Characterization of Chlorophenol 4-Monoxygenase (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-monoxygenase 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 FADH2 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-quinol and then to 5-chlorohydroxyquinol but converted 2,4,6-trichlorophenol only to 2,6-dichloro-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). TtAB, 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-quinol (also known as 2,5-dichloro-p-hydroquinone, 2,5-DiCh) and then to 5-chlorohydroxyquinol (5-CHO) (17, 41). TftG dechlorinates 5-CHO to hydroxyquinol, which is reduced to hydroquinol 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-monoxygenase (17, 41). Recent sequence analysis suggests that TftD may belong to the newly reclassified enzymes, which currently consist of only two characterized enzymes, 4-hydroxyphenylacetate 3-monoxygenase (HpaB) of Escherichia coli W (13, 42) and 2,4,6-trichlorophenol monoxygenase (TepA) ofRalstonia 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 monoxygenase.

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-CTT-GGC-CGA-GCG-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-AAG-ATT-CTT-CCG-GGA-3') was complementary to sequences from base positions 1418 to 1444, had an NdeI site introduced (underlined) by changing one base. The forward primer (5'-TAT-GGA-GAC-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-AAAT-TGC-GCA-AGG-ATT-3') was complementary to sequences from positions 3053 to 3076, has 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 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-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-AAAT-TGC-GCA-AGG-ATT-3') was complementary to sequences from positions 3053 to 3076, has 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-038; Amino, 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 (TftCH). Five hundred milliliters of a cell suspension containing about 10 mg of protein was shaken 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 KPi buffer. The cells were lysed, and the lysate was centrifuged. The supernatant was loaded onto a Cibicron 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 Aqueaide 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 (ε900 = 6,220 M−1 cm−1) at 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 2,4,5-TCP to 2,6-DCH 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 mM 2,4,6-TCP, 0.4% Tween 20, 1 mM acetic acid, 20 μM 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-DCH, and 2,4-DCH were prepared in absolute ethanol.

Kinetic analysis was done by measuring the rate of the substrate. Three independent sets of experiments were performed with at least six substrate concentrations ranging from one-half of Km to four times Km. Data were fitted with the Michaelis-Menten equation, by using GraFit (5.0; J. R. Leaver, Eriticus Software Ltd., Steyne, England, 2001).

Measurement of the dissociation constant. The dissociation constants, Km, 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 TftCH was recorded at 350 nm. The excitation and emission monochromator slit widths were set at 2.5 nm. A 2-ml solution of 1.4 μM TftCH 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·flavin complex was estimated by the following equation:

\[ [\text{TftC·flavin}] = [\text{TftCH}] \times \left[ (I_0 - I_f) / (I_0 - I_i) \right] \]  

In the equation, [TftCH] represents the initial concentration of TftCH at the initial titration point, I0 is the fluorescence intensity of TftCH at a specific titration point, and Ii is the fluorescence intensity at saturating concentrations of flavin. The Km was determined from a plot of [TftC·flavin] (y axis) versus [total flavin] (x axis) fitted with equation 2 (32), by using GraFit 5.0. Cap was the binding capacity of TftCH.

\[ y = (K_m + x + Cap) / (Cap + x + Km)^2 \]  

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-His, 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 mmol min−1 mg of protein−1. The Ni2+-NTA agarose
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 Ni^{2+}-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 Cibicron 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.** TftD 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 and TftD were determined (Table 1), and FAD was a better 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, and 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 FADH_2. 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 FADH_2 (Table 2). When 2,4,5-TCP was used as a substrate, the product 2,5-DiCH was readily degraded 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 TftC_H or Fre in a typical 40-μl reaction mixture containing 10 to 100 U of the flavin reductase.

**Dissociation constants.** The dissociation constant, K_p, was determined by measuring the fluorescent quenching of TftC_H when FAD or FMN was added to the solution. Graft was used to plot the added concentration of flavin versus bound flavin to obtain the K_p values. The K_p values were 2.2 ± 0.1 and 7.8 ± 0.2 μM for TftC-FAD and TftC-FMN complexes, respectively. The K_p 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 transitorily 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 by 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

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**TABLE 1. Kinetic properties of TftC(H)**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K_m (μM)</th>
<th>k_cat (s^{-1})</th>
<th>k_cat/K_m (μM^{-1} s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAD^a</td>
<td>4.8 ± 1.5</td>
<td>16.6 ± 1.3</td>
<td>3.4</td>
</tr>
<tr>
<td>FMN^a</td>
<td>10.3 ± 4.1</td>
<td>18.5 ± 1.9</td>
<td>1.8</td>
</tr>
<tr>
<td>Riboflavin^e</td>
<td>40.1 ± 12.9</td>
<td>30.1 ± 4.4</td>
<td>0.8</td>
</tr>
<tr>
<td>NADH_{FAD}^d</td>
<td>164.0 ± 31.2</td>
<td>11.9 ± 0.9</td>
<td>0.1</td>
</tr>
</tbody>
</table>

^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 μM.

^c No detectable activities.

^d Determined with fixed FAD concentration at 18 μM.

^e Determined with fixed FMN concentration at 20 μM.

