Biodegradation (2005) 16: 353–362

Identification and characterization of hydroxyquinone hydratase activities from *Sphingobium chlorophenolicum* ATCC 39723

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Accepted 11 August 2004

Key words: 2,5-dihydroxyquinol, 2,5-dihydroxyquinone, auto-oxidation, hydratase, hydroxyquinol, hydroxyquinone

Abstract

Hydroxyquinol, a common metabolite of aromatic compounds, is readily auto-oxidized to hydroxyquinone. Enzymatic activities that metabolized hydroxyquinone were observed from the cell extracts of *Sphingobium chlorophenolicum* ATCC 39723. An enzyme capable of transforming hydroxyquinone was partially purified, and its activities were characterized. The end product was confirmed to be 2,5-dihydroxyquinone by comparing UV/Vis absorption spectra, electrospray mass spectra, and gas chromatography–mass spectra of the end product and the authentic compound. We have proposed that the enzyme adds a H_2O molecule to hydroxyquinone to produce 2,5-dihydroxycyclohex-2-ene-1, 4-dione, which spontaneously rearranges to 1, 2,4,5-tetrahydroxybenzene. The latter is auto-oxidized by O_2 to 2,5-dihydroxyquinone. The proposed pathway was supported by the overall reaction stoichiometry. Thus, the transformation of hydroxyquinol to 2,5-dihydroxyquinone involves two auto-oxidation of quinols and one enzymatic reaction catalyzed by a hydratase. The specific enzymatic step did not require O_2, further supporting the assignment as a hydratase. To our knowledge, this is the first identification of a quinone hydratase, enhancing the knowledge on microbial metabolism of hydroxyquinone and possibly leading to the development of enzymatic method for the production of 2,5-dihydroxyquinone, a widely used chemical in various industrial applications.

Introduction

Hydroxyquinol and its oxidized form, hydroxyquinone, are central metabolites in microbial transformation of many compounds, including biopolymers (Rieble et al. 1994), aromatic compounds (Brune & Schink 1990; Chapman & Ribbons 1976; Chauhan et al. 2000; Gaal & Neujahr 1979; Gallus & Schink 1998; Jones et al. 1995; Philipp & Schink 1998; Philipp & Schink 2000; Spain & Gibson 1991; Stolz & Knackmuss 1993; Sze & Dagley 1984) and halogenated aromatic compounds (Apajalahti & Salkinoja-Salonen 1987; Daubaras et al. 1996; Latus et al. 1995; Reddy et al. 1998; Uotila et al. 1995; Zaborina et al. 1995). Spontaneous auto-oxidation of quinonols produces semiquinone radicals (Armstrong, et al. 1993; Guillen et al. 1997), which can damage protein and DNA molecules. Hydroxyquinol has been identified as an agent causing breaks in DNA molecules (Hiramoto et al. 1998; Li et al. 2000). Thus, biotransformation of hydroxyquinol and hydroxyquinone has physiological significance.

Several metabolisms of hydroxyquinol have been reported. Hydroxyquinol 1,2-dioxygenases are often found to cleave the aromatic ring to produce maleylacetate in aerobic degradation pathways. This metabolism has been reported in *Pseudomonas*

putida (Chapman & Ribbons 1976), *Burkholderia cepacia* AC1100 (Daubaras et al. 1996), *Azotobacter* sp. (Latus et al. 1995), *Sporotrichum pulverulentum* (Buswell & Eriksson 1979), *Phanerochaete chrysosporium* (Rieble et al. 1994), and *Trichosporon cutaneum* (Sze & Dagley 1984). Under anaerobic conditions, hydroxyquinol can be metabolized by several different pathways. Some fermenting microorganisms, such as *Pelobacter acidigallici* (Brune & Schink 1990) and *Eubacterium oxidoreductans* (Krumholz & Bryant 1988), degrade hydroxyquinol through phloroglucinol. Hydroxyquinol is transformed by transhydroxylation to phloroglucinol, which is reduced to dihydrophloroglucinol, and further metabolized to three acetate molecules (Brune & Schink 1990; Haddock & Ferry 1993). Recently, a second pathway of hydroxyquinol degradation under anaerobic conditions has been reported. Cell free extracts of *Desulfovibrio inopinatus* reduce hydroxyquinol to 1,2,4-trihydroxycyclohexa-1,3-diene, which is further degraded to acetate and an unknown product (Reichenbecher et al. 2000). A third hydroxyquinol degradation pathway under anaerobic conditions has been reported in *Azoarcus anaerobius* (Philipp & Schink 1998) and *Thauera aromatica* (Gallus & Schink 1998; Philipp & Schink 2000). Hydroxyquinol, a metabolic intermediate of resorcinol degradation in these bacteria, is oxidized to hydroxyquinone by a dehydrogenation reaction with simultaneous reduction of nitrate in cell extracts of *Azoarcus anaerobius*, suggesting hydroxyquinone is a metabolic intermediate. However, little is known about further metabolism of hydroxyquinone.

