Task 4.6 - Biodesulfurization

Topical Report
Jan. 1, 1994 - Sept. 30, 1995

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Work Performed Under Contract No.: DE-FC21-93MC30097

For
U.S. Department of Energy
Office of Fossil Energy
Morgantown Energy Technology Center
Morgantown, West Virginia

By
University of North Dakota
Energy Research Center
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TASK 4.6 - BIODESULFURIZATION

EXECUTIVE SUMMARY

The desire for a clean environment has driven interest in better and more cost-effective methods of producing clean fuels. Sulfur represents one of the more difficult and expensive contaminants in fuels. Conventional hydrodesulfurization technology is effective for many types of sulfur compounds found in petroleum, but is less effective for dibenzothiophenic sulfur. Microbial methods of desulfurizing fuels have the potential to require low operating and capital costs and to be environmentally friendly technology.

Sulfur-specific desulfurization of dibenzothiophene (DBT) has been shown previously at the Energy & Environmental Research Center (EERC) to give two different products, based on growth conditions. Growing microorganisms produce 2'-hydroxybiphenyl-2-sulfonate and 2,2'-dihydroxybiphenyl, while stationary-phase cells produce 2'-hydroxybiphenyl-2-sulfinate and 2-hydroxybiphenyl. Earlier work suggested that dibenzothiophene sulfone is a key intermediate in these pathways. The use of ^{18}O -labeled dibenzothiophene sulfone allowed us to determine the pathways that result in these products. These experiments demonstrated that the sulfone is directly converted to the 2'-hydroxybiphenyl-2-sulfinate. The sulfinate intermediate is then reductively cleaved to 2-hydroxybiphenyl (stationary cells) or is oxidized to 2'-hydroxybiphenyl-2-sulfonate, which is subsequently oxidized to 2,2'-dihydroxybiphenyl (growing cells). The ^{18}O labeling experiments also revealed that the sulfonate is formed via dimerization and disproportionation of the sulfinate.

New methods for isolation of the sulfur-containing anions were developed in this project. Larger amounts of the metabolic intermediates were recovered by anion exchange techniques that would preserve all the labeling from oxygen isotopes by preventing exchange and avoiding displacement during derivatization for the gas chromatography-mass spectrometry (GC-MS) analysis. Previously, the analytical method involved cyclization of the sulfinate and sulfonate intermediates which resulted in the loss of one oxygen. The new method allowed the EERC to obtain better mass balances as well as more reproducible and more interpretable data.

Despite a number of attempts to prepare active immobilized desulfurization enzymes, as described in the milestones for the project, the cell-free systems were inactive. It is not known whether the enzymes were damaged during breakage of the cells, or whether some essential coenzyme or other component was lost in the processing. Further work in obtaining immobilized enzymes is continuing in the subsequent cooperative agreement work.

TASK 4.6 - BIODESULFURIZATION

1.0 INTRODUCTION

Conventional catalytic hydrodesulfurization involves high costs largely because of heavy metal deactivation of the catalysts. A potential lower-cost treatment is microbiological or enzymatic desulfurization. Recent advances at the Energy & Environmental Research Center (EERC) at the University of North Dakota have improved our understanding of sulfur-specific microbial desulfurization pathways in *Rhodococcus* bacteria, but further work is needed to develop a technology based on biodesulfurization.

2.0 OBJECTIVES

The main focus in this task is the application of desulfurization enzymes to remove sulfur from crude oil, diesel fuel, or resid precursors for needle cokes. The most important question to be answered is how to utilize the *Rhodococcus* desulfurization complex in a nonaqueous solvent or medium, such as the oil itself. Successful application of nonaqueous enzymology to this problem will involve finding ways to stabilize the active conformations of the enzymes and provide for easy recovery of the enzymes, perhaps in an immobilized-enzyme packed-bed reactor. Understanding the desulfurization activity will require that we determine the regulatory and mechanistic properties of the enzymes.

3.0 RESULTS

Dibenzothiophene (DBT) has been widely accepted as a suitable model compound for the thiophenic sulfur present in fuels. Microbial metabolism studies of DBT have demonstrated three types of aerobic microbial attacks. The first described metabolism attacks the carbon ring of DBT to form hydroxylated derivatives, ultimately with ring breakage (1). Very little DBT is desulfurized by this method, and the heating value of the fuel is lost during oxidation of the ring carbon (2). This pathway, known as the Kodama pathway, has been shown to be mediated by naphthalene degradative enzymes (3) and is an example of cometabolism, as the microbes cannot grow using DBT (4).

Another carbon-destructive method of DBT metabolism was described by Van Afferden et al. (5). This pathway results in the intermediate formation of benzoate and, ultimately, in mineralization of the DBT. This metabolism is not cometabolic, as the bacterium can grow using DBT.

