

PURIFICATION AND CHARACTERIZATION OF
2'-HYDROXYBIPHENYL-2-SULFINATE DESULFINASE (DSZB)
USING THE SUBSTRATE ANALOG THIOUREA DIOXIDE

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ABSTRACT

PURIFICATION AND CHARACTERIZATION OF 2'-HYDROXYBIPHENYL-2-SULFINATE DESULFINASE (DSZB) USING THE SUBSTRATE ANALOG THIOUREA DIOXIDE

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The DszB enzyme, found in *Rhodococcus erythropolis*, is part of a metabolic desulfurization pathway which can be used to remove harmful sulfur from crude oil. The mechanism of this enzyme was studied using inhibition, inactivation, and alternative substrate studies. The possibility of DszB using a covalent mechanism was dismissed but either a non-covalent mechanism or a reversible covalent mechanism is still a viable option for this enzyme. Further studies are needed to better distinguish between the two current mechanism theories.

Introduction

For each 42 gallons of crude oil pulled out of the ground, only about 12 gallons of distillate are useable for diesel fuel (1). Part of the oil refining process is distillation. This involves heating the oil, which then is evaporated, and when the vapor cools the condensed liquid, or distillate, is now purified of the particles the crude oil contained because the impurities do not melt at the same temperature as the oil compounds. This is done several times to get fractions of purity and these refined fractions are all used in different ways and products ranging from diesel fuel to any product containing petrochemicals. Altogether, sulfur compounds make up 0.03-8 % wt of crude oil (2).

These sulfur compounds can be found in most fossil fuel related materials including gasoline, diesel, and coal. All of those are used by various means of transportation ranging from vehicles to ships with the biggest consumer being electric power stations. When the sulfur compounds in fuel are combusted, harmful products formed with sulfur containing compounds such as sulfur dioxide, are released into the atmosphere. These sulfur compounds contribute to air pollution and acid rain, destroying precious resources and infrastructure through corrosion. Furthermore, almost all of the sulfur emitted into the air after combustion will be SO_2 , which aggravates previously existing respiratory conditions and heart conditions such as asthma and heart disease (3).

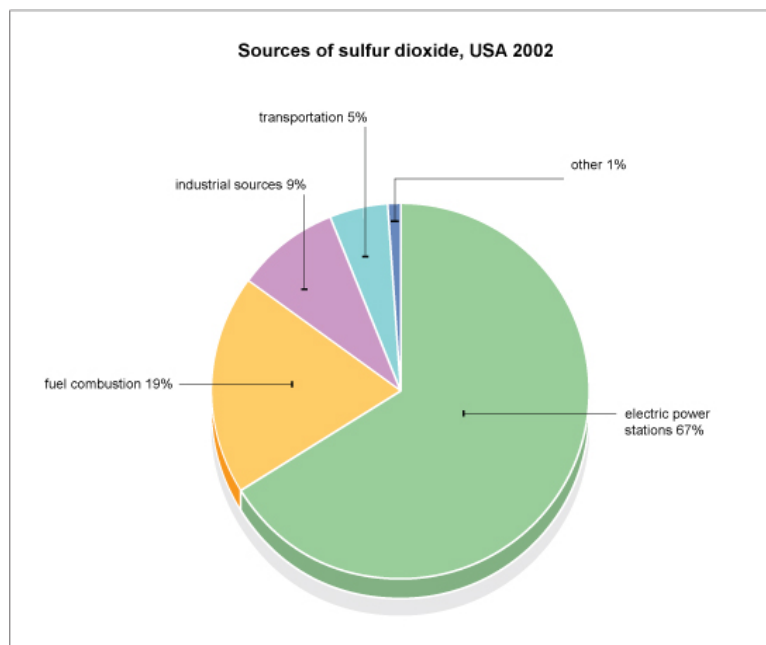


Figure 1. Chart of sources of sulfur dioxide production in the USA as of 2002. Electric power stations use 67%, fuel combustion use 19%, industrial sources use 9%, and transportation use 5%, with other as 1% (4).

Though most cars today are equipped with a catalytic converter, it only removes carbon monoxide, unburned hydrocarbons, and nitric oxides. Sulfur will actually poison the catalytic material and reduce efficiency of the converter (5). The higher sulfur content in the fuel, the more pollution is added to the atmosphere. It follows then, that the sulfur must be removed from the fuel before it can be sold to consumers. The current method of removing sulfur from the fuel is hydrodesulfurization (HDS). This is a physical means of removal involving high temperature and pressure and can remove inorganic or

nonaromatic organic sulfur compounds. But, HDS is not able to remove polycyclic aromatic sulfur compounds such as benzothiophene and dibenzothiophene (DBT) (Figure 2) and derivatives, which comprise up to 60% of the organic sulfur compounds found in fuel (6, 7, 8).

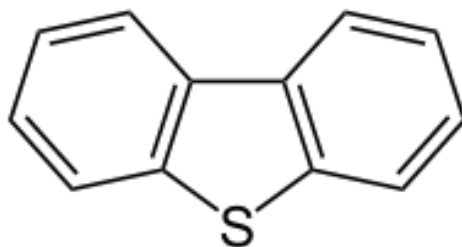


Figure 2. Structure of dibenzothiophene.

This molecule is the root of DBT and its derivatives.

Moreover, recent government regulations required a 97% decrease in sulfur, from the 500 parts per million (ppm) to its current standard of 15 ppm (Ultra Low Sulfur Diesel) for all highway traveling vehicles by 2010. Non-road, locomotive, and marine transportation modes will be subject to these regulations by 2014. Considering the US produces 5 million barrels and imports 9 million barrels of crude oil a day with 42 gallons of oil per barrel, all of which need to be processed, HDS cannot process it all (9). Not only can HDS not handle this strain, it costs consumers more (10). Therefore, new methods of desulfurization are being focused on, with emphasis on biodesulfurization.

Biodesulfurization is defined as the biochemical processes of using biological organisms to remove sulfur from a substance (11). This process can be done by a

number of organisms ranging from fungus to bacteria. The key breakthrough for bacterial biodesulfurization was the discovery of a metabolic pathway of the *Rhodococcus erythropolis* strain sp. IGTS8 (IGTS8). This bacterium was found in coal deposits and can remove sulfur from the polycyclic aromatic sulfur compounds without loss of fuel value as part of its natural metabolism. Other organisms such as *Rhodococcus erythropolis* strain sp. D-1, H-2, *Corynebacterim* sp. strain SY-1, and the *Gordona* sp. Strain CYKS1 have also been found to have DBT-desulfurizing activity, but IGTS8 is the most widely studied (12).