In search for a hydroxyquinol metabolizing enzyme in pentachlorophenol-degrading *Sphingobium chlorophenolicum* (formerly *Flavobacterium* sp.) strain ATCC 39723, we discovered a novel reaction catalyzed by a hydratase that converted hydroxyquinone to 2,5-dihydroxy(benzo)quinone (DHBQ) under aerobic conditions.

Materials and methods

Bacteria and culture conditions

Sphingobium chlorophenolicum strain ATCC 39723 was grown in a medium with glutamate as the carbon source (Saber & Crawford 1985). The cultures were grown in a 20-l carboy at room temperature with moderate aeration. Toward the end of the log phase, the cells were harvested by concentrating the volume to about 2 l using a hollow fiber filtration unit model DC10L (Millipore, Bedford, MA) and then centrifuged at 17, $000 \times g$ for 10 min at 3 °C. The pelleted cells were stored at −80 °C for up to several weeks.

Chemicals

Hydroxyquinol was obtained from Pfaltz & Bauer (Stamford, Conn.), and DHBQ from Alfa Aesar (Ward Hill, MA.). Hydroxyquinone was prepared by non-enzymatic auto-oxidation of hydroxyquinol in 20 mM potassium phosphate (KPi) buffer, pH 7 (more details in the Result Section).

Hydroxyquinone hydratase activity assay

The activity of hydroxyquinone hydratase was determined by monitoring the conversion of hydroxyquinone to DHBQ using a UV/visible spectrophotometer model 4000 (Pharmacia, Alameda, CA). The standard reaction contained 1ml of 20 mM KPi buffer pH 7.0, 100 μM hydroxyquinol, and an appropriate amount of the enzyme. Hydroxyquinol was added 30 min prior to adding the enzyme, to allow the complete auto-oxidation of hydroxyquinol to hydroxyquinone . The enzyme activity was determined from the increase of the peak of the end product at 320 nm. The $\varepsilon_{320\ nm}$ of the authentic DHBQ was determined to be 24, 490. One unit was defined as the amount of protein required to catalyze the formation of 1 nmol of the end product per min. The activity assays of the enzyme in the absence of oxygen were performed in an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI), which contained 96% N_2, 4% H_2, and undetectable amount of O_2 (<0.5 ppm) as shown by a Coy O_2 monitor inside the chamber. Samples were brought into the chamber through a sealed door chamber. After three cycles of vacuuming and pressurizing with N_2, the samples were transferred into the chamber. The samples were further equilibrated inside the chamber for 30 min before reactions were assembled. When 1 ml of 20 mM KPi buffer was treated under the same conditions, we found that the residual amount of O_2 was undetectable by using a flavin reductase (1 μg), FAD (1 μM) and NADH

(100 μM) system (Xun & Sandvik 2000). In the absence of O_2, NADH consumption is not detectable because $FADH_2$ is not oxidized back to FAD for continuous oxidation of NADH. The reaction mixtures were transferred into quartz cuvettes (Fisher Scientific, Pittsburgh, PA), sealed with rubber stoppers, taken out of the chamber, and analyzed spectrophotometrically. All reactions were done at 23–24 °C.

Purification of hydroxyquinone hydratase

All purification steps were performed at 4 °C. All buffers except the buffer for cell lysis and for phenyl-agarose column chromatography contained 0.5 mM dithiothreitol (DTT). Ammonium sulfate saturation levels were at 25 °C.

