The Escherichia coli ssuEADCB gene cluster is required for the utilization of alkanesulfonates as sulfur sources, and is expressed under conditions of sulfate or cysteine starvation. The SsuD and SsuE proteins were overexpressed and characterized. SsuE was purified to homogeneity as an N-terminal histidine-tagged fusion protein. Native SsuE was a homodimeric enzyme of Mr 58,400, which catalyzed an NAD(P)H-dependent reduction of FMN, and was maximal for SsuE/SsuD molar ratios of 2.1 to 4.2 in 10 mM Tris-HCl, pH 9.1. Native SsuD was a homotetrameric enzyme of Mr 130,000. This results demonstrate that SsuD is a broad range FMNH2-dependent monooxygenase catalyzing the oxygenolytic conversion of alkanesulfonates to sulfitic and pentaldehyde and was able to desulfonate a wide range of sulfonated substrates including C-2 to C-10 unsubstituted linear alkanesulfonates, substituted ethanesulfonic acids and sulfonated buffers. SsuD catalysis was absolutely dependent on FMNH2 and oxygen, and was maximal for SsuE/SsuD molar ratios of 2.1 to 4.2 in 10 mM Tris-HCl, pH 9.1. Native SsuD was a homotetrameric enzyme of Mr 181,000. These results demonstrate that SsuD is a broad range FMNH2-dependent monooxygenase catalyzing the oxygenolytic conversion of alkanesulfonates to sulfitic and the corresponding aldehydes. SsuE is the FMN reducing enzyme providing SsuD with FMNH2.

In Escherichia coli, sulfate starvation causes increased synthesis of several proteins involved in scavenging sulfur from alternative sulfur sources (1). Among these proteins are the taurine-transported enzymes required for uptake and desulfonation of taurine (2-aminooethanesulfonic acid) (2, 3) and the proteins SsuE and SsuD of the ssuEADCB gene cluster. We have shown that the ssuEADCB gene cluster, located at 21.4 min on the E. coli chromosome, is specifically involved in the utilization of alkanesulfonates as a source of sulfur for growth (4). Deletion of ssuEADCB resulted in the loss of the ability to utilize alkanesulfonates as a sulfur source but did not affect the utilization of taurine as a sulfur source in this purpose. The amino acid sequences of SsABC exhibit similarity to components of ABC-type transport systems (4, 5). SsuA has a putative signal sequence, indicating that it functions as a periplasmic binding protein, and the sequences of SsB and SsC are significantly similar to those of ATP-binding proteins and membrane components, respectively, of members of the ABC transporter superfamily. It thus appears that the proteins encoded by ssuABC constitute an uptake system for alkanesulfonates.

The ssuD gene product shows 25% sequence identity to a characterized nitritolriocetate two-component monooxygenase of Chelatobacter heintzii (6) and to the pristinamycin II, synthase subunit A of Streptomyces pristinaeprilis (7), suggesting that SsuD is involved in the oxygenolytic release of sulfite from alkanesulfonates. Here we report the purification of the SsuD and SsuE proteins, describe their biochemical properties, and demonstrate that SsuD is a monooxygenase that catalyzes the desulfonation of alkanesulfonates and requires reduced FMN, which is provided by the NAD(P)H flavin oxidoreductase SsuE.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and T4 DNA ligase were obtained from MBI Fermentas. Pfu and TaqPlus DNA polymerases were from Stratagene. DNase I came from Roche Molecular Biochemicals. NADH, NADPH, FMN, riboflavin, and lumiflavin were from Fluka, as were all sulfonated substrates except N-phenylsulfurine, 4-phenyl-1-butenesulfonic acid, 2-bromoethanesulfonic acid (Sigma), and isethionic acid (Aldrich).

Bacterial Strains and Growth Conditions—E. coli strains DH5α (8) and BL21(DE3) (9) were grown aerobically at 37 or 30 °C in Luria-Bertani medium (8) with constant shaking (180 rpm). When necessary, kanamycin was added at 50 μg/ml and ampicillin at 100 μg/ml. Solid media were prepared by addition of 1.5% (w/v) agar.

