a Complex Role of Arg226 in the Proposed Desulfonation Mechanism of Alkanesulfonate Monooxygenase

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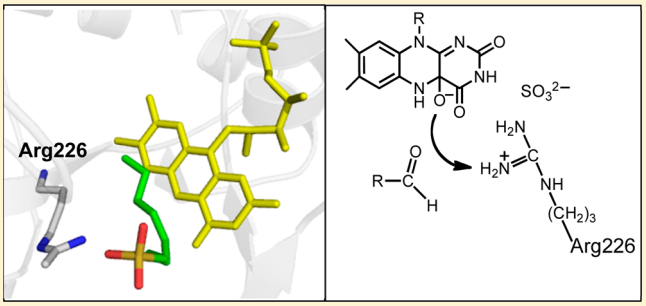
ABSTRACT: The alkanesulfonate monooxygenase system catalyzes the desulfonation of alkanesulfonates through proposed acid−base mechanistic steps that involves the abstraction of a proton from the alkane peroxyflavin intermediate and protonation of the FMN-O^- intermediate. Both solvent and kinetic isotope studies were performed to define the proton transfer steps involved in the SsuD reaction. Substitution of the protium at the C1 position of octanesulfonate with deuterium resulted in an observed primary isotope effect of 3.0 ± 0.2 on the k_{cat} parameter, supporting abstraction of the α-proton from the alkane peroxyflavin as the rate-limiting step in catalysis. Previous studies implicated Arg226 as the acid involved in the reprotonation of the hydroxyflavin intermediate. Solvent isotope kinetic studies gave an inverse isotope effect on $^{D_2O}k_{cat}$ of 0.75 ± 0.04 with no observable effect on $^{D_2O}k_{cat}/K_m$. This resulted in equivalent solvent isotope effects on $^{D_2O}k_{cat}$ and $^{D_2O}(k_{cat})_D$, suggesting a solvent equilibrium isotope effect on a step occurring after the first irreversible step through product release. Data from proton inventory studies on k_{cat} were best fit to a dome-shaped curve consistent with a conformational change to an open conformation during product release. The solvent isotope effect data coupled with the corresponding proton inventory results support and extend our previous observations that Arg226 donates a proton to the FMN-O^- intermediate, triggering a conformational change that opens the enzyme to solvation and promotes product release.



Isotope effects are valuable tools for the evaluation of kinetic mechanisms associated with enzyme-catalyzed reactions. Techniques employing isotopically labeled solvents and substrates have been shown to distinguish several features of an enzyme's mechanism, including, but not limited to, the rate-limiting bond-breaking step(s), the commitments to catalysis, the groups directly involved in catalysis, and any conformational change(s) associated with the enzymatic reaction. Kinetic isotope techniques utilizing deuterium have been demonstrated to be particularly useful in identifying enzyme mechanisms promoting acid−base catalysis. In many reactions that are dependent on acid−base catalysis, the proton transfer step is rate-limiting and can be affected by substituting a deuterium in place of the proton being transferred. The two-component flavin-dependent alkanesulfonate monooxygenase enzyme (SsuD) promotes the oxygenolytic cleavage of a carbon−sulfur bond from sulfonated substrates to yield free FMN, aldehyde, and bioavailable sulfite via acid−base catalysis. Despite extensive investigations, however, the details of the catalytic mechanism employed by the alkanesulfonate monooxygenase system remain an enigma.[1] Deuterium isotope effect studies were performed for SsuD in an effort to further resolve key steps involved in the proposed catalytic mechanism.

In *Escherichia coli*, SsuD is expressed when sulfate becomes limiting. The limitation induces the synthesis of a set of proteins involved in acquiring sulfur from alternative sources, primarily alkanesulfonates.[1−4] The two-component alkanesulfonate monooxygenase system allows the organism to utilize a broad range of alkanesulfonates as alternative sulfur sources.[1,2,4] Unlike a traditional flavin monooxygenase in which both oxidative and reductive half-reactions occur on the same enzyme, only the oxidative half-reaction is catalyzed by SsuD. The $FMNH_2$-bound SsuD then activates dioxygen and cleaves the carbon−sulfur bond of alkanesulfonates, releasing sulfite and the corresponding aldehyde (Scheme 1).[1,2,4]

In the SsuD reaction, a C4a-peroxyflavin intermediate is proposed to perform a nucleophilic attack on the sulfur atom of the alkanesulfonate substrate (Scheme 1, I).[5,6] The resulting intermediate then undergoes a Baeyer−Villiger rearrangement leading to the cleavage of the alkanesulfonate carbon−sulfur bond and the generation of sulfite and an alkane peroxyflavin intermediate (Scheme 1, II). The release of the aldehyde product from the enzyme occurs following abstraction of a proton from the alkane peroxyflavin intermediate by a catalytic base (Scheme 1, III). The abstraction of the α-proton from the alkane peroxyflavin intermediate has been proposed as one of several potential rate-limiting chemical steps in SsuD catalysis. Therefore, substitution of the α-protons of octanesulfonate

