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Hydroxyhydroquinone reductase, the initial enzyme involved in the degradation of hydroxyhydroquinone (1,2,4-trihydroxybenzene) by *Desulfovibrio inopinatus*

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Abstract The recently isolated sulfate reducer *Desulfovibrio inopinatus* oxidizes hydroxyhydroquinone (1,2,4-trihydroxybenzene; HHQ) to 2 mol acetate and 2 mol CO₂ (mol substrate)⁻¹, with stoichiometric reduction of sulfate to sulfide. None of the key enzymes of fermentative HHQ degradation, i.e. HHQ-1,2,3,5-tetrahydroxybenzene transhydroxylase or phloroglucinol reductase, were detected in cell-free extracts of *D. inopinatus*, indicating that this bacterium uses a different pathway for anaerobic HHQ degradation. HHQ was reduced with NADH in cell-free extracts to a nonaromatic compound, which was identified as dihydrohydroxyhydroquinone by its retention time in HPLC separation and by HPLC-mass spectrometry. The compound was identical with the product of chemical reduction of HHQ with sodium borohydride. Dihydrohydroxyhydroquinone was converted stoichiometrically to acetate and to an unknown coproduct. HHQ reduction was an enzymatic activity which was present in the cell-free extract at 0.25–0.30 U (mg protein)⁻¹, with a pH optimum at 7.5. The enzyme was sensitive to sodium chloride, potassium chloride, EDTA, and *o*-phenanthroline, and exhibited little sensitivity towards sulfhydryl group reagents, such as copper chloride or *p*-chloromercuribenzoate.

Key words Trihydroxybenzenes · Hydroxyhydroquinone · Phloroglucinol · Reductive dearomatization · *Desulfovibrio* sp.

Introduction

Hydroxyhydroquinone (1,2,4-trihydroxybenzene; HHQ) is an intermediary product in the aerobic degradation of hydroxylated aromatic compounds such as phloroglucinol, resorcinol, resorcylic acid, *p*-hydroxybenzoate, phenol, and dibenzo dioxin (Chapman and Ribbons 1976; Karasevich et al. 1976; Patel et al. 1990; Stolz and Knackmuss 1993; Armengaud et al. 1999). In some microorganisms, oxygenases have been found which form maleylacetate from HHQ, which is further reduced to 3-oxoadipate. The pathway is known as the HHQ variation of the pathway that usually involves catechol as intermediate (Middelhoven 1993).

In the absence of molecular oxygen, trihydroxybenzenes are degraded by fermenting bacteria such as *Eubacterium oxidoreducens*, *Pelobacter acidigallici*, *Pelobacter massiliensis*, and the homoacetogenic *Holophaga foetida* through the phloroglucinol pathway (Schink and Pfennig 1982; Krumholz et al. 1987; Brune and Schink 1990; Schnell et al. 1991; Kreft and Schink 1993). One or three transhydroxylation reactions transform pyrogallol (1,2,3-trihydroxybenzene) or HHQ to phloroglucinol (1,3,5-trihydroxybenzene; Brune and Schink 1990; Brune et al. 1992), which is subsequently reduced to dihydrophloroglucinol (Samain et al. 1986; Haddock and Ferry 1989; Brune and Schink 1992) and further oxidized to three acetate residues.

Reductive steps are common reactions for the destabilization of aromatic compounds (Schink et al. 1992; Heider and Fuchs 1997). With benzoyl-CoA, reductive dearomatization requires electrons at very low redox potential and ATP as an additional energy source to overcome the mesomery energy barrier (Boll et al. 1997). In resorcinol (1,3-dihydroxybenzene) reduction by fermenting bacteria, the electron donor has not yet been identified; the reaction is measured in vitro with low-potential electron donors such as benzyl viologen (Kluge et al. 1990; Schüler and Schink, unpublished data). Reductive dearomatization of phloroglucinol to dihydrophloroglucinol is catalyzed by an NADPH-dependent enzyme.

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Recently, we isolated a novel sulfate-reducing bacterium, *Desulfovibrio inopinatus*, with HHQ as sole organic substrate in the presence of sulfate (Reichenbecher and Schink 1997). This bacterium does not contain phloroglucinol reductase or any transhydroxylating activity. In the present communication, we describe a new reductive dearomatization reaction in *D. inopinatus* which reduces HHQ to dihydrohydroxyhydroquinone. This enzyme differs substantially from the phloroglucinol reductases of *E. oxidoreducens* (Haddock and Ferry 1989), *P. acidigallici* (Brune and Schink 1992), and *P. massiliensis* (Brune et al. 1992).

