Purification of Hydroxyquinol 1,2-Dioxygenase and Maleylacetate Reductase: the Lower Pathway of 2,4,5-Trichlorophenoxyacetic Acid Metabolism by *Burkholderia cepacia* AC1100

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The enzyme hydroxyquinol 1,2-dioxygenase, which catalyzes *ortho* cleavage of hydroxyquinol (1,2,4-trihydroxybenzene) to produce maleylacetate, was purified from *Escherichia coli* cells containing the *tftH* gene from *Burkholderia cepacia* AC1100. Reduction of the double bond in maleylacetate is catalyzed by the enzyme maleylacetate reductase, which was also purified from *E. coli* cells, these cells containing the *tftE* gene from *B. cepacia* AC1100. The two enzymes together catalyzed the conversion of hydroxyquinol to 3-oxoadipate. The purified hydroxyquinol 1,2-dioxygenase was specific for hydroxyquinol and was not able to use catechol, tetrahydroxybenzene, 6-chlorohydroxyquinol, or 5-chlorohydroxyquinol as its substrate. The native molecular mass of hydroxyquinol 1,2-dioxygenase was 68 kDa, and the subunit size of the protein was 36 kDa, suggesting a dimeric protein of identical subunits.

Aerobic metabolism of chloroaromatic compounds occurs through two pathways. Generally, simple chlorinated aromatic compounds containing one or two chlorine substituents are converted to chlorocatechols, which are further degraded by the enzymes of the modified *ortho* cleavage pathway (6, 7, 10, 11, 22, 30). Compounds containing two or more chlorine substituents are usually converted to hydroxyquinol or chlorohydroxyquinol (1, 13, 18, 20, 24, 27). These trihydroxylated intermediates are metabolized by the enzyme hydroxyquinol 1,2-dioxygenase (4, 18, 19, 21, 26, 33), which is different from the well-studied modified *ortho* pathway enzyme chlorocatechol 1,2-dioxygenase. Both pathways converge at the intermediate maleylacetate (14). Maleylacetate reductase catalyzes the reduction of maleylacetate to 3-oxoadipate, an intermediate common to many pathways of aromatic compound metabolism (12, 15, 25).

Burkholderia (Pseudomonas) cepacia AC1100 uses the recalcitrant herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) as a sole source of carbon and energy (17). Previous evidence shows that the lower pathway of 2,4,5-T degradation proceeds through the intermediate 5-chloro-1,2,4-trihydroxybenzene (5-chlorohydroxyquinol) (5). Recently, the two-subunit chlorophenol 4-monooxygenase responsible for the conversion of 2,4,5-trichlorophenol to 2,5-dichlorohydroquinone and subsequently to 5-chloro-1,2,4-trihydroxybenzene was purified (Fig. 1) (29, 31). Metabolism of the 5-chloro-1,2,4-trihydroxybenzene compound by *B. cepacia* AC1100 until now has not been investigated. This 5-chloro-1,2,4-trihydroxybenzene intermediate accumulates when the 2,4,5-T-negative mutant, *B. cepacia* PT88, grows in the presence of glucose and 2,4,5-T (24). A DNA fragment containing six open reading frames (ORFs) complements *B. cepacia* PT88 for growth on 2,4,5-T as a sole source of carbon (9). In order to determine the lower pathway

of 2,4,5-T degradation, involving the metabolism of 5-chloro-1,2,4-trihydroxybenzene, the genes essential for the complementation of the mutant *B. cepacia* strain, PT88, were cloned and overexpressed in *Escherichia coli*.

Previously, it was suggested that the protein encoded by the *tftH* gene (ORF 6) of *B. cepacia* AC1100 was a catechol 1,2-dioxygenase-like enzyme which may use hydroxyquinol as its substrate rather than catechol (9). The *tftH* gene was overexpressed in *E. coli* cells from the T7 promoter with the construct pDD2QΔ7 (9). Crude cell extracts were assayed for oxygenase activity by monitoring the conversion of hydroxyquinol to maleylacetate at 243 nm. *E. coli* cell extracts had no oxygenase activity toward hydroxyquinol; however, in cell extracts in which the *tftH* gene was overexpressed, there was a change in the absorption spectrum of hydroxyquinol. The absorbance peaks at 225 and 289 nm were gradually depleted, and a new peak at 243 nm appeared. The peak at 243 nm was consistent with previous reports, which indicated that maleylacetate was being produced (4, 28). It should be noted that the oxidation of hydroxyquinol occurs both enzymatically and nonenzymatically (4, 33). The hydroxyquinol compound will spontaneously oxidize to 2-hydroxy-1,4-benzoquinone. Crude cell extracts from *E. coli* inhibit spontaneous oxidation, presumably due to enzymes which reduce 2-hydroxy-1,4-benzoquinone or scavenge oxygen radicals, as discussed by Armstrong et al. (2). Because spontaneous oxidation also causes an increase in the A_{243} , a control reaction without protein was monitored at 243 nm for nonenzymatic oxidation and the result was subtracted from the enzymatic reaction rate.

