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Initial investigations of C4a-(hydro)peroxyflavin intermediate formation by dibenzothiophene monooxygenase

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Abstract

Dibenzothiophene monooxygenase is the initiating enzyme in the *Rhodococcus* 4S biodesulfurization pathway. A member of the Class D flavin monooxygenases, it uses FMN to activate molecular oxygen for oxygenation of the substrate, dibenzothiophene. Here, we have used stopped-flow spectrophotometry to show that DszC forms a peroxyflavin intermediate in the absence of substrate. Mutagenesis of Ser163 and His391 to Ala appears to decrease the binding affinity for reduced FMN and eliminates the enzyme's ability to stabilize the peroxyflavin intermediate.

Keywords

flavin monooxygenase; flavin hydroperoxide; dibenzothiophene; biodesulfurization

Introduction

SO₂ is an atmospheric pollutant generated by both natural and industrial processes, including combustion of organosulfur compounds found as contaminants in fuel. Hydrodesulfurization, the current method of sulfur removal [1], can remove most simple sulfur-containing contaminants, but certain aromatic compounds (*refractory* organosulfur compounds) cannot be effectively treated this way [2]. Several bacterial species are capable of removing sulfur from specific refractory organosulfur compounds such as dibenzothiophene (DBT) [3,4]. In *Rhodococcus erythropolis*, a three-enzyme pathway (DszCAB) removes sulfur from DBT to produce hydroxybiphenyl and sulfite for

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metabolism [5,6,7]. A detailed understanding of catalysis by DszCAB would prove invaluable toward development of biodesulfurization for industrial purposes.

DszC is the monooxygenase of a two-component flavin monooxygenase system that catalyzes two sequential S-oxidations of DBT to dibenzothiophene 5-sulfoxide (DBTO) and then dibenzothiophene 5,5-dioxide (DBTO₂, Supplemental Figure 1). The enzyme uses reduced flavin mononucleotide (FMNH⁻) to activate molecular oxygen for reaction with the sulfenyl (or sulfinyl) sulfur of DBT (or DBTO) [5], possibly by electrophilic oxidation involving the known flavin-oxygen adduct, C4a-hydroperoxyflavin [8]. The FMNH⁻ required for catalysis is produced by DszD [9], the flavin reductase component of the two-component monooxygenase system [10]. Five X-ray crystal structures have shown DszC to be a tetrameric protein with sequence and structural similarity to the acyl-CoA dehydrogenases [8,11,12,13]. Based on its sequence homology and catalytic requirements, DszC is a Class D monooxygenase [14,15], along with p-hydroxyphenylacetate hydroxylase (HpaH) [16], chlorophenol-4-monooxygenase [17], nitrososynthase [18], and others [19,20,21,22]. DszC interacts with flavin as do other Class D monooxygenases [8,11,12,13], and two loops adjacent to the active site, both of which adopt distinct “open” and “closed” conformations, may control access to the active site pocket [8,13]. Mutagenesis of individual active site residues, including H92, S163, H388, H391 and D392 (Supplemental Figure 2), abolishes enzymatic activity [8,12]. The specific catalytic roles of each of these residues are currently unknown.

Here, we begin our study of catalysis by DszC and the functional roles of two residues in the DszC active site, S163 and H391. Based on studies of HpaH, S163 and H391 are likely to be involved in formation of C4a-hydroperoxyflavin [23]. H391 is suspected to facilitate reaction with O₂ by decreasing the kinetic barrier to electron transfer to O₂ by protonating the C4a-peroxyflavin anion [8,23,24], while S163 provides stabilizing interactions to the peroxyflavin through a hydrogen bond to N5 of the isoalloxazine [23]. To investigate these proposals, both residues were mutated to alanine and studied in comparison to wild-type DszC by steady state activity assays and stopped-flow kinetics to examine the reaction of reduced DszC with O₂.