Construction of ssuE Expression Plasmids—For the production of wild type SsuE, the ssuE gene was placed under the control of the T7 RNA polymerase promoter of vector pET-24a (+) (Novagen). The ssuE gene was amplified by PCR1 from plasmid pME4180 (4) with the oligonucleotide primers EE7 (5'-AGGGAGGACATATGCGTGTCAT-3') and EE8 (5'-CTATAACGTAAAGCTTACCGGAG-3'), with the changes to introduce Ndel and HindIII restriction sites, respectively, underlined. The 672-base pair PCR product was digested with Ndel and HindIII and the resulting 655-base pair fragment was ligated in pUC19 (10), generating plasmid pME4145. The Ndel-HindIII fragment from pME4145 was subsequently cloned in pET-24a (+) resulting in plasmid pME4146.

For the production of SsuE as an N-terminal histidine-tagged fusion protein, the Ndel-HindIII SsuE-encoding fragment from plasmid pME4145 was ligated into Ndel-HindIII digested pET-28a (+) (Novagen) leading to plasmid pME4287. The ssuE sequence of plasmid pME4287 was sequenced to confirm that no changes had been introduced during PCR amplification.

Construction of a ssuD Expression Plasmid—The complete ssuD gene was PCR-amplified with TaqPlus DNA polymerase from genomic DNA prepared from E. coli EC1250 (11) as described elsewhere (12). The oligonucleotide primers used were EEs1 (5'-GGAAACACATATGAGTCACGTGTAT-3') and EEs2 (5'-ATGCGTGTTTGACCCCGGGA-3') with the changes to introduce Ndel and HindIII restriction sites, respectively, underlined. Ndel-HindIII digested pET-24a (+) was ligated into Ndel-HindIII digested pET-28a (+) (Novagen) leading to plasmid pME4150. The ssuD gene sequence of plasmid pME4150 was sequenced to confirm that no changes had been introduced during PCR amplification.
Alkanesulfonate Monoxygenase from E. coli

sites underlined. The resulting 1289-base pair PCR product was di-
gested with Ndel and SalI and ligated into pUC19, resulting in plasmid
pME4281. Finally, the Ndel-HindIII fragment from pME4281 was
cloned in PET-24a (+), leading to the ssuD expression vector pME4282.

Protein Production—For the production of SusE and SusD, E. coli BL21(DE3) containing the appropriate overexpression plasmid was grown at 30 °C and 180 rpm in a 5-liter Erlenmeyer flask containing 1000 ml of growth medium. To minimize the formation of insoluble protein aggregates, which were observed when protein production was carried out at 30 °C, cultures grown to 110,000 x g, 4 °C, induced by the addition of isopropyl-1-thiogalactopyranoside to a final concentration of 50 μM and incubated for a further 5 h at 16 °C with constant shaking (180 rpm). Cells were collected by centrifugation for 20 min at 5800 x g and 4 °C, washed in an excess of 20 ml Tris-HCl buffer, pH 8.0, and stored at −20 °C as frozen pellets until further use. About 4.5 g fresh weight of cells were collected from a 1000-ml culture.

Purification of SusE—For the production of crude extracts containing the histidine-tagged SusE fusion protein, 1.8 g of induced E. coli BL21(DE3)(pME4287) cells were resuspended in 4 ml of binding buffer (5 mM imidazole, 0.5 mM NaCl, 20 mM Tris-HCl, pH 7.9) supplemented with 25 μg/ml DNase I and disrupted by three passages through a French Pressure cell at 5.5 megapascals. After clarification by ultra-
centrifugation (40 min, 110,000 x g, 4 °C), the SusE enzyme was purified on a BioCAD apparatus using 20 mM sodium phosphate, pH 6.0, and loaded at a flow rate of 4 ml/min on a 4.5 ml Fractogel EMD TMAE (S) anion exchange column (Merck) equilibrated with the same buffer. The enzyme was eluted between 100 and 0 mM (NH4)2SO4. The eluted fractions were collected from the anion exchange column were pooled SsuD fractions collected from the anion exchange column were pooled and loaded onto a 7.5-ml phenyl-
(NH4)2SO4, at the concentrations indicated below. The crude extract was first loaded at a flow rate of 5 ml/min on a 3.5-ml Fractogel EMD SO3
(S) cation exchange column (Merck) equilibrated with 20 mM sodium phosphate, pH 6.0. The sample was washed with 2 column volumes with 70 mM NaCl and SusD was eluted with 160 mM NaCl. The SusD fractions were pooled, diluted twice with 20 mM sodium phosphate, pH 6.0, and loaded at a flow rate of 4 ml/min on a 4.5 ml Fractogel EMD TMAE (S) anion exchange column (Merck) equilibrated with the same buffer. After a wash step with 20 mM sodium phosphate, pH 6.0, for 2 column volumes followed by washing with 90 mM NaCl (6 column volumes), SusD was eluted with 150 mM NaCl. In a third step, the pooled SusD fractions collected from the anion exchange column were diluted twice with 1 mM (NH4)2SO4, and loaded onto a 7.5-ml phenyl-
Sepharose Fast Flow 66 hydrophobic interaction column (Amersham Pharmacia Biotech) at a flow rate of 5 ml/min and washed with 2 column volumes of 500 mM (NH4)2SO4. Proteins were eluted with a linear gradient from 500 to 0 mM (NH4)2SO4 in 5 column volumes, followed by washing with 20 mM sodium phosphate, pH 6.0. SusD eluted between 100 and 0 mM (NH4)2SO4.