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**TABLE 2. Kinetic properties of TftD**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K_m (μM)</th>
<th>k_cat (s^{-1})</th>
<th>k_cat/K_m (μM^{-1} s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4,5-TCP</td>
<td>35.8 ± 3.9</td>
<td>0.67 ± 0.03</td>
<td>0.019</td>
</tr>
<tr>
<td>2,5-DiCH</td>
<td>4.3 ± 1.1</td>
<td>0.10 ± 0.01</td>
<td>0.025</td>
</tr>
<tr>
<td>2,4,6-TCP</td>
<td>39.9 ± 7.6</td>
<td>0.41 ± 0.03</td>
<td>0.010</td>
</tr>
<tr>
<td>2,6-DiCH^b</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^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.
triacetylated glycerol, and the second peak was identified as monoacetylated 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 triacetylated 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 triacetylated 6-CHQ gave a different retention time of 10.65 min by the GC-MS analysis, the compound was assigned as triacetylated 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, \( \text{H}_2\text{O}_2 \) production, and 2,4,6-TCP degradation. For 100 \( \mu\text{M} \) TCP, the concentration of \( \text{H}_2\text{O}_2 \) was consumed, 66 \( \mu\text{M} \) \( \text{O}_2 \) was consumed. A 34 \( \mu\text{M} \) concentration of \( \text{H}_2\text{O}_2 \) was produced because 17 \( \mu\text{M} \) \( \text{O}_2 \) was released upon addition of catalase. Since one \( \text{O}_2 \) reacted with one FADH\(_2\) to generate one \( \text{H}_2\text{O}_2 \), this left 32 \( \mu\text{M} \) \( \text{O}_2 \) for TCP oxidation. A 31 \( \mu\text{M} \) 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 FADH\(_2\) 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\(_\text{Tt}\). 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, FADH\(_2\) was rapidly oxidized back to FAD without any apparent decrease of FAD (10 \( \mu\text{M} \)) (Fig. 2B). In the presence of TftD (Fig. 2C), the FAD concentration was 1.95 \( \mu\text{M} \) when estimated from the remaining absorption at 450 nm with the subtraction of the FADH\(_2\) absorption or it was 1.6 \( \mu\text{M} \) when calculated by the Michaelis-Menten equation with the rate of NADH oxidation and the determined \( K_m \) (1.4 \( \mu\text{M} \)) and \( V_{\text{max}} \) (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 \( \mu\text{M} \) FAD when the TftD concentration was increased to 5 \( \mu\text{M} \) (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 \( \text{H}_2\text{O}_2 \) was produced from one FADH\(_2\) oxidation. When catalase was added, two \( \text{H}_2\text{O}_2 \) were converted immediately back to one \( \text{O}_2 \). Therefore, the reaction with catalase should theoretically consume approximately 510 \( \mu\text{M} \) NADH, while the reaction without catalase should consume about half of that, assuming that \( \text{O}_2 \) concentration is about 255 \( \mu\text{M} \) (41). When the reaction without catalase completely depleted \( \text{O}_2 \), NADH consumption stopped at about 125 \( \mu\text{M} \) 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 \( \text{O}_2 \) 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 FADH\(_2\) (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 μM and TftD was used from 0 to 5 μM. The rate of NADH oxidation (○) was determined by monitoring the decrease of NADH at 340 nm. The FAD concentrations (■) were estimated with the Michaelis-Menten equation by using the rates of NADH oxidation and the determined kinetic parameters of Fre.

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 FADH2-utilizing mono- 
oxygenase. 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 TftC14 did not have bound flavin as a prosthetic group, it belongs to class I. The TftC14 had a calculated molecular weight of 21,194, which agrees with the SDS-PAGE analysis (Fig. 1). TftC14 was active for FAD reduction with NADH as the reductant, and it did not use NADPH or ribo- flavin. Kinetic (Table 1) and binding properties show that TftC14 prefers FAD to FMN. Its catalytic properties are different from those of other characterized flavin reductases. Although supplying FADH2 to HpaB, which uses FADH2 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).

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 FADH2-utilizing monoxygenases, 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 FADH2 to function. FADH2 was supplied by flavin reductases. The fact that Fre successfully replaced TftC14 to provide TftD with FADH2 supports the idea that TftD is an FADH2-utilizing monoxygenase. Fre has also been used to supply FADH2 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-Dichloroacetate but not 2,6-Dichloroacetate, 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-Chloroacetate and 2,6-Dichloroacetate as a possible intermediate (28). The stoichiometric analysis of 2,4,6-TCP oxidation by TftD and Fre shows the consumption of one O2 and two NADH for one 2,4,6-TCP oxidized to 2,6-Dichloroacetate. Thus, we propose that the direct product of 2,4,6-TCP oxidation by TftD is 2,6-Dichloroacetate, which is chemically reduced to 2,6-Dichloroacetate by either NADH or FADH2 (Fig. 5). The formation of quinone after removal of a chlorine or a nitro group from substituted phenols by monoxygenases 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 product based on HPLC analysis (41), a recent report stated that 5-CHQ was not detectable by HPLC analysis (30). The mass spectrum of triacetylated 5-CHQ 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 FADH$_2$ by O$_2$.

\[
FADH_2 + O_2 \rightarrow FADHOOH \rightarrow FAD + H_2O_2.
\]

In this equation, FADH$_2$ and O$_2$ form FAD-peroxide. In the absence of TftD, $k_1$ is faster than $k_2$ (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 FMN$_2$-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.

FADH$_2$-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 classified as FADH$_2$-utilizing monooxygenases because of sequence similarities (1, 7, 8, 14, 20, 37, 38, 40). The characterization of these proteins as FADH$_2$-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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