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A Flavin Reductase Stimulates DszA and DszC Proteins of *Rhodococcus erythropolis* IGTS8 *in Vitro*

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Rhodococcus erythropolis **IGTS8 is a gram positive bacterium, which can catabolize dibenzothiophene to 2-hydroxybiphenyl and inorganic sulfur without the cleavage of carbon-carbon bonds. Three structural genes, *dszA, dszB,* and *dszC,* have been cloned and shown to be necessary for this phenotype. Here, we demonstrate that a FMN:NADPH oxidoreductase from *Vibrio harveyi* complements activities of purified DszA and DszC proteins. Furthermore, we propose that DszA and DszC are oxygenase units that do not use NAD(P)H directly, but instead use $FMNH_2$ from a FMN:NADPH oxidoreductase for oxygenation.**

Sulfur containing heterocyclics in petroleum are a major pollution source when they are combusted (1,2). In the fractions of crude oil boiling between 500 to 700 °C (the "middle distillate"), the major form of the heterocyclics is dibenzothiophene (DBT) and its derivatives. These comprise up to 60% by weight of the sulfur-containing compounds and resist cleavage by conventional chemical methods (3). *Rhodococcus erythropolis* IGTS8 has the ability to selectively cleave sulfur from DBT to form 2-hydroxybiphenyl (HBP) and inorganic sulfur (4). More importantly this catabolic pathway does not destroy carbon-carbon bonds, and therefore the fuel value is retained (4,5).

Three structural genes, namely *dsz*A, *dsz*B and *dsz*C, have been cloned, sequenced, and shown to confer on DSZ^- derivatives of the wild type organism the ability to carry out desulfurization (6,7,8). Deletion and mutation studies have shown that DszC is responsible for converting DBT to $DBTO_2$, and DszA and DszB together convert $DBTO_2$ to HBP (7,8).

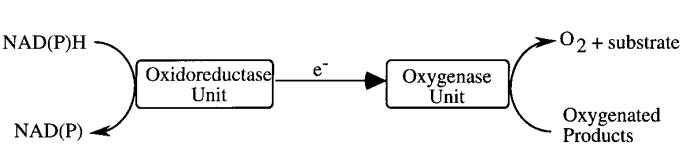
It has been shown that desulfurization in *R. erythropolis* D-1 requires NADH, which is tentimes more efficient than NADPH (9). More recently it was shown that both FAD and FMN are involved in the reactions from DBT to HBP and that the optimal concentration of FMN was found to be around 10 μM with higher concentrations of FMN or FAD being inhibitory (10). DszA is also capable of converting biphenylene sultone (BPsu) to 2,2′-bihydroxy-biphenyl (BHBP) (11). This reaction provides a valuable alternative approach to analyze DszA activity (11). In this article, we demonstrate that an FMN:NADPH oxidoreductase (flavin reductase, E.C. 1.68) is required for DszC- and DszA-catalyzed reactions and propose that $FMNH_2$, reduced by a flavin reductase, is the reductant used by the terminal oxygenases, DszA and DszC. A preliminary account of these findings has been presented on 14th Enzyme Mechanisms Conference, Scottsdale, Arizona, January 4-8, 1995.

MATERIALS AND EXPERIMENTAL DETAILS

Chemicals. FMN, NADH and NADPH were from Sigma Chemical Company. DBT, $DBTO_2$, BPsu and BHBP were from Aldrich. Purified *Vibrio harveyi* flavin reductase (Frp) was obtained from Dr. S.-C. Tu (12). One unit will reduce 1.0 μmole of FMN in the presence of NADPH per min at pH 7.5 at room temperature. Purified DszA and DszC proteins were obtained from Dr. K. A. Gray (13). Protein concentrations were determined spectrophotometrically (14)

Enzyme assays. Enzyme assays were done at 30 °C in 300 μl 0.1 M phosphate buffer, pH 7.5, containing 10 μM FMN, 5 mM NADPH, 500 μM DBT or BPsu, 200 μg/ml (4.03 μM) DszA or 200 μg/ml (4.41 μM) DszC and 0, 0.25, 0.5, or 1.0 unit/ml of Frp. At 0, 2 (or 2.5), 5, 10, 15, and 20 min, 50 μl was withdrawn and quenched by adding an equal volume of acetonitrile. The products were quantitated by HPLC (15).