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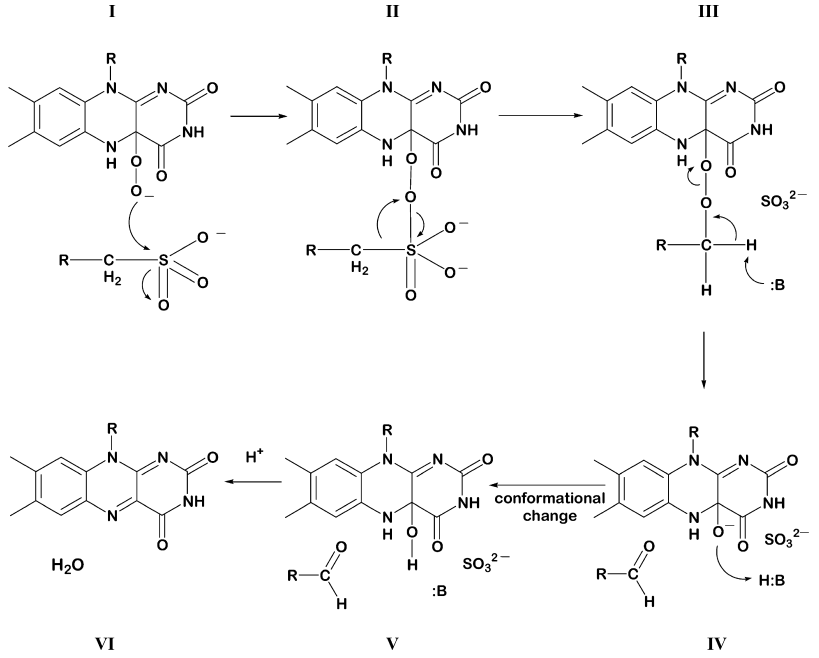
Scheme 1. Detailed Catalytic Mechanism of SsuD Catalysis



with deuterium should result in a kinetic isotope effect on the proton abstraction step (Scheme 1, III). Additionally, results from previous studies suggest that Arg226 is the active-site acid in the SsuD reaction donating a proton to the hydroxyflavin intermediate triggering a proposed conformational change that opens the enzyme to solvation and promotes product release (Scheme 1, IV). The reprotonation step should be susceptible to solvent deuterium kinetic isotope effect studies. Therefore, both solvent and kinetic isotope effect studies were performed to define the proton transfer steps involved in the SsuD reaction.

MATERIALS AND METHODS

Materials. Potassium phosphate (monobasic and dibasic), flavin mononucleotide phosphate (FMN), reduced nicotinamide adenine dinucleotide phosphate (NADPH), Trizma base, Bis-Tris, glycine, ammonium sulfate, ampicillin, ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), streptomycin sulfate, glucose, glucose oxidase, 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), and lysozyme were purchased from Sigma (St. Louis, MO). Isopropyl β-D-thiogalactoside (IPTG), sodium chloride, and glycerol were obtained from Fisher Biotech (Pittsburgh, PA). Dithiothreitol (DTT) and 1-octanesulfonate were purchased from Fluka (Milwaukee, WI). Deuterium oxide (D_2O), deuterium chloride, and sodium deuteroxide were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). 1-Bromooctane-1,1-d_2 was purchased from CDN Isotopes (Pointe-Claire, QC). Expression and purification of recombinant SsuD and SsuE were performed as previously described.[7] The concentrations of SsuD and SsuE proteins were determined from A_{280} measurements using molar extinction coefficients of 47.9 and 20.3 mM^{-1} cm^{-1}, respectively.[7]

Synthesis of 1-Octanesulfonate-1,1-d_2. The synthesis of 1-octanesulfonate-1,1-d_2 was performed as previously described with the following modifications.[8] A solution of sodium sulfite (13.5 mmol, 1.69 g) in 12.5 mL of water was slowly added to a refluxing solution of 1-bromooctane-1,1-d_2 (12 mmol, 2.32 g) in an ethanol/water mixture (75 mL/12.5 mL). After a reaction time of 48 h, the solution was cooled to room temperature, and the precipitate was removed. The remaining ethanol/water mixture was removed by evaporation, and the resulting precipitate was extracted six times with 25 mL of pentane. The remaining solid was recrystallized from ethanol three times to give 0.47 g (2 mmol, 16.7%). The product was identified by ^{1}H nuclear magnetic resonance. Chemical shifts obtained for the octanesulfonate ion in DMSO-d_6, RSO_3^-: 3.38 (d, 2H), 1.60 (apparent quintet, 2H), 1.25 (m, 10H), 0.85 (t, 3H). Chemical shifts obtained for the octanesulfonate ion, 1-octanesulfonate-1,1-d_2: 1.7 (apparent t, 2H), 1.25 (m, 10H), 0.85 (t, 3H). The absence of the chemical shift at RSO_3^- (1) [3.38 (d, 2H)] for 1-octanesulfonate-1,1-d_2 was used to estimate that the degree of deuteration was >90%. The absence of excess or unidentified chemical shifts was used to conclude that the overall purity of the synthesized 1-octanesulfonate-1,1-d_2 was >95%. The purchased 1-octanesulfonate and the synthesized 1-octanesulfonate-1,1-d_2 were heated for several minutes at 120 °C to produce the anhydrate form of each salt. The resulting loss of mass from each hydrate upon being heated was used to determine that each was present as a monohydrate. All 1-octanesulfonate and 1-octanesulfonate-1,1-d_2 concentrations were determined using the molecular weight of the respective monohydrate salt.