NAD(P)/FMN Oxidoreductase Activity—Appropriate amounts of SusE were incubated at 30 °C in a reaction mixture (1 ml final volume) containing either 500 μM NADPH and 3 μM FMN, or 1 mM NADH and 0.5 mM FMN in 20 mM imidazole, 50 mM NaCl, 20 mM Tris-HCl, pH 7.9. Reactions were started by addition of enzyme to the reaction mixture; activity was followed online with a UVikon 900 spectrophotometer. An assay mixture without FMN was used as a blank. One unit was defined as the amount of enzyme catalyzing the reduction of 1 μmol of flavin substrate per minute at 30 °C under anaerobic conditions. Activity was calculated from the slope of the absorbance decrease at 375 nm using ε375 = 9.3 mM−1 cm−1 for NADPH and ε375 = 10.4 mM−1 cm−1 for FMN. The obtained values were corrected for residual NADH absorption at 375 nm using ε375 = 2.76 mM−1 cm−1 for NADH.

Alkanesulfonate Monoxygenase Assay—SusD activity was routinely assayed with Ellman’s reagent (5,5′-dithiobis(2-nitrobenzoic acid)) which produces a bright yellow color upon reaction with sulfite (13, 14). The assay mixture (1.5 ml final volume) contained 500 μM NADPH, 3 μM FMN, 500 μM sulfonated substrate, SusE and SusD to a SusE:SsuD molar ratio of 3:0, in 10 mM Tris-HCl, pH 9.1, and was stirred on a magnetic stirrer at 30 °C. Reactions were started by addition of SsuD to the reaction mixture and stopped by the addition of 200-μl samples to a spectrophotometric cuvette containing 100 μl of 5,5′-dithiobis(2-nitrobenzoic acid) (1 mg/ml in 100 mM sodium phosphate buffer, pH 7.0) to which 700 μl of deionized water was finally added. The colorimetric reaction was allowed to develop at room temperature for 2 min. Sulfite determination with Ellman’s reagent was linear up to 250 μM sulfite. One unit of activity was defined as the amount of enzyme forming 1 μmol of sulfite per minute at 30 °C under standard assay conditions, calculated from the slope of absorbance increase at 430 nm with reference to sulfite calibration curves. These were prepared daily from fresh solutions of sodium sulfite in deionized water. When assayed under anaerobic conditions, all reagents were made anaerobic by at least 10 cycles of alternate flushing with 95% N2, 5% H2 and vacuum pulling. Reaction mixtures were prepared in an anaerobic chamber and sulfite detection was carried out as described above, but using anaerobized 5,5′-dithiobis(2-nitrobenzoic acid).

Analysis of Enzyme Reaction Products—The presence of penta-
led hydrolysis of reaction mixtures using 20 mM imidazole, 50 mM NaCl, 20 mM Tris-HCl, pH 7.9. All reagents and buffers were therefore made anaerobic by at least 10 cycles of alternate flushing with 95% N2, 5% H2 and vacuum evacuating. Reaction mixtures were prepared in an anaerobic chamber and reactions were initiated by enzyme addition. An assay mixture in which NADH was omitted was used as a blank. One unit was defined as the amount of enzyme catalyzing the reduction of 1 μmol of flavin substrate per minute at 30 °C under anaerobic conditions. Activity was calculated from the slope of the absorbance decrease at 375 nm using ε375 = 9.3 mM−1 cm−1 for FAD and ε375 = 10.4 mM−1 cm−1 for FMN. The obtained values were corrected for residual NADH absorption at 375 nm using ε375 = 2.76 mM−1 cm−1 for NADH.