Extraction of cells
The cells (about 15 g wet weight) were harvested from a 15-l culture and resuspended in 30 ml of 20 mM KPi buffer, pH 7.0, containing 1 mM EDTA. The protease inhibitor phenylmethylsulphonyl fluoride freshly prepared in absolute ethanol was added to a final concentration of 0.5 mM. The cells were then broken by passing three times through a French pressure cell model FA-030 (Aminco, Urbana, IL) at 260 MPa. The lysate was then centrifuged at $17,000 \times g$ for 10 min to remove the cell debris and unbroken cells. The supernatant was saved and stored at −80 °C.

Ammonium sulfate fractionation
Ammonium sulfate was added to cell free extracts to 30% saturation with constant stirring. The pH of the solution was not adjusted. The mixture was centrifuged at $17{,}000 \times g$ for 10 min and the pellet was discarded. Additional ammonium sulfate was added to the supernatant to 70% saturation with constant stirring. The mixture was then centrifuged again at $17{,}000 \times g$ for 10 min, and the precipitate was saved.

Phenyl agarose chromatography
The precipitate was dissolved in 20 mM KPi buffer (pH 7.0) with 25% saturation of ammonium and loaded onto a phenyl agarose (Sigma, St. Louis, MO) column (1.5 × 12.5 cm) equilibrated with the same buffer. The proteins were eluted with a linear gradient of ammonium sulfate (25 to 0% saturation in 200 ml). The active fractions were pooled and solid ammonium sulfate was added to bring to 70% saturation. The sample was centrifuged and the pellet was saved.

Large scale DEAE column chromatography
The pellet from phenyl agarose column fractions was resuspended in 20 mM KPi (pH 7.0) buffer containing 0.5 mM DTT and dialyzed against the same buffer for 2 h. The dialyzed sample was loaded onto a DEAE Sepharose (Sigma) column (1.5 × 15 cm) equilibrated with the same buffer. The proteins were eluted with a linear gradient of NaCl (0 – 1 M, 100 ml). The active fractions were pooled and ammonium sulfate was added to 70% saturation. The mixture was centrifuged at 17, $000 \times g$ for 10 min and the pellet was saved.

Small scale DEAE column chromatography
The pellet from large scale DEAE purification step was resuspended in 1 ml of 10 mM Tris buffer (pH 8.0) containing 0.5 mM DTT and dialyzed against the same buffer. The sample was loaded onto a Bio Scale DEAE2 column (Bio Rad) equilibrated with the same buffer and eluted with a step and linear gradient of NaCl (concentration of NaCl: 0 mM, 5 ml; 0–500 mM, 20 ml; 1, 000 mM, 5 ml; 0 mM, 5 ml) using a BioLogical Chromatography System (BioRad). The active fractions were pooled.

Hydroxyapatite column chromatography
The buffer of the active fractions was changed to 8 mM KPi (pH 6.6) containing 0.3 mM $CaCl_2$ and 0.5 mM DTT by using Centriprep-10 (Millipore). The mixture was then loaded onto a hydroxyapatite column CHT2-I (Bio-Rad) equilibrated with the same buffer. The proteins were eluted with a step and linear gradient of KPi (concentration of KPi: 8 mM, 5 ml; 8–250 mM, 22 ml; 500 mM, 5 ml; 8 mM, 5 ml) using the BioLogic Chromatography System. The active fractions were pooled and concentrated to less than 1 ml by using Centriprep-10.

Gel filtration chromatography
The concentrated fractions from CHT2-I column were loaded onto a Superdex 75 column (10 × 300 mm, Pharmacia), equilibrated with 20 mM KPi, 150 mM NaCl buffer, pH 7.0. The proteins

were eluted with the same buffer, using the Bio-Logical FPLC system. The active fractions were pooled and saved.

SDS-PAGE

SDS-PAGE was performed as previously described (Laemmli, 1970). Low range molecular weight protein markers (BioRad) were used as reference. The gels were stained with Gel Code Blue stain (Pierce, Rockford, IL).