Dependence of Kinetic Parameters on pL. The activity of SsuD was routinely assayed at 25 °C as previously described.[4,5] Reactions employed a range of 1-octanesulfonate or 1-octanesulfonate-1,1-d_2 concentrations (10−5000 μM) in either 50 mM Bis-Tris (pL range of 5.8−7.2), 50 mM Tris-HCl (pL range of 7.2−9.0), or 50 mM glycine (pL range of 9.0−10.0) with the following modifications. Each buffered solution was supplemented with 100 mM sodium chloride to maintain the ionic strength, and overlapping assays were performed in Bis-Tris and Tris-HCl at pL 7.2 and in Tris-HCl and glycine at pL 9.0 to ensure activity was independent of the buffer used. When the viscosity experiments were performed, assays were also supplemented with 9% glycerol. All assays were performed

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in triplicate, and steady-state kinetic parameters were determined by fitting the data to the Michaelis–Menten equation. The pL dependence of k_{cat} and k_{cat}/K_m was best fit to a single-ionization model (eq 1) or a double-ionization model (eq 2):

$$\log y = \log[C/(1 + H/K_1)] \quad (1)$$

$$\log y = \log[C/(1 + H/K_1 + K_2/H)] \quad (2)$$

where H is $[H^+]$, y is k_{cat} or k_{cat}/K_m, C is the pH-independent value of y, and K_1–K_3 represent the dissociation constant for groups on the SsuD–$FMNH_2$ complex. Both SsuD and SsuE were found previously to be stable throughout the pH range.[6] The activity of SsuE was determined previously to be pH-independent.[6]

To determine the effect of deuterium oxide on the kinetic parameters of SsuD, modifications were made to the standard activity assay. The reaction mixtures were prepared with 99.8% D_2O. Buffer salts were dissolved in D_2O and adjusted with DCl or NaOD to the appropriate pH value (where pD is equal to the pH meter reading + 0.4). Anhydrous FMN, NADPH, SsuD (20 μM), SsuE (60 μM), 1-octanesulfonate, and 1-octansulfonate-1,1-d_2 were suspended in 50 mM Tris-DCl and 100 mM NaCl (pD 7.5). Steady-state kinetic parameters were determined by fitting the resulting plots to the Michaelis–Menten equation, and solvent isotope effects at a given pL were calculated as the ratio of k_{cat} and k_{cat}/K_m values in H_2O to those in 99.8% D_2O.

For proton inventory measurements, assays were performed as previously described over the experimental pL range. For each pL, different deuterium fractions (n = 0–0.998) were achieved by combining varying volumes of H_2O and D_2O buffers as previously described.[9] The pH dependence of wild-type SsuD was determined over the pL range in the buffered solutions containing varying isotopic fractions of deuterium. Assays were performed as previously described in triplicate, and steady-state kinetic parameters were determined by fitting the data to the Michaelis–Menten equation.[6] The pL dependence of k_{cat} and k_{cat}/K_m for each isotopic fraction of deuterium was best fit to a single-ionization model (eq 1) and a double-ionization model (eq 2), respectively. The pH-independent kinetic parameters for SsuD in each deuterium fraction concentration (n = 0, 0.124, 0.247, 0.372, 0.495, 0.619, 0.743, 0.870, and 0.998) were obtained from these assays, plotted against deuterium fraction n, and best fit to a dome-shaped curve using the following variation of the Gross–Butler equation:

$$k_n = k_0(1 + n + n\phi^T)Z_k{}^n \quad (3)$$

where k_n is the k_{cat} in a mixed isotopic solvent with a deuterium atom fraction n, k_0 is the k_{cat} in the absence of a deuterated solvent, ϕ^T is the transition-state fractionation factor, and Z_k is the medium effect contribution.[9–11] All reported K_m values are for the octanesulfonate or 1-octansulfonate-1,1-d substrate.

Data Analysis. The upper pK_a value for the pD dependence of k_{cat} was determined as follows. The upper pK_a value for each fraction of D_2O was determined from their respective pL profiles. $[H^+]$ was calculated for each of these pK_a values and then plotted against deuterium fraction n. The resulting scatter was best fit to line $y = mn + z$, where y is the K_a at deuterium fraction n, z is the K_a in pure H_2O, and m is the slope of the line. Slope m was determined by linear regression, and the K_a at n = 0.998 was calculated.

RESULTS

Substrate Kinetic Isotope Effects on Steady-State Kinetic Parameters. The kinetic parameters for the wild-type enzyme were established with different concentrations of 1-octanesulfonate-1,1-d_2 as a substrate and plotted as a function of pH from pH 5.8 to 10.0 to probe the presence of a deuterium kinetic isotope effect on the proposed proton abstraction step (Scheme 1, III). The SsuD enzyme with 1-octanesulfonate-1,1-d_2 showed optimal catalytic activity between pH 7.5 and 9.5 where the pH-independent value was found to be 31 ± 2 min^{-1} for k_{cat} and 1.9 ± 0.3 μM^{-1} min^{-1} for k_{cat}/K_m (Figure 1A,B). These pH-independent values were

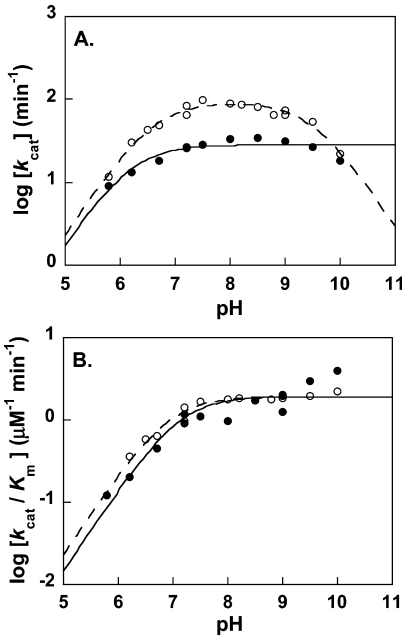

Figure 1. Substrate kinetic isotope effects on SsuD kinetic parameters. Reactions were initiated by the addition of NADPH (500 μM) to a reaction mixture containing SsuD (0.2 μM), SsuE (0.6 μM), FMN (2 μM), and a range of labeled 1-octanesulfonate-1,1-d_2 concentrations (10–5000 μM) in either 50 mM Bis-Tris (pH range of 5.8–7.2), 50 mM Tris-HCl (pH range of 7.2–9.0), or 50 mM glycine (pH range of 9.0–10.0) and 100 mM sodium chloride at 25 °C: (A) k_{cat} and (B) k_{cat}/K_m values for SsuD reactions in H_2O (●). The corresponding kinetic parameters for the reaction of SsuD supplemented with unlabeled 1-octanesulfonate and H_2O (○) were included in each plot as a reference. Each point is the average of at least three separate experiments.

