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FINAL REPORT

TITLE: Biotic and Abiotic Carbon to Sulfur Bond Cleavage

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OBJECTIVES

The microbial desulfurization of organosulfur compounds occurs by unprecedented and largely unexplored biochemical processes. A study of such biotic desulfurizations can be expected to give rise to new and useful chemistry and enzymology. The potential value of understanding and harnessing these processes is seen in relation to the need for methods for the removal of organically bound sulfur from coal and the degradation of organic sulfur-containing pollutants. Our research effort has been directed towards an examination of desulfurization ability in well characterized microorganisms, the isolation of bacteria with desulfurization ability from natural sources, the characterization and mechanistic evaluation of the observed biocatalytic processes, the development of biomimetic synthetic organic chemistry based on biotic desulfurization mechanisms and the design and preparation of improved coal model compounds for use in microbial selection processes. A systematic approach to studying biodesulfurizations was undertaken in which organosulfur

compounds have been broken down into classes based on the oxidation state of the sulfur atom and the structure of the rest of the organic material. Microbes have been evaluated in terms of ability to degrade organosulfur compounds with sulfur in its sulfonic acid oxidation state. These compounds are likely intermediates in coal desulfurization and are present in the environment as persistent pollutants in the form of detergents. It is known that oxygen bonded to sulfur lowers the carbon-sulfur bond energy, providing a thermodynamic basis for starting with this class of compounds.

RESULTS AND DISCUSSION

Escherichia coli K12 has been examined for sulfonic acid desulfurization ability. The strain has been well characterized, allowing for ready handling and possible genetic manipulation. The *E. coli* strain was observed to catalyze a carbon-sulfur bond cleaving reaction on aliphatic sulfonic acids. *E. coli* K12 were seen to grow on minimal medium¹ plates containing methanesulfonic acid as the sole sulfur source. No growth was observed on control plates containing no sulfur source. Single colonies from methanesulfonic acid plates were used as inoculants for liquid cultures containing various sulfonic acids as the sulfur source (Table 1). The medium supported growth when primary aliphatic sulfonates were supplied and did not support growth when the secondary sulfonate 1-phenyl-2-propanesulfonic acid or the arylsulfonate, benzenesulfonic acid, was used as the sulfur source.

The organic fragment of the aliphatic sulfonate desulfurization reaction was found with one exception to be the alcohol corresponding to the supplied sulfonate. The alcohols were isolated from acidified culture supernatant by chloroform extraction. Structure assignments were based on gas chromatographic and GC-MS analyses and ¹H and ¹³C spectra of the extracts. Small, variable amounts of the corresponding aldehydes and carboxylic acids were also detected. The degradation of methanesulfonic acid was the

Table 1. Aliphatic Organosulfonate C-S Bond Cleavage Reactions Catalyzed by *E. coli* K-12

organosulfonate	growth ^a	specific ^b activity 10 ⁻⁴ units/mg	products ^c	
			intact cells	lysate
¹⁴ CH ₃ SO ₃ H	+	0.35	¹⁴ CO ₂	ND ^d
CH ₃ (CH ₂) ₃ SO ₃ H	+	3.3	CH ₃ (CH ₂) ₃ OH	CH ₃ (CH ₂) ₄ CO ₂ H
-	X	X	X
-	0.29	X

n=1	+	5.4	n=1	n=0
n=2	+	9.0	n=2	n=1
n=3	+	6.4	n=3	n=2

a + growth, - no growth. b 1 unit = mmole SO₃⁻²/min. c Chloroform extracts of culture supernatants and cell lysates were analyzed by gaschromatography and GC-MS. d not determined

exceptional case. Cells grown on [¹⁴C]-methanesulfonic acid in a sealed tube were observed upon GC analysis of the headspace to have produced [¹⁴C]-carbon dioxide. No other volatile radioactive materials were detected.

A sample of 2-phenyl-1,1-dideuteroethanesulfonic acid was prepared from ethyl phenylacetate to serve as a mechanistic probe of the biocatalytic process. The alcohol

formed in microbial desulfurization, 2-phenyl-1-deuteroethan-1-ol retained one of the deuterium atoms present in the sulfonic acid (Scheme 1).