Oxygen consumption assay

Oxygen consumption was measured using YSI 5301B standard bath system (YSI, Yellow Springs, OH). The probe was calibrated using a previously described method (Robinson & Cooper 1970); 3 ml of experimental reaction was prepared by adding hydroxyquinol into 20 mM KPi buffer to a final concentration of 100 μM, and letting it oxidize to hydroxyquinone. The mixture was preincubated to determine endogenous oxygen consumption. The reaction was started by injection of appropriate amount of protein. The amount of hydrogen peroxide produced during hydroxyquinol auto-oxidation and the enzymatic reaction was also measured using the YSI system. After the reaction was completed, 3 ml was transferred into the oxygen probe chamber. After 10 min equilibration, 90 U of catalase (Sigma) were added and the amount of oxygen released from hydrogen peroxide was measured. The amount of hydrogen peroxide present in the reaction was calculated as twice of the oxygen released (Robinson & Cooper 1970).

Analytical methods

For end product identification, the enzymatic reaction was stopped by filtration through Centriprep-10 to remove proteins. The end product was then partially purified using an HPLC system (Waters, Milford, MA) equipped with Nova-Pak C^{18} column (3.9 × 150 mm, Waters) with isocratic flow of 10 mM ammonium acetate (pH 4.8, adjusted with acetic acid) at 0.5 ml per min. The end product was eluted off in the first peak at 2.23 min. The fraction was collected and injected directly in an electrospray mass spectrometer (MS) (ZQ4000, Waters). The capillary voltage was 3.75, and the cone voltage 30. The source block temperature was 100 °C, and desolvation temperature 250 °C for the MS. Gas chromatography (GC)–MS analysis of the end product was performed on QP5050A GC–MS system (Shimadzu,: Columbia, MD) equipped with a capillary column (30 m × 0.25 mm). The derivatization of the samples by acetylation was carried out using a previously described method (Knapp 1979). The solutions were acidified to pH 4, and extracted into ethyl acetate. The organic phase was dried, and the remaining solid dissolved in a mixture of pyridine and acetic anhydride (1:3). The solution was heated to 45 °C for 20 min, and then analyzed directly by GC–MS at a flow rate of 0.8 ml min^{-1} of helium. The oven parameters were 50 °C for 3.5 min, with a 30 °C min^{-1} increase to final temperature of 300 °C for 3 min; the injector temperature was 250 °C. The sample was analyzed with scan interval of 0.34 s and m/z range of 40 to 400.

Results

The activities of S. chlorophenolicum cell free extracts against hydroxyquinol and hydroxyquinone

When hydroxyquinol was added to a reaction mixture containing *S. chlorophenolicum* ATCC 39723 cell extracts, a product was produced that had an absorption maximum at 320 nm (Figure 1).

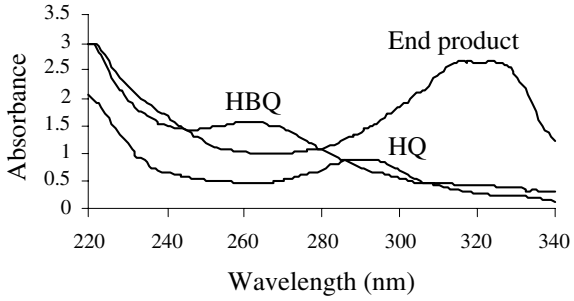

Figure 1. Absorption spectra of hydroxyquinol, hydroxyquinone, and the end product. Reaction was in 20 mM KPi buffer, pH 7. Hydroxyquinol spectrum (HQ) was taken immediately after 100 μM hydroxyquinol was added into the buffer; hydroxyquinone spectrum (HBQ) was taken 30 min later when the auto-oxidation of hydroxyquinol was complete. Cell extracts containing 2.6 mg of protein was added to the solution, the reaction was completed in 30 min. The end product spectrum was then taken.

In the absence of the cell extracts, hydroxyquinol was unstable in aqueous solutions, and autooxidized by O_2 to hydroxyquinone (Figure 1) within 5 min. The cell extracts also converted hydroxyquinone to the product with an absorption peak at 320 nm (Figure 1). When the reaction mixture was incubated under anaerobic conditions to prevent the auto-oxidation of hydroxyquinol, hydroxyquinol was not metabolized by the cell extracts. However, when hydroxyquinol was first auto-oxidized to hydroxyquinone and then O_2 was removed by degassing, transferring into an anaerobic chamber, and equilibrating for 30 min, the cell extracts still converted hydroxyquinone to the end product under anaerobic conditions. Thus, we concluded that the enzyme degrades hydroxyquinone, but not hydroxyquinol. The spectrum of the end product was a double peak with the absorption maxima of 315 and 325 nm and a center at 320 nm (Figure 1).