A recent study done by Torkamani, S. *et al* investigated biodesulfurization properties of the fungus species *Stachybotrys* of strains WS4, WK4, and WK2. While this species is detrimental to health, these strains of fungi grow in oil contaminated soil and use the large amount of sulfur in the oil as part of the necessary nutrients for growth. They found that these strains removed 76% and 64.8% of the sulfur of select fuels with initial sulfur content of 5 wt% and 7.6 wt% within 72 and 144 h, respectively. They also compared the WS4 strain with a bacterium, labeled as b-MS1-1. Both the fungus and bacterium reached a sulfur removal efficiency of 76%, but the bacterium took twice as long as the fungus to reach the same efficiency. Since these results are the first to report a fungi as having desulfurization properties more research needs to be done before it can be commercially implemented (13). Until then, bacteria are still the most studied and best source of biodesulfurization to-date. Once the bacteria are fully understood they may reach the same desulfurization potential as the fungi but with greater ease of use and quicker growth rates.

Through its metabolic process, the IGTS8 bacterium removes the sulfur from the aromatic compounds without loss of fuel value using four different enzymes; dszA, dszB, dszC, dszD. In this pathway, DszC (DBT-monoxygenase or DBT-MO) coupled with DszD (NADH-FMN oxidoreductase) oxidizes DBT (dibenzothiophene) to DBTO (DBT sulfone). The second oxidative reaction is catalyzed by another DszD coupled reaction but with Dsz A (dibenzothiophene-5,5'-dioxide monoxygenase or DBTO2-MO) instead, converting DBTO to HPBS (2-(2'-hydroxyphenyl)benzenesulfinate). Finally, the rate limiting step of the desulfinase enzyme (DszB) cleaves the carbon-sulfur bond to form HPB (2-hydroxybiphenyl) and sulfite (Figure 3) (8, 14).

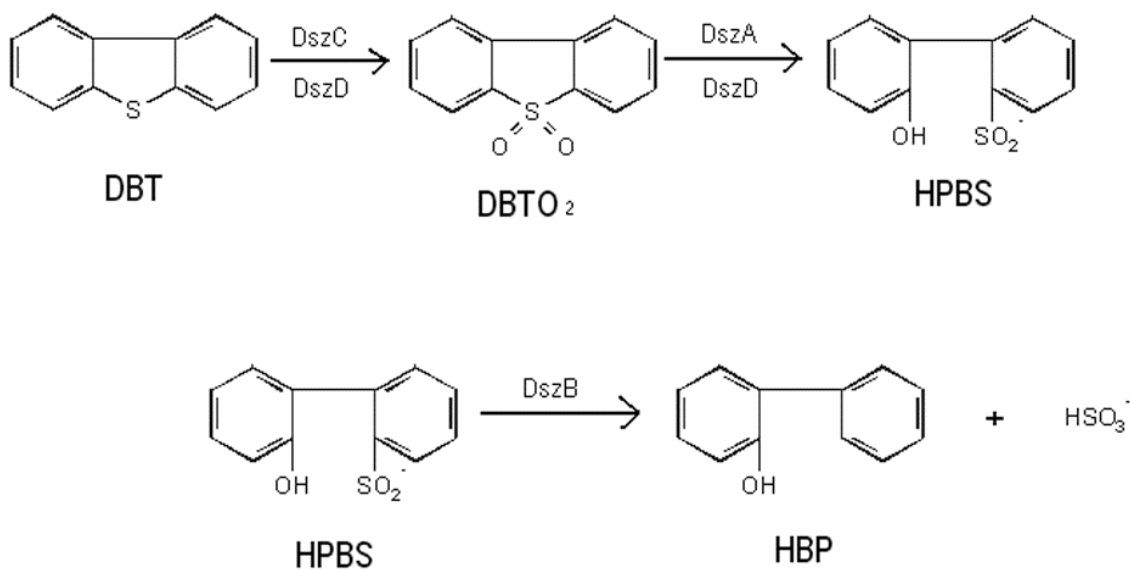


Figure 3. The Dsz metabolic pathway for desulfurization. DszB converts DBT to DBTO₂, DszA converts DBTO₂ to HPBS and DszB converts HPBS into HBP with sulfite release.

DszA codes for a protein of 453 amino acids that add up to 49,579 Da. DszB has 365 amino acids and those come out to 39,001 Da. DszC is coded into 417 amino acids and has a molecular weight of 44,977 Da (10). DszD, the flavin reductase, has 157 amino acids and those add up to 33,858 Da (15). The structure of the crucial enzyme, DszB, besides being amphipathic, has an overall fold similar to periplasmic substrate-binding proteins. These proteins consist of two globular domains, which together resemble a Venus fly trap. The enzyme is 46% helical with 23 helices and 15% beta sheets with 14 strands. The two domains almost evenly share the amino acids with domain 2DE2A0 having 152 residues and domain 2DE2A1 having 195 residues split into two fragments. The active site residues are Leu²²⁶, Gly¹⁸³, Trp¹⁵⁵, Phe²⁰³, Leu¹⁵², Pro²⁸, Phe⁶¹, Gly⁷³, Ser²⁵, Arg⁷⁰, Cys²⁷, and His⁶⁰ through conformational change (7).