Scheme 1

Cultured cells were disrupted with a French press. Sulfonic acids whose desulfurization had been examined with intact cells were then exposed to cell lysate. The primary aliphatic sulfonic acids were each transformed to the corresponding carboxylic acid (Table 1), with small amounts of alcohol observed. The secondary sulfonate 1-methyl-2-phenylethanesulfonic acid was converted to phenyl-2-propanone. No reaction was observed with benzenesulfonic acid.

The cell lysate desulfurization process was found to require reduced nicotinamide adenine dinucleotide (NADH) and dioxygen. Desalted cell lysate catalyzed the sulfonic acid to carboxylic acid transformation only after NADH was added. When cell lysate was degassed and stored under argon prior to incubation with NADH and a substrate, no reaction was observed. The introduction of oxygen into the lysate restored the carboxylic acid production.

The form of inorganic sulfur produced in the cell lysate process was found to be sulfite ion. The progress of the conversion of 2-phenyl-1-ethanesulfonic acid was followed with Ellmann's reagent, 5-5'-dithiobis(2-nitrobenzoic acid). Spectrophotometric

observation of 5-mercapto-2-nitrobenzoic acid indicated that sulfite was produced over time. In a control experiment, sulfate conversion to sulfite under transformation conditions was not observed.

Several mechanistic formulations were proposed to account for the biocatalyzed desulfurization (Scheme 2). The loss of a deuterium atom in the biodesulfurization of 2-phenyl-1,1-dideuteroethanesulfonic acid eliminated from consideration direct hydrolysis (pathway A) or a direct oxygen insertion process (pathway B). The NADH and oxygen requirement for the cell lysate process effectively eliminated sulfene intermediacy (pathway

Scheme 2

C) from consideration. The observed formation of sulfite is inconsistent with oxygen insertion processes (pathways B and E), which would produce sulfate. An α -hydroxylation mechanism, with subsequent aldehyde intermediacy followed by oxidation or reduction to the observed product, is the only remaining viable reaction mechanism for the transformation.

The *E. coli* strain examined for organosulfonate desulfurization ability degraded primary and secondary aliphatic sulfonic acids but did not desulfurize benzenesulfonic acid. In the search for a microbe which would desulfurize arylsulfonates the approach adopted entailed screening strains present in activated sludge obtained from the West Lafayette, IN sewage treatment plant. The sludge was used as an inoculant for cultures subsequently spread on solid growth medium where *p*-nitrobenzenesulfonic acid was the only source of sulfur. Single colonies surrounded by a yellowish halo were subcultured leading to the isolation of strain KS3D. The strain was determined to be *Klebsiella oxytoca*.

A wide range of arylsulfonates including variously substituted benzene-, naphthalene- and biphenylsulfonates supported growth of *Klebsiella oxytoca* KS3D when used as the sole source of sulfur in minimal liquid suspension medium² (Table 2). Arylsulfonates were converted into corresponding phenols which accumulated in the culture supernatant. Acidified supernatant was extracted with ethyl acetate and the extract was analyzed by gas chromatography and GC-MS analysis. Three strains of *Klebsiella oxytoca* were obtained from the American Type Culture Collection, each of which were capable of degrading aromatic sulfonates. Strains ATCC 8724, ATCC 43075 and ATCC 1538 were capable of using *p*-toluene-, *p*-nitrobenzene- and 2-naphthalenesulfonic acid as sole sources of sulfur in cell cultures. The coal model compounds dibenzothiophene and dibenzothiophene-5,5-dioxide did not support KS3D growth.