Purification of hydroxyquinone hydratase

Hydroxyquinone was used as the substrate to detect the enzyme activity. The enzyme was produced in a very small amount in the cell. Production of the enzyme is not induced by the addition of PCP in the medium and appears to be constitutive. We have tried several growth media, but failed to find a growth medium that enhanced the production of the hydroxyquinone hydratase. Even after 1000-fold purification, the enzyme was not purified to homogeneity. The protein eluted from phenol agarose column at 18–15% saturation of ammonium sulfate, from DEAE-Sepharose column at 100–300 mM of NaCl, and from DEAE-2 column at 150–200 mM NaCl. It eluted from gel filtration column with the retention volume of 12 ml, and from hydroxyapatite column at 100 mM KPi. Table 1 summarizes the purification steps.

Characterization of hydroxyquinone hydratase

The enzyme transformed hydroxyquinone, but not hydroxyquinol. The reaction with hydroxyquinone as a substrate produced about 40% product under anaerobic conditions in comparison to the same reaction under aerobic conditions. When the anaerobic sample was directly analyzed by HPLC analysis, hydroxyquinol was detected with a retention time of 3.06 min. When the anaerobic reaction was exposed to oxygen, it resumed the production of the end product. The final amount of the end product reached to the same level as the reaction incubated aerobically. The addition of EDTA to the reaction mixture did not prevent the enzymatic activity, suggesting that the enzyme does not use free cations as cofactors. The optimal pH was 8.5 (measured in the range of pH 4 to pH 10). Even the purest sample of this enzyme obtained in this study still contained several proteins with 3 dominant bands of 20, 000, 36, 000, and 71, 000 Da detected on SDS-PAGE gels (Data not shown). Native PAGE analysis also reveal several bands and did not reveal any clues on which band is the active one. Gel filtration indicated that the native enzyme was larger than 66 kDa. Although the intensity of a 71, 000-Da band by SDS-PAGE analysis corresponded to the level of activity of the fractions after gel filtration, we cannot confidently conclude that it was the protein of interest. The enzyme retained the same activity after one-month storage at −80 °C. No activity was observed against 1,4-benzoquinone, 6-chloro-2-hydroxybenzoquinone, and 5-methyl-2-

Table 1. Purification steps of hydroxybenzoquinone hydratase from *S. Chlorophenolica* ATCC 39723

Purification step	Total protein (mg)	Total activity (U)[a]	Spec. activity (U/mg)	Yield (%)
Cell free extract	1400	15,267	11	100
Ammonium sulfate	635	10,563	17	69
Phenol agarose.	240	4433	18	29
First DEAE column	26	3809	147	25
Second DEAE column	4.0	3300	825	22
Gel filtration	0.53	2083	3930	14
Hydroxyapatite	0.14	1555	11107	10

[a]One unit is defined as the production of 1 nmol of DHBQ per min.

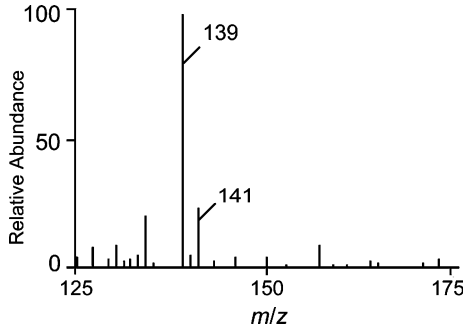

Figure 2. Negative ion mass spectrum of the end product. Hydroxyquinone was converted by purified hydratase to the end product with 320 nm peak, and the end product was purified by HPLC. The sample was then directly injected in an electrospray MS to obtain the mass spectrum.