SDS-Polyacrylamide Gel Electrophoresis—SDS-PAGE was performed on a Mini-PROTEAN II system (Bio-Rad) with 12% polyacryl-
Agarose gels under denaturing conditions (8). Protein concentrations were measured using the method of Bradford (15) with Bio-Rad reagent dye concentrate, following the manufacturer’s instructions. Bovine se-
rum albumin was used as a standard.

Analytical Methods—The native Mr of SusE was estimated by gel filtration on Superdex 75 HR 10/30 (Amersham Pharmacia Biotech). The column was equilibrated (2 column volumes) and eluted with 50 mM sodium phosphate, pH 8.0, containing 150 mM NaCl at a flow rate of 1.0 ml/min. The column was calibrated with appropriate known Mr protein standards: thyroglobulin (670,000), ferritin (440,000), catalase (232,000), aldolase (158,000), apo-
transferrin (77,000), bovine serum albumin (67,000), ovalbumin (43,000), and RNase A (13,700).

RESULTS

Purification of NAD(P)/FMN Oxidoreductase—The wild type SusE enzyme produced from the overexpression plasmid pME4146 lost its NAD(P)/FMN-dependent FMN reduction activity within 2–3 h in crude extracts, when kept on ice. When partially purified by ammonium sulfate fractionation (30–40% saturation) stored at −20 °C with 20% glycerol, the enzyme could be maintained for a few days. However, cation or anion exchange chromatography on Fractogel EMD SO3− (S) and EMD TMAE (S) (Merck) or SP- and Q-Sepharose (Amersham Pharmacia Biotech), respectively, or dye-ligand affinity chromatography on Green A-agarose (Millipore AG) led to significant protein inactivation. Affinity chromatography with FMN-agarose (Sig-
was therefore done with the histidine-tagged SsuE fusion protein. Enzyme characterization of both extracts was identical, indicating that the histidine tag had no influence on SsuE activity. Purification of the two components of the alkanesulfonate monoxygenase system from E. coli is shown in Fig. 1. Protein samples (30 μg) were analyzed at different stages of purification on 12% SDS-PAGE gels under reducing and denaturing conditions, and stained with Coomassie Brilliant Blue. A. Purification of NAD(P)H:FMN oxidoreductase SsuE. M, molecular weight markers (with molecular masses indicated on the left in kDa); lane 1, extract of uninduced E. coli BL21(DE3)(pME4287); lane 2, extract of cells producing wild type SsuE from pME4146; lane 3, extract of induced E. coli BL21(DE3)(pME4287) cells producing histidine-tagged SsuE; lane 4, wash with binding buffer; lane 5, wash with 60 mM imidazole; lane 6, purified SsuE. B. Purification of alkanesulfonate monoxygenase SsuD. M, molecular weight markers (with molecular masses indicated on the left in kDa); lane 1, cell extract of uninduced E. coli BL21(DE3)(pME4287); lane 2, extract of induced cells; lane 3, fraction after Fractogel EMD SO3+ chromatography; lane 4, fraction after Fractogel EMD TMAE (S) chromatography; lane 5, purified SsuD obtained after phenyl-Sepharose Fast Flow 66 chromatography.

Characterization of NAD(P)H:FMN Oxidoreductase—Densitometric scanning of lanes 2 and 3 of the SDS-PAGE gel (Fig. 1A) showed that wild type SsuE represented around 18%, whereas the histidine-tagged SsuE amounted to approximately 13% of the total soluble protein in crude extracts. The relative specific activities calculated as NADPH oxidation rates of both extracts were identical, indicating that the histidine tag had no influence on SsuE activity. Enzyme characterization was therefore done with the histidine-tagged SsuE fusion protein.

The effect of pH on NAD(P)H:FMN oxidoreductase activity was investigated over a range from 4.5 to 10.8 using appropriate buffer systems (16). SsuE activity was maximal in 20 mM Tris-HCl buffer, pH 7.9, supplemented with 50 mM NaCl and 50 mM Na acetate, bis-Tris, and glycine-NaOH. SsuE specific activity was less than 5% of the maximal activity. This was also the case when the enzyme was dialyzed or subjected to buffer exchange using PD10 gel filtration columns (Amersham Pharmacia Biotech). It is thus likely that a cofactor essential for activity was lost upon dialysis or gel filtration. The pure SsuE protein showed no typical absorption spectrum of flavin-containing flavoproteins, suggesting that the enzyme does not contain any bound flavin cofactor.