compared with those obtained with wild-type SsuD with unlabeled octanesulfonate to reveal kinetic isotope effects of 3.0 ± 0.2 for $^Dk_{cat}$ and 1.0 ± 0.2 for $^Dk_{cat}/K_m$ (Table 1).[6] The pH dependence of k_{cat} for SsuD with deuterated substrate revealed a single titratable residue with a pK_a value of 6.3 ± 0.1 (Table 2). The pH dependence of k_{cat}/K_m with a labeled substrate revealed a single titratable amino acid group with a pK_a value of 7.1 ± 0.1 (Table 2). This pK_a value and the pH-independent value were consistent with those for the unlabeled substrate, indicating that the deuterated substrate had no effect on the k_{cat}/K_m for SsuD.

Solvent Isotope Effects on Steady-State Kinetic Parameters. The kinetic parameters for the wild-type enzyme were established in 99.8% D_2O and plotted as a function of pD from pD 5.8 to 10.0 to probe the presence of a deuterium

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Table 1. Summary of the Kinetic Isotope Effects[a]

$^{D}k_{cat}$	3.0 ± 0.2
$^{D}k_{cat}/K_m$	1.0 ± 0.2
$^{D_2O}k_{cat}$	0.75 ± 0.04
$^{D_2O}k_{cat}/K_m$	0.95 ± 0.07
$^{D_2O}(k_{cat})_D$	0.79 ± 0.07
$^{D_2O}(k_{cat}/K_m)_D$	0.83 ± 0.17
$^{D}(k_{cat})_{D_2O}$	3.1 ± 0.2
$^{D}(k_{cat}/K_m)_{D_2O}$	0.87 ± 0.12

[a]Isotope effects were determined using pL-independent C values for k_{cat} and k_{cat}/K_m after pL profiles were best fit to a single-ionization model (eq 1) or a double-ionization model (eq 2).

Table 2. pL Dependence of Kinetic Parameters for SsuD

	k_{cat}		k_{cat}/K_m
	pK_1	pK_2	pK
1-octanesulfonate			
H_2O[a]	6.6 ± 0.1	9.5 ± 0.1	6.9 ± 0.1
D_2O	6.8 ± 0.1	10.1 ± 0.2[b]	7.3 ± 0.1
9% glycerol	6.6 ± 0.2	9.5 ± 0.1	6.4 ± 0.1
1-octanesulfonate-1,1-d_2			
H_2O	6.3 ± 0.1	–[c]	7.1 ± 0.1
D_2O	6.6 ± 0.1	–[c]	7.0 ± 0.1

[a]Previously reported.[13] [b]Value calculated from proton inventory measurements. [c]Value could not be determined within the experimental pL range.

kinetic isotope effect on the proposed catalytic acid-mediated reprotonation step (Scheme 1, IV). The optimal catalytic activity for SsuD was observed in D_2O between pD 8.5 and 10.0, where the pD-independent value was found to be 123 ± 3 min^{-1} for k_{cat} and 2.0 ± 0.1 μM^{-1} min^{-1} for k_{cat}/K_m. These pD-independent values were compared with those obtained with wild-type SsuD in H_2O to reveal solvent isotope effects equal to 0.75 ± 0.04 for $^{D_2O}k_{cat}$ and 0.95 ± 0.07 for $^{D_2O}k_{cat}/K_m$ (Table 1).[6] While no isotope effect on k_{cat} was observed from pL 5.8 to 7.5, an inverse isotope effect was observed from pL 8.0 to 10.0 (Figure 2A). The isotope effect on k_{cat} became more inverse with an increasing pD, reaching a value of 0.25 ± 0.2 at pL 10.0. There was no isotope effect observed on k_{cat}/K_m at any pL (Figure 2B). The inverse isotope effect only on k_{cat} indicates that deuteration affects only a catalytic step occurring after the first irreversible step in catalysis through product release.[12]

The resulting pD dependence of SsuD obtained in 99.8% D_2O was compared to the pH dependence previously obtained in H_2O.[13] The pD dependence of k_{cat} revealed a single titratable amino acid residue with a pK_a value of 6.8 ± 0.1 (Table 2). While this single pK_a value measured in 99.8% D_2O exhibits a small ΔpK_a value of 0.2 upon comparison to the lower pK_a value obtained in H_2O, the absence of the upper pK_a value integral to its H_2O counterpart suggests an outward shift of this pK_a value in D_2O.[9−12] It was determined from the proton inventory measurements, however, that the value of the upper pK_a for k_{cat} in D_2O was 10.1 ± 0.2 (Figure 3, inset, and Table 2). This pK_a value would fall into the normal limit for an isotope effect on ionization of a catalytic group (ΔpK_a value of 0.4−0.6).[9−11] The pD dependence of k_{cat}/K_m revealed a single titratable amino acid group with an apparent pK_a value of 7.3 ± 0.1, exhibiting a normal ΔpK_a value of 0.4 compared to the value obtained in H_2O (Table 2).[13]