Materials and Methods

Cloning and purification

The codon-optimized *Rhodococcus erythropolis* DszC gene was obtained from GenScript USA Inc. (Piscataway, NJ) in pUC57 and subcloned into the *E. coli* expression vector pBG100 (Vanderbilt University's Center for Structural Biology), which confers a cleavable N-terminal 6-histidine tag and kanamycin resistance. The S163A- and H391A-DszC mutant plasmids were created using the Phusion Site-Directed Mutagenesis Kit (Thermo) and custom primers (Supplemental Table 1). DNA was propagated in *E. coli* DH5α cells, purified from cells using the GeneJET Plasmid Miniprep Kit (Thermo) and sequenced to confirm single codon replacement (Laragen, Inc). Recombinant DszC was expressed in *E. coli* BL21(DE3) cells at 18 °C using 1.0 mM IPTG to induce DszC synthesis. Cells were lysed by sonication in Buffer A (0.02M Tris, 0.2 M NaCl, 1mM DTT, pH 7.5) supplemented with 1 mM PMSF, 0.01 mg/mL DNase and 0.01 mg/mL RNase. The clarified cell lysate was

fractionated on a nickel column (GE Healthcare) using Buffer A with 2.5 mM imidazole to bind and Buffer A with 500 mM imidazole to elute pure DszC. The final DszC sample was stored in 0.02 M Tris, 0.2 M NaCl, 1mM DTT, 5% glycerol, pH 7.5. The oligomerization state of each DszC protein was corroborated by size exclusion chromatography on a HiPrep 26/60 Sephacryl S-300 High Resolution column (GE Healthcare) and native polyacrylamide gel electrophoresis.

Steady state activity assays

DszC activity was quantitated using a modification of established methods¹. DBT stock solutions (0.010 M) were made in ethanol immediately prior to use. DszC (22.2 μ M) was incubated for 20 minutes in a 500 μ L assay mixture containing 100 μ M flavin mononucleotide (FMN), 1 U/mL NAD(P)H:FMN oxidoreductase from *Photobacterium fischeri* (Fre; Sigma-Aldrich), 4 mM NADH, 0.4 U/ μ L catalase (MP Biochemicals), 0.61 mM dissolved O₂ and 100 μ M DBT in 100mM sodium phosphate, 100mM NaCl at pH 6.2-8.2. Solutions were oxygenated by equilibration with 100% O₂ gas for 8 min prior to assembling the assays. After incubation at 22 °C, the reactants and products were extracted into hexane and analyzed on an Agilent 1100 HPLC system using a ZORBAX Eclipse Plus C18 column (GE Healthcare, 4.6 $\times$ 150mm, 3.5 μ m) eluted with 40% acetonitrile in water (first 3 minutes of the run) followed by 60% acetonitrile in water. DBT, DBTO and DBTO₂ were detected at A₂₈₅ and quantitated using standard curves.

Quantitation of flavin binding

The affinity of wildtype-, S163A- or H391A-DszC for reduced FMN was quantitated by spectrophotometric titration at 25°C. A solution containing 20 μ M FMN in 50 mM Tris-H₂SO₄, pH 7.5 was made anaerobic in a glass titration cuvette by cyclic evacuation and equilibration with argon that was passed through an oxygen removal column (Labclear). The anaerobic FMN was reduced with sodium dithionite (~5mg/mL in 50 mM Tris-H₂SO₄, pH 7.5). DszC solutions (234 μ M wild-type DszC, 143 μ M S163A-DszC or 197 μ M H391A-DszC) were made anaerobic in a glass tonometer by repeated cycles of vacuum and equilibration with anaerobic argon, and titrated into the reduced FMN solution (15-30 minutes total titration time) via a gastight Hamilton syringe. Spectra were recorded after each addition of enzyme and corrected for turbidity using the increasing absorbance at 600 nm with each protein addition. The extent of the perturbation of reduced FMN's absorption spectrum by each enzyme was used to quantitate binding affinities. Separately, anaerobic wild-type DszC was added to anaerobic, reduced FMN (20 μ M) to a final concentration of 53 μ M enzyme, and spectra were recorded every 10 minutes for four hours to identify longer-term time-dependent spectral changes.

