Characterization of Chlorophenol 4-Monooxygenase (TftD) and NADH:Flavin Adenine Dinucleotide Oxidoreductase (TftC) of *Burkholderia cepacia* AC1100

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Burkholderia cepacia AC1100 uses 2,4,5-trichlorophenoxyacetic acid, an environmental pollutant, as a sole carbon and energy source. Chlorophenol 4-monooxygenase is a key enzyme in the degradation of 2,4,5-trichlorophenoxyacetic acid, and it was originally characterized as a two-component enzyme (TftC and TftD). Sequence analysis suggests that they are separate enzymes. The two proteins were separately produced in *Escherichia coli*, purified, and characterized. TftC was an NADH:flavin adenine dinucleotide (FAD) oxidoreductase. A C-terminally His-tagged fusion TftC used NADH to reduce either FAD or flavin mononucleotide (FMN) but did not use NADPH or riboflavin as a substrate. Kinetic and binding property analysis showed that FAD was a better substrate than FMN. TftD was a reduced FAD (FADH₂)-utilizing monooxygenase, and FADH₂ was supplied by TftC. It converted 2,4,5-trichlorophenol to 2,5-dichloro-*p*-quinol and then to 5-chlorohydroxyquinol but converted 2,4,6-trichlorophenol only to 2,6-dichloro-*p*-quinol as the final product. TftD interacted with FADH₂ and retarded its rapid oxidation by O₂. A spectrum of possible TftD-bound FAD-peroxide was identified, indicating that the peroxide is likely the active oxygen species attacking the aromatic substrates. The reclassification of the two enzymes further supports the new discovery of FADH₂-utilizing enzymes, which have homologues in the domains *Bacteria* and *Archaea*.

2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) is an herbicide and a major component of Agent Orange (3, 11, 27). As a chlorinated pollutant, it is recalcitrant to degradation in the environment (11). *Burkholderia cepacia* AC1100 is the first bacterium in pure culture shown to use 2,4,5-T as a sole carbon and energy source (21, 22). The 2,4,5-T degradation pathway has been completely elucidated for AC1100 (44). TftAB, a 2,4,5-T oxygenase, converts 2,4,5-T to 2,4,5-trichlorophenol (2,4,5-TCP) (5, 43). TftCD catalyzes the oxidation of 2,4,5-TCP to 2,5-dichloro-*p*-quinol (also known as 2,5-dichloro-*p*-hydroquinone, 2,5-DiCH) and then to 5-chlorohydroxyquinol (5-CHQ) (17, 41). TftG dechlorinates 5-CHQ to hydroxyquinone, which is reduced to hydroxyquinol by a quinone reductase (44). TftH breaks the aromatic ring of hydroxyquinol to yield maleylacetate; TftE reduces maleylacetate to 3-oxoadipate, which is further channeled into the tricarboxylic acid cycle for complete mineralization (6).

TftC and TftD were originally characterized as a two-component chlorophenol 4-monooxygenase (17, 41). Recent sequence analysis suggests that TftD may belong to the newly discovered reduced flavin adenine dinucleotide (FADH₂)-utilizing monooxygenases, which currently consist of only two characterized enzymes, 4-hydroxyphenylacetate 3-monooxygenase (HpaB) of *Escherichia coli* W (13, 42) and 2,4,6-trichlorophenol monooxygenase (TcpA) of *Ralstonia eutropha* JMP134 (28). Surprisingly, a new report stated that TftD alone, without

TftC, catalyzes NADH-dependent oxidation of chlorophenols and questioned whether TftD is an FADH₂-utilizing monooxygenase (30). To support the assignment by sequence analysis and to clarify the discrepancy, we characterized TftC and TftD as two separate enzymes: an NADH:FAD oxidoreductase and an FADH₂-utilizing monooxygenase.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* strains NovaBlue and BL21(DE3) were grown in Luria-Bertani (LB) medium or on LB agar (36) with kanamycin (30 µg/ml) at 37°C. Strain BL21(DE3) was also incubated at room temperature when used to overproduce recombinant proteins.