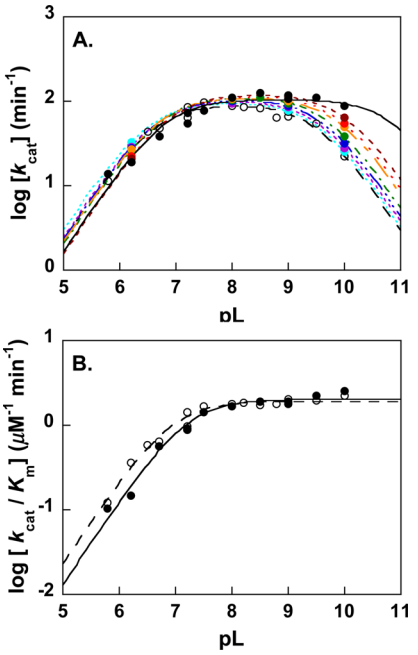

Figure 2. Solvent isotope effects on SsuD kinetic parameters. Reactions were initiated by the addition of NADPH (500 μM) to a reaction mixture containing SsuD (0.2 μM), SsuE (0.6 μM), FMN (2 μM), and a range of unlabeled 1-octanesulfonate concentrations (10−5000 μM) in either 50 mM Bis-Tris (pL range of 5.8−7.2), 50 mM Tris-HCl (pL range of 7.2−9.0), or 50 mM glycine (pL range of 9.0−10.0) and 100 mM sodium chloride. pH dependence of (A) k_{cat} and (B) k_{cat}/K_m values for SsuD reactions at 25 °C supplemented with 0.00% (white), 12.4% (cyan), 24.7% (purple), 37.2% (blue), 49.5% (green), 61.9% (orange), 74.3% (red), 87.0% (brown), and 99.8% D_2O (black). The corresponding kinetic parameters for the reaction of SsuD supplemented with unlabeled 1-octanesulfonate and H_2O (○) were included in each plot as a reference. Each point is the average of at least three separate experiments.

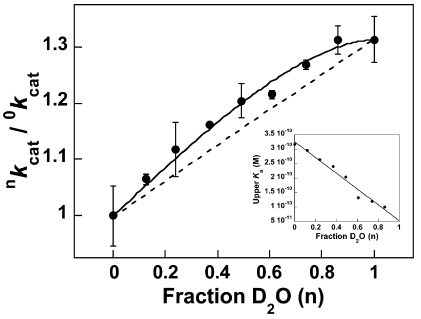

Figure 3. Proton inventory measurements. The ratio of k_{cat} in fraction n of 99.8% D_2O ($^{n}k_{cat}$) to that in 100% H_2O ($^{0}k_{cat}$) is plotted as a function of the mole fraction of 99.8% D_2O in the reaction. Each point is the average of results from at least four separate experiments. The dashed line is theoretical for a linear proton inventory. The inset shows a plot of the upper K_a value vs the fraction of D_2O (n). Upper K_a values were determined from respective pL profiles (Figure 2A). The upper pK_a for SsuD in 99.8% D_2O was determined by calculating the K_a at n = 0.998.

Proton Inventory Measurements. Proton inventories were conducted in an effort to determine the number and nature of hydrogenic sites involved in the inverse isotope effect on k_{cat}. Because of the difference in pL optima for k_{cat} in H_2O (pH 7.2−8.5) compared to D_2O (8.0−9.5), a clear pL-independent value necessary to perform a proton inventory

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could not be identified. As a result, the pL dependence of SsuD was determined for various fractions of D_2O (Figure 2A). From these pL dependencies, the pL-independent values of k_{cat} and k_{cat}/K_m for SsuD were determined and used to obtain an accurate proton inventory for each catalytic parameter. Inventory curves were obtained by plotting the pL-independent values of k_{cat} and k_{cat}/K_m as a function of the fraction of D_2O (n = 0−0.998) present in the activity assays. While proton inventories for k_{cat}/K_m did not demonstrate any curvature and were consistent with plots representing residual error due to the absence of an SIE (data not shown), the proton inventories for k_{cat} at each pL were best described as inverse and dome-shaped or bulging upward (Figure 3). While the pattern has not previously been observed for inverse solvent isotope effects, dome-shaped proton inventory curves indicate the presence of offsetting normal and inverse contributions to the overall solvent isotope effect.[9−11] An ϕ^T of 0.53 ± 0.03 and a Z_k of 2.46 ± 0.10 were extrapolated from the proton inventory curve after fitting the data to the best-fit form of the Gross−Butler equation (eq 3). Therefore, the results suggest a normal transition-state contribution offset by a large inverse medium effect. Proton inventories of the catalytic parameters of SsuD employing 1-octanesulfonate-1,1-d_2 were indistinguishable from those employing unlabeled octanesulfonate.