Stopped-Flow Experiments

Stopped-flow experiments were performed using a Hi-Tech Scientific KinetAsyst SF-61 DX2 stopped-flow spectrophotometer at 4°C in single mixing mode. To maintain anaerobic conditions in the instrument, the flow system was rinsed and then equilibrated overnight with an oxygen scrubbing solution (25 μ M protocatechuate and 0.06 unit/ml protocatechuate dioxygenase in 50 mM sodium phosphate, pH 7.0), which was removed by flushing the system with anaerobic buffer prior to experimentation [25]. Solutions of 400 μ M DszC and

40 μ M FMN in 50 mM Tris- H_2SO_4 , pH 7.5 were made anaerobic in a tonometer, reduced stoichiometrically with dithionite, and then loaded into the stopped-flow instrument. The enzyme and reduced FMN were incubated for a total of at least two hours before data were collected, and the anaerobic enzyme solution was kept at 4°C during data collection using an ice bath. Oxygenated buffers of varying O_2 concentrations were generated by equilibrating the buffer solutions with air or with certified O_2/N_2 gas mixes (5, 10, 50 and 100% O_2) for 8 minutes. Glycerol was included in the O_2 -containing buffers at 3-5% in order to minimize mixing artifacts due to the high protein concentrations used. Reaction traces were fit to sums of exponentials in KaleidaGraph to determine apparent rate constants (k_{obs}).

Results and discussion

Cloning, mutagenesis, purification and analysis of wild-type and mutant DszC

The *dszC* gene was subcloned into plasmid pBG100 and verified by DNA sequencing (Supplemental Information). S163A-DszC and H391A-DszC were made by site-directed mutagenesis and were also confirmed by sequencing. Wild-type and mutant DszC expressed at high levels and were purified to apparent homogeneity using nickel affinity chromatography (Supplemental Figure 3) at yields of 60-70 mg of purified protein per liter of culture. Native PAGE (Supplemental Figure 4) and size exclusion chromatography results are consistent with DszC existing as a tetramer in solution, as observed by others [8,11,12,13].

Steady state product formation

DszC activity is determined by monitoring conversion of DBT (or DBTO) to DBTO_2 , using HPLC to quantitate reaction rates [5,8,12]. Fre and excess NADH are included in our assays to provide a sufficient amount of FMNH^- for catalysis and catalase is included to minimize any nonenzymatic DBT oxidation due to reaction with free H_2O_2 generated by autocatalytic oxidation of flavin.

DszC can oxidize both DBT and DBTO (Figure 1). Under our conditions, DBT is converted completely to DBTO_2 with no DBTO observed when wildtype-DszC is assayed. Maximal activity occurs at pH 7.5 with NaCl concentrations of up to 100 mM at 35 – 40°C. Purified wild-type DszC had an average apparent turnover number of $4.4 \pm 0.5 \text{ s}^{-1}$ when assayed under the conditions described in Methods. Our purified wild-type DszC, which has a turnover number of $2.7 \pm 0.5 \text{ s}^{-1}$ when assayed under the conditions reported by Lei and Tu [5], has almost 1,000-fold higher activity than the enzyme used in their study (turnover number of 0.0033 s^{-1} , calculated from the reported specific activity). This inconsistency is likely due to differences in protein oligomerization state. The enzyme studied in the earlier report was dimeric, as shown by thorough size exclusion experiments [5], which is at odds with current data on DszC and the oligomerization state expected based on its sequence similarity and fold type. The authors of the previous study may have had a mixture of small amounts of active, tetrameric DszC with a larger amount of inactive, dimeric enzyme, leading to a lower apparent turnover number.

Mutation of S163 or H391 to alanine destroys the enzyme's ability to generate DBTO₂ from DBT (Figure 1), though the H391A mutant was able to produce small amounts of DBTO (which were too small to quantitate). Our results are consistent with previous reports in which both of these mutations virtually abolish enzymatic activity [8,12]. Because these residues are implicated in the interactions made by DszC with FMNH⁻ to form and maintain C4a-hydroperoxyflavin, we next examined each mutant's affinity for flavin and ability to catalyze the formation of C4a-hydroperoxyflavin.