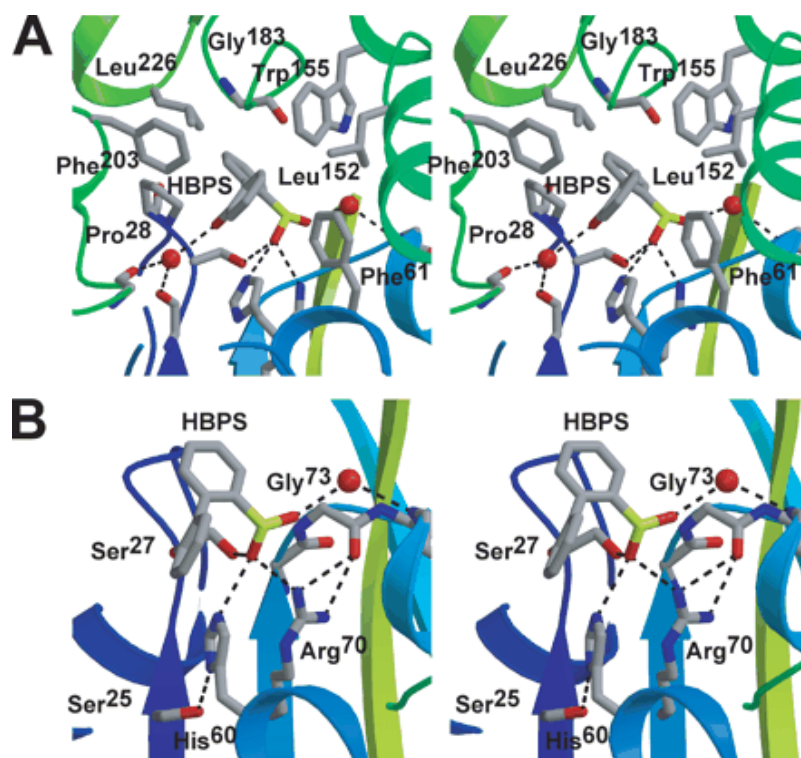


Figure 4. Crystal image of active site of DszB. A, shows the hydrophobic amino acids and their interactions with HPBS. B, shows the hydrogen bonding of the sulfinate group of HPBS. The active site residues participating in the specific interaction are shown as sticks while the domains are shown as ribbons.

As part of substrate binding, residues 55-62 and 187-204, which were loops in the pre-binding state, change into alpha helices to allow the bulky phenyl rings room in the active site. This conformational change brings the His⁶⁰ into the active site and near the Ser²⁵ (7).

Previous site-directed mutagenesis studies have indicated that the sole cysteine in the DszB active site is necessary for enzymatic activity, and thus plays a role in catalysis or binding (14). There are two main proposed mechanisms for the electrophilic substitution of the aryl sulfinate. The first mechanism (Fig 4) showed a base temporarily removing the proton off the hydroxyl group of the second aryl ring. This catalyzes electron relocalization and allows the sulfinate to be replaced by a proton via electrophilic substitution. The base is possibly thought to be histidine as it is the only possible base around the sulfinate (16).

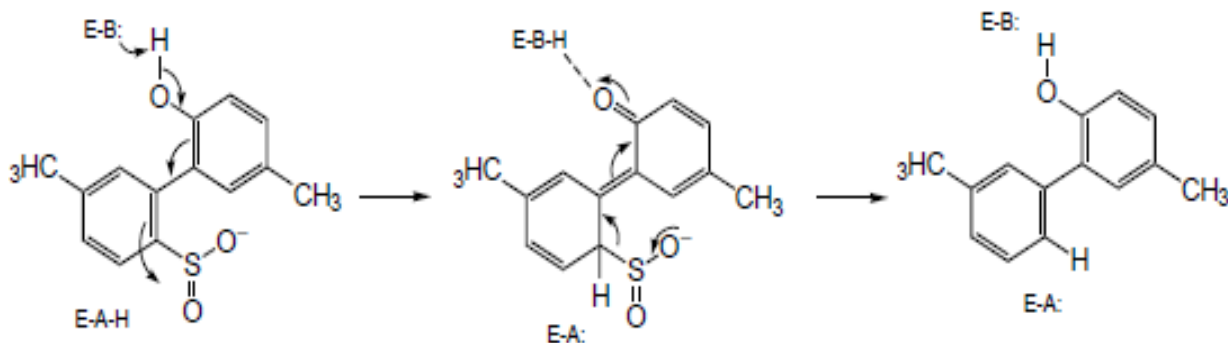


Figure 5. The non-covalent mechanism theory for desulfinase activity. An amino acid from DszB would act as a base and deprotonate the hydroxyl which would cause electron relocalization, making the SO_2 an excellent leaving group to be replaced with a hydrogen.

The second mechanism (Fig. 6) was first proposed after the histidine was mutated and some activity still remained. It was suggested that the cysteine, which is primed for nucleophilic attack on the sulfinate sulfur, is close enough for the

sulfinate group to act as the general base. In this mechanism the histidine is proposed to keep the sulfinate group in optimal positioning and orientation as to maximize the basicity of the sulfinate group. This combination of the sulfinate group and the cysteine forms a thiosulfonate-like intermediate as the first step of the reaction. Next, the unstable intermediate is protonated then hydrolyzed resulting in the release of the sulfinate group (14).

