Biodesulfurization of fossil fuels
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Biotechnological techniques enabling the specific removal of sulfur from fossil fuels have been developed. In the past three years there have been important advances in the elucidation of the mechanisms of biodesulfurization; some of the most significant relate to the role of a flavin reductase, DszD, in the enzymology of desulfurization, and to the use of new tools that enable enzyme enhancement via DNA manipulation to influence both the rate and the substrate range of Dsz. Also, a clearer understanding of the unique desulfirase step in the pathway has begun to emerge.

Introduction

The development of biotechnologies aimed at upgrading the quality of fossil fuels has been an area of interest for at least two decades (for recent reviews see [1,2]). Much of this attention has focused on microbiological/biochemical means of desulfurizing fossil-fuel streams. The impetus for this work was the discovery of a highly specific metabolic pathway in the organism Rhodococcus erythropolis strain IGTS8. This organism is capable of obtaining nutritional sulfur from common heterocyclic molecules in a fuel without causing oxidative loss of carbon from the fuel itself. A biological approach to desulfurization has also been motivated by difficulties in reducing sulfur to very low levels using current petroleum refinery practices (hydrodesulfurization). These low levels are required by governmental regulations, and in the next few years the regulations are expected to become even more stringent. Crude oil contains a large number of molecules that contain sulfur; however, the major sulfur-containing molecules in the middle distillate fraction (diesel fuel) are alkylated dibenzo thiophenes (DBT). The complete removal of sulfur from DBT-like molecules requires four enzymes. Two of these enzymes, DBT monoxygenase (DszC or DBT-MO) and DBT-sulfone monoxygenase (DszA or DBTO2-MO), are flavin-dependent. These both require a third enzyme (the flavin reductase DszD) for activity. The fourth enzyme, HPBS desulfinase (DszB), completes the reaction sequence, which results in a phenolic product and SO3<sup>2-</sup> (Figure 1). The genes for these enzymes have been cloned, sequenced and engineered from a variety of microorganisms.

Within the past few years several new flavin reductases, including thermo-tolerant enzymes, have been discovered and a more comprehensive picture of how these enzymes participate in the oxygenation reaction is emerging. In addition, the least well understood enzyme in the pathway, HPBS desulfinase, is becoming better characterized. On the basis of both published and unpublished data, we present a speculative model of the mechanism of action of this enzyme as a starting point for further research.

A significant stumbling block to the commercialization of biodesulfurization is the rate at which whole bacterial cells can remove sulfur. The throughput of substrates in this pathway may be hindered at several steps, including substrate acquisition [3], the supply of reducing equivalents [4,5] and enzyme turnover rates for specific substrates [6]. Recently, directed evolution techniques have been applied to improve both the rate and extent of biodesulfurization by derivatives of R. erythropolis strain IGTS8 [7,8]. Application of these techniques, together with the progress cited above, promises to improve both the range and robustness of this microbiologically catalyzed chemical transformation.

In this review, we summarize published and unpublished results of the past three years on the flavin reductase required for monoxygenase activity, the recent discovery of additional Dsz pathways and enzymes, the use of selective and in vitro genetic tools to recover improved or novel Dsz phenotypes, and on the final enzyme in the pathway, which has a novel catalytic activity and for which we propose some enzyme reaction mechanisms.

Abbreviations

DBT dibenzothiophene
DBT-MO DBT monoxygenase
DBTO2-MO DBT-sulfone monoxygenase
FMNH<sub>2</sub> reduced flavin mononucleotide
HPB 2-hydroxybiphenyl
HPBS 2-(2'-hydroxyphenyl)benzenesulfonic acid
PBS 2-phenylbenzene sulfinate
RACHITT random chimeragenesis on transient templates
TPL tyrosine-phenol lyase

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DszD: the flavin reductase

Oxidative desulfurization of dibenzothiophene requires reducing equivalents in the form of NADH and reduced flavin mononucleotide (FMNH$_2$). As with other biological reactions that involve oxygen insertion, reducing equivalents are necessary to activate molecular oxygen. Presumably, the activated oxygen species is a flavin hydperoxide that catalyzes the transformation of DBT to DBT sulfoxide and DBT sulfone via DBT-MO and the subsequent conversion of DBT sulfone to HPBS via DBTO$_2$-MO. Neither DBT-MO nor DBTO$_2$-MO are activated oxygen species is a flavin monooxygenase.

