Purification and characterization of enzymes involved in desulfurization of dibezothiophene in fossil fuels

Yoshikazu Izumi *, Takashi Ohshiro

Faculty of Engineering, Department of Biotechnology, Tottori University, Koyama, Tottori 680-8552, Japan

Abstract

DszC and DszA, dibenzothiophene (DBT) and DBT sulfone monooxygenases, respectively, involved in DBT desulfurization, were purified to homogeneity from *Rhodococcus erythropolis* D-1. The two enzymes were crystallized and enzymologically characterized. We found a high activity of flavin reductase in the non-DBT-desulfurizing bacterium, *Paenibacillus polymyxa* A-1, which is essential for DszC and A activities, and purified to homogeneity and characterized the enzyme. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

As the regulation of sulfur in petroleum is becoming stringent, microbial desulfurization has attracted attention as an alternative and innovative process. Dibenzothiophene (DBT) is regarded as a model compound for microbial desulfurization, and some bacteria have been found to metabolize DBT to 2-hydroxybiphenyl (2-HBP) without breaking DBT's carbon skeleton [1]. We also isolated the promising desulfurization bacterium, *Rhodococcus erythropolis* D-1 [2], and elucidated some essential factors for the desulfurization enzyme activity [3–5]. In DBT desulfurizing bacteria including strain D-1, DBT is desulfurized by four consecutive reactions catalyzed by three enzymes, DBT monooxygenase (DszC), DBT sulfone monooxygenase (DszA) and sulfinase (DszB) (Fig. 1). DszC catalyzes two consecutive steps: DBT → DBT sulfoxide → DBT sulfone. DszA catalyzes the conversion of DBT sulfone to 2′-hydroxybiphenyl 2-sulfinic acid (HBPSi). DszB catalyzes the final step: HBPSi → 2-HBP + SO_3^{2-}. In addition, flavin reductase (DszD) has been shown to be essential for the enzyme activities of DszC and DszA [6]. Although the enzymes involved in DBT desulfurization were purified [7], detailed properties were not investigated. We demonstrate the purification, crystallization, and characterization of DszC and A from *R. erythropolis* D-1. Concerning flavin reductase, we searched non-DBT-desulfurizing microorganisms producing more active enzyme than that of the desulfurizing bacterium, *R. erythropolis* D-1, and isolated *Paenibacillus polymyxa* A-1 as the promising strain.

* Corresponding author. Tel.: +81-857-31-5267; fax: +81-857-31-5267.

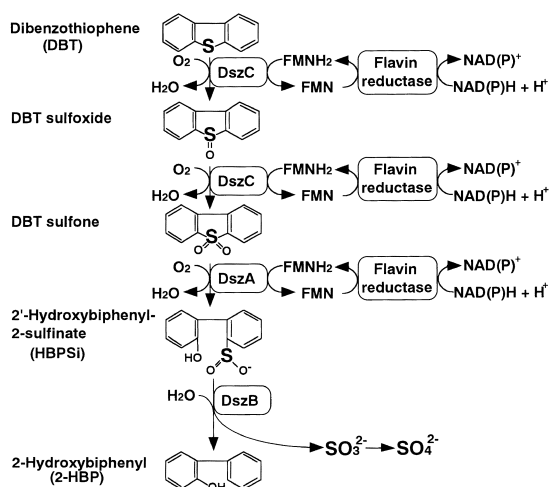*E-mail address:* izumi@bio.tottori-u.ac.jp (Y. Izumi).

Fig. 1. Pathway of microbial DBT desulfurization and involvement of flavin reductase.

We also report the purification and characterization of flavin reductase from *P. polymyxa* A-1.

2. Results and discussion

2.1. Purification and characterization of DszC from R. erythropolis D-1

We purified DszC to homogeneity. The subunit and native molecular masses were 45 and 250 kDa, respectively, indicating a homo-hexameric structure for the enzyme. The substrate range of DszC was investigated by measuring the decrease in the amounts of substrate. As shown in Table 1, DszC acted on not only DBT but also thioxanthen-9-one as well as DBT derivatives, 2,8-dimethyl DBT, 4,6-dimethyl DBT, and 3,4-benzo DBT, which are recalcitrant to chemical desulfurization. On the contrary, DszC showed no activity toward biphenyl and other heterocyclic compounds, carbazole, dibenzofurane, and fluorene where other atoms such as N, O, and C are substituted for a sulfur atom of DBT, respectively. These results suggest that DszC specifically recognized sulfur atom and catalyzed the mono-oxygenation reaction. Thiol reagents and metal chelators inhibited the enzyme activity from DBT to DBT sulfone, and a thiol reagent, *p*-chlore-

mecuribezoic acid, also inhibited flavin reductase. Therefore, at least, metal ions might be involved in DszC activity. Crystals of DszC in the form of flat bars or plates were detected.