The third metabolic pathway for DBT was postulated early as the 4S pathway (6). This pathway was shown to involve oxidation of DBT to the sulfoxide, sulfone, and finally to mono- or dihydroxybiphenyl and sulfate. A sulfonate intermediate was postulated as the fourth "S" (sulfoxide, sulfone, sulfonate, sulfate). This pathway has been of interest because the sulfur is specifically attacked and removed, preserving the heating value of the fuel (7).

Desulfurization investigators had no difficulty demonstrating the production of sulfoxide and sulfone from DBT. In fact, these compounds can be readily formed from DBT by oxidizing agents such as hydrogen peroxide and hemoglobin and by liver mixed-function oxidases (8-10). The final, desulfurized product has variously been described as a mono- or a dihydroxybiphenyl (11, 7). However, until recently, the identity of the third "S" in the pathway was elusive. Olson et al. (12) showed that under nongrowth conditions, the products of sulfur-specific DBT metabolism were the sulfoxide, sulfone, 2'-hydroxybiphenyl-2-sulfinate and 2-hydroxybiphenyl, while growing cells produced the sulfoxide, sulfone, 2'-hydroxybiphenyl-2-sulfonate, and 2,2'-dihydroxybiphenyl. Based on the identity of these desulfurization intermediates and growth experiments, a pathway was proposed (Figure 1, from Gallagher et al. [13]).

The identity of the sulfinate and sulfonate intermediates in DBT metabolism filled a gap, but left a number of unanswered questions. Since the actual compounds detected were the cyclic esters of these acids, viz., dibenz[*c,e*][1,2] oxathiin-6-oxide (sultine) and dibenz[*c,e*][1,2] oxathiin-6,6-dioxide (sultone), the actual form of the metabolite was not known. Additionally, although the data show formation of either the sulfonate and the corresponding phenol (2-hydroxybiphenyl) or the sulfinate and the corresponding phenol (2,2'-dihydroxybiphenyl), the mechanism of these reactions is not known.

4.0 RESULTS AND DISCUSSION

Desulfurization in Rhodococci

The experiments reported here were performed using *Rhodococcus* sp. IGTS8 (ATCC 53968). We have also tested several other rhodococci strains (UMX9, courtesy B. Ward, University of Mississippi; Q1a-22 and N1-43, courtesy S. Krawiec, Lehigh University) and have identified the same intermediates and products from these microbes. Other investigators have reported on similar sulfur-specific microorganisms. Omori et al. (14) reported *Corynebacterium* sp. strain SY1 that produced the sulfoxide, sulfone, sulfite, and 2-hydroxybiphenyl from DBT. Izumi et al. (15) reported that *Rhodococcus erythropolis* D-1 metabolized DBT to the sulfone and 2-hydroxybiphenyl. Further work with cell-free extracts of this strain demonstrated that NADH is required for desulfurization (16).

Cyclic Ester or Acid Intermediates

The sulfinate and sulfonate intermediates can be present either as the cyclic esters (sultine and sultone, respectively), or they may be present as the acid form. These forms, shown in Figure 2, are in equilibrium with a very acidic pKa. Analysis of culture supernatants for intermediates was routinely accomplished by extraction with ethyl acetate. Extractions conducted at neutral pH yield unmetabolized DBT, sulfoxide, sulfone and 2-hydroxybiphenyl. Acidifying the culture supernatant to a pH of about 1 followed by extraction yields the sultine and/or sultone. Since the sultine and sultone will readily partition into the ethyl acetate at neutral pH, the intermediates are present in the culture media as the sulfinate and sulfonate.

Figure 1. Pathway of desulfurization in *Rhodococcus* sp. IGTS8 (ATCC 53968) (13).

Figure 2. Equilibrium between cyclic ester and acid forms of the biphenylene sulfinate and sulfonate.

Inducibility

Cells are routinely grown using low, but sufficient, amounts of sulfur (0.2 mM). Cells grown with sulfate as the sulfur source are capable of desulfurizing DBT, but cells grown with DBT are about 4 times more active in desulfurizing. Other organic sulfur compounds serve as suitable sulfur sources, but may or may not be induced to desulfurize DBT. For example, cysteine-grown cells do not appreciably desulfurize DBT, while cells grown with dimethylsulfoxide desulfurize at rates similar to DBT-grown cells.

Mechanism of Formation of the Sulfinic/Sulfonate

In order to elucidate further the pathway on DBT metabolism, we synthesized ^{18}O -labeled DBT sulfone. If the reaction pathway from sulfone were via Reaction G, the sultone produced after acid-catalyzed cyclization would contain a single labeled oxygen. However, if the reaction proceeded via B, half of the molecules would be singly labeled, while the other half would be unlabeled as a result of oxygen loss and subsequent displacement during cyclization in the analysis. Isotope exchange or disproportionation can result in deviations from predicted distributions.