Gene cloning and protein expression. PCR primers were designed to clone *tftC* into pET-30 LIC vector (Novagen, Madison, Wis.) with a C-terminal six-His tag. The forward primer (5'-GAA-CGA-AGG-AGG-TTC-ATA-TGC-ATG-CCG-3') was from the beginning of the *tftC* gene starting at position 875 of the deposited sequence (GenBank accession no. U83405), and an *NdeI* site was introduced (underlined) by changing one base. The reverse primer (5'-GGG-GAA-TTC-AGG-CTT-ATT-CCG-CGA-GCG-3'), complementary to sequences from base positions 1418 to 1444, had an *EcoRI* site introduced (underlined) by altering two bases, which also fused the C terminus of *tftC* into a six-His tag on the vector. The gene was amplified from *B. cepacia* AC1100 genomic DNA for 30 PCR cycles of 94°C for 40 s, 55°C for 30 s, and 72°C for 40 s. The amplification yielded a 542-bp PCR product. The product was cut with *NdeI* and *EcoRI* and then ligated into a similarly digested pET-30 LIC vector. The ligation product was electroporated into *E. coli* strain NovaBlue, and the clones were recovered and sequenced for confirmation (36). The correct clone was then transformed into *E. coli* BL21(DE3) for protein production.

To clone *tftD*, PCR primers were synthesized. The forward primer (5'-TAT-GGA-GAC-TGC-ATA-TGC-GCA-CTG-3') started at base position 1457 (GenBank accession no. U83405), and an *NdeI* site was introduced (underlined) by changing three bases. The reverse primer (5'-CGG-AAT-TCG-CTA-CAC-TCT-TGG-TAA-3'), complementary to sequences from positions 3053 to 3076, had three bases changed to introduce an *EcoRI* site (underlined). The gene was amplified by PCR as described for *tftC* amplification, and a 1.6-kb product was obtained. It was cloned into pET-30 LIC vector as a nonfusion gene and transformed into *E. coli* as described for *tftC* cloning.

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Protein purification. Cells were grown in LB medium at 37°C to a turbidity of 0.5 at 600 nm, induced with 1 mM isopropyl-β-D-thiogalactopyranoside, and then incubated at room temperature for 3 h. The cells were harvested by centrifugation and suspended in 20 mM potassium phosphate (KPi) buffer (pH 7.0). Freshly prepared phenylmethylsulfonyl fluoride in absolute ethanol was added to a final concentration of 0.5 mM. The cells were disrupted by passage through a French pressure cell (model FA-030; Aminco, Urbana, Ill.) three times at 260 MPa. The lysate was ultracentrifuged at 50,000 × *g* for 1 h to remove cell debris and membrane fragments. All purification steps were performed at 4°C. Fre, a general flavin reductase from *E. coli*, was purified as previously reported (42).

(i) **Purification of His-tagged TftC (TftC_H).** Five hundred milliliters of cells was harvested and resuspended in 10 ml of the KPi buffer. The cells were lysed. Four milliliters of the extract containing about 10 mg of protein was shaken with 1 ml of Ni²⁺-nitrilotriacetic acid (NTA) agarose beads (Qiagen, Valencia, Calif.) for 1 h at 4°C for binding. The resin was packed into a small column by gravity and was washed with 3 ml of the wash solution. TftC_H was then eluted off the column with 4 ml of elution buffer. The wash solution contained 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 1 mM dithiothreitol (DTT), 10% glycerol, and 40 mM imidazole; the elution buffer contained all of the above with 140 mM imidazole. The samples were stored at -80°C.

(ii) **TftD purification.** Cells from 2 liters of culture were harvested and suspended in 14 ml of the KPi buffer. The cells were lysed, and the lysate was centrifuged. The supernatant was loaded onto a Cibicon Blue 3GA agarose column (1.5 by 18 cm; Sigma, St. Louis, Mo.) equilibrated with the KPi buffer (pH 7) containing 1 mM DTT. The unbound proteins were washed with 3 bed volumes of the KPi buffer, and TftD was eluted with 3 bed volumes of the KPi buffer containing 1 mM DTT and 1 M NaCl. The sample was concentrated to about 2 ml by dialysis against dry Aquacide II (Calbiochem, La Jolla, Calif.) and dialyzed for 2 h against the KPi buffer containing 1 mM DTT. The dialyzed sample was loaded onto a 2-ml Bio-Scale ceramic hydroxyapatite 2-I (CHT) column (Bio-Rad Laboratories, Hercules, Calif.) equilibrated with the KPi buffer. TftD did not bind to the column and came off in a 4-ml wash with the equilibrating buffer. The sample was stored at -80°C.

Enzyme assays. NADH:FAD oxidoreductase activity was determined spectrophotometrically by measuring NADH oxidation ($\epsilon_{340} = 6,220 \text{ M}^{-1} \text{ cm}^{-1}$) in 20 mM KPi buffer (pH 7.0) containing 20 μM FAD and 300 μM NADH at room temperature (24°C). One unit of reductase activity was defined as the amount required to catalyze the oxidation of 1 nmol of NADH per min.