Arylsulfonate degradation has not been observed in cell lysate. Cultured cells were lysed either in a French pressure cell, by ultracentrifugation or by osmotic shock. Cells

Table 2. Desulfurization Products Resulting from Growth of *Klebsiella oxytoca* KS3D in Medium with Arylsulfonates as the Sole Sulfur Source

Organosulfonate	Growth Conditions	Product
a

a,b

c

a

a

a

a

a

a

a

a

^a Cultured under $^{16}\text{O}_2$ in H^{16}OH containing arylsulfonate along with minimal salts.³ ^b Cultured under $^{16}\text{O}_2$ in H^{18}OH containing arylsulfonate along with minimal salts. ^c Cultured under $^{18}\text{O}_2$ in H^{16}OH containing arylsulfonate along with minimal salts.

were broken into phosphate buffer containing a variety of cofactors, salts and trace metal ions.³ Upon incubation with arylsulfonates, neither phenol nor sulfite increase was detected.

Mechanistic analysis of the biodesulfurization has been thus based on studies with intact cells. KS3D grown on *p*-toluenesulfonic acid in a sealed Erlenmeyer containing [¹⁸O]-dioxygen produced [¹⁸O]-*p*-cresol, while cultures grown in media enriched with [¹⁸O]-water produced [¹⁶O]-*p*-cresol. Sulfite ion was detected in the culture supernatant.

Biomimetic transformations were designed based on the findings that the arylsulfonate carbon-sulfur bond was replaced by a carbon-oxygen bond and that the reactive species was derived from molecular oxygen. Reactants were chosen which represented or mimicked oxygen species formed in oxygen fixation.

Table 3. Single Electron Reduction of an Arylsulfonate and Arylsulfonamide and Reaction of the Resulting Radical Anions with Molecular Oxygen.

Organosulfur Starting Material	Reaction Conditions	Isolated Yields (%)

R = O ⁻ N(C ₆ H ₅) ₂	KO ₂ (25 eq.), O ₂ 18-crown-6 C ₆ H ₆ , 60 h, RT	55	-
R = N(C ₆ H ₅) ₂	KO ₂ (4 eq.), O ₂ 18-crown-6 C ₆ H ₆ , 18 h, RT	35	14
R = N(C ₆ H ₅) ₂	1. SmI ₂ 2. O ₂ , THF, -20 °C, 1 h	31	10
R = N(C ₆ H ₅) ₂	1. 2. O ₂ , THF, -20 °C, 1 h	12	trace

Potassium superoxide in benzene in the presence of 18-crown-6 reacted slowly at 20 °C with either tetrabutylammonium-*p*-nitrobenzenesulfonate or N,N-diethyl-*p*-nitrobenzenesulfonamide to produce *p*-nitrophenol (Table 3). Because potassium superoxide reacts with *p*-nitro-bromobenzene via an SRN1 mechanism involving the radical anion of the aromatic bromide,⁴ a similar intermediate was proposed to be involved here. N,N-diethyl-*p*-nitrobenzenesulfonamide was reacted sequentially in THF at -20 °C with a single electron transfer reductant then with molecular oxygen to form *p*-nitrophenol (Table 3). Benzene-, toluene- and naphthalenesulfonamide derivatives did not react under these conditions.

Intramolecular attack of alkoxy radicals was chosen as a model for the type of chemistry accessible to metal-oxo complexes, which are often electrophilic and have radical character. Pursuit of such a chemical model was based on the carbon-sulfur bond cleavage observed by Speckamp⁵ and Motherwell⁶ in intramolecular, carbon-centered radical reactions on arylsulfone, arylsulfonate and arylsulfonamide model systems. Three alcohols, mono(4-methylbenzenesulfonyl)-1,2-ethanediol, 2-(phenylsulfonyl)ethanol and catechol monotosylate were chosen as radical precursors. The reaction conditions employed and products isolated are summarized in Table 4. The reactions observed are characteristic of alkoxy radical chemistry⁷ and no carbon-sulfur bond cleavage reactions were observed. Alkoxy radicals were produced from the two primary alcohols by *in situ* hypiodide preparation and photolysis.⁸ Photolysis of mono(4-methylbenzenesulfonyl)-1,2-ethanediol in benzene afforded (4-methylbenzenesulfonyl)-2-phenoxyethanol, formed by intermolecular, electrophilic reaction of the alkoxy radical with the solvent. No products indicative of alkoxy attack on the aromatic ring of the arylsulfonate were formed when carbon tetrachloride was used as the solvent. Phenyl ether formation was the primary product of photolysis of 2-(phenylsulfonyl)ethanol in benzene. A small amount of 2,3-dihydro-1,4-benzoxathiin-4,4-dioxide was formed indicating intramolecular attack on aromatic carbon *ortho* to the sulfone. This product became the dominant product when the

Table 4. Reaction of Arylsulfonates and Arylsulfones with Alkoxy Radicals.