2.2. Purification and characterization of DszA from R. erythropolis D-1

We purified DszA to homogeneity. The subunit and native molecular masses were 50 and 97 kDa, respectively, indicating that DszA was a homodimer. It was shown that, by using the cell-free extract of the recombinant *E. coli* expressing *dszA* of *R. erythropolis* IGTS8, DszA catalyzed the conversion of dibenz[*c,e*][1,2]oxathiin 6,6-dioxide (sultone) to 2,2′-dihydroxybiphenyl (DHBP) [8]. In this study, we also demonstrated that, by using the purified DszA of *R. erythropolis* D-1, sultone showed 54% activity as a substrate of DszA compared with DBT sulfone (Table 2), and DHBP was formed as a product. In addition, dibenz[*c,e*][1,2]oxathiin 6-oxide (sultine) showed 23% activity and gave DHBP as a product. However, DszA did not act on DBT and HBPSi. Although sultine was nonenzymatically hydrolyzed to form HBPSi, it was also oxidized to sultone especially with shaking. It was thought that once sultone was nonenzymatically formed from sultine, it was immediately converted to DHBP by DszA. DszA may recognize the sulfone moiety within the structure of DBT sulfone and sultone. The exper-

Table 1
Substrate specificity of DszC

Compound	Relative activity (%)
DBT	100
3,4-Benzo DBT	6.3
2,8-Dimethyl DBT	129
4,6-Dimethyl DBT	52.1
Biphenyl	0
Carbazole	0
Dibenzofurane	0
Fluorene	0
Thioxanthen-9-one	45.2

The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 6 mM NADH, 10 μM FMN, 0.18 units/ml partially purified flavin reductase, 8 units/ml DszC, and 1 mM DBT derivatives. The amounts of substrates were determined with HPLC.

Table 2
Substrate specificity of DszA

Compound	Relative activity (%)
DBT sulfone	100
Sultone	54.4
DBT	0
HBPSi	0

The reaction mixtures contained 100 mM potassium phosphate buffer (pH 7.0), 6 mM NADH, 10 μM FMN, 4 units/ml flavin reductase, 27 units/ml DszA, and 0.25 mM substrates. The amounts of the products from substrates were determined with HPLC. Other reaction conditions were the same as those for the measurement of DszA.

sultine   sultone
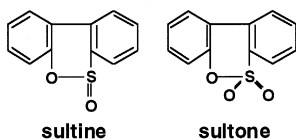
iments using various inhibitors indicated that DszA also possibly had metal ion(s) in the active center. Polygonal crystals of DszA were observed.

2.3. Purification and characterization of flavin reductase from P. polymyxa A-1

As we screened for a flavin reductase in about 100 microorganisms which were unable to desulfurize DBT, we isolated *P. polymyxa* A-1 as the promising strain. Flavin reductase was purified to homogeneity by column chromatographies including FMN-agarose. The subunit molecular mass of the enzyme was 25 kDa and the K_m values for FMN and NADH were 1.7 and 425 μM, respectively. The enzyme activity was strongly inhibited by dicumarol and 4-hydroxycoumarin. Although it was not inhibited by a thiol reagent, *N*-ethylmaleimide (NEM), when FMN, NADH, and the enzyme were simultaneously combined, the preincubation of the enzyme with NEM and NADH in the absence of FMN caused a significant inhibition of the enzyme reaction. This observation indicated that NADH reduced the disulfide bond at the active site of the enzyme with formation of the thiols, which were attacked by NEM. The interconversion of disulfide bonds and thiols must occur at the active center. In addition, the detailed kinetics studies revealed that the enzyme reaction proceeded according to the sequential ordered bi bi mechanism.