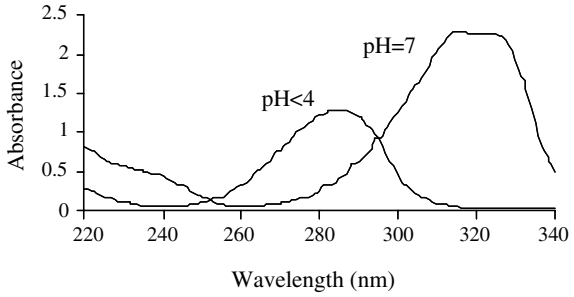

Figure 3. Absorption spectra of 100 μM 2,5-DHBQ in neutral and acidic buffers.

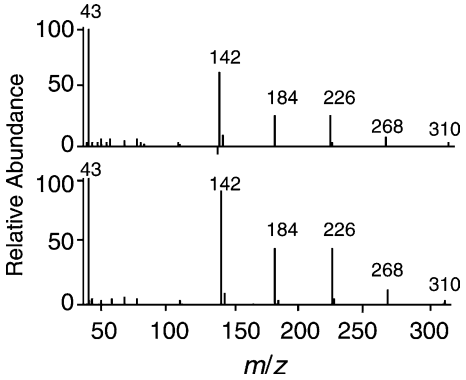

Figure 4. Mass spectra of (A, top) the acetylated end product and (B) the acetylated 2,5-DHBQ. The end product and 2,5-DHBQ were separately extracted into ethyl acetate, dried, and acetylated. The derivatized end product and 2,5-DHBQ were subject to GC–MS analysis, giving the same retention time at 11.21 min and virtually identical mass spectra.

hydroxybenzoquinone (the oxidized form of 2,4,5-trihydroxytoluene).

Analysis of the end product

The end product with the absorption peak at 320 nm was stable when stored in −80 °C, and it was resistant to short-term boiling. Its absorption maximum changed gradually to lower wavelengths when pH was lowered. Finally, at pH 4 and lower, the absorption maximum remained at 285 nm (Data not shown). The product was eluted off in a major peak centered at 2.23 min, which likely contained several hydrophilic compounds that have very low affinity to the column. The HPLC peak was collected and analyzed on an electrospray MS by direct injection. Two m/z peaks were observed at 139 and 141, with the peak at 139 being dominant (Figure 2). The m/z peak of 139 [M^-] represents a molecule of 140 Da, and the m/z peak of 141 [M^-] corresponds to a molecule of 142 Da. The two peaks apparently represented a molecule in its oxidized and reduced states. The 142 Da-molecule is equivalent to the addition of an H_2O molecule to hydroxyquinone (molecular weight of 124), indicating that the enzyme is a hydratase. H_2O_2 was produced during the reaction. For the auto-oxidation of 100 nmol of hydroxyquinol to hydroxyquinone alone, 79 ± 0.7 (average and SD of 3 samples) nmol of H_2O_2 was produced. Together with the reaction of hydroxyquinone hydratase, 171.4 ± 7.25 (3 samples) nmol of H_2O_2 was detected from 100 nmol of hydroxyquinol. The approximately stoichiometrical production of H_2O_2 indicates that the autooxidation of the direct reaction product is taking place as well as the auto-oxidation of hydroxyquinol.

Several isomers of dihydroxybenzoquinone could correspond to the molecule with a molecular weight of 140, giving an m/z peak of 139 [M^-]. The end product was compared to authentic DHBQ. DHBQ also absorbed maximally at 320 nm (Figure 3). After acidification to pH 4 or lower, the absorption maximum shifted to 285 nm (Figure 3), the same as the end product at pH lower than 4. HPLC analysis gave the same retention times for the end product and DHBQ at 2.23 min. After extraction into ethyl acetate and acetylation, both molecules had the same retention time of 11.21 min and identical mass spectra (Figure 4) by GC–MS analysis. The peak at m/z of 310 repre-

sented the fully derivatized molecule of tetrahydroxybenzene, produced from DHBQ by reduction during acetylation with pyridine and acetic anhydride, the same as previously reported (Farfan et al. 1986). The peaks at m/z of 268, 226, 184, and 142 represented the molecule after the loss of 1, 2, 3, and 4 acetyl groups, respectively. The peak at m/z 43 represented acetyl moiety. The data support that the end product is DHBQ. Electrospray MS of the authentic DHBQ gave a single m/z peak at 139 [M^-], which corresponds to the 139 peak of the end product (Figure 2). The $\varepsilon_{320\ nm}$ of the authentic DHBQ was $24,490 \pm 140$ (5 samples). Using this determined molar extinction coefficient, it was determined that 88.3 ± 3.3 (3 samples) μM DHBQ was produced from 100 μM HBQ after the completion of the reaction.