Under these experimental conditions, the concentration of reduced



SCHEME I

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Abbreviations: DBT, dibenzothiophene; DBTO, dibenzothiophene monoxide; $DBTO_{i2i}$, dibenzothiophene sulfone; PPS, phenylphenol sulfinate; HBP, 2-hydroxybiphenyl; BPsu, biphenylene sultone; BHBP, 2,2′-bihydroxybiphenyl.

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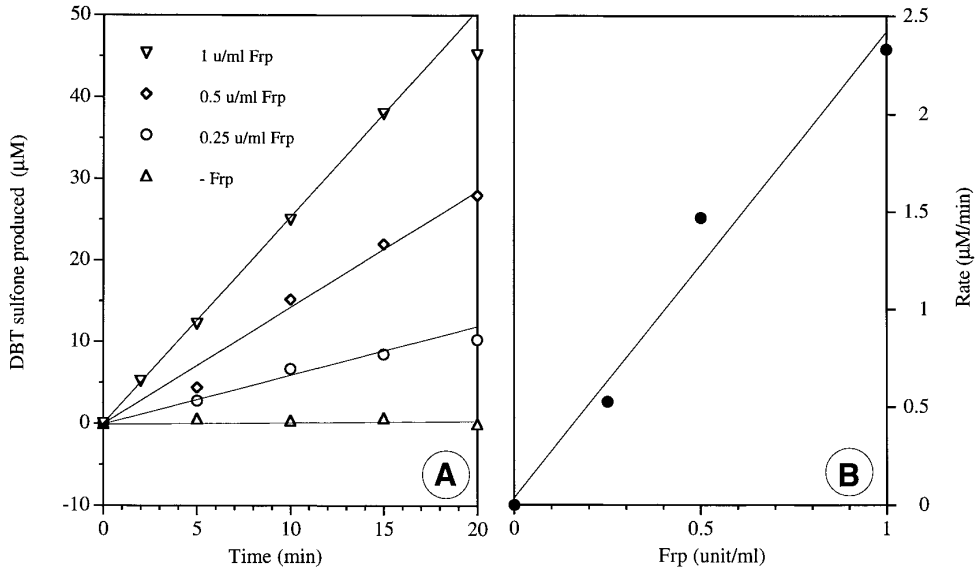


FIG. 1. The effect of Frp on the formation of $DBTO_2$ by DszC. The reaction conditions are described in Materials and Methods. In B the reaction rate is plotted as a function of Frp concentration.

FMN at steady state was proportional to the concentration of Frp. A high concentration of NADPH was used in all reactions to ensure this proportionality.

RESULTS AND DISCUSSION

Typically, oxygenases are divided into two functional units: an oxidoreductase and a terminal oxygenase (16) as shown in Scheme I.

These two functions can reside in the same protein either in the same peptide chain like such as salicylate hydroxylase with a very simple electron transport chain consisting of only flavins, or in separate peptide chains such as benzoate dioxygenase or naphthalene dioxygenase with one or two metal ions in addition to flavins (17). There is also a group of oxygenases including the bacterial luciferase system (17), actinorhodin synthase system (18), and PII_A synthase system (19) where the two functions are in separate proteins. These proteins are loosely associated units, with the oxy-