Previously, it was shown that the protein encoded by the *tftE* gene (ORF 1) of *B. cepacia* AC1100 was a maleylacetate reductase that reduced maleylacetate to 3-oxoadipate with NADH as a cofactor (9, 14). The *tftE* gene was overexpressed in *E. coli* cells from the *tac* promoter by use of the construct pMMD0 (9). Crude extracts from cells grown in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG) showed a fourfold increase in NADH oxidation in the presence of maleylacetate compared with cells grown without IPTG. On the basis of preliminary results, including molecular mass, subunit size,

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FIG. 1. Pathway of 2,4,5-T degradation. The *tftA* and *tftB* genes encode two subunits of the 2,4,5-T oxygenase enzyme responsible for the conversion of 2,4,5-T to 2,4,5-trichlorophenol (8, 32). A two-component flavin-containing monooxygenase encoded by the *tftC* and *tftD* genes catalyzes the *para*-hydroxylation of 2,4,5-trichlorophenol to yield 2,5-dichlorohydroquinone (29, 31). A second hydroxylation step by the same enzyme converts 2,5-dichlorohydroquinone to 5-chloro-1,2,4-trihydroxybenzene (5-chlorohydroxyquinol), which is dechlorinated to yield 1,2,4-trihydroxybenzene (hydroxyquinol). Ring cleavage of 1,2,4-trihydroxybenzene to yield maleylacetate is catalyzed by hydroxyquinol 1,2-dioxygenase, encoded by the *tftH* gene. Maleylacetate reductase, encoded by the *tftE* gene, catalyzes the reduction of maleylacetate to 3-oxoadipate, which ultimately is converted to tricarboxylic acid cycle intermediates.

FIG. 2. SDS-PAGE (12% polyacrylamide) of crude soluble protein and Ni^{2+} -NTA agarose-purified protein. Lanes 1 to 3 correspond to the hydroxyquinol 1,2-dioxygenase (HQO). Lanes 5 to 7 correspond to the maleylacetate reductase (MAR). Lanes 1 and 7 contain equal amounts of crude soluble protein (66.0 μg). Lanes 2 and 3 contain 6.0 and 2.0 μg of purified hydroxyquinol 1,2-dioxygenase, respectively. Lanes 5 and 6 contain 0.5 and 1.5 μg of purified maleylacetate reductase, respectively. The protein concentration was determined by the Bradford method (3). Lane 4 contains the low-molecular-mass protein standards of 14,400, 21,500, 31,000, 45,000, 66,200, and 97,400 Da.

substrate range, and inhibition studies, this protein is similar to other maleylacetate reductases that have been purified.

PCR (using the constructs pMMD0 and pDD2Q47 as template DNA) was used to generate both *tftE* and *tftH* genes containing six histidine codons at the 3' end of each gene to enable protein purification by Ni^{2+} -nitrilotriacetic acid (NTA)-agarose affinity chromatography (Qiagen). Overproduction of maleylacetate reductase from the *tftE* PCR-generated clone was not sufficient for protein purification. In order to increase the overproduction of maleylacetate reductase, its putative Shine-Dalgarno sequence was mutated during PCR by changing nucleotides in the 5' end PCR primer to an *E. coli* consensus Shine-Dalgarno sequence. This strategy increased the overproduction of maleylacetate reductase as visualized by Coomassie blue staining of protein from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The PCR products were cloned into the PCR II (*lacZ'*; M13 intergenic region; Ap^r Km^r) expression vector for overproduction of the protein (Invitrogen). The proper clones were transformed into *E. coli* TG1 [K-12(*lac-pro*) *supE thi hsdΔ5 F'* (*traD36 proAB⁺ lacI^a lacZΔM15*)] according to standard procedures (23) or into *E. coli* BL21DE3/pLysS [(F⁻ *ompT hsdS_B* (*r_B⁻ m_B⁻*) *gal dem*) (λ DE3: *lacI lacUV5 T7* RNA polymerase gene) (pLysS-T7 lysozyme gene)] (Novagen). *E. coli* transformants of TG1 or BL21DE3/pLysS were grown at 30°C in Luria broth supplemented with 75 μg of ampicillin per ml. Expression of the histidine-tagged genes from the *lac* promoter or the T7 promoter of the PCR II vector was induced by the addition of 0.5 to 1.0 mM IPTG. Induced cells were lysed in native Ni^{2+} -NTA agarose binding buffer (described below) by sonication with a Branson Sonifier 450, and cell debris was removed by centrifugation. Overproduction of the hydroxyquinol 1,2-dioxygenase and the maleylacetate reductase was roughly fourfold greater when the genes were expressed from the *lac* promoter in the PCR II vector rather than from the T7 promoter in the same vector. Thus, the genes were overexpressed from the *lac* promoter for protein purification purposes.