Chlorophenol 4-monooxygenase activity was measured by analyzing the conversion of 2,4,6-TCP to 2,6-DiCH with a high-performance liquid chromatography (HPLC) system. A 40-μl assay mixture contained 20 mM KPi buffer (pH 7.0), 10 μM FAD, 100 μM 2,4,6-TCP, 0.4% Tween 20, 1 mM ascorbic acid, 20 U of TftC or Fre, 2.5 mM NADH, and 10 U (μmol/min) of catalase (Sigma). The reaction was started by the addition of NADH, the reaction mixture was incubated for 3 min at room temperature, and the reaction was terminated by adding 40 μl of an acetonitrile-acetic acid (vol/vol, 9:1) mixture. The samples were centrifuged, and the supernatants were analyzed by HPLC. Stock solutions of 100 mM 2,4,5-TCP, 2,4,6-TCP, 2,5-DiCH, and 2,6-DiCH were prepared in absolute ethanol.

Kinetic analysis was done by measuring the decrease of the substrate. Three independent sets of experiments were performed with at least six substrate concentrations ranging from one-half of K_m to four times K_m . Data were fitted with the Michaelis-Menten equation, by using Grafit 5.0 (R. J. Leatherbarrow, Erithicus Software Ltd., Staines, England, 2001).

Measurement of the dissociation constant. The dissociation constants, K_d , of the TftC · FAD and TftC · flavin mononucleotide (FMN) complexes were measured by using a fluorometer (Luminescence Spectrometer LS50B; Perkin-Elmer, Shelton, Conn.). The excitation wavelength was set at 280 nm, and the fluorescence emission of TftC_H was recorded at 350 nm. The excitation and emission monochromator slit widths were set at 2.5 nm. A 2-ml solution of 1 μM TftC_H in 50 mM KPi buffer (pH 7.0) was titrated with flavin from a 1 mM stock solution, and the fluorescence was measured after each addition. The concentration of TftC_H · flavin complex was estimated by the following equation:

$$[\text{TftC}_H \cdot \text{flavin}] = [\text{TftC}_H] \times [(I_0 - I_c)/(I_0 - I_f)] \quad (1)$$

In the equation, $[\text{TftC}_H]$ represents the initial concentration of TftC_H, I_0 is the fluorescence intensity of TftC_H at the initial titration point, I_c is the fluorescence intensity of TftC_H at a specific titration point, and I_f is the fluorescence intensity at saturating concentrations of flavin. The K_d was determined from a plot of $[\text{TftC}_H \cdot \text{flavin}]$ (*y* axis) versus $[\text{total flavin}]$ (*x* axis) fitted with equation 2 (32), by using Grafit 5.0. *Cap* was the binding capacity of TftC_H.

$$y = \frac{-(K_d + x + \text{Cap}) + \sqrt{(K_d + x + \text{Cap})^2 - 4x\text{Cap}}}{2} \quad (2)$$

pH, ionic strength, and temperature optima. TftC_H activity was measured at pH levels ranging from 5 to 6.2 in 40 mM sodium succinate buffer and from 6 to 7.6 in 40 mM sodium phosphate (NaPi) buffer in a total volume of 1 ml at 30°C. The reaction mixture was the same as described for the enzyme assay. The enzyme activity at ionic strengths ranging from 10 to 160 mM NaPi (pH 7.0) was tested in a similar way. The temperature optimum was determined within the range of 23 to 45°C in 20 mM NaPi (pH 7.0).

Oxygen consumption. Oxygen consumption was measured in a closed reaction vessel (0.650-ml total volume) fitted with a Clark-type oxygen electrode (Instech, Plymouth Meeting, Pa.). The electrode was calibrated by using *N*-methylphenazonium methosulfate and NADH to quantitatively consume O₂ (35). The reaction mixture contained 2.2 μg of Fre alone or Fre with 6.8 μM TftD. The reaction mixture also contained 2 μM FAD, 100 μM NADH, 1 mM ascorbic acid, and 100 μM 2,4,6-TCP. Catalase (99 U) was added after the oxygen consumption stopped. After the reaction was completed, the consumption of 2,4,6-TCP was measured by HPLC.

Analytical methods. A UV-visible light spectrophotometer (Ultrospec 4000; Amersham Pharmacia, Piscataway, N.J.) was used to analyze absorption spectral changes during FAD reduction. The data were recorded with a SWIFT program (Amersham Pharmacia) on a personal computer and transferred to Microsoft (Redmond, Wash.) Excel format. The influence of a compound on the spectrum of a mixture can be removed by subtracting the spectrum of the single compound from that of the mixture. The subtraction and generation of figures with digital data were done by using Microsoft Excel.