Discussion

The physiological importance of hydroxyquinone hydratase for the cells has not been determined in this study. Hydroxyquinol was originally postulated to be an intermediate in pentachlorophenol degradation, as for 2,4,5-trichlorophenol degradation by *Burkholderia cepacia* AC1100 (Daubaras et al. 1996). It has recently been reported that 2,6-dichloro-*p*-hydroquinone 1,2-dioxygenase converts 2,6-dichloro-*p*-hydroquinone directly to 2-chloromaleylacetate in *S. chlorophenolicum* (Ohtsubo et al. 1999; Xun et al. 1999), bypassing the step of hydroxyquinol. However, the possible participation of hydroxyquinol in PCP degradation initiated our study on hydroxyquinol metabolism in *S. chlorophenolicum*. The metabolism of hydroxyquinol to a product that has an absorption peak at 320 nm was observed with the cell extracts of *S. chlorophenolicum* ATCC 39723 (Figure 1), and the observed specific activity was quite high (Table 1). The enzyme concentration in the cell must be very low, which prevented us from purifying the protein (Table 1). Nonetheless, we believe that the enzyme is real because of the high specific activity for DHBQ production at 10, 798 n mol min^{-1} mg^{-1} of the partially purified proteins.

The enriched enzyme was used to characterize the reaction and the end product. We showed evidence that the end product is DHBQ. The end product and DHBQ have matched UV/Vis absorption spectra (Figures 1 and 3), and both spectra respond to pH changes in the same way (Figure 3). The most convincing evidence is the matched retention times and MS spectra of the derivatized end product and DHBQ (Figure 4).

Given the end product is DHBQ, the reactions proposed in Figure 5 is most logic pathway. Hydroxyquinol is first auto-oxidized by O_2 to hydroxyquinone with the concurrent formation of H_2O_2. The enzyme adds an H_2O molecule to hydroxyquinone to produce 2,5-dihydroxycyclohex-2-ene-1, 4-dione that rearranges to 1,2,4,5-tetrahydroxybenezene. The latter is finally auto-oxidized by O_2 to DHBQ and H_2O_2. Several lines of evidence support the proposed pathway. First, DHBQ is identified as the end product. Second, stoichiometry of the overall reaction, the conversion of one hydroxyquinol to one DHBQ and two H_2O_2, agrees with the proposed pathway. Third, electrospray MS also detected a major molecule of 140 Dalton ([M^-] = 139) and a minor molecule of 142 Dalton ([M^-] = 141) from the reaction mixture (Figure 2). The 140-Dalton molecule likely represents DHBQ, while the 142-Dalton molecule could be either 2,5-dihydroxycyclohex-2-ene-1,4-dione or 1,2,4,5-tetrahydroxybenezene. The presence of both DHBQ and 1,2,4,5-tetrahydroxybenezene could not be verified by the GC–MS analsysis, as the derivatization for GC–MS analysis reduced DHBQ back to 1,2,4,5-tetra-

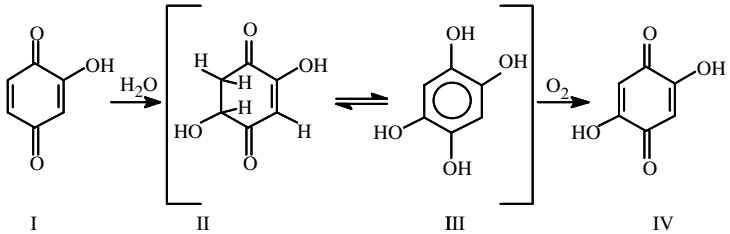

Figure 5. Proposed reactions of converting hydroxyquinone to 2,5-DHBQ by hydroxyquinone hydratase and auto-oxidation. Compound I is hydroxyquinone; II, 2,5-dihydroxycyclohex-2-ene-1, 4-dione; III, 1,2,4,5-tetrahydroxybenzene, and IV, 2,5-DHBQ.