Affinity of wild-type and mutant DszC for reduced FMN

Two distinct spectral changes occur upon FMNH⁻ binding by DszC (Figure 2). The first change, which is larger, occurs immediately upon addition of DszC (Figure 2a) and is maximal at 451 nm (Figure 2b). The second change is slow and clearest after the addition of protein (Figure 2c). It is associated with an increase in absorbance over 330-430 nm (maximal at 350-360 nm) and comes to completion in approximately two hours. These observations show that FMNH⁻ binding by DszC involves a slow step.

The spectral change at 451 nm was used to calculate apparent dissociation constants for FMNH⁻ binding of $53 \pm 11 \mu\text{M}$, $147 \pm 49 \mu\text{M}$ and $64 \pm 12 \mu\text{M}$ for the wild-type, S163A and H391A DszC monomers, respectively (Figure 2d). However, the values obtained from these calculations do not account for the suspected time-dependent binding step.

Therefore, these values only approximate the dissociation constant. Notably, mutation of S163 to alanine results in an increase in the apparent dissociation constant for FMNH⁻, consistent with its role in hydrogen bonding the isoalloxazine N5 atom. The apparent K_d of the H391A mutant is similar to wild-type DszC, which suggests H391 does not play a significant role in flavin binding.

The apparent dissociation constants determined here are on the micromolar level, as is the dissociation constant of TftD for FADH₂ [17]. However, these values differ significantly from those reported previously by Lei and Tu ($K_d < 10^{-8}$ M per DszC homodimer) [5]. Though this difference may reflect our use of a different construct (N-terminally his-tagged versus untagged) or improper oligomerization of the enzyme used in the previous study, the magnitude of the difference combined with our observation of a time-dependent spectral change points to a slow binding step that yields an increased affinity. Further investigation is required to clarify these results.

Formation of the C4a-(hydro)peroxyflavin intermediate by wild-type DszC

Catalysis by Class D monooxygenases involves formation of C4a-(hydro)peroxyflavin [26]. To demonstrate DszC's ability to generate this intermediate, we carried out time-resolved studies of the reaction between reduced FMN and O₂ in the presence of a 10-fold molar excess of DszC. DszC-bound FMN was reduced with dithionite and mixed with oxygenated buffers (with 0.033, 0.065, 0.13, 0.31 and 0.61 mM O₂) in the stopped-flow instrument as described above. Changes in the state of the flavin were monitored at 365 and 450 nm and occurred in three phases (Figure 3). In the first phase, which occurs between 0.01 and 0.1 seconds, there was a rapid increase in the absorbance at 365 nm with no increase at 450 nm. This was followed by a simultaneous increase in absorbance at both wavelengths from 0.1 to

10 seconds. In the third phase, occurring over 5-10 to 500 seconds, the absorbance at 450 nm underwent a large increase with no corresponding increase at 365 nm.

The k_{obs} of the first stage of the reaction is dependent on the concentration of O_2 , making the characteristics of this step consistent with formation of the C4a-(hydro)peroxyflavin intermediate. The step occurs with a second order rate constant of $1.69 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (Figure 3, inset). This is similar to others observed in Class D monooxygenase systems, which range from $2.97 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ for ActVA [22], to between $4.8 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $1.66 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ for p-hydroxyphenylacetate monooxygenase from *P. aeruginosa* [26], *A. baumannii* [27] and *P. putida* [28].

The third phase of the reaction between FMNH^- and O_2 in the presence of DszC is consistent with elimination of H_2O_2 to yield oxidized flavin. This phase was independent of O_2 concentration. According to these data, the DszC-bound C4a-(hydro)peroxyflavin has a half-life of 66 s ($k = 1.05 \times 10^{-2} \text{ s}^{-1}$) at 4°C, which is similar to the value for H_2O_2 elimination by other Class D monooxygenases such as p-hydroxyphenylacetate monooxygenase (80 - ~340 s) [26,28]. Our results suggest that DszC, while capable of stabilizing C4a-(hydro)peroxyflavin, forms the intermediate more slowly and stabilizes it for a slightly shorter amount of time in comparison to p-hydroxyphenylacetate monooxygenase, the prototypical Class D monooxygenase.