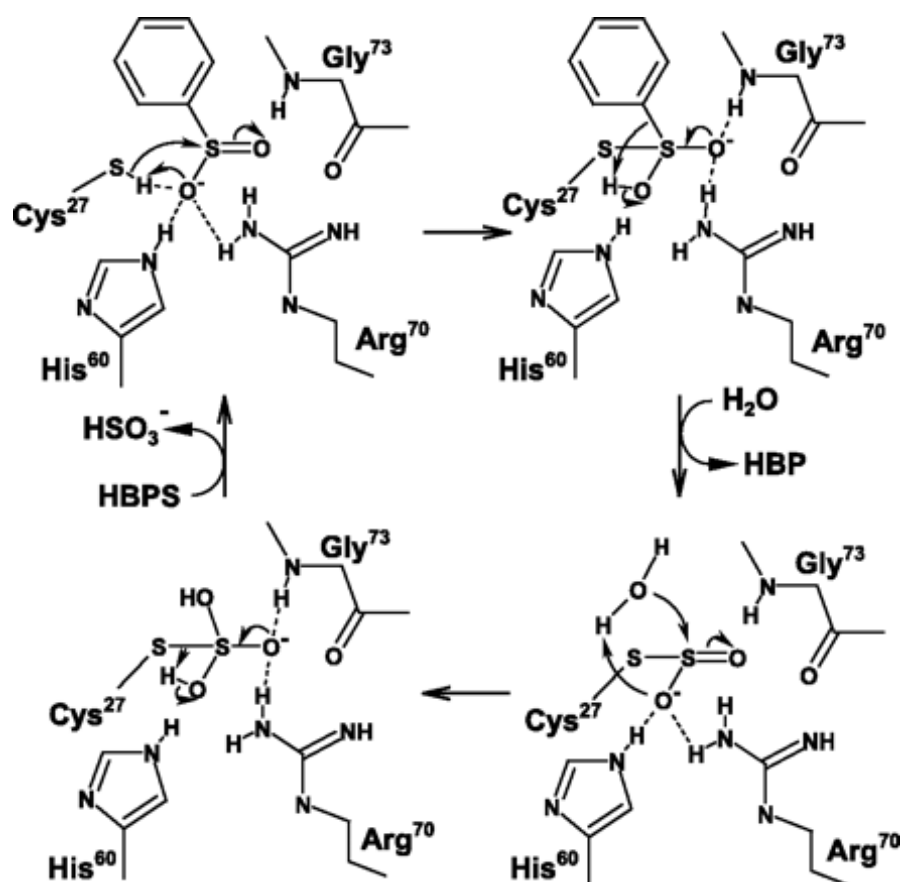


Figure 6. The covalent catalysis mechanism theory for desulfinase activity. This mechanism shows the His⁶⁰ holding the sulfinate group in position, allowing for an unusual sulfur attack on the sulfur of the sulfite. This forms an intermediate which collapses and cleaves the C-S bond, releasing sulfite.

Thiourea dioxide (TD), or aka Formamidine sulfinic acid, is a slow reductant in the presence of a base (Figure 7). It has a molecular weight of 108.11 and is usually found in the form of a powdery white crystal. It can be used as a destaining agent for fibers, ore melting, and metal refining in general. TD is similar structurally to urea but has a sulfur instead of an oxygen at the base of the double bonded oxygen (17).

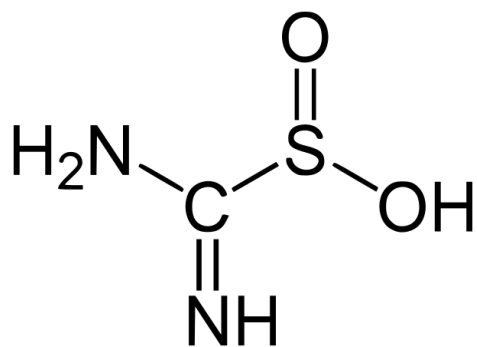


Figure 7. Thiourea dioxide

TD was chosen as an alternate substrate for DszB because of the sulfur-carbon bond (Figure 7). That bond closely resembles the bond HBPS has and the same product will be released, sulfite, if the bond is cleaved. If the enzyme follows the proton shuffling theory then either the bond will be cleaved and sulfite released, or the enzyme will be

slightly inhibited by the TD. On the other hand, if the enzyme follows the covalent catalysis theory, DszB will be completely inhibited by TD and the enzyme will show no activity (Figure 8).



Figure 8. Chart of possible mechanisms and results of inhibition using TD. If the results show product or inhibition, the mechanism is most likely the acid/base mechanism. If the results show complete inhibition, the mechanism is most likely the covalent mechanism.

The goal of this project was to use TD to further understand the mechanism of DszB to enhance the rate of desulfurization for commercialization. In a coupled assay, the release of sulfite, which directly corresponds to the amount of HBP produced by the DszB catalyzed reaction, will be quantitatively measured using cytochrome c and sulfite oxidase recombinantly expressed and purified from *Escherichia coli*. Thiourea dioxide

was used to test whether DszB uses a covalent (Figure 6) or noncovalent (Figure 5) catalysis mechanism.

MATERIALS AND METHODS

Supplies and Providers

The *Nocardia asteroides* A3H1 DszB gene was a gift from Energy Biosystems (The Woodlands, TX). The pREP4 plasmid was a gift from Dean Appling at University of Texas at Austin (Austin, TX). The pTAC-MAT-Tag®-1 Expression Vector, egg white lysozyme, nitrocellulose filter, imidazole, thiourea, 30% hydrogen peroxide, sodium sulfite (Na₂SO₃), LB broth, cytochrome c from equine heart, benzenesulfinic acid, and sodium monophosphate (NaH₂PO₂) were purchased from Sigma Aldrich (St. Louis, MO). BL21(DE3) ((F⁻ ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) *E. coli* cells were obtained from New England Biolabs (Ipswich, MA). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was acquired from BD Biosciences (San Jose, CA). Allegra 25R centrifuge was bought from Beckman Coulter (Brea, CA). The 400 MHz FT NMR was acquired from Bruker (Madison, WI). Ampicillin (Amp) was purchased from Amresco (Solon Industrial Parkway). Kanamycin (kan) and phenylmethanesulfonylfluoride (PMSF) were obtained from IBI Scientific (Peosta, IA). Vibra-cell Sonicator was acquired from Sonics (Newtown, CT). Sodium chloride (NaCl) was acquired from Mallinckrodt (Hazelwood, MO). Slide-A-lyzers were bought from Pierce (Rockford, IL). Sodium acetate was purchased from VWR (San Dimas, CA). Bovine serum albumin (BSA) was a gift from the lab of Dr. Rachel Booth at Texas State University. ProBond™ Nickel-Chelating Resin was obtained from Invitrogen (Carlsbad, CA). HPBS precursor was a gift from Herbert L. Holland at Brock University (St. Catharines, Ont – Canada). The Varian Spectrophotometer and Cary Eclipse software

program came from Varian, Inc. (Palo Alto, CA). The 96 well plates were bought from Evergreen Scientific (Los Angeles, CA). The NanoDrop 1000 Spectrophotometer V3.7 was procured from Thermo Fisher Scientific (Waltham, MA). The micro plate reader and Coomassie Brilliant Blue G-250 dye came from Bio Rad (Hercules, CA). The VisualEnzymics software was bought from SoftZymics (Princeton, NJ). The human sulfite oxidase clone pTG918 in *E. coli* strain TP1000 (MC4100; *AmobAB::kan* (Kn^R) was a gift from BiooScientific (Austin, TX). The UV/Visible spectrophotometer was acquired from Beckman Coulter (Brea, CA). The distilled deionized water (ddH₂O) was attained using a Milipore Water Filtration system from EMD Millipore (Darmstadt, Germany). The wash buffer was composed of 500 μM NaCl, 50 μM NaH₂PO₂, and 20 μM imidazole. The binding buffer was composed of the same components except the imidazole concentration was 10 μM. The elution buffer was composed of the same components except the imidazole concentration was 250 μM. Buffer A was composed of 20 μM NaH₂PO₂ and 500 μM NaCl. All buffers were at pH 8.0.