hydroxybenezene and then acetylated the latter. Thus, both DHBQ and 1,2,4,5-tetrahydroxybenezene in the sample would both be identified as derivatized 1,2,4,5-tetrahydroxybenezene by the GC–MS analysis. Nonetheless, the possible presence of either 2, 5-dihydroxycyclohex-2-ene-1,4-dione or 1,2,4,5-tetrahydroxybenezene in the reaction mixture also support the proposed pathway and the assignment of the enzyme as hydroxyquinone hydratase. Fourth, the enzymatic reaction occurred under anaerobic conditions, but the reaction was incomplete. The limited production of DHBQ is due to the lack of free O_2 to oxidize 1,2,4,5-tetrahydroxybenezene. When the reaction is exposed to O_2, the reaction proceeded to completion. Under anaerobic conditions, the produced 1,2,4,5-tetrahydroxybenezene may also react with hydroxyquinone in the reaction mixture to produce hydroxyquinol and DHBQ, which were detected in the anaerobic reaction mixture by HPLC and UV/Vis spectrometry. Quinones and quinols are known to equilibrate under anaerobic conditions, and we have previously reported a similar phenomenon (Zaborina et al. 1998).

The enzyme is assigned as a hydroxyquinone hydratase in the proposed overall conversion of hydroxyquinol to DHBQ. Further evidence supports the assignment is that the enzymatic step occurs under both aerobic and anaerobic conditions, ruling out the possibility of an oxygenase reaction. Since there were no other added co-substrates, the only enzymatic reaction that could occur is hydration.

The reported hydroxyquinone hydratase activity is novel. Quinones are known to be reduced to quinols for further metabolism. To our knowledge, this is the first identification of a quinone hydratase. The documentation of this novel enzymatic reaction may provide guidance for the understanding of microbial metabolism of hydroxyquinone, which has been reported as a metabolic intermediate in several microorganisms (Gallus & Schink 1998; Philipp & Schink 1998; Philipp & Schink 2000; Valli et al. 1992). Inside cells, 1,2,4,5-tetrahydroxybenezene may be further metabolized without the formation of DHBQ, especially under anaerobic conditions. Tetrahydroxybenzene can be used in transhydroxylation reactions with hydroxyquinol, pyrogallol and phloroglucinol, or cleaved by a ring-cleavage dioxygenase, as previously reported for the metabolism of related compounds (Armstrong & Patel 1993; Haddock & Ferry 1993; Krumholz & Bryant 1988; Reichenbecher & Schink 1999). Thus, the presence of a hydroxyquinone hydratase in *S. chlorophenolicum* indicates the possible presence of an as yet undiscovered aromatic catabolism pathway in this organism. Alternatively, DHBQ and its derivatives have been reported to inhibit processes such as primary photochemical events in *Rhodospirillum rubrum* (Bering et al. 1981) and to quench chlorophyll *a* fluorescence (Natanajan & Blankenship 1983). Therefore, DHBQ or its derivatives could be anabolic compounds produced as metabolites acting as antibiotics for both bacteria and plants. Such questions encourage the sequencing of the complete genome of *S. chlorophenolicum* ATCC 39723 to shed light on the novel pathways participating in the metabolism of this catabolically unusual strain.

DHBQ and its direct derivatives are important molecules used in many industrial applications. It is used as a stabilizer of polycaprolactam yarns and films (Matusevitch et al. 1982), as a component of direct hair dyes (Lang et al. 1983), as a suppressor of hydration of ferromagnetic ions in magnetic recording media (Suzuki et al., 1984), as a component of stain-removing compositions (Gogek 1965), as a component in aqueous drilling fluids which improves dispersion and reduces viscosity (Kolaian 1967), as a component of antiallergics, especially antiasthmatics in medicine (Iwaki et al. 1985), and as a component of photoconducting layer of electrostatic copiers (Aftergut 1996). Thus, the discovery of this enzymatic activity may also lead to the development of enzymatic method for the production of DHBQ.

Acknowledgments

This research was supported by NSF grant MCB-0323167.

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