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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.

1. Introduction

SO_2 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 metabolism [5–7]. 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 ($DBTO_2$, Supplemental Fig. 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 [8,11–13]. 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 [19–22]. DszC interacts with flavin as do other Class D monooxygenases [8,11–13], 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 Fig. 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

Abbreviations: DszC, dibenzothiophene monooxygenase; DBT, dibenzothiophene; DBTO, dibenzothiophene 5-sulfoxide; $DBTO_2$, dibenzothiophene 5,5-dioxide; Fre, NAD(P)H:FMN oxidoreductase from *Photobacterium fischeri*.

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facilitate reaction with O_2 by decreasing the kinetic barrier to electron transfer to O_2 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 O_2.

2. Materials and methods

2.1. 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 (0.02 M Tris, 0.2 M NaCl, 1 mM 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, 1 mM 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.

2.2. Steady state activity assays

DszC activity was quantitated using a modification of established methods [5]. DBT stock solutions (0.010 M) were made in ethanol immediately prior to use. DszC (22.2 μM) was incubated for 20 min in a 500 μL assay mixture containing 100 μM flavin mononucleotide (FMN), 1 U/mL NAD(P)H:FMN oxidoreductase from *Photobacterium fischeri* (Fre; Sigma-Aldrich), 4 mM NADH, 0.4 U/μL catalase (MP Biochemicals), 0.61 mM dissolved O_2 and 100 μM DBT in 100 mM sodium phosphate, 100 mM NaCl at pH 6.2–8.2. Solutions were oxygenated by equilibration with 100% O_2 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 × 150 mm, 3.5 μm) eluted with 40% acetonitrile in water (first 3 min of the run) followed by 60% acetonitrile in water. DBT, DBTO and $DBTO_2$ were detected at A_{285} and quantitated using standard curves.

2.3. 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 μM FMN in 50 mM Tris-H_2SO_4, 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 (~5 mg/mL in 50 mM Tris-H_2SO_4, pH 7.5). DszC solutions (234 μM wild-type DszC, 143 μM S163A-DszC or 197 μM 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 min 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 μM) to a final concentration of 53 μM enzyme, and spectra were recorded every 10 min for 4 h to identify longer-term time-dependent spectral changes.

2.4. 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 μM 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 μM DszC and 40 μM 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 2 h 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 min. 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}).

3. Results and discussion

3.1. 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 Fig. 3) at yields of 60–70 mg of purified protein per liter of culture. Native PAGE (Supplemental Fig. 4) and size exclusion chromatography results are consistent with DszC existing as a tetramer in solution, as observed by others [8,11–13].

3.2. 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 (Fig. 1). Under our conditions, DBT is converted completely to $DBTO_2$ with no DBTO observed when wild-type-DszC is assayed. Maximal activity occurs

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