The second phase, between C4a-(hydro)peroxyflavin formation and decay, is attributed to the autocatalytic oxidation of unbound FMNH^- . Thus the k_{obs} values associated with this phase increased with O_2 concentration, and large absorbance increases occurred at both wavelengths, which is expected for the conversion of FMNH^- to FMN. The oxidation of unbound FMNH^- is an obfuscating reaction that poses a challenge in the study of Class D monooxygenases, e.g., p-hydroxyphenylacetate monooxygenase [29].

Effect of mutagenesis of S163 and H391 on DszC-catalyzed reaction between FMN and O_2

Experiments described here and elsewhere [8,12] indicate that mutation of S163 and H391 to alanine destroys enzymatic activity, though notably, a small amount of product is formed by H391A-DszC in our experiments (Figure 1). We expected that the decreased activity would be due to disruption of the enzyme's ability to form C4a-(hydro)peroxyflavin. To investigate this possibility, we carried out stopped-flow experiments examining the reaction of anaerobic mutant-bound FMNH^- with O_2 (Figure 4). Both mutant enzymes appear to oxidize FMNH^- without an observable accumulation of transiently stabilized C4a-(hydro)peroxyflavin, and the time scale of the DszC-catalyzed reaction between FMNH^- and O_2 was altered significantly by both mutations (Figure 4a,b).

In the absence of substrate, a typical enzyme-bound C4a-hydroperoxyflavin intermediate will decay by H_2O_2 elimination. This elimination step proceeds by loss of the flavin N5 proton to the departing H_2O_2 [30]. The active site residue that hydrogen bonds to N5 (S163 in DszC) therefore plays a part in protecting C4a-hydroperoxyflavin from H_2O_2 elimination, and mutation of this residue should increase the rate of C4a-hydroperoxyflavin decay [23]. This is indeed what we observe in the DszC system. The S163A-DszC-catalyzed oxidation of flavin (red traces) is shown in comparison with the wild-type-catalyzed formation of C4a-

(hydro)peroxyflavin (black traces) in Figure 4a. The reaction of S163A-DszC was modeled with two phases: a shallow absorbance increase beginning at ~0.1 seconds (most likely autocatalytic flavin oxidation), and a larger increase occurring from 0.4 – 40 seconds (corresponding to the oxidation of the enzyme-bound flavin). The 365 and 450 nm absorbance changes overlay well, indicating that the enzyme is not stabilizing C4a-(hydro)peroxyflavin. This is in contrast to the behavior of HpaH from *A. baumannii*, in which the equivalent mutation increases the rate of H₂O₂ elimination (by 1,400-fold) but does not abrogate formation of the intermediate [23]. The oxidation of FMNH⁻ by S163A-DszC has a second-order rate constant of $1.01 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ (Figure 4c), ~15-fold lower than that of peroxyflavin formation by wild-type DszC (Figure 4a). The kinetic behavior observed here is consistent with the proposed role for S163, with weaker FMNH⁻ binding by S163A-DszC and a higher rate of H₂O₂ elimination due to loss of the hydrogen bonding interaction at the flavin N5.

The one-electron reduction of oxygen to superoxide by flavin is kinetically slow, and many flavin-dependent oxidases and monooxygenases use a positively charged active site species to enhance the rate of this step [24,31]. This role is often played by a protonated histidine, lysine or a substrate-based cation [31], and in most cases is thought to decrease the kinetic barrier for formation of superoxide anion and/or act as a Lewis acid [24]. We expected H391 to provide this necessary positive charge in the DszC active site. As in the S163A-DszC experiments, the reaction of H391A-DszC proceeds in two phases, with simultaneous absorbance changes at both wavelengths (Figure 4b). The first phase at ~0.2-10 seconds is most likely the autooxidation of free FMNH⁻. The second phase, corresponding to a larger absorbance change over 10-300 seconds, reflects oxidation of the enzyme-bound FMNH⁻, occurring with a second order rate constant of $35.7 \text{ M}^{-1}\text{s}^{-1}$ (Figure 4d). This represents a dramatic decrease (>400-fold) in the rate of the reaction with O₂ compared to that of wild type enzyme, comparing reasonably well with HpaH (which sees a 100-fold decrease in the rate constant of the reaction with O₂ with the equivalent mutation) [23]. These results are consistent with our proposal that H391 facilitates the formation of C4a-(hydro)peroxyflavin by providing a positive charge in the active site and thereby enhancing the rate of electron transfer to O₂.