An HPLC system (Waters, Milford, Mass.) with a Biosep Sec-S3000 size-exclusion column (7.8 by 300 mm; Phenomenex, Torrance, Calif.) was used to determine the native molecular weights of the proteins. The column was equilibrated with 100 mM KPi (pH 7.0) at a flow rate of 0.5 ml/min. The standards (Bio-Rad) were blue dextran (2,000,000 Da), bovine serum albumin (66,000 Da), carbonic anhydrase (29,000 Da), cytochrome *c* (12,400 Da), and aprotinin (6,500 Da). The HPLC system with a Nova-Pak C₁₈ column (3.9 by 150 mm; Waters) was used to analyze 2,4,5-TCP, 2,4,6-TCP, 2,5-DiCH, and 2,6-DiCH. The compounds were eluted by an 11 mM H₃PO₄-acetonitrile gradient. The percentages of acetonitrile were as follows: 5 to 70%, 5-ml linear gradient; 70%, 6 ml; and 100%, 3 ml. The absorption spectra from 240 to 350 nm were monitored by a Waters 996 photodiode array detector operated with the Millennium 2010 version 2.1 program (Waters) on a personal computer. The compounds were identified by comparing their retention times and absorption spectra to those of authentic standards, and the peak areas were used for quantification.

The end product of 2,4,5-TCP oxidation by TftD was determined by mass spectrometry (MS). An 0.6-ml reaction mixture contained 2 μM FAD, 1 mM ascorbic acid, 100 μM 2,4,5-TCP, 2.5 mM NADH, 60 U of catalase, 2.2 μg of Fre, 10% glycerol, and 4.25 μM TftD in 20 mM KPi (pH 7.0). The reaction was carried out at room temperature for 20 min, and HPLC analysis confirmed the complete degradation of 2,4,5-TCP. The organic compounds were extracted into ethyl acetate, dried, derivatized with acetic anhydride, and analyzed on a QP5050A gas chromatography (GC)-MS system (Shimadzu, Columbia, Md.) as previously described (28).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (24), and gels were stained with GelCode Blue (Pierce, Rockford, Ill.). Protein concentrations were determined by using a protein dye reagent (2) with bovine serum albumin as the standard.

RESULTS

Protein production and purification. To determine whether TftC and TftD are separate enzymes, the *tftC* and *tftD* genes were cloned separately into pET-30 LIC vector (Novagen) and transformed into *E. coli* strain BL21(DE3) for overexpression. When induced at 37°C, the proteins were mainly insoluble. However, the cells produced soluble TftC and TftD proteins when incubated at room temperature after induction.

TftC was produced as a C-terminally His-tagged fusion protein, TftC_H, and was purified with Ni²⁺-NTA agarose beads. When 2.7 g (wet weight) of cells was disrupted, 23.1 mg of protein was obtained in the cell extract with a specific activity of 7,040 nmol min⁻¹ mg of protein⁻¹. The Ni²⁺-NTA agarose

FIG. 1. SDS-PAGE of TftC_H and TftD. Lane 1, molecular mass standards in kilodaltons (Bio-Rad); lane 2, 2.5 μ g of purified TftC_H; lane 3, 2 μ g of purified TftD.

purification was sufficient to purify TftC_H to apparent homogeneity (Fig. 1). When 9.2 mg of cell extract protein was applied to 1 ml of Ni²⁺-NTA agarose beads, 0.17 mg of TftC_H was purified with a specific activity of 49,000 nmol min⁻¹ mg of protein⁻¹. TftD was produced as a nonfusion protein and purified with Cibicon Blue 3GA resin and a prepacked hydroxyapatite column. From 7.8 g (wet weight) of cells, 57.3 mg of protein was obtained in the cell extract. After purification, 4.0 mg of TftD was purified to apparent homogeneity (Fig. 1). The specific activity of purified TftD was 280 nmol min⁻¹ mg of protein⁻¹ for 2,4,6-TCP oxidation. The TftD was shown to be 58 kDa and TftC_H was shown to be 22 kDa by SDS-PAGE. Both TftD and TftC_H were monomers as estimated by size-exclusion chromatography analysis. Pure TftC_H was unstable in the elution buffer containing 140 mM imidazole on ice, losing about 50% activity in 2 h. It was relatively stable when kept at -80°C, where it lost about 50% activity after 2 months. TftD was relatively stable on ice for several hours and did not show any apparent loss of activity after storage at -80°C for 2 months. The purified TftC_H and TftD were both colorless, indicating that they do not have bound flavin prosthetic groups.