Dependence of Kinetic Parameters of SsuD on Viscosity. Because the viscosity of D_2O is higher than that of H_2O, activity assays were conducted over the experimental pH range in buffers supplemented with 9% glycerol to determine the effect of viscosity on the kinetic parameters of SsuD. It has been demonstrated previously that the viscosity of D_2O is comparable to that of a solution supplemented with 9% glycerol.[14] The resulting viscosity-dependent values of k_{cat} and k_{cat}/K_m for SsuD were compared to the values obtained for pH and pD dependencies (Figure 4A,B). The viscosity dependence of k_{cat} revealed two titratable amino acid residues with pK_a values consistent with those measured in H_2O (Figure 4A). The pH-independent value for k_{cat} was determined to be 80 ± 4 min^{-1}, resulting in a normal viscosity effect (H_2O/glycerol) of 1.2. The result indicated that the inverse isotope effect observed with D_2O was independent of viscosity. The viscosity dependence of k_{cat}/K_m revealed a single ionizable amino acid residue with an apparent pK_a value of 6.4 ± 0.1 with a pH-independent value of 1.2 ± 0.1 μM^{-1} min^{-1} (Figure 4B). This value resulted in a normal viscosity effect of 1.6 on k_{cat}/K_m upon comparison to the k_{cat}/K_m value in H_2O (Table 1). These effects on k_{cat} and k_{cat}/K_m support a change in the rate of one or more non-pH-dependent conformational changes during catalysis as a result of the increased viscosity.[9,11,12,14,15]

Multiple-Isotope Effect Studies of the Steady-State Kinetic Parameters. The kinetic parameters for the wild-type enzyme with 1-octanesulfonate-1,1-d_2 were established in H_2O and 99.8% D_2O to evaluate multiple kinetic isotope effects on the SsuD mechanism. The pD dependence of k_{cat} for SsuD with a deuterated substrate revealed a single titratable residue with a pK_a value of 6.6 ± 0.1 consistent with the lower pK_a value in the pH profile for SsuD with the unlabeled substrate in H_2O (Table 2).[6] The upper pK_a value associated with the pH profile for SsuD with unlabeled substrate in H_2O appeared to have shifted outside of the experimental pH range with use of the labeled substrate in D_2O (Figure 5A).[6] The pH dependence of k_{cat}/K_m with the labeled substrate revealed a single titratable amino acid group with a pK_a value of 7.0 ± 0.1, consistent with the pK_a value in the pH profile for SsuD with the unlabeled

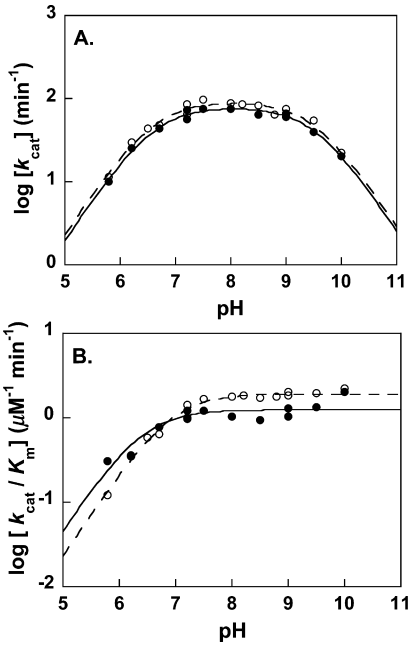

Figure 4. Effects of viscosity on SsuD kinetic parameters. Reactions were initiated by the addition of NADPH (500 μM) to a reaction mixture containing SsuD (0.2 μM), SsuE (0.6 μM), FMN (2 μM), and a range of unlabeled 1-octanesulfonate concentrations (10−5000 μM) in either 50 mM Bis-Tris (pH range of 5.8−7.2), 50 mM Tris-HCl (pH range of 7.2−9.0), or 50 mM glycine (pH range of 9.0−10.0) and 100 mM sodium chloride at 25 °C: (A) k_{cat} and (B) k_{cat}/K_m values for SsuD reactions with 9% glycerol (●). The corresponding kinetic parameters for the reaction of SsuD supplemented with unlabeled 1-octanesulfonate and H_2O (○) were included in each plot as a reference. Each point is the average of at least three separate experiments.

substrate in H_2O (Table 2).[6] SsuD with the labeled substrate showed optimal catalytic activity in D_2O between pH 8.0 and 10.0, where the pH-independent value was found to be 39 ± 2 min^{-1} for k_{cat} and 2.3 ± 0.3 μM^{-1} min^{-1} for k_{cat}/K_m (Figure 5A,B). The solvent isotope effect on $^{D_2O}(k_{cat})_D$ with 1-octanesulfonate-1,1-d_2 was 0.79 ± 0.07, and the effect on $^{D_2O}(k_{cat}/K_m)_D$ was 0.83 ± 0.17 (Table 1). While the solvent isotope effect on $^{D_2O}(k_{cat}/K_m)_D$ was near unity (within error), the solvent kinetic isotope effects on $^{D_2O}k_{cat}$ with 1-octanesulfonate-1,1-d_2 or 1-octanesulfonate were equivalent. The results support a stepwise mechanism for the isotope-sensitive steps.[16,17]

■ DISCUSSION

The proposed catalytic mechanism for SsuD involves the abstraction of the α-proton from the alkane peroxyflavin intermediate (Scheme 1, III to IV). This step has been proposed to be the rate-limiting catalytic step. Therefore, the protium at this position was substituted with deuterium to limit the rate of this abstraction step during catalysis. The lack of an isotope effect on k_{cat}/K_m over the experimental pH range indicates that the labeled octanesulfonate substrate does not affect any catalytic steps up through the first irreversible step.[11,12] Alternatively, the k_{cat} pH profile encompasses all steps occurring after the first irreversible step through product release, which would include the proton abstraction step. Therefore, the kinetic isotope effect of 3 on k_{cat} supports proton abstraction being the rate-limiting step. Additionally, compar-