Materials and methods

Source of strain and growth conditions

Desulfovibrio inopinatus strain HHQ20 (DSM 10711) was described in detail earlier (Reichenbecher and Schink 1997) and was taken from our own culture collection. The strain was cultivated in a bicarbonate-buffered, sulfide-reduced anoxic mineral medium with 1% salinity and 20 mM sulfate (Widdel and Pfennig 1981). The medium was dispensed in 50-ml serum bottles sealed with butyl rubber stoppers, or in 1-l infusion bottles sealed with rubber septa with a N₂/CO₂ (90:10 v/v) headspace. Substrates were added from concentrated stock solutions. Solutions of oxygen-sensitive compounds were prepared anoxically and sterilized by filtration. Purity was checked microscopically in cultures grown in mineral media with 2 mM HHQ and in media containing 0.05% (w/v) yeast extract, 5 mM glucose, and 5 mM fumarate.

Enzyme assays in cell-free extracts

Cells of a 1-l culture in the late exponential growth phase were harvested in an anoxic chamber (Coy, Ann Arbor, Mich., USA) with an N₂/H₂ atmosphere (95:5 v/v), centrifuged at 13,000×g for 20 min, and washed with 250 ml anoxic 100 mM potassium phosphate buffer, pH 7.0. Extracts were prepared anoxically by passing resuspended cells twice through a French press cell at 138 MPa. Cell debris was removed by centrifugation at 20,000×g for 20 min. This preparation is subsequently referred to as the cell-free extract. The cytoplasmic and membrane fractions were separated by ultracentrifugation at 160,000×g for 30 min.

A six-fold enriched HHQ reductase preparation was obtained by passing a 160,000×g supernatant fraction over a HiTrap Q anion exchange column and eluting with a linear NaCl gradient (0–500 mM).

Enzyme activities were measured anoxically at 25°C, either discontinuously by HPLC or continuously by photometry. In routine tests, reduction of HHQ with NADH was measured photometrically as the decrease in NADH at 365 nm wavelength in oxygen-free 100 mM potassium phosphate buffer, pH 7.0, in the presence of 0.2 mM dithioerythritol. Protein was quantified with bovine serum albumin as standard (Bradford 1976).

Degradation of HHQ and formation of the product was monitored discontinuously by HPLC analysis. The assay was performed under strictly anoxic conditions. An assay mixture used for HPLC-MS analysis contained 50 mM potassium phosphate buffer (pH 7.0), 0.3 mM NADH, and 100 µl cell-free extract (0.4–0.5 mg protein) and was started by the addition of 1 mM HHQ. Samples (100 µl) were taken from the reaction mixture at regular intervals and immediately transferred to 400 µl phosphoric acid (100 mM) in order to stop all biological activities.

HPLC analysis

Aromatic compounds and NAD(H) were routinely analyzed by HPLC equipped with a C₁₈ reversed phase column (Grom, Herrenberg, Germany), a UV detector and an autosampler (Brune and Schink 1990). For separation of HHQ and the reduction product dihydrohydroxyhydroquinone, a gradient with ammonium acetate buffer (10 mM, pH 3.8 adjusted with acetic acid) as eluent A and methanol as eluent B was used. One separation run took 8 min and started with 10% B. Eluent B was increased at 0.5 min to 20% within 0.3 min, at 1.5 min to 35% within 3.4 min, and was decreased to 10% at 6.0 min within 0.2 min. The flow rate was 1 ml min⁻¹ and the detection wavelengths were 206 nm and 260 nm. UV/VIS on-line scans were acquired with a Beckman 168 diode-array detector. Alternatively, HHQ reductase reaction products were analyzed by HPLC ion exchange chromatography. The HPX 87-H-ion exchange column from Biorad (Munich, Germany) was eluted with 5 mM sulfuric acid at 0.6 ml min⁻¹ and connected to a refraction index detector. With this method, acetate and an unidentified product eluting at 31 min were separated.

HPLC/mass spectroscopy

Mass spectra were acquired on a Platform LC (Micromass, Manchester, UK), using negative ion electrospray. The needle voltage was set to 3.75 kV, the cone voltage to 26 V, giving soft ionization conditions. Nitrogen gas was used as nebulizer and drying gas (150°C). The mass spectrum was scanned from 90 to 130 Da at one scan per second. HPLC analysis was performed under the same conditions as described above for separation of HHQ and its reduction product, using a Hewlett Packard 1100 system.

Chemical reduction of HHQ

For chemical reduction of HHQ, 2.5 ml of freshly and anoxically prepared 10 mM NaBH₄ was added dropwise to 500 µl of 50 mM HHQ within 10 min while stirring. The reaction was performed under strictly anoxic conditions in a 5-ml serum bottle sealed with a butyl rubber stopper, and the reaction mixture was incubated at room temperature for 3–4 h. HHQ reduction and product formation were monitored discontinuously as described above for the HHQ reductase assay.