Enzyme activity and substrate specificity. TftC_H used NADH to reduce either FAD or FMN, but it did not use NADPH or riboflavin as a substrate. The kinetic parameters of TftC_H were determined (Table 1), and FAD was a better

TABLE 1. Kinetic properties of TftC(H)^a

Substrate	K_m (μ M)	k_{cat} (s ⁻¹)	k_{cat}/K_m (μ M ⁻¹ s ⁻¹)
FAD ^b	4.8 $\pm$ 1.5	16.6 $\pm$ 1.3	3.4
FMN ^b	10.3 $\pm$ 4.1	18.5 $\pm$ 1.9	1.8
Riboflavin ^c			
NADH _{FAD} ^d	40.1 $\pm$ 12.9	30.1 $\pm$ 4.4	0.8
NADH _{FMN} ^e	164.0 $\pm$ 31.2	11.9 $\pm$ 0.9	0.1
NADPH ^c			

^a Experiments were done in 20 mM KPi buffer (pH 7.0) at 24°C. Values are means of three experiments with standard deviations.

^b Determined with fixed NADH concentration at 300 μ M.

^c No detectable activities.

^d Determined with fixed FAD concentration at 18 μ M.

^e Determined with fixed FMN concentration at 20 μ M.

TABLE 2. Kinetic properties of TftD^a

Substrate	K_m (μ M)	k_{cat} (s ⁻¹)	k_{cat}/K_m (μ M ⁻¹ s ⁻¹)
2,4,5-TCP	35.8 $\pm$ 3.9	0.67 $\pm$ 0.03	0.019
2,5-DiCH	4.3 $\pm$ 1.1	0.10 $\pm$ 0.01	0.025
2,4,6-TCP	39.9 $\pm$ 7.6	0.41 $\pm$ 0.03	0.010
2,6-DiCH ^b			

^a Experiments were done in 40 mM KPi buffer (pH 7.0) at 24°C. Values are means of triplicate experiments with standard deviations. TftD was the limiting factor with TftC_H in excess, and so the k_{cat} values are calculated with the molecular weight of TftD.

^b No detectable activities.

substrate than FMN. The highest enzyme activity was observed at pH 6.0 in NaPi buffer with 87, 82, 65, and 61% of the activity retained at pH 6.4, 6.8, 7.2, and 7.6, respectively. Sodium succinate buffer was used for the pH range of 5.0 to 6.2. Compared to the activity in the pH 6.0 NaPi buffer, 52, 64, 81, and 75% of its activity was retained at pH 5.0, 5.4, 5.8, and 6.2, respectively. The optimal temperature was seen at 30°C with 85, 92, 82, and 70% activity at 23, 35, 40, and 45°C, respectively. The optimal ionic strength ranged from 10 to 120 mM NaPi (pH 7), supporting similar activity, which was reduced by 28% at 160 mM NaPi.

TftD required a flavin reductase to supply FADH₂. TftD oxidized 2,4,5-TCP, 2,4,6-TCP, and 2,5-DiCH but not 2,6-DiCH. The kinetic properties of TftD were determined with TftD to supply FADH₂ (Table 2). When 2,4,5-TCP was used as a substrate, the product 2,5-DiCH was readily degraded to 5-CHQ, whereas 2,4,6-TCP was quantitatively converted to 2,6-DiCH. Thus, the TftD activity was usually determined by measuring the oxidation of 2,4,6-TCP to 2,6-DiCH. To determine whether TftD was active with any flavin reductase or only with TftC, Fre was used to replace TftC_H. The rates of 2,6-DiCH production were similar when TftD was coupled with either TftC_H or Fre in a typical 40- μ l reaction mixture containing 10 to 100 U of the flavin reductase.

Dissociation constants. The dissociation constant, K_d , was determined by measuring the fluorescent quenching of TftC_H when FAD or FMN was added to the solution. Grafit was used to plot the added concentration of flavin versus bound flavin to obtain the K_d values. The K_d values were 2.2 $\pm$ 0.1 and 7.8 $\pm$ 0.2 μ M for TftC-FAD and TftC-FMN complexes, respectively. The K_d values correlated well with the K_m values for FAD and FMN (Table 1).