Gel filtration chromatography on Superdex 75 was used to estimate a Mr of 58,400 ± 100 for the native enzyme. The subunit molecular mass calculated from the histidine-tagged SsuE amino acid sequence of 23.7 kDa was estimated by SDS-PAGE analysis as 25.4 ± 0.7 kDa. These results therefore suggest a homodimeric structure for the SsuE enzyme.

### Table I

| Substrate | K_m (μM) | V_max (μkat/mg) | k_cat/K_m (μkat/μM) |
|-----------|---------|----------------|---------------------|
| FMN (0.04-7.2 μM) | 0.054 | 0.54 | 474.1 |
| FAD (0.08-17.3 μM) | 2.7 | 0.96 | 16.9 |
| NADPH (7-424 μM) | 46.0 | 32.4 | 33.4 |
| NADH (32-670 μM) | 555.5 | 1.3 | 0.1 |

The substrate range and kinetic constants of NAD(P)H:FMN oxidoreductase SsuE are shown in Table I. When affinities for FMN or FAD were explored, FMN was the preferred flavin substrate of SsuE, but FAD and riboflavin were also reduced at significant rates, whereas lumiflavin was not (Fig. 2). When NADH was the pyrimidinic substrate, a distinct activity maximum was obtained at an FMN concentration of 0.5 μM, whereas concentrations higher than 2.5 μM led to more than 60% decrease in specific activity. On the contrary, the SsuE specific activity increased with increasing FAD concentrations and reached saturation at 10 to 25 μM FAD. When NADPH was supplied as pyrimidinic substrate, maximal reductase activity was obtained with 2.5–10 μM FAD, while higher FAD concentrations led to 15% decrease in SsuE activity. These results showed the necessity of performing the determination of kinetic constants for NADH in reaction mixtures containing only 0.5 μM FMN, whereas 3 μM FMN was used when the kinetic constants for NADPH were investigated. When affinities for FMN or FAD were explored, measurements were done under anaerobic conditions using NADH because a strong abiotic FMN reduction was observed when NADPH was given as pyrimidinic substrate. Table I
summarizes the kinetic parameters for SsuE with regard to NADH, NADPH, FMN, and FAD. It can be concluded that SsuE is a NAD(P)H:flavin oxidoreductase.

**Purification of SsuD**—The SsuD protein initially produced from an overexpression construct in which the ssuD gene originating from plasmid pME4180 (4) was under the control of the T7 RNA polymerase promoter, was not active. Sequencing of the ssuD gene of plasmid pME4180 revealed a single point mutation leading to an arginine to cysteine exchange at position 298 of the SsuD sequence, which was responsible for the complete loss of activity of the enzyme. The SsuD protein was therefore produced from the overexpression plasmid pME4282, which contains the ssuD gene sequence amplified from E. coli EC1250 genomic DNA. Using the three-step procedure summarized in Table II, the ssuD gene product was purified to near homogeneity with a yield of 35% from the overexpression plasmid pME4282 as shown in Fig. 1B. The band corresponding to a 32-kDa protein which co-purified with SsuD and which represented less than 2% of the phenyl-Sepharose purified protein preparation could not be removed by any other chromatography technique tested. After an additional purification step on hydroxyapatite, the SsuD protein amounted to only 90% of total protein, and a net increase in intensity of the 32-kDa protein band was observed at the expense of SsuD. After storage for 24 h at −20 °C of the hydroxyapatite-purified protein, SsuD was only 70% of the total protein, and the remaining 30% being represented by the 32-kDa protein. We therefore presume that this 32-kDa protein is a degradation product of the native SsuD protein. For this reason, we chose to perform SsuD characterization with the enzyme preparation obtained after phenyl-Sepharose chromatography, in which no further degradation of SsuD was observed. Densitometric scanning indicated that SsuD was over 98% pure (Fig. 1B, lane 5).

**Characterization of SsuD**—The purified enzyme was stable upon storage at −20 °C in buffer with 15% glycerol and it had a specific activity of 2.5 units/mg. The activity increased slightly during the first 2 to 3 weeks of storage.

