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Purification and characterization of a flavin reductase from the biodesulfurizing bacterium *Mycobacterium goodii* X7B

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ABSTRACT

Dibenzothiophene (DBT) in fossil fuels can be efficiently biodesulfurized by a thermophilic bacterium *Mycobacterium goodii* X7B. Flavin reductase DszD, which catalyzes the reduction of oxidated flavin by NAD(P)H, is indispensable for the biodesulfurization process. In this work, a flavin reductase DszD in *M. goodii* X7B was purified to homogeneity, and then its encoding gene *dszD* was amplified and expressed in *Escherichia coli*. DszD is a homodimer with each subunit binding one FMN as cofactor. The K_m values for FMN and NADH of the purified recombinant DszD were determined to be 6.6 ± 0.3 μM and 77.9 ± 5.4 μM, respectively. The optimal temperature for DszD activity was 55 °C. DszD can use FMN or FAD as substrate to generate $FMNH_2$ or $FADH_2$ as product. DszD was coexpressed with DBT monooxygenase DszC, the enzyme catalyzing the first step of the biodesulfurization process. It was indicated that the coexpressed DszD could effectively enhance the DszC catalyzed DBT desulfurization reaction.

1. Introduction

Emitting of sulfur oxides during the combustion of fossil fuels is one of the most important reasons for the acid rain [1]. Desulfurization through biotechnological process is an alternative to decrease the environmental problems caused by the utilization of the fossil fuels. Compared with chemical hydrodesulfurization process, biodesulfurization is rather promising for its mild reaction conditions [2] and attracting attentions [3].

Dibenzothiophene (DBT) and its derivatives are the most abundant organic sulfur compounds in petroleum oils [4]. Most DBT-degrading microbes possess the "4S" biodesulfurization pathway that was first well illustrated in *Rhodococcus erythropolis* IGTS8 [5]. In *R. erythropolis* IGTS8, DBT-sulfone monooxygenase (DszA) and DBT monooxygenase (DszC) catalyze DBT to hydroxyphenyl benzene sulfonate (HPBS), and then HPBS desulfinase (DszB) catalyzes HPBS to 2-hydroxybiphenyl (2-HBP). Both DszC and DszA need $FMNH_2$, which is provided by flavin reductase DszD, to activate oxygen (Supplementary Fig. S1).

Flavin reductases catalyze the reduction of the oxidated flavin using NAD(P)H and participate in many biological processes such as bioluminescence, antibiotic synthesis and DNA repairing [6–8]. Compared with the flavin reductases found in those processes, the flavin reductases in biodesulfurization were only studied in several model strains such as *R. erythropolis* IGTS8 [5] and *R. erythropolis* D-1 [9]. On the other hand, desulfurization is one step of the oil refining process [10]. To match the conditions of biodesulfurization with other refining steps that conduct under high temperature, it is essential to introduce a thermostable biodesulfurization process [11]. However, little study related to the thermostable biodesulfurization enzymes, especially the flavin reductase, has been reported [12].

Mycobacterium also has the biodesulfurizing ability [13]. *Mycobacterium goodii* X7B is a thermophilic biodesulfurizing bacterium isolated from petroleum-contaminated soils [14]. It also employs the "4S" pathway to metabolize DBT under the assistance of the flavin reductase. In this study, flavin reductase DszD in *M. goodii* X7B was firstly purified to homogeneity, and then expressed and fully characterized. It was demonstrated that the DszD enzyme could activate the monooxygenase DszC from a biodesulfurizing *R. erythropolis* strain XP [15].

2. Materials and methods

2.1. Materials

FMN, FAD, riboflavin, NADPH and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma. NADH was purchased from Amresco. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) and dithiothreitol (DTT) were purchased from Merck. The FMN-Sepharose 6B column was prepared under the instruction of GE Healthcare. For genetic manipulation, TIANprep Midi Plasmid Kit and TIANgel Midi

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Purification Kit from TIANGEN were used for plasmid extraction and DNA purification, respectively. Other reagents used were of the highest grade commercially available.

2.2. Bacterial strains, plasmids and cultivation

Bacterial strains and plasmids used are listed in Supplementary Table S1. *M. goodii* X7B was grown in the sulfur-free medium as described previously [16,17]. The medium contained 1 mM dimethylsulfoxide as the sole sulfur source and was shaken on a rotary shaking at 45 °C for 16 h. Lysogenic broth medium (1%, w/v tryptone, 0.5%, w/v yeast extract and 1%, w/v NaCl, pH 7.5) [18] was used for the cultivation of *Escherichia coli*.