Organosulfur Starting Material	Reaction Conditions	Isolated Products
	Hg(OAc)2 C6H6 hv 12 h, RT	 15%
	Hg(OAc)2 C6H6 hv 12 h, RT	 14% 3%
	Hg(OAc)2 CCl4 hv 12 h, RT	 10% 2%
	Ca(NH4)2(NO2)6 CH3CN RT, 30 min	 11% 18%

photolysis was carried out in carbon tetrachloride. Iodomethyl phenyl sulfone was also formed in this reaction. Generation of this product is consistent with loss of formaldehyde from the alkoxy radical followed by reaction of the resulting radical with iodine. When the phenoxy radical was produced from catechol monotosylate by oxidation with ceric ammonium nitrate,⁹ nitration instead of either *ipso* or *ortho* attack of the radical was observed. When the tosylate was reacted with potassium ferricyanide under biphasic conditions,¹⁰ insoluble polymeric material was formed.

Scheme 3

As was the case for the aliphatic sulfonate desulfurization process, a number of plausible reaction mechanisms exist for the biocatalyzed transformation of arylsulfonates to phenols (Scheme 3). The isotopic labelling studies with intact *Klebsiella oxytoca* cells rule out hydrolysis, which could be viewed as a biological version of the alkali fusion reaction. Mesitylenesulfonic acid was used as a sulfur source specifically to examine a mechanism involving the direct loss of sulfite to form a benzyne intermediate. The observed conversion of the sulfonate, which has no *ortho* protons, to mesitol eliminates this mechanism from consideration. Three mechanisms based on reactive species derived from dioxygen have been postulated, based respectively on superoxide, peroxide and a metal-oxo compound as the reactive species. Chemical precedent exists for the transformation of arylsulfonates to phenol via a peroxide intermediate.¹¹ Desulfurization of arylsulfonates with superoxide has now been demonstrated, provided that a nitro group is present on the substrate. While no examples of arylsulfonate desulfurization by a metal-oxo species are known, this mechanistic alternative may not be ruled out.

Scheme 4

Dibenzothiophene has been used as the sulfur source in microbial desulfurization selection processes.¹² A potential liability in such dibenzothiophene usage is that it is insoluble in culture media. A phosphonyldibenzothiophene could be used in place of dibenzothiophene. The phosphonate would exhibit increased aqueous solubility and could be used as both the sulfur and phosphorus source in a selection process. Regioselective phosphonylation of dibenzothiophene at the 2- and 4-positions has been achieved (scheme 4). Diethyl-4-phosphonodibenzothiophene was prepared by lithiation of dibenzothiophene in THF followed by reaction with excess diethyl chlorophosphate. Diethyl-2-phosphonodibenzothiophene was prepared by reaction of 2-bromodibenzothiophene with triethyl phosphite and a stoichiometric amount of nickel chloride.

The chemical stability of dibenzothiophene is another limitation of its utility as a microbial selection agent. Most dibenzothiophene carbon-sulfur bond cleavage has been accomplished after multiple transformations. Hydroxylated dibenzothiophene might be useful for the isolation of thiophene carbon-sulfur bond cleaving microbes which cannot degrade dibenzothiophene. A preparation of each of the four hydroxydibenzothiophene regioisomers exists in which the use of an autoclave is required to complete the synthesis.¹³ Methodology has been developed for the preparation of hydroxylated

Scheme 5

dibenzothiophene at C-2 and C-4 which involves lithium metallation. Reaction of the lithiated dibenzothiophene with trimethyl borate followed by hydrogen peroxide oxidation yields the 2-hydroxy- and 4-hydroxydibenzothiophene (Scheme 5).