Table 1 shows the predicted and found distribution of ^{18}O -label in two metabolic experiments. Initial experiments with the labeled substrate were conducted with stationary-phase cells using a 4-hour incubation time. The mass spectrum of the sultone isolated from the culture supernatant showed that 73% of the sultone was singly labeled, eliminating Reaction B (less than 50% expected). Thus the pathway must proceed directly from sulfone to the sulfinic acid (Reaction G), rather than as a reduction to sulfoxide and oxidation to the sulfinic acid. Since somewhat less than the theoretical percentage of singly labeled sultone was observed (87% based on the actual enrichments in the starting material), some exchange of ^{18}O with water or other species must have occurred.

Reaction G is actually a reduction with respect to the sulfur. Electrons from the cleavage of the carbon-sulfur bond flow to the sulfur, forming the sulfinic anion. Precedence for this biochemistry comes from reactions where attack of nucleophilic oxygen occurred at the ring carbon at high temperatures in the presence of crown ether to form 2'-hydroxybiphenyl-2-sulfinate (17). Biochemically, the mechanism probably involves addition of oxygen at the ring carbon via a hydroperoxyflavin form of activated oxygen.

The formation of sulfonate by oxygen addition to the sulfinic would not be expected to change the number of labeled oxygens on the sulfur, resulting in a doubly labeled sulfonate. The loss of one oxygen during the cyclization to sultone should give one-third double-labeled and two-thirds single-labeled, assuming 100% isotopic purity in the starting material. The distribution of the label in the sultone from this experiment suggested that the sulfonic acid must have been primarily triply labeled. In addition, the results shown as Trial 1 in Table 1 revealed a small amount of oxygen exchange, as shown by the decreases in ^{18}O and increases in ^{16}O (18).

Since cyclization of the sulfinic and sulfonate results in the loss of one oxygen, a method was needed for recovery and mass spectral analysis of the oxygen in the sulfinic and sulfonate that does not lose labeled oxygen during the analysis. The method we have developed uses a strong

TABLE 1

Results of Metabolism of ^{18}O -Labeled Dibenzothiophene Sulfone
(Distribution of label is based on the mechanism outlined in Figure 3.)

Trial	Compound	Predicted Enrichment		Actual	
				Enrichment	
	DBTO ₂ - Starting material	---		$^{18}\text{O}_2$	77%
				$^{18}\text{O}_1^{16}\text{O}_1$	20%
				$^{16}\text{O}_2$	2%
1	Biphenylene sultine	$^{18}\text{O}_1^{16}\text{O}_1$	87%	$^{18}\text{O}_1^{16}\text{O}_1$	73%
		$^{16}\text{O}_2$	12%	$^{16}\text{O}_2$	27%
1	Biphenylene sultone	$^{18}\text{O}_2^{16}\text{O}_1$	76%	$^{18}\text{O}_2^{16}\text{O}_1$	57%
		$^{18}\text{O}_1^{16}\text{O}_2$	20%	$^{18}\text{O}_1^{16}\text{O}_2$	33%
		$^{16}\text{O}_3$	2%	$^{16}\text{O}_3$	10%
2	Methylated sulfinate - methylated on resin	$^{18}\text{O}_2$	77%	$^{18}\text{O}_2$	75%
		$^{18}\text{O}_1^{16}\text{O}_1$	20%	$^{18}\text{O}_1^{16}\text{O}_1$	23%
		$^{16}\text{O}_2$	2%	$^{16}\text{O}_2$	2%
2	Sultone - acidified off resin	$^{18}\text{O}_2^{16}\text{O}_1$	76%	$^{18}\text{O}_2^{16}\text{O}_1$	76%
		$^{18}\text{O}_1^{16}\text{O}_2$	22%	$^{18}\text{O}_1^{16}\text{O}_2$	22%
		$^{16}\text{O}_3$	2%	$^{16}\text{O}_3$	2%

anion exchange resin in the incubation mix. Anionic intermediates are bound to the resin as they are formed or as they are released from the cells. At the conclusion of the incubation, the resin is removed from the aqueous suspension; the cells are removed by washing; and the resin is treated with methyl iodide in benzene. Derivatized intermediates were analyzed by gas chromatography-mass spectroscopy (GC-MS). Methylation of the sulfinate gave methylhydroxybiphenylsulfone. The sulfonate was not methylated by these reactions and was recovered as the sultone.