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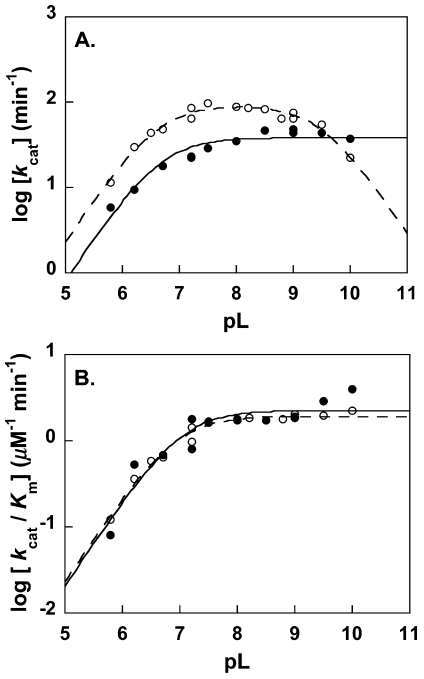

Figure 5. Substrate kinetic and solvent isotope effects on SsuD kinetic parameters. Reactions were initiated by the addition of NADPH (500 μM) to a reaction mixture containing SsuD (0.2 μM), SsuE (0.6 μM), FMN (2 μM), and a range of labeled 1-octanesulfonate-1,1-d_2 concentrations (10−5000 μM) in either 50 mM Bis-Tris (pL range of 5.8−7.2), 50 mM Tris-HCl (pL range of 7.2−9.0), or 50 mM glycine (pL range of 9.0−10.0) and 100 mM sodium chloride at 25 °C: (A) k_{cat} and (B) k_{cat}/K_m values for reactions at 25 °C of SsuD supplemented with labeled 1-octanesulfonate-1,1-d_2 in D_2O (●). The corresponding kinetic parameters for the reaction of SsuD supplemented with unlabeled 1-octanesulfonate and H_2O (○) were included in each plot as a reference. Each point is the average of at least three separate experiments.

ison of the k_{cat} pH profile of the labeled substrate with that of the unlabeled substrate revealed an acidic shift of this lower pK_a value from 6.6 to 6.3, while the upper pK_a value associated with unlabeled octanesulfonate appeared to have shifted outside of the experimental pH range with the labeled substrate (Table 2).[6] An outward shift or broadening of pK_a values observed only in the k_{cat} pH profile is indicative of a non-pH-dependent, but normally rate-limiting, slow step following the chemical catalytic reaction.[9−11] Previously, this step was reported as a conformational change linked to product release.[6] Therefore, the results suggest that the overall substrate isotope effect originates, at least partially, from product release. Because both α-carbon protons have been replaced with deuterium in the labeled substrate, an α-secondary isotope effect (α-2°) must also be considered. Because the hybridization of the isotopically labeled carbon changes from sp^3 to sp^2 during the reaction (Scheme 1, III to IV), a normal α-2° would be expected and would account for some of the observed KIE value.[18]

Solvent isotope effect studies were undertaken to probe the reprotonation step in the SsuD reaction (Scheme 1, step IV). The pD profile of k_{cat} was fit to eq 1 to yield a single pK_a value of 6.8 ± 0.1 and an inverse solvent isotope effect of 0.75 ± 0.04. When compared to the lower pK_a value in H_2O, the ΔpK_a value of 0.2 unit in D_2O is consistent with that expected for a cysteine amino acid group.[10,11,19] Previously, the substitution of Cys54 with alanine in SsuD was shown to cause the proton associated with the lower pK_a value to become sticky during catalysis.[13] These combined results support Cys54 making an at least partial contribution to this lower pK_a value along with the proton at the N1 position of the FMN.[13] Alternatively, the upper pK_a value in D_2O exhibited the expected shift for a simple carboxylic or ammonium acid group (ΔpK_a = 0.4−0.6) compared to its value in H_2O (Table 1). Interestingly, previous studies were unable to determine whether Cys54 was the sole group contributing to this upper pK_a value or whether Cys54 was serving to lower the pK_a of Arg226 during catalysis.[6] The normal shift of this pK_a value in D_2O supports Cys54 serving to lower the pK_a of Arg226 as the ΔpK_a value for a cysteine amino acid group contributing exclusively to the upper pK_a would be much smaller.[6] Additionally, cysteine residues like Cys54 have been shown to promote reverse protonation that can lead to an inverse solvent isotope effect like the one seen on the SsuD k_{cat} parameter.[9]

In a reverse protonation mechanism, the amino acid residue contributing to the lower pK_a value must be protonated while the amino acid residue contributing to the upper pK_a value must be deprotonated for catalysis to occur. For the D_2O solvent to favor the deprotonated form of the group contributing to the upper pK_a value, this group would need to have a fractionation factor that is less than unity (ϕ < 1) in a mixed isotopic solvent.[9−11] Cysteine residues have been implicated in several reverse protonation mechanisms as the group contributing to the upper pK_a value, with fractionation factors for cysteine residues ($\phi \sim 0.5$) being less than unity in a mixed isotopic solvent.[9−11,19−21] Protonated Cys54 was previously shown to be involved in stabilizing the C4a-(hydro)peroxyflavin in SsuD either through direct interactions with the flavin or by helping to maintain the active-site environment.[22] In addition, the inverse kinetic isotope effect was still observed with the C54S SsuD variant at pL 8.6 (SIE = 0.63 ± 0.03), suggesting that Cys54 is not involved in a reverse protonation event (unpublished data). Therefore, the observed inverse solvent isotope effect is likely the result of a normal protonation event.