2.3. Purification of DszD from M. goodii X7B

Approximately 20 g (wet weight) of *M. goodii* X7B cells were collected by centrifugation at 8000 × *g* for 10 min. The pellet was washed, resuspended in basal buffer (50 mM Tris–HCl, pH 8.5, 10 mM ethylenediaminetetraacetic acid, 1 mM DTT, 1 mM PMSF and 20% glycerol), and disrupted with a Vibra-Cell VCX 500 (Sonics and Materials, USA) sonicator. The crude extract was collected by centrifugation at 12,000 × *g* for 20 min for the following protein purification steps.

(i) *Ammonium sulfate fractionation*. Saturated ammonium sulfate solution was added to the crude extract to 40% saturation. Then the solution was stirred for 1 h on ice and the precipitation was removed by centrifugation at 12,000 × *g* for 20 min.
(ii) *Butyl-Sepharose column chromatography*. The supernatant from ammonium sulfate precipitation was applied to a Butyl-Sepharose Fast Flow column (1.6 cm × 10 cm, GE Healthcare, USA) that had been equilibrated with basal buffer containing 40% saturated ammonium sulfate. After washing the column with basal buffer containing 5% saturated ammonium sulfate, the fractions containing the flavin reductase activity were eluted with basal buffer.
(iii) *Source 30Q column chromatography*. The active fractions from the last step were applied to a Source 30Q column (1.6 cm × 10 cm, GE Healthcare) that had been equilibrated with basal buffer. The column was eluted by a linear 0–1 M KCl gradient in basal buffer. Eluants with the flavin reductase activity were pooled for the next chromatography process.
(iv) *FMN-Sepharose 6B affinity chromatography*. Potassium phosphate buffer (PPB, 3 M, pH 8.0) was added to the eluants from the last step to a final concentration of 1.8 M. Then the solution was applied to an FMN-Sepharose 6B column (1.6 cm × 5 cm) that had been equilibrated with 1.8 M PPB. The column was eluted by a linear 1.8–0 M PPB gradient in basal buffer. Eluants with the flavin reductase activity were pooled and concentrated by ultrafiltration. Purified enzyme was desalted using a HiTrap desalting column (5 ml, GE Healthcare).

2.4. Protein sequencing

The *N*-terminal amino acid sequence of the purified flavin reductase was determined using the Edman degradation method by a Procise 492cLC protein sequencer (Applied Biosystems, USA). For the microsequencing of the internal peptides, the purified DszD was trypsin-digested and analyzed by MALDI-TOF/TOF MS on an ABI 4700 Proteomics Analyzer (Applied Biosystems). MS/MS data search was processed using an online MS/MS ion search program (MASCOT tool).

2.5. Gene cloning, expression and purification of DszD

Genomic DNA of *M. goodii* X7B was extracted using the method described by Andor et al. [19]. The *dszD* gene encoding the flavin reductase was amplified by PCR using primers *dszD*-1 and *dszD*-2 (Supplementary Table S1). Resulting PCR fragments were cloned into the pACYCDuet-1 vector (Novagen, Germany) with *Nde*I and *Xho*I restriction sites, and the resulting vector was transformed into *E. coli* BL21(DE3).

E. coli BL21(DE3) cells containing recombinant plasmids were cultured and induced by the addition of 0.4 mM IPTG when $OD_{620\,nm}$ reached 0.6–0.8. Approximately 2 g (wet weight) of cells were collected and lysed by sonication. After centrifugation, the supernatant was precipitated by 60–80% saturated ammonium sulfate, and then the pellet was redissolved in 1.8 M PPB (pH 8.0) and applied to an FMN-Sepharose 6B column (1.6 cm × 5 cm) equilibrated with 1.8 M PPB (pH 8.0). The active fractions were eluted and concentrated as described above. The flavin reductase pool was then applied to a Superdex 75 GL column (1 cm × 30 cm, GE Healthcare). The fractions containing flavin reductase activity were used for the characterization of DszD.