Activity Assay Protocol

Unless otherwise noted, all DszB activity assays followed the same procedure. In one well of a 96 well plate the following were added in order for each protein sample: Buffer A (25 μM NaH₂PO₂, 100 μM NaCl), 0.01 μM HPBS, and 20 μL DszB of varied concentration for a total volume of 200 μL. This sample was immediately assayed by the fluorescence spectrophotometer at an excitation wavelength of 280 nm and emission

wavelength of 414 nm for 0.13 s at 1 min increments for 10 min total. The activity of the sample is the linear slope of fluorescence change per min.

DszB Purification

The DszB gene from *Nocardia asteroides* (A3H1) had been previously cloned into the pTac MAT Tag expression vector by the Watkins lab at Texas State University (12). This plasmid was transformed into the BL21(DE3) cells already containing the pREP4 plasmid. The pREP4 plasmid was coexpressed to produce GroES and GroEL chaperone proteins needed for proper protein folding. The DszB gene containing cells were grown and the expressed protein (DszB) was purified according to the following protocol modified from Vaz (18). One colony of cells grown on a LB plate with 0.1 mg/mL of amp and kan at 37 °C were incubated overnight in 50 mL of the same media and antibiotic concentration at 37 °C shaking at 250 rpm. Five mL of the overnight culture were added to 500 mL of LB with the same antibiotic concentration and allowed to grow under the previously mentioned conditions till the OD₆₀₀ reached 0.6-0.8. The cells were then induced with 1 mM IPTG and gently stirred overnight at RT. The cells were then centrifuged at 4 °C, 6,000 rpm, for 15 min and the resulting pellet was resuspended in 10 mL of binding buffer. Next, 2 mg each of lysozyme and PMSF were added to the solution, which was swirled and incubated on ice for 30 min. The solution was sonicated at 50% amplitude for 10 s with 10 s on ice for a total of 4 times and centrifuged at 4 °C, 12,500 rpm for 30 min. The supernatant was filtered through a 0.2 µm nitrocellulose filter. The following column purification was done according to

Invitrogen's ProBond Purification System under native conditions (19). The column was prepared by washing it with 6 mL of ddH₂O then 12 mL of binding buffer. After the protein solution was applied, the column was gently shaken every 5-10 min for 30-60 min. The excess solution was released and the protein was eluted with 8 mL of elution buffer. An activity assay was done on each sample immediately.

DszB Quantification

The extinction coefficient of DszB was determined using Beer's law with an assumed pathlength of 1 nm, $A_{280} = \epsilon lc$. The A_{280} of each eluted protein sample was taken on a nanodrop spectrophotometer according to the protocol used for the NanoDrop 1000 Spectrophotometer V3.7 (20). The concentration of each sample was determined by a Bradford assay according to a modified protocol of the Bio-Rad protein microplate standard assay as follows (21). The Coomassie Brilliant Blue G-250 dye was first diluted to one-fourth concentration before 200 μ L were added to each microplate well for the entire assay. For the standard, the BSA was added and its concentration in each well ranged from 0 to 10 μ g/mL for a total of six wells. For the sample, 10 μ L of each were added to the 200 μ L of the dye for a total of eight wells. The mixtures were incubated at RT for 10 min. and the A_{595} was read on a microplate reader. The extinction coefficient was calculated using Beer's law as follows. $A = \epsilon lc$ with an assumed constant pathlength of 1 nm. The extinction coefficient was calculated for each sample and averaged.

Thiourea Dioxide Based Experiments

Thiourea dioxide (TD) was made and used in assays according to the following protocols. TD was synthesized by mixing 30 mg of sodium acetate and 0.5 g of thiourea in 9 mL of water and was brought to 5 °C. While stirring and on ice, 1.5 mL of 30% hydrogen peroxide was added dropwise over 30 min. The crystals were separated by gravity filtration and washed with 10 mL of methanol at 65 °C. The TD was dried in a desiccator for several days. The identity of the synthesized TD was analyzed by Carbon-13 NMR by the use of a 400 MHz FT NMR. This TD was used in a timed dialysis experiment and a substrate concentration experiment. The timed dialysis experiment was done according to the following protocol. Initial activity of the DszB sample to be used was assayed. TD was added to a 500 µL DszB sample at 0.029 mM for 700 µL total volume. Buffer A (71 mM NaH₂PO₂, 286 mM NaCl) was added to another 500 µL DszB sample for a total volume of 700 µL. These samples were incubated at RT for 10 min and assayed. Then each sample was dialyzed in Buffer A (25 mM NaH₂PO₂, 100 mM NaCl) using slide-a-lyzers at 4° C for 18 h. The activity of both samples was measured after dialysis and the results were graphed using Excel. The substrate concentration experiment testing the effect of HBPS vs TD concentration on DszB was done according to the subsequent protocol. Twelve DszB activity assays were run in total with the following modifications. For each assay the HBPS concentrations were either 10 µM, 20 µM, or 40 µM, and for each HBPS concentration, the TD concentration would be either 0 µM, 5 µM, 10 µM, or 20 µM with Buffer A (5 mM NaH₂PO₂, 100 mM NaCl) for a total volume of 200 µL per assay.

Benzenesulfinic Acid Assay

An activation experiment was done using benzenesulfinic acid. A total of twelve DszB activity assays were run in total with the following modifications. For each assay the HBPS concentration was either 5 μM , 10 μM , or 20 μM , then for each HBPS concentration, the benzenesulfinic acid concentration was either 0 μM , 5 μM , 10 μM , 15 μM , 20 μM or 25 μM with Buffer A (5 mM NaH_2PO_2 , 100 mM NaCl) for a total volume of 200 μL per assay.

Substrate Inhibition Experiment

An experiment to determine the K_m of HBPS was done according to the following procedure. Six activity assays were done with the following modifications. The HBPS concentration for each assay was 0.25 μM , 1 μM , 2.5 μM , 5 μM , 10 μM , 20 μM , 40 μM , 50 μM , and 55 μM plus 30 μL of DszB for a total volume of 200 μL for each assay. The data was fitted to the Michaelis-Menten equation and the substrate inhibition equation using the VisualEnzymics software. From the data, the best fit K_m , k_{cat} , and k_i were given for each equation used.