As DszD uses FMN as a dissociable substrate it has been classified as a member of the class I flavin reductases [13*]. This class is further subdivided on the basis of sequence similarities. One subgroup, typified by the well-characterized Escherichia coli Fre, is involved in the activation of ribonucleotide reductase [13*,14,15]. Other members of this subgroup include the flavin reductases from bioluminescent bacteria [16,17,18*]. A second subgroup, to which DszD belongs, contains enzymes involved in the synthesis of certain antibiotics; these enzymes include ActVB [17,19,20], SnaC [21], VlmR [22], HpaC for the oxidation of 4-hydroxyphenylacetate in E. coli [23] and cB for the degradation of nitroloacetic acid in Chelatobacter heintzii [24,25]. Detailed structural and mechanistic information is becoming available for these classes of flavin reductases [13*,14,15]. The data suggest that the enzymatic mechanism of the ActVB and DszD proteins is ordered-sequential, where NADH binds first [13*] (J Emmanuelle, personal communication). Free reduced flavin is very unstable and autooxidizes extremely quickly; it is therefore being proposed [18*,26] that some sort of protein–protein interaction occurs between the reductase and its cognate oxygenase to stabilize the reduced flavin and allow oxygen insertion.

Recently, Ishii et al. [27] have isolated and cloned a flavin reductase (tdsD) involved in desulfurization by the thermophilic desulfurizing bacterium, Paenibacillus sp. A11-2. TdsD has high sequence similarity to other flavin reductases like Fre, but there is no detectable sequence similarity between TdsD and the Rhodococcus DszD.
In addition, the tdsD gene is not located in the flanking region of the tds operon encoding TdsA and TdsC monoxygenases.

In certain cases it has been shown that the rate-limiting factor in complete desulfurization is flavin reductase activity; hence, overexpression of an appropriate reductase enhances desulfurization. Interestingly, Ohshiro et al. [28] report that different flavin reductases have different abilities to couple to the monooxygenases. In fact, they show that the flavin reductases from a non-desulfurizing bacterium are superior to those from a DBT-desulfurizing bacterium, suggesting more efficient transfer of FMNH₂ from the reductase to the monooxygenases. This information may be beneficial in developing the most efficient enzymatic desulfurization system and in elucidating the mechanisms of interaction between the monooxygenases, reductases and reduced flavin species.

Recent discovery of additional dsz pathways and enzymes

Although R. erythropolis is the most extensively studied example, several other mesophilic microorganisms have also been shown to catalyze oxidative desulfurization; these include Sphingomonas [29], Corynebacterium [30], Gordona [31], Klebsiella [32] and Nocardia [33]. Duarte et al. [34] have performed genetic analyses (using PCR and denaturing gel electrophoresis) of the bacterial community found in oil-contaminated soils. They observed that highly polluted samples contain organisms related to actinomycetes, Arthrobacter and an unidentifiable microorganism. The dsz genes (A, B and C) were present in all soil samples and the dszA sequences obtained following PCR revealed a great similarity (>95%) to dszA from strain IGTS8. However, the PCR primers used in these experiments were designed using Rhodoctococcus dsz sequences, potentially biasing the results. These genes did not appear to be present in experiments on non-contaminated soils.

In the past few years it has been recognized that the use of a thermophilic organism might be extremely advantageous for a large-scale industrial process. Elevated temperatures may provide the following advantages: first, the potential for improved enzymatic rates; second, a decrease in bacterial contamination; and third, overall improvements in biocatalyst stability. Thermophilic desulfurization has been shown to occur in Paenibacillus sp. [27,35–38] and Mycobacterium phlei [39–41]. Both of these strains function at temperatures >50°C. It is interesting to note, however, that the dszABC genes of M. phlei GTIS10 are identical in DNA sequence to those from IGTS8 even though the temperature optima and stability of the strains differ by almost 20°C [39]. This implies that there are other factors in the whole-cell reaction that contribute to the thermostability of the reaction pathway.

The use of selective and in vitro genetic tools to recover improved or novel Dsz phenotypes

The emergence of new in vitro tools for mutation and genetic rearrangement [42,43] and the clever application of some older ones [7] have enabled the limits of the biodesulfurization system to begin to be explored. Fossil fuels contain, in addition to alkylated DBTs, a variety of alkyl and aryl sulfides, thiophenes and benzo thiophenes [44,45]. Arensdorf et al. [7] used model compound assays to explore the limits of biodesulfurization for these classes of non-DBT sulfur compounds. They chose several models (5-methyl benzo thiophene [5-MBT], 2,3,5-trimethyl thiophene, and octyl sulfide) that were not substrates for the R. erythropolis strain IGTS8 Dsz system. Gain-of-function mutations were isolated for octyl sulfide utilization (dszA Q345A) and 5-MBT utilization (dszC V261F). Further, saturation mutagenesis of dszC at amino acid residue 261 showed that none of the other 19 possible substitutions resulted in the ability to use 5-MBT.