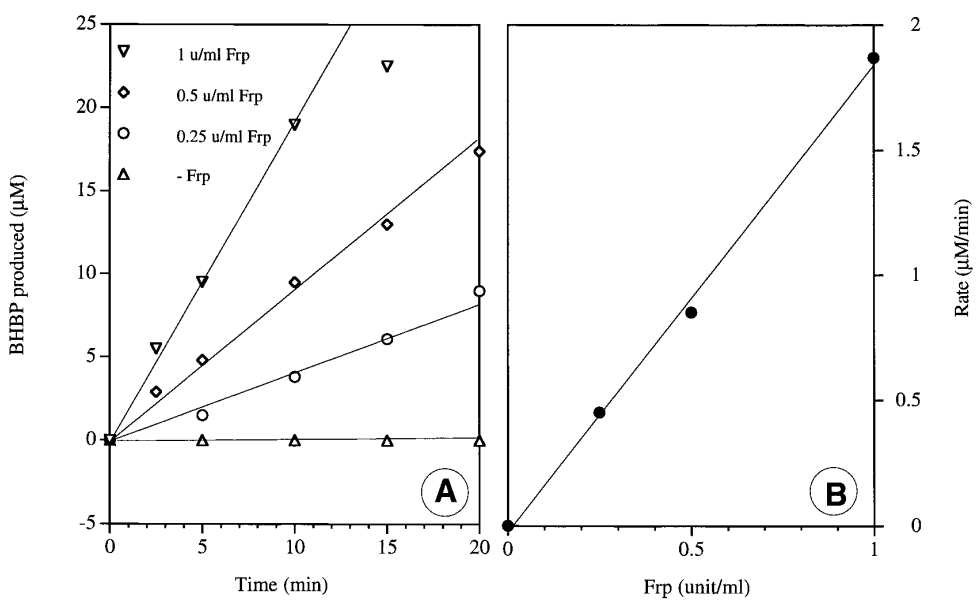
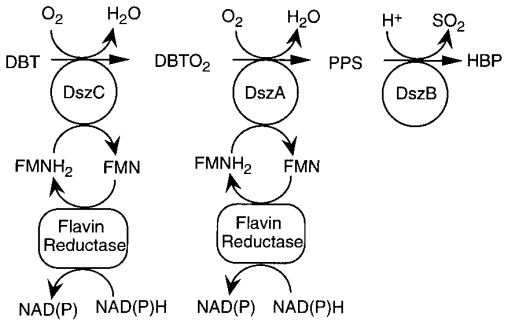


FIG. 2. The effect of Frp on the formation of BHBP by DszA. The reaction conditions are described in Materials and Methods. In B the reaction rate is plotted as a function of Frp concentration.



SCHEME II

genase unit taking $FMNH_2$, reduced by a flavin reductase, as the direct energy source.

Purified DszC or DszA proteins exhibit no activity in the presence of 10 μM FMN, 5 mM NADPH, oxygen, and organosulfur substrates (Fig. 1 and 2) (13). A possible explanation for this observation is that a required component for both of these reactions, was lost during purification. The most likely component is an oxidoreductase As heterologous flavin reductases can complement PII_A synthase activity (19,20), the following experiments were done to test if the activities could be complemented by a heterologous flavin reductase. When Frp protein was added to the reaction mixtures containing either DszC or DszA protein, the respective oxygenase activity was restored. The amount of $DBTO_2$ or BHBP produced increased with time; the reaction rates were linear for 10 to 15 min with 1 unit/ml of Frp, and for 20 min or longer with lower concentrations of Frp. (Fig. 1 and 2). Increasing the amount of Frp protein in the reaction mixture linearly increased the rate of reaction for both DszC (Fig. 1, Panel B) and DszA (Fig. 2, Panel B). These results suggest that both DszC and DszA are terminal oxygenases and that another protein, a flavin reductase, is required for full activity of both enzymes. Our results also suggest that flavin is not a cofactor of DszA or DszC protein, but that the reduced form of flavin serves as a substrate of DszA and DszC and that neither protein uses NAD(P)H directly. These results and the finding that phenyl-phenol sulfinate is the product of DszA (15) suggest the enzymatic scheme for desulfurization shown in Scheme II.

Consistent with this scheme , we have also found that a recombinant *Pseudomonas putida* strain KT2440 with a plasmid encoding *dsz*A, *dsz*C, and a flavin reductase gene is much more active than a strain with only *dsz*A and *dsz*C (data not shown). This indicates that for efficient desulfurization *in vivo* a flavin reductase is required. Thus, a reductase must be present in *R. erythropolis* IGTS8, and that any reductases normally present in alternative hosts such as *P. putida* are inadequate for full expression of the desulfurization phenotype. We are cloning a native flavin reductase encoding gene from IGTS8 to test the effect of its overexpression on the Dsz phenotype.

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