Sulfite Oxidase

The cells that expressed the sulfite oxidase enzyme (S.O.) were grown and the enzyme was purified according to the same protocol used for DszB purification except the sulfite oxidase plasmid is only amp resistant. The sulfite oxidase activity assay was done in a cuvette and the absorbance was measured by a UV/Vis

spectrophotometer. This assay was done according to a modified protocol (22). Each assay contained 0.02 μM TD, Buffer B (50 mM NaH_2PO_2 , 100 mM NaCl), 60 μM cytochrome c, 300 μL DszB, and 300 μL purified sulfite oxidase for a total volume of 1 mL. The activity of the sample was measured by monitoring the change in absorbance at 549 nm over 10 min.

RESULTS

The absorbance of each of the eluted protein samples from the Probond column were quantified using the nanodrop, a spectrophotometer, to check the absorbance at 280 nm (Figure 9). The samples showed a steady increase in absorbance until the last three samples which had a lower absorbance. The highest absorbance point reached was 0.636. These samples were blanked against a sample of the elution buffer used for that purification.

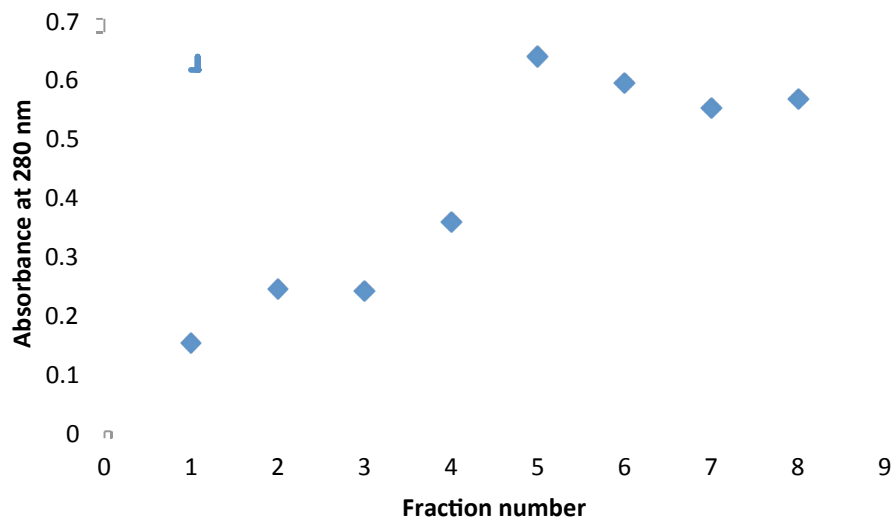


FIG. 9. DszB absorbance quantification by A_{280} . The absorbance at 280 nm was read for each of the eight fractions collected. The peak protein absorbance came from the last four eluted fractions.

A Bradford assay was run on the eluted protein fractions to precisely determine the DszB concentration (Figure 10). The protein concentrations of the BSA ranged from 0 – 10 $\mu\text{g}/\text{mL}$ and the BSA and DszB samples were read at an absorbance of 595 nm. The

A_{595} were adjusted for the blank (0 $\mu\text{g/mL}$) reading. The equation of the best fit trend line produced from the graph of [BSA] vs A_{595} gave an equation of $y = 0.0043x + 0.0033$.

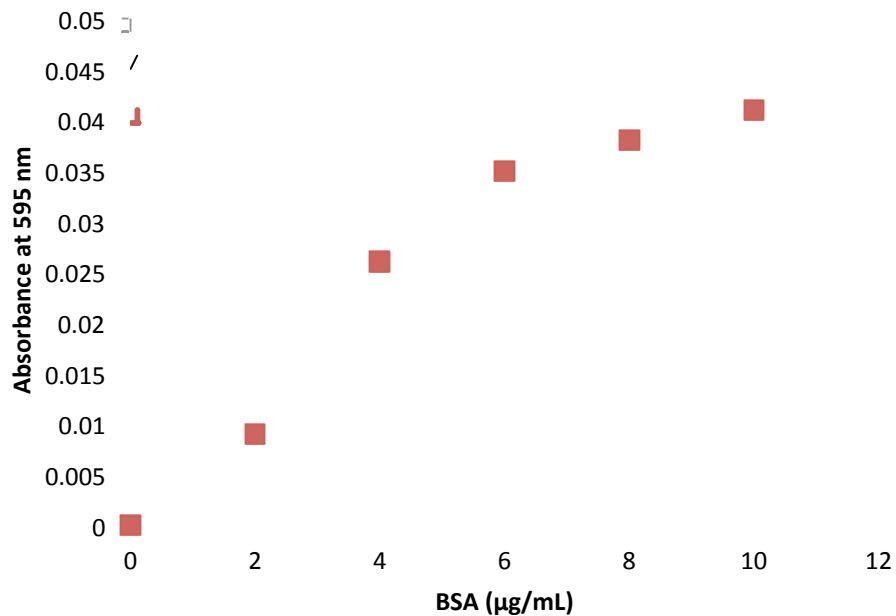


FIG. 10. DszB concentration quantification by Bradford assay. The absorbance at 595 nm was plotted against each of the corresponding BSA concentrations. The resulting equation of the best-fit line was $y = 0.0043x + 0.0033$ with an R^2 value of 0.92.

The eluded DszB yield ranged from 76.21 – 113.42 ($\mu\text{g/mL}$). The averaged extinction coefficient was 0.00432 [$\text{ml}/(\text{nm}\cdot\mu\text{g})$].

The timed dialysis experiment was done to determine if TD binds covalently to DszB's active site (Figure 11). This was done by assaying the original DszB activity, incubating DszB with TD for 10 min, assaying, then dialyzing the solution for 18 h and assaying again. A DszB sample without TD was used as a control. The sample containing TD showed significantly decreased activity after the first 10 min. followed by a slight

decrease in activity after dialysis. In contrast the control only showed a slight decrease in activity after the first 10 min. but a significant decrease after dialysis. After dialysis both sample's enzymes no longer showed activity.