Coco et al. [8] introduced a method of in vitro recombination to generate libraries of evolved enzymes — random chimeragenesis on transient templates (RACHITT). In this method, randomly cleaved parental DNA fragments are annealed to a transient polynucleotide scaffold. This technique appears to have advantages in the diversity it can generate, the fact that closely linked alleles are easily recombined, and the fact that parental sequences are virtually assured to be absent in the recombinant pool [43]. Coco et al. demonstrated the method by carrying out in vitro recombination of dszC genes derived from two distinct genera, Rhodococcus and Nocardia. The DBT-MO derived from Nocardia had been shown to have a higher substrate affinity and/or substrate range for complex alkylated derivatives of dibenzo thiophene that were otherwise poorly converted. The DBT-MO from Rhodococcus, by comparison, had been shown to have a higher specific reaction rate for sparsely alkylated DBTs [7]. Coco et al. were able to isolate recombinant clones from a dszC-family recombinant library encoding altered DBT-MO proteins with significantly improved rates of substrate turnover. Of 175 unselected recombinants tested, the six best were confirmed by further testing to have significantly higher activity than the parents. All were found to be chimeras by DNA sequencing. One clone was found to be better at desulfurizing diesel, but not the model compound, DBT. Variants from the same library were isolated that displayed both higher rates and more extensive substrate oxidation, showing the ability of RACHITT to generate clones with multiple simultaneous improvements.

HPBS desulfinase

The last step in the pathway, which is catalyzed by HPBS desulfinase, is the actual desulfurization of condensed thiophenic substrates. A unique aspect of this reaction is
that it results in carbon–sulfur bond breakage between a phenyl and a sulfonic acid group. There are reports in the literature of enzymatic carbon–sulfur bond breakage between a phenyl and a sulfonic acid group; however, this is proposed to be catalyzed by a monooxygenase [32]. There are also reports of enzymatic carbon–sulfur bond breakage between aliphatic carbon and the sulfonic acid group; however, this tends to be the result of alternative activities of enzymes that have an absolute catalytic requirement for pyridoxal 5'-phosphate (PLP) [47,48]. HPBS desulfinase is not a monooxygenase, nor does it require PLP.

Nakayama et al. [49**] cloned, expressed, purified and characterized an HPBS desulfinase from R. erythropolis strain KA2-5-1. They also report on the inactivation of the enzyme by mutation of the sole cysteine to serine. Chemical modification of cysteine has also been performed, which resulted in inactivation of the enzyme except in the presence of HPBS, 2-hydroxybiphenyl(HPB) and 2-(2'-hydroxyphenyl)benzenesulfonate (HPBSO) (GT Mrachko, unpublished data) [50]. Therefore, a catalytic or binding role is implicated for cysteine.

The use of alternative substrates and/or inhibitors of the desulfinase can provide a substantial amount of information. 2-Phenylbenzene sulfinate (PBS) is a substrate, as evidenced by the measurement of the product biphenyl by GC-MS [49**]. The $k_{cat}$ was similar to that measured for HPBS, but the $K_m$ was nearly eightfold greater. An interesting inhibition result in light of the catalytic efficiency of the desulfinase on PBS is the observation that naphthosultam inhibits the desulfinase, whereas naphthosultone is not an inhibitor [50]. The specific difference between the sultam and sultone lies in the fact that the sultam has a proton available for hydrogen bonding with an active site residue. The fact that the catalytic efficiency of the desulfinase is eightfold diminished with