3. Materials and methods

R. erythropolis D-1 was cultivated in the synthetic medium supplemented with DBT as the sole source of sulfur at 30°C for 36 h with reciprocal shaking. All operations for the enzyme purification were performed at 4°C and 50 mM Tris–HCl buffer (pH 8.0) containing 10% glycerol and 1 mM dithiothreitol was used as the basal buffer. The cell-free extract was prepared by disrupting the cells by ultrasonic oscillator. After the centrifugation, the supernatant was dialyzed against the basal buffer and applied onto a DEAE-Sepharose column. All four enzymes for DBT desulfurization were separated by this column chromatography. Subsequently, DszC was purified to homogeneity by Phenyl-Toyopearl, MonoQ, and Superdex column chromatographies. DszA was purified to homogeneity by Q-Sepharose, Phenyl-Toyopearl, and Superdex column chromatographies. Crystals of DszC and A were obtained using the hanging-drop method with Linbro plates at 4°C. The well solutions consisted 30% polyethylene glycol, 0.1 M sodium citrate (pH 5.6), and 0.2 M ammonium acetate for DszC, and 12% polyethylene glycol 400 and 0.2 M $CaCl_2$ for DszA, respectively. The enzyme reaction mixtures for DszC and A contained 100 mM potassium phosphate buffer (pH 7.0), 6 mM NADH, 10 μM FMN, flavin reductase separated by DEAE-Sepharose column chromatography, and 0.25 mM DBT or DBT sulfone. The reactions were done at 30°C for DszC and at 35°C for DszA, and both with shaking. The amounts of DBT sulfone formed for DszC and HBPSi formed for DszA were determined by HPLC with an LC-10AS pump and an SPD-10A UV spectrophotometric detector (both from Shimadzu, Kyoto) with a Lichrospher RP-18 column (4 × 250 mm, E. Merck, Darmstadt, Germany). The mobile phase contained 20 mM KH_2PO_4 which was adjusted to pH 2.5 with H_3PO_4, and methanol. The ratios of potassium phosphate solution to methanol were 15:85 for the measurement of DszC activity (retention time: DBT sulfone, 2.7 min; DBT, 8.7 min), 40:60 for that of DszA activity (retention time:

HBPSi, 3.2 min; DBT sulfone, 5.4 min). The flow rate was 1 ml/min and the detection was done at 280 nm. One unit of DszC and A activities was defined as the amount of the protein that catalyzed the formation of 1 nmol of DBT sulfone and HBPSi, respectively.

For the screening of a flavin reductase with a high activity, we cultivated approximately 100 kinds of microorganisms in the nutrient broth and the cell-free extracts were prepared. We examined for activities converting DBT to DBT sulfone coupled with DszC from *R. erythropolis* D-1. The enzyme reaction was done according to the method for DszC as described above.

P. polymyxa A-1 was cultivated in the nutrient broth at 30°C for 48 h with reciprocal shaking. The basal buffer used was the same buffer as described above. The cell-free extract was also prepared by the same method as described above, and the enzyme solution was applied onto a Q-Sepharose column. After the step by Phenyl-Toyopearl column chromatography, flavin reductase was finally purified to homogeneity by FMN-agarose column chromatography. The reaction mixture for the measurement of enzyme activity contained 40 mM citric acid–potassium phosphate buffer (pH 5.5), 20 μM FMN, and 0.5 mM NADH. The decrease in absorbance at 340 nm was followed at 30°C to determine the enzyme activity. One unit of flavin reductase activity was defined as the amount of enzyme utilizing 1 μmol of NADH per minute.

Acknowledgements

References

[1] T. Ohshiro, Y. Izumi, Biosci., Biotechnol., Biochem. 63 (1999) 1.

[2] Y. Izumi, T. Ohshiro, H. Ogino, Y. Hine, M. Shimao, Appl. Environ. Microbiol. 60 (1994) 223.

[3] T. Ohshiro, Y. Hine, Y. Izumi, FEMS Microbiol. 118 (1994) 341.

[4] T. Ohshiro, Y. Kanbayashi, Y. Hine, Y. Izumi, Biosci., Biotechnol., Biochem. 59 (1995) 1349.

[5] T. Ohshiro, K. Suzuki, Y. Izumi, J. Ferment. Bioeng. 83 (1997) 233.

[6] L. Xi, C.H. Squires, D.J. Monticello, J.D. Childs, Biochem. Biophys. Res. Commun. 230 (1997) 73.

[7] K.A. Gray, O.S. Pogrebinsky, G.T. Mrachko, L. Xi, D.J. Monticello, C.H. Squires, Nat. Biotechnol. 14 (1996) 1705.

[8] C. Oldfield, O. Pogrebinsky, J. Simmond, E.S. Olson, C. Kupla, Microbiology 143 (1997) 2961.