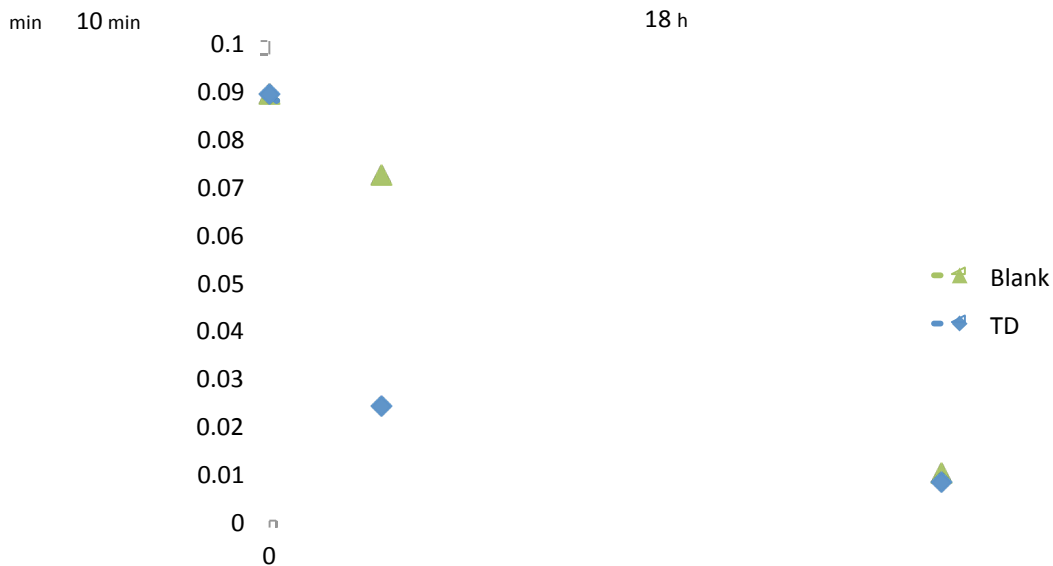


FIG. 11. Timed dialysis of DszB incubated with TD. The three data points represent the initial activity of DszB, activity after 10 min, then activity after 18 h dialysis. The TD sampled showed immediate decreased activity but only a minimal decrease in activity after dialysis.

The substrate concentration experiment was done to test the effect of HPBS vs TD concentration on DszB activity and determine TD's effect on DszB activity (Figure 12a and 12b). This was done by a series of activity assays with varying HPBS and TD concentration. At constant HPBS concentration and initial TD concentration the activity decreased but as the TD concentration increased the activity increased back to the activity level of the sample before the addition of TD (Figure 12a). The same general pattern was

observed with the fixed TD levels (Figure 12b). The activity of each sample initially decreased but then increased as HPBS levels rose.

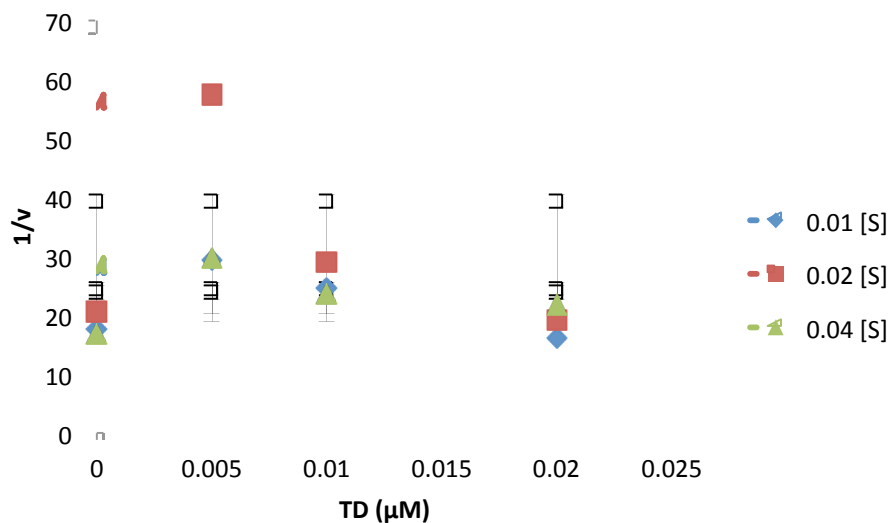


FIG. 12a. **DszB assay with fixed HPBS concentration and increasing TD concentrations.** The activity of DszB was monitored at various HPBS and TD concentrations. When the HPBS level is held constant, the activity initially decreased but then increased with increasing TD levels.

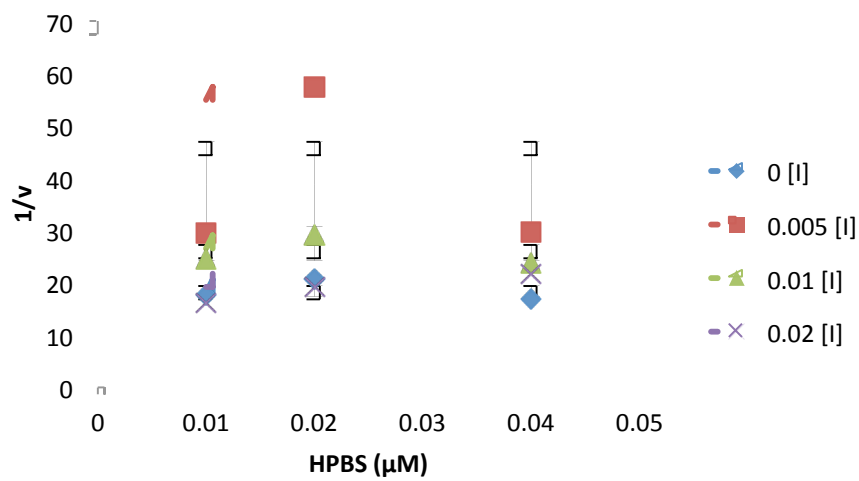


FIG. 12b. DszB assay with fixed TD concentrations and increasing HPBS concentrations. The activity of DszB was monitored at various HPBS and TD concentrations. When the TD level is held constant, the activity initially decreased but then increased with increasing HPBS levels.

The benzenesulfinic acid assay was done to test if the product of the TD and DszB reaction was activating the enzyme to examine why TD activated the DszB in the previous assay (Figure 13). This assay was done with varying HPBS and benzenesulfinic acid concentrations. Overall, the benzenesulfinic acid did not appear to activate the DszB enzyme.