End product analysis. HPLC analysis was used to show the complete consumption of 2,4,5-TCP by TftD and the formation of the end product. 2,4,5-TCP gave a peak at 9.0 min, and the end product had a retention time of 5.08 min with an absorption maximum at 293 nm; thus, it is proposed to be 5-CHQ because of its retention time and absorption maximum (41). The peak at 6.9 min for 2,5-DiCH was transiently observed during the reaction and was not detectable at the end of the reaction. The organic compounds were extracted into ethyl acetate, and the aqueous solution was analyzed with HPLC to confirm that the end product was completely extracted. The organic phase was dried and acetylated for GC-MS analysis. Three peaks were detected, with retention times of 8.72, 10.09, and 11.03 min. The first peak had a mass spectrum typical of

triacylated glycerol, and the second peak was identified as monoacylated ascorbate. Glycerol and ascorbate were reagents in the reaction mixture. The third peak had a mass spectrum that was typical for a molecule with one chlorine and was similar to the spectrum of triacylated 6-CHQ as previously reported (28). A molecular ion peak was seen at m/z 286, and its fragments were at m/z 244 (loss of $-\text{COCH}_3$), 202 (loss of two $-\text{COCH}_3$), and 160 (loss of all three $-\text{COCH}_3$). Since triacylated 6-CHQ gave a different retention time of 10.65 min by the GC-MS analysis, the compound was assigned as triacylated 5-CHQ.

The stoichiometry of TCP oxidation. The reaction stoichiometry of TCP oxidation by TftD coupled with Fre was analyzed by measuring NADH consumption, oxygen consumption, H₂O₂ production, and 2,4,6-TCP degradation. For 100 μM NADH consumed, 66 μM O₂ was consumed. A 34 μM concentration of H₂O₂ was produced because 17 μM O₂ was released upon addition of catalase. Since one O₂ reacted with one FADH₂ to generate one H₂O₂, this left 32 μM O₂ for TCP oxidation. A 31 μM concentration of TCP was consumed in the reaction, giving a ratio of 1:1 for oxygen consumption and TCP oxidation. The ratio of NADH used for TCP oxidation was approximately 2:1.

Spectral analysis of the effect of TftD on Fre activity. TftD required FADH₂ as a cosubstrate, which was supplied by a flavin reductase. The flavin reductase reduces FAD with NADH. In this study, the changes of NADH and FAD were monitored during the course of a reaction by scanning the absorption from 300 to 550 nm. Fre was used as the flavin reductase in this experiment because it was more stable than TftC_H. The NADH consumption rate was higher in a reaction with Fre alone than in the same reaction also containing TftD (Fig. 2). When the reaction mixture contained only Fre, FADH₂ was rapidly oxidized back to FAD without any apparent decrease of FAD (10 μM) (Fig. 2B). In the presence of TftD (Fig. 2C), the FAD concentration was 1.95 μM when estimated from the remaining absorption at 450 nm with the subtraction of the FADH₂ absorption or it was 1.6 μM when calculated by the Michaelis-Menten equation with the rate of NADH oxidation and the determined K_m (1.4 μM) and $V_{\max}$ (69,764 nmol/min/mg) for Fre under the assay condition.

Fre activity was analyzed by measuring the rate of NADH oxidation with various amounts of TftD in the reaction mixture. When TftD was absent in the reaction mixture, Fre activity was the highest (Fig. 3). As TftD concentration increased, the activity of Fre decreased, due to decreased FAD available in the reaction mixture. By the Michaelis-Menten equation, the FAD concentration in the reaction was estimated based on the rate of NADH oxidation and the kinetic parameters of Fre. The estimated available FAD concentration decreased from 1.65 to 0.10 μM FAD when the TftD concentration was increased to 5 μM (Fig. 3). Thus, TftD decreased FAD concentration during its reduction by Fre (Fig. 2 and 3), resulting in lower rates of NADH oxidation (Fig. 3).

A potential flavin-peroxide intermediate. The absorption spectrum of a reaction with TftD and Fre was analyzed to identify whether FAD-peroxide was present. The absorption spectrum of a reaction was monitored at different time points during the reaction. In the reaction, one H₂O₂ was produced from one FADH₂ oxidation. When catalase was added, two

FIG. 2. UV-visible light spectra of NADH oxidation and FAD reduction by Fre alone and by Fre with TftD. The reaction mixture contained 2.2 μg of Fre, 400 μM NADH, 10 μM FAD, and 99 U of catalase in 0.7 ml of 20 mM KPi buffer (pH 7.0). In reaction mixtures containing TftD, 16.9 μM TftD was used. (A) Time course of NADH oxidation in a reaction with Fre alone (■) or Fre with TftD (◇). (B) The FAD spectrum of the reaction with Fre alone. (C) The FAD spectrum of Fre and TftD. Arrows indicate the spectrum at time zero before the addition of Fre.