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**Figure 2**

The proposed mechanism for TPL [52], which is used as a model for two working hypotheses for the desulfinase. (a) The proposed mechanism for TPL. (b) One working hypothesis for the desulfinase, general acid-catalyzed desulfination, in which the substrate hydroxyl group is involved in substrate recognition and binding. (c) Another hypothesis, proton-transfer-assisted desulfination, in which the hydroxyl group is involved in catalysis. The reaction is shown using the substrate 5,5'-dimethyl-2-hydroxybiphenyl-2'-sulfinate to illustrate the inductive effect of a para methyl group on the stabilization of the carbocation formed during the proposed protodesulfination.
HPBS as a competitive inhibitor, with a Ki comparable to that of naphthosultam inhibits the enzyme, is a possible indication that the hydroxyl group of HPBS has a role in binding, at least, as depicted in Figure 2b. Another result in support of this working hypothesis is the finding that HPBSO is a competitive inhibitor, with a Ki comparable to that of HPBS (GT Mrachko, unpublished data), but that biphenyl-2-carboxylate was found not to be an inhibitor [50]. A catalytic role for the substrate hydroxyl involving electron localization by a proton-transfer step to facilitate electrophilic substitution of the sulfinate by a proton, as described in Figure 2c, may not be the case, given the comparable $k_{cat}$ for HPBS and PBS and the apparent requirement of a proton-donating substituent for enzyme inhibition. The alkyl-substituted HPBS compounds 3,3′-dimethyl-2-hydroxybiphenyl-2′-sulfinate and 5,5′-dimethyl-2-hydroxybiphenyl-2′-sulfinate, which are derived from the action of the two Dsz monoxygenases on 4,6-dimethyl dibenzothiophene and 2,8-dimethyl-dibenzo thiophene, respectively, are eight times more reactive with the desulfinase than is HPBS (GT Mrachko, unpublished results). These results lend themselves in support of the reaction mechanisms depicted in Figure 2, as a methyl group ortho to para to the substitution site is an activating substituent for this proposed protodesulfination electrophilic substitution. The regiosomeric distribution of methylated HBP produced from asymmetrically methylated DBT could provide further enlightenment with respect to the working hypotheses in Figure 2 [38].

A couple of working hypotheses are presented to describe an electrophilic substitution of the aryl sulfinate by a proton, with or without the assistance of a proton transfer involving the substrate hydroxyl group. The enzyme mechanism proposed for tyrosine-phenol lyase (TPL), as illustrated in Figure 2a, has been used as a model for such an enzyme reaction mechanism [51,52]. The results in the literature summarized here favor the reaction mechanism without a proton transfer step involving the hydroxyl group of the substrate; however, several mechanistic details remain to be elucidated to complete the proposal for the desulfinase modeled after TPL. A crucial experiment missing from the data is the determination of a pH-rate profile describing the dependence of $V/K$ and $V_{max}$ on $pH$; such a profile would indicate the $pK_a$ values for binding and catalysis, which might aid in the elucidation of structure–function relationships. A comparison of the $pH$ dependence of the chemical modification of cysteine with the dependence of $V/K$ and $V_{max}$ on $pH$ would aid in the illumination of the role for cysteine in the enzyme reaction mechanism. Furthermore, a pH-rate profile for the desulfination of PBS compared with HPBS, in addition to a determination of the $pH$ dependency of the inhibition by HPBSO, could aid in the description of the involvement of the substrate hydroxyl in binding. The catalytic base and the catalytic acid proposed in the TPL reaction are arginine and tyrosine, respectively [51,52]. Tyrosine has been implicated by chemical modification studies in the reaction catalyzed by the desulfinase [49,50]. Further experiments aimed at identifying a tyrosine as the catalytic acid in the desulfinase, which generate mutations at that position and couple these results to $pH$ dependence studies, should provide the next level of understanding of this unique enzyme. Naturally, a crystal structure, preferably with a bound inhibitor such as HPBSO, would provide a detailed picture of structure–function relationships.

**Conclusions**

Our understanding of how microorganisms metabolize sulfur heterocyclic compounds through the Dsz pathway is improving rapidly. However, we still need a far better understanding of all aspects of this pathway and of the attendant substrate-acquisition issues if this pathway is ever to be turned into a commercial process. It is evident that a more in-depth view of the enzymology of the process will be required, particularly as this relates to the supply of reducing equivalents to the monooxygenase steps. To some extent this knowledge will dictate the genetic approaches required to improve both the rate of desulfurization of the multiple thiophenic species present in fossil fuels and the extent to which these can be catalytically removed. Enzymology and microbiology together may offer a unique path to the total removal of sulfur from these petroleum streams if these problems can be overcome.

**References and recommended reading**

Papers of particular interest, published within the annual period of review, have been highlighted as:

* of special interest
** of outstanding interest


generating highly recombined genes and evolved enzymes.


9.  

A powerful new technique of molecular evolution that the authors use to efficiently isolate improved DBT monooxygenase variants using two related dzuC genes as parents. This work points the way to future improvements to the Dsz system.