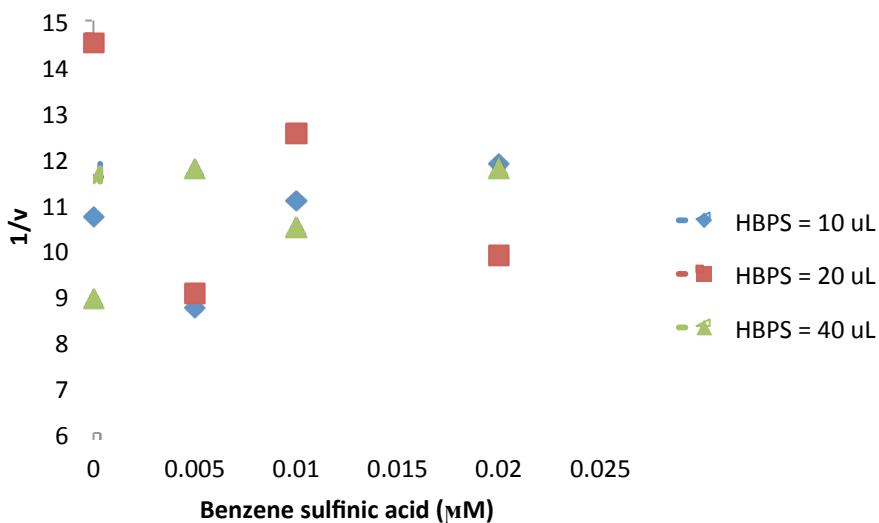


FIG. 13. DszB activity assay with benzenesulfinic acid. The activity of DszB at various HPBS concentrations was monitored at increasing benzenesulfinic acid levels. There was no significant change in activity.

To investigate the substrate inhibition observation, the DszB K_m and k_i were investigated (Table 1). This was done using data from activity assays with increasing

HPBS concentration. The data was fitted to both the Michaelis-Menten equation and the substrate inhibition equation using VisualEnzymics.

Table 1. Determination of constants from kinetic data fitting

<u>Constants</u>	<u>Michaelis-Menten</u>	<u>Substrate Inhibition</u>
	$v = (k_{cat} \cdot S)/(K_m + S)$	$v = (k_{cat} \cdot S)/(K_m + S + S^2/K_m)$
k_{cat}	0.13178 ± 0.00916	0.13934 ± 0.02204
K_m	0.90586 ± 0.52195	1.1616 ± 0.89178
k_i	N/A	846.67 ± 2282.5

The sulfite oxidase assay was performed to determine if the reaction between TD and DszB produced sulfite as a product (Table 2). This was done by monitoring the absorbance of a coupled assay using TD, DszB, sulfite oxidase, and cytochrome c. A negative control assay using the same concentrations of TD and cytochrome c only was also performed.

Table 2. Change in absorbance of cytochrome c at 549nm over 10 min.

	<u>Sulfite oxidase assay</u>	<u>Negative control</u>
Trial 1	0.210	0.306
Trial 2	0.080	0.150

DISCUSSION

The nanodrop and Bradford were performed to analyze the concentration of the purified DszB and to calculate the extinction coefficient (Figure 9,10). The DszB purification using the ProBond resin yielded a varied concentration of protein in the unexpected order of lower to higher concentration. This was later fixed by changing the elution buffer loading technique allowing the most concentrated sample to elude first, maximizing protein collection. The overall low yield may be increased by renewing or regenerating the column between each use or by loading more supernatant on the column for the same elution for more concentrated samples.

The timed dialysis experiment was done to test if TD, the HPBS analog, would covalently bind to the DszB active site, which would test the covalent catalysis mechanism theory (Figure 11). The activity after the first ten minutes showed the TD had significantly decreased activity which indicates the TD was somehow preventing the active site chemistry. But, after 18 h dialysis both the TD and non-TD sampled showed the same minimal activity. This indicated that the dialysis had weakened the effect of TD on DszB and allowed HPBS to kick out the TD from the DszB active site. Altogether this may indicate that DszB binds to TD non-covalently since the dialysis was able to weaken or negate the TD-DszB interaction. A possible explanation for both samples to have had the same activity was the dialysis time was too long, the enzymes became inactive, and the activity readings did not provide reliable results.

The substrate concentration assay was performed to further explore the effect TD had on DszB's activity by analyzing the activity with HPBS and TD of varying concentrations. When the HPBS concentration was held constant and the TD concentration increased, the activity initially increased but then decreased back to initial activity without TD (Figure 12a). This may be a result of TD acting as an activator but then being overwhelmed by possible product inhibition. When the TD concentration was held constant but the HPBS concentration increased, the activity also initially increased due to increase substrate but then the activity declined back to initial activity with the lower HPBS concentration (Figure 12b). This may be due to substrate inhibition, an inhibitory intermediate, or product inhibition.

The benzenesulfinic acid assay was done to test if the TD was activating the enzyme, specifically the essential cysteine of the active site. Benzenesulfinic acid was used because it is a structural analog to the supposed product of TD and DszB. The results of the assay did not show any significant increase or decrease in activity due to the addition of the benzenesulfinic acid (Figure 13). Therefore, the observed increase in activity was not due to intermediates or products of the TD-DszB reaction. The reason why TD initially activated DszB is still unknown.

The kinetic constants experiment was performed to explore the possible substrate inhibition result from the substrate concentration assay (Table 1). Using VisualEnzymics, the fitted data reported that the k_i for the substrate inhibition model was two orders of magnitude higher than the K_m for that model. Thus, the substrate concentration would

have to be excessively high, having already surpassed the required K_m concentration, before any inhibition could occur.

The sulfite oxidase assay was done to determine if the reaction between TD and DszB produced sulfite as a product (Table 2). The results of the assay and controls concluded that the TD alone reduced the cytochrome c more than any sulfite that could have been possibly produced. Therefore, any sulfite production remained undetected and the assay was considered unusable.

Overall, the preliminary data indicated that DszB interacts with TD either by a non-covalent mechanism or a reversible covalent mechanism, which in this case means no permanent modification to the essential cysteine at the active site. While a non-reversible interaction has been ruled out, the interference from TD's reactivity with the chemicals used in the experiments prevented interpreting the data for further conclusions. The data also showed that TD can react with DszB as an activator or inhibitor. This difference in activity may be concentration dependent. Further experiments are needed to explore the results of this study and differentiate between the non-covalent and the reversible covalent mechanism. Specifically, a urea assay which would monitor the other product, urea, which would be formed if TD reacts with DszB as a substrate. If urea is formed, then by default sulfite is also formed and the non-covalent mechanism theory has been supported.

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