¹ Minimal media contained purified agarose (Type II, Medium EEO, Sigma Chemicals, 15g/L), dibasic sodium phosphate (42 mM), monobasic potassium phosphate (22 mM), ammonium chloride (19 mM), sodium chloride (10 mM), magnesium chloride (1 mM), glucose (6 mM) thiamine hydrochloride (15 μM) and an aliphatic sulfonic acid (0.25 mM). Liquid culture medium was prepared the same way with the agarose omitted.

² Minimal medium was the same as for *E. coli* growth but with the thiamine hydrochloride omitted.

³ Cells were broken into phosphate buffer (64 mM, pH = 7) containing NAD (1 mM), NADH (1 mM), NADP (1 mM), NADPH (1 mM), FAD (2.75 μM), FMN (2.75 μM), riboflavin (2.75 μM), MgCl_2 (1 mM), FeCl_2 (0.25 mM), and trace metals including MnCl_2 (0.37 μM), B(OH)_3 (0.15 μM), CoCl_2 (0.84 μM), NiCl_2 (0.084 μM), CuCl_2 (0.059 μM), Na_2MoO_4 (0.12 μM), and $\text{Ca(NO}_3)_2$ (0.21 μM). Neither phenol nor sulfite was detected. Activity was also not obtained when cells were lysed by French press into phosphate buffer (64 mM, pH = 7) containing ATP (2 mM), coenzyme A (2 mM), MgCl_2 , FeCl_2 , NAD, (1 mM), NADH (1 mM), NADP (1 mM), and NADPH (1 mM).

⁴ Frimer, A.; Rosenthal, I. *Tetrahedron Lett.* 1976, 2809

- ⁵ (a) Kohler, J. J.; Speckamp, W. N. *Tetrahedron Lett.* 1977, 631. (b) Kohler, H. J.; Speckamp, W. N. *J. Chem. Soc. Chem. Commun.* 1980, 142.
- ⁶ (a) Motherwell, W. B.; Pennell, A. M. K. *J. Chem. Soc. Chem. Commun.* 1991, 877. (b) Motherwell, W. B. *Aldrichimica Acta* 1992, 25, 74.
- ⁷ Kochi, J. K. In *Free Radicals*, Kochi, J. K. Ed., John Wiley and Sons: New York, 1973; Chapter 23.
- ⁸ (a) Kraus, G. A.; Thurston, J. *Tetrahedron Lett.* 1987, 28, 4011. (b) Concepcion, J. I.; Fransisco, C. G.; Hernandez, R.; Salazar, J. A.; Suarez, E. *Tetrahedron Lett.* 1984, 25, 1953.
- ⁹ Chaudhuri, K.; Chawla, H. M. *Indian J. Chem.* 1987, 26B, 272.
- ¹⁰ (a) Wasserman, H. H.; Brunner, R. K.; Buynak, J. D.; Carter, C. G.; Oku, T.; Robinson, R. P. *J. Am. Chem. Soc.* 1985, 107, 519. (b) Stoessel, A. *Can. J. Chem.* 1967, 45, 1745.
- ¹¹ (a) Crovatt, L. W.; McKee, R. L. *J. Org. Chem.* 1959, 24, 2031. (b) Fichter, F.; Stocker, E. *Helv. Chim. Acta* 1924, 7, 1064. (c) Dannley, R. L.; Ternstrom, P. K. *J. Org. Chem.* 1975, 40, 2278.
- ¹² (a) Gallagher, J. R.; Olson, E. S.; Stanley, D. C. *FEMS Microbiol. Lett.*, 1993, 107, 31. (b) Kilbane, J. J.; Jackowski, K. *Biotechnol. Bioeng.* 1992, 40, 1107. (c) Omori, T.; Monna, L.; Kodama, T. *Appl. Envir. Microbiol.*, 1992, 58, 911. (d) Krawiec, S. *Dev. Ind. Microbiol.*, 1990, 31, 103. (e) Kim, H. Y. *Biotechnol. Lett.*, 1990, 12, 761.
- ¹³ Kudo, H.; Castle, R. N.; Lee, M. L. *J. Heterocyclic Chem* 1985, 22, 215.

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