For large batch purifications, the cleared crude cell extracts containing the overproduced histidine-tagged proteins in native binding buffer (50 mM sodium phosphate buffer, pH 8.0,

300 mM sodium chloride, 20 mM imidazole) were incubated for 1 h with the Ni^{2+} -NTA agarose (Qiagen). To remove any contaminating proteins, the Ni^{2+} -NTA agarose was washed with native wash buffer containing 50 mM sodium phosphate buffer, pH 7.0, 300 mM sodium chloride, and 40 to 60 mM imidazole until the A_{280} was below 0.01. Native elution buffer containing 50 mM sodium phosphate buffer, pH 7.0, 300 mM sodium chloride, and 500 mM imidazole was used to elute the histidine-tagged proteins from the Ni^{2+} -NTA agarose. For smaller protein preparations, the Ni^{2+} -NTA Spin Kit was used with the same binding, wash, and elution buffers described above.

The one-step nickel-affinity chromatography procedure, specific for the 6-histidine-residue tag at the carboxyl-terminal end of each protein, produced a 95% homogeneous preparation of both enzymes (Fig. 2). Purification of the hydroxyquinol 1,2-dioxygenase yielded 10% of the initial protein activity with a specific activity of 3.71 U/mg after a 48-fold enrichment. The molar extinction coefficient for maleylacetate was reported by Tiedje et al. to be 42,000 $\text{M}^{-1} \cdot \text{cm}^{-1}$ (28). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1.0 μmol of maleylacetate per min. Purification of the maleylacetate reductase yielded 11% of the initial protein activity with a specific activity of 34.37 U/mg after a 195-fold enrichment.

Hydroxyquinol 1,2-dioxygenase in crude cell extracts was unstable and could not be stored at 4°C or frozen at -20°C without significant loss of activity. Purified protein was stable for 2 to 3 weeks at 4°C in elution buffer (50 mM sodium phosphate buffer, pH 7.0, 300 mM sodium chloride, 500 mM imidazole). Presumably, the enzyme stability was due to the high salt concentration. The enzyme was active in buffers of pH 6.0 to 7.2, but optimal activity was at pH 6.6. The enzyme was active between 23 and 52°C, with an optimal activity at 37°C.

Pure hydroxyquinol 1,2-dioxygenase was applied to a Superose-12 fast-protein liquid chromatography column under non-denaturing conditions and eluted in 50% elution buffer. The molecular mass of the native histidine-tagged enzyme, determined by a linear regression curve of the standard-molecular-

mass proteins (1,350 to 670,000 Da), was 68 kDa. The molecular mass of the denatured histidine-tagged protein was determined by SDS-PAGE to be 36 kDa with a linear regression curve of the standard low-molecular-mass proteins (14,400 to 97,400 Da). The molecular mass calculated from the amino acid sequence encoded by the *tftH* gene was 32.2 kDa (9). Thus, hydroxyquinol 1,2-dioxygenase appears to be a homodimer.

The K_m and V_{max} for hydroxyquinol 1,2-dioxygenase were determined from a Lineweaver-Burk plot ($1/S$ versus $1/V$). The k_{cat} value was calculated with the subunit molecular mass of 36 kDa. The K_m for hydroxyquinol as a substrate was $7 \mu\text{M}$, and the V_{max} was 5.25 U/mg . The k_{cat} value was 6.35 s^{-1} , and the calculated specificity constant was $0.907 \mu\text{M}^{-1} \cdot \text{s}^{-1}$.