Although the change in viscosity that results from substituting D_2O for H_2O can account for apparent solvent isotope effects, k_{cat} values in increased viscosity are normal compared to values observed in H_2O over the experimental pH range (Figure 4A,B). A solution containing 99.8% D_2O has the same relative viscosity ($\eta_{rel} \sim 1.24$) as a solution containing 9% glycerol.[9−11,14,19,23−25] However, the k_{cat} pH profile shows that increased viscosity reduces k_{cat} over the experimental pH range, suggesting that the inverse isotope effect on k_{cat} is not the result of increased solvent viscosity (Figure 4A). Nevertheless, this normal viscosity effect on the k_{cat} parameter does indicate viscosity affects the rate-limiting step of the mechanism.[15,26] Increased viscosity often affects an enzyme mechanism that is dependent on a conformational change by favoring closed conformations and slowing the rate of return to the open conformation.[24,25] Therefore, increased solvent viscosity may be serving to limit a proposed conformational change from a closed to an open conformation in SsuD.

Alternatively, the proton inventories on k_{cat} suggest that D_2O is contributing to the overall inverse isotope effect by favoring a conformational change to the open conformation during the product release step. The proton inventory was best fit to dome-shaped curves suggesting a normal transition-state contribution (ϕ^T) opposing multiple-inverse effect contributions (Z_k) to yield the overall inverse isotope effect.[9−11,14,19,23]

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The transition-state contribution (ϕ^T) is consistent with the solvation of a catalytic proton bridge commonly observed for proton transfers among O, N, and S groups (ϕ^T = 0.4−0.6).[11] Previously, Arg226 was proposed to be responsible for protonating the FMN-O$^-$ intermediate in the catalytic mechanism for SsuD; the data suggest that the ϕ^T contribution arises from the transfer of a proton from the N group of Arg226 to the O group of FMN-O$^-$.[6] The Z term contribution is indicative of multiple reaction-state contributions (ϕ^R) occurring on various protein structural sites (ϕ^R = 1.05−1.16) as a result of a conformational change.[11] These effects in combination with the normal transition-state contribution (ϕ^T) support the formation of a transition-state intermediate in which tighter, stiffer hydrogen bonds (like those corresponding to deuterium bonds) are favored in the committed step for product release. The protonation of FMN-O$^-$ by Arg226 would serve as the committed step. Additionally, the transfer of a proton from an arginine group to a group with a potentially greater fraction factor (FMN-O$^-$) would be faster with deuterium than with protium. If the fractionation factors of FMN-OOH and FMN-OH are great and above unity, then the current results would support Scheme 1 as well as account for the inverse solvent isotope effect observed on the k_{cat} parameter.

Additionally, because the inverse solvent isotope effect is seen in the k_{cat} pL profile and not the k_{cat}/K_m pL profile, the results indicate a step occurring after the first irreversible step through product release is affected by the deuterated solvent. The lack of an observable isotope effect on k_{cat}/K_m over the experimental pL range indicates that this ionizable group is not involved in a proton transfer event contributing to an overall rate limitation. Previously, SsuD has been shown to follow a steady-state ordered binding mechanism with $FMNH_2$ binding first to SsuD, followed by either octanesulfonate or O_2 prior to formation of the C4a-peroxyflavin intermediate proposed to govern the desulfonation reaction.[5] Because $FMNH_2$ must bind to SsuD first, its binding will represent an on-rate constant that will be insensitive to deuterium isotopic substitution and the effect on k_{cat}/K_m for octanesulfonate and k_{cat}/K_m for dioxygen will be unity by definition.[27] Because both the substrate isotope effect and solvent isotope effect on k_{cat}/K_m are within experimental error of 1, the octanesulfonate could be added to SsuD prior to O_2 under steady-state conditions.[27] Alternatively, the release of SO_3^{2-} occurs prior to abstraction of a proton from the peroxyalkane intermediate in the proposed mechanism and could represent the irreversible step in the desulfonation mechanism (Scheme 1, II and III).

The determination of SsuD kinetic parameters in D_2O and H_2O with the labeled substrate was performed in an effort to extrapolate the intrinsic substrate and solvent isotope effect from the combined isotope effect data. The pD profile of k_{cat} with the deuterium-labeled substrate exhibited features from profiles of the labeled substrate in H_2O and unlabeled substrate in D_2O, while the profile for k_{cat}/K_m remained unaffected by the combined isotope effects (Figure 5A,B). The reduced pL-independent value of k_{cat} combined with the outward shift of the pK_a values for the labeled substrate in D_2O is consistent with the reductions and shifts seen for the labeled substrate in H_2O (Table 1). The $^{D_2O}(k_{cat})_D$ and $^{D_2O}k_{cat}$ solvent isotope effects were equivalent, consistent with a stepwise mechanism for proton abstraction and subsequent proton donation by Arg226. These results affirm a kinetic isotope effect upon abstraction of a proton from the peroxyalkane intermediate, and an equilibrium isotope effect on a proton donation by Arg226 in the proposed desulfonation mechanism of SsuD.[16,17]

Altogether, the results presented here support the proposed mechanism for SsuD. The observed isotope effect of 3.0 ± 0.2 on the k_{cat} parameter when deuterium-labeled octanesulfonate is used as a supplement in the steady-state reaction supports the abstraction of the α-proton from the alkane peroxyflavin intermediate being the rate-limiting chemical step in SsuD catalysis (Scheme 1, III). Additionally, the solvent isotope effect data coupled with the corresponding proton inventory results support the idea that Arg226 donates a proton to the FMN-O$^-$ intermediate triggering a proposed conformational change that opens the enzyme to solvation and promotes product release (Scheme 1, IV).

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Notes

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ABBREVIATIONS

DTNB, 5,5-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; FMN, flavin mononucleotide; $FMNH_2$, reduced flavin mononucleotide; NADPH, nicotinamide adenine dinucleotide (phosphate); D_2O, deuterium oxide; SsuE, alkanesulfonate flavin reductase; SsuD, alkanesulfonate monooxygenase.

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