The data from Trial 2 show that the distribution of label on the recovered methylated sulfinate was very close to what was predicted by Reaction G (Table 1). Further, the label distribution in the sultone isolated from the resin was in agreement with that of the earlier experiment. These data are readily explained by dimerization and disproportionation of the sulfinic acid (19). During the disproportionation, one of the labeled oxygens is transferred from one sulfur to the other. The label enrichment predicted by this mechanism, shown in Figure 3, was found exactly (Table 1). Note that the disproportionation reaction predicts the formation of a sulfenic acid. We have not yet found evidence for a sulfenic acid; however, this compound may be rapidly oxidized to the sulfinate by the desulfurization enzymes. The production of sulfonate via this disproportionation reaction suggests that this reaction is nonenzymatic.

Note that the amount of oxygen exchange observed in intermediates isolated from the anion exchange resin is very small. Thus this method has been shown to be excellent at accumulating intermediates, both anionic and phenolic, and reducing isotope exchange prior to analysis.

Figure 3. Mechanism of formation of biphenylene sulfinate and sulfonate.

Formation of 2,2'-Dihydroxybiphenyl

Although both Reactions C and F are desulfurization reactions that lead to the production of phenolic end products, the two reactions are very different. Reaction C is a reductive cleavage that leaves a hydrogen in place of the sulfur. Reaction F, however, is an oxidative attack that leaves a hydroxyl in place of the sulfur. In the observed chemistry, Reaction F is very similar to Reaction G, which forms the sulfinic acid from sulfone. Since the desulfurization pathway has only three enzymes, it is possible that the same enzyme that catalyzes Reaction G also desulfurizes the sulfonate (20). Under stationary-phase conditions, the enzyme catalyzing Reaction C is present and active. This enzyme rapidly desulfurizes sulfinic acid, preventing its accumulation. Under growth conditions, the Reaction C enzyme is absent or inhibited. Sulfinic acid accumulates and undergoes disproportionation to form sulfonate. Accumulated sulfonate is then available to be metabolized by the sulfinic acid-forming enzyme.

This hypothesis provides a reasonable explanation for the fact that mono- and dihydroxybiphenyl have both been found as the products of sulfur-specific desulfurization. This suggests that the biochemistry of microorganisms producing the two different end products is the same. Microorganisms lacking Enzyme C produce dihydroxybiphenyl, while those having this enzyme produce monohydroxybiphenyl.

Form of Inorganic Sulfur

We previously reported on efforts to determine the form of sulfur released during desulfurization (13). Although the desulfurization mechanism postulated suggests that sulfite should be formed, we have not been able to demonstrate sulfite. Only small, substoichiometric amounts of sulfate have been detected. We suggest that sulfite is rapidly oxidized, probably intracellularly, to sulfate. Much of the sulfate produced is used for growth. Sulfite as a product of sulfur-specific desulfurization has been reported by several other investigators (14, 21).

Desulfurization Pathway

Based on the ^{18}O label experiments, we suggest a revised sulfur-specific desulfurization pathway, shown in Figure 4.

Immobilized enzymes

Despite a number of attempts to prepare active immobilized desulfurization enzymes, as described in the milestones for the project, the cell-free systems were inactive. It is not known whether the enzymes were damaged during breakage of the cells, or whether some essential coenzyme or other component was lost in the processing. Further work in obtaining immobilized enzymes is continuing in the subsequent cooperative agreement work.

Figure 4. Revised pathway of desulfurization by *Rhodococcus* sp. IGTS8.

5.0 SUMMARY AND CONCLUSIONS

Sulfur-specific desulfurization of DBT is a property distributed among a number of taxonomically related bacteria. Desulfurization proceeds via stepwise oxygenation to the sulfoxide and sulfone. The sulfone sulfur is reductively cleaved to form the sulfinic acid. The sulfinic acid is desulfurized to form 2-hydroxybiphenyl. Some sulfinic acid can dimerize and disproportionate to form sulfonic acid. The sulfonic acid is desulfurized by a mechanism similar to the carbon-sulfur cleavage of the sulfone to form the 2,2'-dihydroxybiphenyl. The regulation mechanism for the formation of 2,2'-dihydroxybiphenyl under growth and 2-hydroxybiphenyl under nongrowth conditions is not yet known. However, based on the mechanism of formation of the sulfonic acid, it is likely that the activity of the final enzyme in the pathway is inhibited. Inhibition of this enzyme will result in accumulation of sulfinic acid and formation of sulfonate. The sulfonate formed is desulfurized by an enzyme similar to the enzyme catalyzing Reaction B (formation of the sulfinic acid).

The differing products of desulfurization are readily explained by microorganisms that lack the final enzyme (2,2'-dihydroxybiphenyl is formed) or possess the final enzyme (2-hydroxybiphenyl is formed).

6.0 ACKNOWLEDGMENT

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