The purified hydroxyquinol 1,2-dioxygenase was specific for hydroxyquinol as its substrate and was unable to catalyze ring cleavage of catechol, tetrahydroxybenzene, or 5-chloro-1,2,4-trihydroxybenzene. In addition, the product of hydroxyquinol spontaneous oxidation, 2-hydroxy-1,4-benzoquinone, was not a substrate for the purified enzyme. The 5-chlorohydroxyquinol (5-chloro-1,2,4-trihydroxybenzene) compound was synthesized according to a procedure described by Joshi and Gold (13) but modified to improve the yield and purity of the compound. The purified product was characterized by nuclear magnetic resonance and mass spectrometry. The purity of the compound was 99.8%, as determined by capillary gas chromatography. The inability of purified hydroxyquinol 1,2-dioxygenase to use 5-chloro-1,2,4-trihydroxybenzene as a substrate suggests that a reductive dechlorination of 5-chloro-1,2,4-trihydroxybenzene may be necessary prior to ring cleavage by hydroxyquinol 1,2-dioxygenase (Fig. 1). Preliminary evidence to support this assumption was found recently, when another gene required for 2,4,5-T metabolism was overexpressed in *E. coli*. The product of the *B. cepacia* AC1100 *tftG* gene (ORF 5) (9) overproduced in *E. coli* cell extracts was shown to alter 5-chloro-1,2,4-trihydroxybenzene, as determined by the absorbance spectrum of the autooxidized reaction product and by high-performance liquid chromatography analysis of the reaction product. In addition, crude cell extracts overproducing the *tftG* gene product, as well as the *tftH* gene product (hydroxyquinol 1,2-dioxygenase), were able to catalyze a decrease in the characteristic 5-chloro-1,2,4-trihydroxybenzene absorbance peaks. Purification and further characterization of the *tftG* gene product is under way to definitively determine its role in the 2,4,5-T metabolic pathway.

The product of the hydroxyquinol 1,2-dioxygenase reaction was proposed to be maleylacetate, on the basis of the appearance of a new absorbance peak at 243 nm. In order to confirm that maleylacetate was being produced from hydroxyquinol and that it served directly as the substrate for the *tftE*-encoded maleylacetate reductase, the product of the purified hydroxyquinol 1,2-dioxygenase assay was used as the substrate for the purified maleylacetate reductase assay. For the assays, the hydroxyquinol reaction was allowed to proceed for at least 15 min, and then 0.40 ml of this reaction mixture was added to 0.55 ml of Tris-HCl buffer, pH 8.0 (bringing the final pH to 7.5). Then, the purified maleylacetate reductase was added and the reaction was started by the addition of NADH and monitored at 340 nm. The results revealed that there was significant oxidation of NADH when purified maleylacetate reductase was added to the product of the hydroxyquinol dioxygenase assay. However, no oxidation of NADH was observed when maleylacetate reductase enzyme was omitted from the reaction nor when maleylacetate reductase was incubated with hydroxyquinol in the absence of hydroxyquinol dioxygenase enzyme. These results suggest that no other enzymes are required

for the conversion of hydroxyquinol to 3-oxoadipate in the lower pathway of 2,4,5-T degradation.

The NH_2 -terminal sequence of hydroxyquinol 1,2-dioxygenase was determined by electrophoresing the enzyme on an SDS-13% polyacrylamide gel and electroblotting the protein band onto a polyvinylidene difluoride hydrophobic membrane. The Coomassie blue R250-stained protein was sequenced by modified Edman degradation. The NH_2 -terminal 21 amino acids of hydroxyquinol 1,2-dioxygenase matched 100% the predicted NH_2 -terminal amino acid sequence encoded by the *tftH* gene (9). Comparison of the NH_2 -terminal amino acid sequences of hydroxyquinol 1,2-dioxygenases from *B. cepacia* AC1100, *Streptomyces rochei* 303, and *Azotobacter* sp. strain GP1 (33) revealed that the *B. cepacia* AC1100 and *Azotobacter* sp. strain GP1 NH_2 -terminal amino acid sequences share the highest identity (57%), whereas the NH_2 -terminal amino acid sequence from *S. rochei* 303 has similar identities to both the *B. cepacia* AC1100 and the *Azotobacter* sp. strain GP1 hydroxyquinol 1,2-dioxygenases (33 and 38%, respectively).

Although hydroxyquinol 1,2-dioxygenases have been purified from organisms which degrade chlorinated aromatic compounds (19, 33), none of these enzymes was shown to be directly involved in the pathways through genetic techniques. In addition, the purified maleylacetate reductases reported so far have been from microorganisms which degrade chlorinated aromatic compounds through the common catechol pathway, not the hydroxyquinol pathway (15, 25). The enzymes reported here were initially shown to be involved in the degradation of 2,4,5-T through genetic studies involving gene cloning and then were purified. The 2,4,5-T hydroxyquinol 1,2-dioxygenase gene is the first of its kind to be fully sequenced (9), and the deduced amino acid sequence should be useful in comparisons with catechol dioxygenases as well as for defining its critical domains through site-directed mutations. In addition, we have genetically and biochemically determined two steps of the lower 2,4,5-T pathway.

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