The effect of the pH on enzyme activity was examined over a range from 4.5 to 10.8 using appropriate buffer systems (16). SsuD showed a distinct activity maximum in 10 mM Tris-HCl, pH 9.1.
SsuD desulfonation activity was measured in 10 mM Tris-HCl, pH 9.1, containing 500 μM sulfonated substrate, 500 μM NADPH, 3 μM FMN, with SsuE to SsuD at a molar ratio of 3.0. Activity measurements with TauD were performed in 10 mM imidazole, pH 6.9, containing 500 μM sulfonated substrate, 600 μM α-KG, 100 μM Fe(IIISO), 200 μM sodium ascorbate. Activity was calculated as sulfite released after incubation for 3 min at 30 °C. A relative specific activity of 100 corresponded to 4.1 units/mg with both enzymes and with regard to their preferred substrate.

### Table III

| Sulfonated substrate | SsuD | TauD |
|----------------------|------|------|
| Taurine              | 0.0  | 100.0|
| N-Phenyltaurine      | 65.6 | 0.0  |
| 4-Phenyl-1-butanesulfonic acid | 42.4 | 2.5 |
| HEPES                | 10.6 | 5.0  |
| MOPS                 | 36.4 | 34.2 |
| PIPES                | 29.2 | 3.1  |
| 2-(4-Pyridyl)ethanesulfonic acid | 87.4 | 0.5 |
| 1,3-Dioxo-2-isoidolinoethanesulfonic acid | 100.0 | 30.1 |
| Sulfoacetic acid     | 19.8 |
| L-Cysteic acid       | 0.0  |
| Isethionic acid      | 14.3 |
| Methanesulfonic acid | 0.7  |
| Ethanesulfonic acid  | 0.7  |
| Propanesulfonic acid | 14.0 |
| Butanesulfonic acid  | 17.8 |
| Pentanesulfonic acid | 40.4 |
| Hexanesulfonic acid  | 43.8 |
| Octanesulfonic acid  | 46.3 |
| Decanesulfonic acid  | 43.2 |
| Dodecanesulfonic acid | 20.1 |
| Tetradecanesulfonic acid | 2.9 |

* —, not determined.

**DISCUSSION**

Several reports on the utilization of primary aliphatic sulfonates by bacteria as carbon or sulfur sources have appeared (2, 17–19). Apart from the previously characterized α-ketoglutarate-dependent taurine dioxygenase TauD from *E. coli* (9), the enzymology of the desulfonation of these compounds remains largely unexplored. Crude extracts of 1-octanesulfonate-grown cells of a *Pseudomonas* strain were reported to desulfonate 1-alkanesulfonates by monoxygenation, giving a putative 1-hydroxyalkane sulfonate which spontaneously rearranged to give the corresponding aldehyde and sulfite (18). An inducible, NADH-specific methanesulfonic acid monoxygenase was detected in a methylotrophic bacterium growing with methanesulfonic acid as a sole source of carbon and energy. This three-component oxygenase was partially purified and shown to

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TABLE III

| Sulfonated substrate | SsuD | TauD |
|----------------------|------|------|
| Taurine              | 0.0  | 100.0|
| N-Phenyltaurine      | 65.6 | 0.0  |
| 4-Phenyl-1-butanesulfonic acid | 42.4 | 2.5 |
| HEPES                | 10.6 | 5.0  |
| MOPS                 | 36.4 | 34.2 |
| PIPES                | 29.2 | 3.1  |
| 2-(4-Pyridyl)ethanesulfonic acid | 87.4 | 0.5 |
| 1,3-Dioxo-2-isoidolinoethanesulfonic acid | 100.0 | 30.1 |
| Sulfoacetic acid     | 19.8 |
| L-Cysteic acid       | 0.0  |
| Isethionic acid      | 14.3 |
| Methanesulfonic acid | 0.7  |
| Ethanesulfonic acid  | 0.7  |
| Propanesulfonic acid | 14.0 |
| Butanesulfonic acid  | 17.8 |
| Pentanesulfonic acid | 40.4 |
| Hexanesulfonic acid  | 43.8 |
| Octanesulfonic acid  | 46.3 |
| Decanesulfonic acid  | 43.2 |
| Dodecanesulfonic acid | 20.1 |
| Tetradecanesulfonic acid | 2.9 |

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TABLE IV

Determination of $K_m$ values was performed in 10 mM Tris-HCl, pH 9.1, containing 55 mM SsuD, 165 mM SsuE, 500 μM NADPH, 3 μM FMN, the sulfonated substrates over the concentration range indicated. Sulfite production was followed for 3 min by withdrawing samples every 15 or 30 s. Initial rates were calculated from the slope of absorbance increase at 430 nm by regression analysis. Kinetic parameters were determined from double-reciprocal plots according to Lineweaver-Burk. Each value represents the mean of at least two independent sets of experiments.