2.6. Coexpression of DszC and DszD

R. erythropolis XP was cultured as previously reported [20]. Genomic DNA of *R. erythropolis* XP was extracted as that of *M. goodii* X7B. The *dszC* gene was amplified from *R. erythropolis* XP by PCR using primers *dszC*-1 and *dszC*-2 (Supplementary Table S1). The *dszC* gene was ligated with the vector pACYCDuet-1 or pACYC-*dszD*, and the resulting plasmids were designated as pACYC-*dszC* and pACYC-*dszD*-*dszC* with *Eco*RI and *Hind*III restriction sites, respectively. Both of the resulting plasmids were transformed into *E. coli* BL21(DE3). To evaluate the role of DszD in DBT degradation, cells of recombinant *E. coli* BL21(DE3) containing pACYC-*dszC* or pACYC-*dszD*-*dszC* were cultured and induced as described above. Cells were harvested by centrifugation at 8000 × *g* for 10 min, washed twice with 100 mM PPB (pH 7.0), and suspended in the same buffer. Reaction with 100 ml of mixture in 500 ml Erlenmeyer flasks at 37 °C and 180 rpm was performed. The 100 ml reaction mixture consisted of 100 mM PPB (pH 7.0), 0.5 mM DBT, 2% glucose and cells of *E. coli* BL21(DE3) containing pACYC-*dszC* or *E. coli* BL21(DE3) containing pACYC-*dszD*-*dszC* with a final concentration of $OD_{620\,nm}$ = 4.8. The reaction solution of 0.5 ml was mixed with 1 ml of ethanol and centrifuged at 12,000 × *g* for 10 min. The concentration of DBT in the resultant supernatant was measured by high-performance liquid chromatography (HPLC, Agilent 1100 series, Hewlett-Packard, USA) with a reverse-phase C18 column (4.6 mm × 150 mm, Hewlett-Packard) [16]. The mobile phase was 80% methanol at a flow rate of 0.5 ml min^{-1}, and the eluent was monitored by a variable-wavelength detector at 254 nm.

2.7. Molecular weight determination

The molecular mass of native DszD was determined using gel filtration chromatography with a Superdex 75 GL column on an ÄKTA Basic 10. The column was washed with a buffer containing 50 mM Tris–HCl (pH 8.5), 10 mM ethylenediaminetetraacetic acid (EDTA), 1 mM DTT, 1 mM PMSF and 150 mM NaCl at a flow rate of 0.4 ml min^{-1}. Molecular mass standards included ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), albumin (67 kDa) and blue dextran 2000 (2000 kDa). Both molecular mass standards and purified DszD were eluted under identical conditions.

2.8. Enzyme assays

Flavin reductase activity was measured spectrophotometrically by monitoring the NADH oxidation at 340 nm (ε_{340} = 6.2 mM^{-1} cm^{-1}) on an Ultrospec™ 2100 *pro* spectrophotometer (GE Healthcare) at 55 °C. The reaction mixture contained 100 mM PPB (pH 7.0), 300 μM NADH, 40 μM FMN and enzyme. One unit of activity was defined as the amount of flavin reductase needed for oxidizing of 1 μmol NADH per minute. Protein concentrations were measured by the Bradford method using bovine serum albumin as the standard [21].

2.9. FMN binding analysis

The FMN saturation of the enzyme was determined by measuring the FMN concentration in the enzyme solution. One milliliter of purified protein (0.2 mM) was heated at 100 °C for 10 min in dark to release FMN and then cooled on ice. The denatured protein was removed by centrifugation at 13,000 × *g* for 10 min. The concentration of FMN in the supernatant was analyzed using HPLC equipped with a reverse-phase C18 column. The column was eluted with 50 mM ammonium bicarbonate and methanol (82%:18% by volume) at a flow rate of 0.5 ml min^{-1}. FMN was monitored by a variable-wavelength detector at 450 nm. To saturate DszD, FMN was added to the purified DszD until the molarity of FMN was twice of that of the subunit of DszD. The FMN-bound DszD and free flavins were then separated by a Superdex 75 GL column. The FMN concentration in the FMN-saturated enzyme was determined as same as in the purified protein. Absorption spectra of the FMN-saturated protein and free FMN were taken from 250 to 550 nm with Ultrospec™ 2100 *pro* spectrophotometer.

2.10. Nucleotide sequence accession number

The *dszD* gene sequence has been deposited in the GenBank database under GenBank ID: EU154996.

3. Results

3.1. Purification of DszD

DszD was purified to homogeneity from *Mycobacterium* strain X7B (1300 fold) as described in Section 2. The purification results are summarized in Table 1. As shown in Fig. 1, the purified DszD protein has an apparent molecular mass of approximately 17 kDa according to the SDS-PAGE analysis. Based on the result of the gel filtration chromatography, the molecular mass of native DszD was about 39 kDa, suggesting that the DszD protein might be a homodimer. Edman degradation analysis revealed the *N*-terminal amino acid sequence of DszD to be MSATDLSPTSL. One peptide in DszD was identified to be SDI(L)VI(L)NEAVPPI(L)VFHR.