H₂O₂ were converted immediately back to one O₂. Therefore, the reaction with catalase should theoretically consume approximately 510 μM NADH, while the reaction without catalase should consume about half of that, assuming that O₂ concentration is about 255 μM (41). When the reaction without catalase completely depleted O₂, NADH consumption stopped at about 125 μM NADH (Fig. 4, line 2). At the same NADH concentration, the reaction with catalase at 130 s of NADH oxidation still had a sufficient amount of O₂ due to regeneration by catalase, and NADH consumption continued (Fig. 2A and C and Fig. 4, line 1). Fig. 4, line 4 was obtained by subtracting the spectrum of FADH₂ (Fig. 4, line 2) from the

FIG. 3. The effect of TftD on NADH oxidation by Fre. The reaction mixture was similar to that described in the Fig. 2 legend except that the initial FAD concentration was 2 μ M and TftD was used from 0 to 5 μ M. The rate of NADH oxidation ($\diamond$) was determined by monitoring the decrease of NADH at 340 nm. The FAD concentrations ($\blacksquare$) were estimated with the Michaelis-Menten equation by using the rates of NADH oxidation and the determined kinetic parameters of Fre.

spectrum of an FAD derivative (Fig. 4, line 1) in the presence of O_2 . The result was a peak with an absorbance maximum from 392 to 400 nm, which was masked by the absorption of NADH in line 1. When NADH was completely depleted, FAD was regenerated due to complete oxidation (Fig. 4, line 3).

DISCUSSION

TftC and TftD were originally characterized as a two-component flavin-dependent monooxygenase (41). Our new data reclassify them as two individual enzymes: TftC is an NADH:FAD oxidoreductase, and TftD is an $FADH_2$ -utilizing monooxygenase. NAD(P)H:flavin oxidoreductases are divided into two classes: the class I enzymes do not have bound flavin prosthetic groups, and the class II enzymes do (4, 10, 12, 23, 39). Since TftC_H did not have bound flavin as a prosthetic group, it belongs to class I. The TftC_H had a calculated mo-

FIG. 4. Spectral analysis of a potential flavin intermediate during the reaction in the presence of TftD. Reaction conditions were essentially the same as described in the Fig. 2 legend with 16.9 μ M TftD in the presence or absence of 99 U of catalase. Line 1, the spectrum of a reaction with catalase in the mixture was taken at 130 s of NADH oxidation; line 2, the spectrum of a reaction without catalase was recorded when O_2 was completely depleted at 200 s of the reaction; line 3, the spectrum of the reaction with catalase was shown after NADH was used up and the FAD was regenerated at 350 s of the reaction; line 4, the spectrum obtained by subtracting line 1 from line 2.

FIG. 5. Proposed conversion of 2,4,6-TCP to 2,6-DiCH. The first step is catalyzed by TftD, and the second step is a chemical reduction by either NADH or $FADH_2$. $FADH_2$ is produced by TftC_H at the expense of NADH.

lecular weight of 21,194, which agrees with the SDS-PAGE analysis (Fig. 1). TftC_H was active for FAD reduction with NADH as the reductant, and it did not use NADPH or riboflavin. Kinetic (Table 1) and binding properties show that TftC_H prefers FAD to FMN. Its catalytic properties are different from those of other characterized flavin reductases. Although supplying $FADH_2$ to HpaB, which uses $FADH_2$ to oxidize 4-hydroxyphenylacetate, HpaC reduces FMN faster than it reduces FAD (13). Fre, an *E. coli* general flavin reductase, can reduce riboflavin, FMN, and FAD with either NADH or NADPH (10). However, Fre reduces FMN faster than it does FAD when assayed individually, whereas FAD is the preferred substrate when both FAD and FMN are present due to the high affinity of Fre for FAD (29). *Streptomyces viridifaciens* has an NADPH:FAD oxidoreductase that reduces both FAD and FMN with either NADPH or NADH, with the highest rate being obtained for FAD reduction with NADPH as the reductant (33). Several flavin reductases have also been characterized from *Vibrio* sp., and they all prefer to reduce FMN (19, 26, 45). Thus, to our knowledge, TftC_H is the only characterized NADH:FAD oxidoreductase.