The results presented here confirm that DszC transiently stabilizes C4a-(hydro)peroxyflavin as its oxygenating species, and that H391 and S163 play critical roles in formation and stabilization of the intermediate during catalysis. Current research focuses on investigating these and other active site residues in more detail. This work will lead to better understanding of catalysis by DszC, which will enable development of the biodesulfurization pathway for industrial use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DszC	dibenzothiophene monooxygenase
DBT	dibenzothiophene
DBTO	dibenzothiophene 5-sulfoxide
DBTO₂	dibenzothiophene 5,5-dioxide
Fre: NAD(P)H	FMN oxidoreductase from <i>Photobacterium fischeri</i>

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Highlights

Dibenzothiophene monooxygenase forms and stabilizes a C4a-peroxyflavin intermediate.

S163 and H391 are required for stabilization of the peroxyflavin intermediate.

H391's positive charge is likely involved in catalyzing the flavin - oxygen reaction.

S163 likely stabilizes the peroxyflavin to elimination of hydrogen peroxide.

Figure 1.
DszC steady state activity. HPLC traces obtained after incubating wildtype-, S163A- or H391A-DszC with DBT (left panel) or DBTO (right panel) are shown. The peaks corresponding to DBT, DBTO and DBTO₂ are indicated above the traces.

Figure 2.

Quantitation of wild-type DszC FMNH⁻ binding by spectrophotometric titration. (a) Absorbance spectra of a solution of 20 μM FMN in 50 mM Tris-H₂SO₄, pH 7.5 were collected after the addition of known amounts of DszC. Here, the spectrum of FMNH⁻ with no DszC is shown in black. The spectra after the addition of 9, 11, 17, 21, 25, 30, 36, 39, and 45 μM DszC are shown in color, with the earliest titration point in red and the last point in pink (limited points are shown for clarity). (b) Difference spectra for each of the selected points on the titration curve are displayed as in the larger figure. (c) Absorbance spectra of 20 μM FMN with 53 μM DszC monitored over a four-hour period. Spectra are shown in rainbow color with the first spectrum in red and last spectrum in blue. Arrows indicate the

direction of absorbance changes. (d) The wildtype-DszC (black), S163A-DszC (red) and H391A-DszC (blue) titration curves.

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Figure 3.

Kinetics C4a-(hydro)peroxyflavin intermediate formation by wild-type DszC. Reactions contained 200 μM DszC, 20 μM FMN and various oxygen concentrations (0.033, 0.065, 0.13, 0.31 and 0.61 mM O₂) after mixing in the stopped-flow spectrophotometer. Traces recorded at 365 nm (solid black lines) and 450 nm (dashed black lines) are shown with oxygen concentrations increasing from the rightmost to the leftmost trace. Inset: k_{obs} of the first phase of the reaction was plotted versus O₂ concentration and fit to a straight line.

Figure 4.

Effect of S163A and H391A mutations on the reaction of FMNH⁻-bound DszC with O₂. (a) S163A-DszC (red traces) or (b) H391A-DszC (blue traces) and FMNH⁻ were reacted with oxygenated buffer (0.61 mM O₂ final concentration) and monitored at 365 nm (solid lines) and 450 nm (dashed lines) to observe changes in the flavin oxidation state. All reactions consisted of 200 μ M enzyme and 20 μ M FMN after mixing. Equivalent traces corresponding to the wild-type DszC reaction with 0.61 mM O₂ are shown in black for comparison. (c) S163A-DszC or (d) H391A-DszC and FMNH⁻ were mixed with buffers of various oxygen concentration (0.033, 0.065, 0.13, 0.31 and 0.61 mM O₂ after mixing). Traces recorded at 450 nm are shown as solid black lines, with oxygen concentrations increasing from the rightmost to the leftmost trace. Insets: k_{obs} was plotted versus O₂ concentration and fit to a straight line.