| Sulfonated substrate | $K_m$ (μM) | $V_{max}$ (units/mg) | $k_{cat}/K_m$ (min$^{-1}$ μM$^{-1}$) |
|----------------------|------------|----------------------|-------------------------------------|
| Decanesulfonic acid  | 35         | 1.4                  | 6.7                                 |
| Octanesulfonic acid  | 44         | 1.6                  | 6.1                                 |
| 1,3-Dioxo-2-isoidolinoethanesulfonic acid (33–500 μM) | 114 | 4.1 | 6.0 |
| 2-(4-Pyridyl)ethanesulfonic acid (33–500 μM) | 139 | 3.8 | 4.6 |
| Hexanesulfonic acid  | 95         | 2.3                  | 4.0                                 |
| N-Phenyltaurine      | 237        | 4.8                  | 3.2                                 |
| 4-Phenyl-1-butanesulfonic acid (70–500 μM) | 110 | 1.8 | 2.7 |
| Pentanesulfonic acid | 189        | 2.0                  | 1.8                                 |
| MOPS (70–1000 μM)    | 617        | 4.1                  | 1.1                                 |
| Butanesulfonic acid  | 870        | 3.3                  | 0.6                                 |
| PIPES (335–5000 μM)  | 1110       | 2.4                  | 0.4                                 |

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FIG. 4. Overall reaction catalyzed by the SsuD/SsuE two-component alkanesulfonate monoxygenase system from *E. coli*.
desulfonate unsubstituted C-1 to C-3 1-alkanesulfonates but not substituted short-chain 1-alkanesulfonates such as isethionnic acid, taurine, and L-cysteic acid (19).

The SsuDE two-component alkanesulfonate monooxygenase characterized in this study is, to our knowledge, the first purified enzymatic system reported that is capable of oxygenolytic cleavage of the C-S bond of 1-alkanesulfonates by monooxygenation. In contrast to the monooxygenase systems for alkanesulfonate desulfonation mentioned above, it does not play a role in carbon metabolism, and its synthesis is regulated by the sulfur supply to the cell (4). Analogous systems involved in the utilization of sulfur from aliphatic sulfonates in *Bacillus subtilis* (20) and from methanesulfonic acid in *Pseudomonas aeruginosa* (21) were recently identified in our laboratory, but are not characterized as purified enzymatic systems.

Based on their biochemical properties, the SsuD and SsuE proteins represent a two-component system that can be assigned to the group of FMNH$_2$-dependent monooxygenases. Besides luciferase, the most extensively studied flavin-monooxygenase, several two-component monooxygenase systems belonging to this group have already been studied. These are involved in nitrolitriacetate hydroxylation (6), EDTA oxidation (22), pristinamycin II$_A$ biosynthesis (7), and dibenzothiophene desulfurization (23). These two-component systems consist of an oxidoreductase providing FMNH$_2$ for the oxygenation reaction catalyzed by the second component.

The SsuE enzyme is different from the flavin reductases characterized in the systems mentioned above. SsuE is an NAD(P)/H:FMN oxidoreductase whose preferred pyrimidinic substrate is NADPH, for which it showed a 12 times higher affinity and a 25 times higher turnover rate than for NADH. Furthermore, the SsuE enzyme does not seem to be a flavoprotein since the visible spectrum of the purified enzyme did not show absorption at wavelengths typical for flavoproteins. These results set the enzyme clearly apart from the characterized NADH:FMN oxidoreductases of the nitrilotriacetate and EDTA monooxygenases as well as from the two-enzyme system involved in pristinamycin II$_A$ synthesis. Their substrate specificity for NADH was strict, and they were shown to be flavoproteins. At the amino acid sequence level, no significant homology of the SsuE protein was found with either of these flavoproteins, or to the Fre-type enzymes, which are flavin reductases that do not contain any light-absorbing prosthetic group and utilize flavin only as a substrate and not a coenzyme (24). However, SsuE showed amino acid sequence identity to an iron-sulfur flavoprotein of *Methanosarcina thermophila* (25). A sequence alignment (Fig. 5) shows a small conserved motif at the N terminus and over 40% identity within a 45-amino acid internal region. This conserved domain could be involved in flavin binding. The archaeal flavoproteins contain a 4 cysteine motif forming an iron-sulfur cluster. Since this motif is not present in SsuE, electron transfer from the pyrimidinic substrate to the flavin substrate in the SsuE-catalyzed reaction does not occur via an iron-sulfur cluster, but perhaps, as has been shown for the Fre protein, directly from NAD(P)H to FMN by an ordered mechanism. In this case NADPH binds first to the active site, followed by the flavin. After electron transfer, the first product released is the reduced flavin, followed by NADP$^+$ (24).