TftD has a calculated molecular weight of 57,451, and it was shown to be a monomer by size-exclusion chromatography analysis. Compared to other $FADH_2$ -utilizing monooxygenases, TftD is similar in size and conserved in sequence. TcpA of *R. eutropha* JMP134 is a monomer of 60 kDa (28), and HpaB of *E. coli* W is a homodimer with subunits of 59 kDa (34). TftD had a broad substrate range and required oxygen and $FADH_2$ to function. $FADH_2$ was supplied by flavin reductases. The fact that Fre successfully replaced TftC_H to provide $FADH_2$ for TcpA (28) and HpaB (34). The kinetic properties of TftD were determined, and the parameters for 2,4,5- and 2,4,6-TCP are similar (Table 2). However, TftD degraded 2,5-DiCH but not 2,6-DiCH, the direct products from the oxidation of 2,4,5-TCP and 2,4,6-TCP, respectively. In contrast to TftD, TcpA of *R. eutropha* JMP134 degrades 2,4,6-TCP to 6-CHQ with 2,6-DiCH as a possible intermediate (28). The stoichiometric analysis of 2,4,6-TCP oxidation by TftD and Fre shows the consumption of one O_2 and two NADH for one 2,4,6-TCP oxidized to 2,6-DiCH. Thus, we propose that the direct product of 2,4,6-TCP oxidation by TftD is 2,6-dichloro-*p*-quinone (2,6-DiCB), which is chemically reduced to 2,6-DiCH by either NADH or $FADH_2$ (Fig. 5). The formation of quinone after removal of a chlorine or a nitro group from substituted phenols by monooxygenases has been reported

elsewhere (15, 18). We further speculate that TftD oxidizes 2,4,5-TCP to 2,5-DiCH and then converts 2,5-DiCH to 5-CHQ with reactions similar to those shown in Fig. 5, i.e., quinones are the direct products following each dechlorination step.

The end product of 2,4,5-TCP oxidation by TftD was shown to be 5-CHQ by GC-MS analysis. The mass spectrum of triacetylated 5-CHQ is very similar to that of triacetylated 6-CHQ as previously reported (28) under the same GC-MS conditions. The different retention times of triacetylated 5-CHQ (11.03 min) and triacetylated 6-CHQ (10.65 min) are likely due to the difference in chlorine positions. The intermediate, 2,5-DiCH, was only transitorily accumulated and was completely converted to 5-CHQ when the reaction was complete. The observation is consistent with our kinetic data showing that 2,5-DiCH is a better substrate for TftD (Table 2) than is 2,4,5-TCP, so that it is not accumulated when 2,4,5-TCP is used up. Although 5-CHQ was previously reported as the end product based on HPLC analysis (41), a recent report stated that 5-CHQ was not detectable by HPLC analysis (30). The report also stated that TftD purified from *B. cepacia* AC1100 alone oxidizes chlorophenols. However, the reported SDS-PAGE results clearly indicate that TftD is not purified to homogeneity, and the reported specific activity is much lower than that originally reported (41) or reported in this study. The data reported here confirm the original characterization of TftD and TftC for the oxidation of 2,4,5-TCP to 5-CHQ. The assignment of 5-CHQ as the end product of 2,4,5-TCP degradation by TftD is also consistent with the proposed pathway for 2,4,5-T degradation, as enzymes converting 5-CHQ into tricarboxylic acid cycle metabolic intermediates have been identified in *B. cepacia* AC1100 (44).

The analysis of the absorption spectra in a reaction with TftD and Fre reveals the presence of a potential FAD-peroxide (Fig. 4). The absorbance maximum of the possible FAD-peroxide was from 392 to 400 nm, which is consistent with the absorption spectra of other flavin-peroxides (9). Equation 3 shows the two-step oxidation of FADH₂ by O₂.

In this equation, FADH₂ and O₂ form FAD-peroxide. In the absence of TftD, k_2 is faster than k_1 (31), and FAD-peroxide is not accumulated. Thus, the observed FAD-peroxide in the presence of TftD must be bound to TftD, and TftD slows down k_2 so that FAD-peroxide is accumulated and detectable by the spectrophotometer. The accumulation of FAD-peroxide is also consistent with the observed decrease of FAD (Fig. 2 and 3), which reduces Fre's activities by lowering the concentration of its substrate FAD in the reaction mixture. The binding and protection of FMN-peroxide by a bacterial luciferase, an FMNH₂-utilizing monooxygenase, have been well documented; the enzyme uses the FMN-peroxide to attack its aldehyde substrate (16). Thus, it is likely that TftD attacks its aromatic substrate with the bound FAD-peroxide.

FADH₂-utilizing monooxygenases are important enzymes in bioremediation for the degradation of aromatic compounds. Although there are only three enzymes characterized in this group to date, several proteins are candidates for being clas-

sified as FADH₂-utilizing monooxygenases because of sequence similarities (1, 7, 8, 14, 20, 37, 38, 40). The characterization of these proteins as FADH₂-utilizing monooxygenases will help us to understand further the genetics and biochemistry of this unique enzyme family and to advance their application to bioremediation of pollutants or biosynthesis of specialty chemicals (25).

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