The homotetrameric structure for SsuD is rather uncommon among flavin-dependent monooxygenases. Bacterial luciferase and pristinamycin II$_A$ synthase were shown to be $\alpha\beta$ heterodimers of $M_r$ 79,000 and 85,000, respectively (7), whereas component A of the nitrolitriacetate monooxygenase was
shown to be a homodimeric enzyme of $M_r 99,000$ (6). The only other tetrameric flavin-dependent monooxygenase reported as homotetrameric enzyme is component A of the EDTA oxidizing system of bacterial strain DSM 9103 with a $M_r$ of 210,000 (22).

Whatever their quaternary structures, around 25% amino acid identity was found between the pristinamycin IIA synthase subunit A (SnaA), component A of the nitrilotriacetate monooxygenase (NtaA), the dibenzothiophene desulfurization enzyme SoxA (DszA) (26), and SsuD (20) (not shown). The sequence of SsuD was also similar to those of LuxA and LuxB subunits, which are components of heterodimeric FMNH$_2$-dependent bacterial luciferases (27). From Mer indicated that these proteins might fold similarly as bacterial luciferase, whose three-dimensional structure has been determined (27). Arginine 298, which is conserved in SnaA, NtaA, and SoxA but not in LuxA, LuxB, or Mer, was essential for SsuD catalysis.

Comparison of the substrate range of the previously characterized $\alpha$-ketoglutarate-dependent taurine dioxygenase TauD with the substrate range of alkanesulfonate monooxygenase SsuD shows that TauD is specific for taurine, whereas SsuD, whose preferred substrate was decanesulfonic acid, desulfonated nearly all substrates tested with the exception of taurine, methanesulfonic acid, aromatic sulfonates, and L-cysteic acid (Table III). Thus, SsuD and TauD are complementary. Taken together, the substrate ranges of the two alkanesulfonate desulfonation systems are in accordance with the range of alkanesulfonates, that are used as sulfur sources by *E. coli* (4). However, they do not explain the reported ability of *E. coli* to utilize

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**Fig. 6. Alignment of SsuD with the flavin-dependent enzymes Mer and LuxB.** The following sequences were aligned with the program ClustalW (30): SsuD from *E. coli* (Swiss-Prot accession number P80645), NtaA from *C. heintzi* (Swiss-Prot accession number P54989), SnaA from *S. pristinaespiralis* (Swiss-Prot accession number P54991), SoxA from *Rhodococcus sp.* strain IGT88 (Swiss-Prot accession number P54995), Mer from *M. thermoautotrophicum* (TrEMBL accession number Q90744), and LuxB and LuxA from *Vibrio harveyi* (Swiss-Prot accession numbers P07739 and P07740). Only the alignment between SsuD, Mer, and LuxB is shown. Identical residues in all three sequences are in *white* on a *black* background, identical residues occurring in two sequences are in *white* on a *gray* background. The secondary structure elements of LuxB (27) are shown below the sequence as *arrows* ($\alpha$-helices) or *thick lines* ($\beta$-sheets). The predicted secondary structure elements of SsuD and Mer were calculated with the program PredictProtein (31) and are indicated by *h* for $\alpha$-helices, *e* for $\beta$-sheets, and *l* for loops. Dots represent lack of defined secondary structure.
methanesulfonic acid and L-cysteic acid as sulfur sources (17). Characterization of the two systems has given insight into the completely different biochemical mechanisms responsible for sulfite liberation in \textit{E. coli} from taurine on the one hand and from a wide range of aliphatic sulfonates on the other. Sulfite liberated from sulfonate-sulfur has been shown to enter the sulfate reduction pathway to cysteine (29) and thereby enables growth in the absence of the preferred sulfur sources sulfate and cysteine. In line with their function in metabolism, the proteins encoded by the \textit{tau} and \textit{ssu} gene clusters have a lower than average content of sulfur-containing amino acids. There is one cysteine residue in SsuD and none are present in SsuE and TauD. Economizing on sulfur-containing amino acids is metabolically sensible since these proteins are specifically synthesized when growth is limited by the biosynthesis of cysteine.

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