Chemicals

All chemicals were of analytical or reagent grade quality and were obtained from Biomol (Ilvesheim, Germany), Boehringer (Mannheim, Germany), Eastman Kodak (Rochester, NY), Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Pharmacia (Freiburg, Germany), Serva (Heidelberg, Germany), and Sigma (Deisenhofen, Germany). Gases were purchased from Messer-Griesheim (Darmstadt, Germany), and Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

Results

Reduction of HHQ

Cell-free extract of *D. inopinatus* does not contain phloroglucinol reductase or HHQ: 1,2,3,5-tetrahydroxybenzene transhydroxylase activity (Reichenbecher and Schink 1997). Instead, cell-free extracts (20,000×g supernatant) contained a HHQ reductase activity that used NADH as electron donor. The measurable activity was strictly proportional to the amount of extract protein provided in the

Fig. 1 HHQ reduction by cell-free extract of *Desulfovibrio inopinatus*. The assay contained 0.4 mg cell-free extract, 0.8 mM NADH (□) and 1 mM HHQ (○) at time zero. NAD⁺ (■) and an unknown product eluting at 3.83 min (●) were formed. NADH and HHQ are shown at their respective concentrations, the unknown compound by its HPLC peak area

assay (results not shown). The specific activity was 0.25–0.45 U (mg protein)⁻¹, which is substantially higher than the metabolic activity of growing cells [0.07 μmol min⁻¹ (mg protein)⁻¹]. When cells were grown with fructose or lactate, HHQ reductase activity was only 0.003 U or 0.005 U (mg protein)⁻¹, respectively, indicating that this enzyme is not constitutively expressed. SDS-PAGE analysis revealed the presence of a 70-kDa and a 45-kDa protein band in a cell-free extract of HHQ-grown cells; these bands were absent in cell-free extracts of cells grown with fructose or lactate (results not shown). When cell-free extract was passed over a HiTrap Q anion exchange column, the 70-kDa and the 45-kDa protein band were both enriched (results not shown). The enzyme activity was localized nearly exclusively in the cytoplasmic fraction, which contained more than 80% of the total activity.

HHQ was stoichiometrically reduced with NADH to form NAD⁺ and an unidentified reduction product (Fig. 1); the ratio of HHQ to NADH varied between 0.931 and 1.06. With NADPH as electron donor, the specific activity was 87% of that obtained with NADH. Other hydroxylated aromatic compounds such as catechol, resorcinol, hydroquinone, pyrogallol, phloroglucinol or 2,5-dihydroxyaniline were not reduced at significant rates.

Identification of the reaction product and its further degradation

When HHQ was reduced with NADH, small amounts of a product accumulated transiently during the first 10 min of the reaction (Fig. 1). This product shared properties with dihydroxyhydroquinone, which was chemically produced from HHQ with sodium borohydride. The products of biochemical and chemical reduction both eluted from the HPLC column at 3.83 min (Fig. 2A, B), both compounds exhibited an absorption maximum at 260 nm (Fig. 2A–C), and both compounds had a mass of 127 when characterized as deprotonated mono-anions by mass

Fig. 2A–C HPLC Chromatograms and absorption spectra of the products of biochemical and chemical reduction of HHQ. The straight line was recorded at 206 nm, the dotted line at 260 nm wavelength. **A** Chromatogram of a sample taken from the biochemical reaction after 14 min. About 1 mM HHQ was reduced with about 1 mM NADH by 0.4 mg cell-free extract. **B** Chromatogram of a sample withdrawn from the chemical reaction after 1 h. The reduction was carried out by adding 1 mM sodium borohydride dropwise to 1 mM HHQ. Samples taken from both reactions were diluted five-fold prior to chromatography. **C** Absorption spectra of the chemical assay at time zero (solid line) and after 1 h (dotted line) and of the biochemically produced compound eluting at 3.83 min from the reversed phase column (dashed line). Spectra were recorded in ammonium phosphate buffer, pH 2.6

spectroscopy (Fig. 3). These data, together with those on the reaction stoichiometry, allow the conclusion that dihydroxyhydroquinone (1,2,4-trihydroxy 1,3-cyclohexadiene) is the primary reduction product of the HHQ reductase reaction.

Dihydroxyhydroquinone was not stable in cell-free extracts but reacted further (Fig. 1). Two more stable products were found that were formed mainly between

Fig. 3 Negative-ion mass spectra of the product of biological (A) and chemical (B) HHQ reduction. The compounds were separated by HPLC prior to mass spectrometric analysis. Spectra A1 and B1 correspond to the reduction product with a mass of 127 Da, spectra A2 and B2 to HHQ

5 min and 15 min after start of the HHQ reductase reaction (Fig. 4A). One product was identified as acetate, which was formed stoichiometrically from HHQ at a 1:1 ratio. The second, so far unknown product appeared concomitant with acetate (Fig. 4A). It exhibited absorption peaks at 223 and 293 nm and a broader absorption band at 429 nm wavelength (Fig. 4B). HPLC comparison with an authentic reference compound indicated that this compound was not identical with succinate semialdehyde, which could be the coproduct of a hypothetical hydrolytic ring cleavage reaction.

Reaction conditions and enzyme stability

The dependence of the HHQ reductase activity on the prevailing pH was tested in a buffer mixture containing 25 mM MES and 25 mM MOPS. The activity exhibited a clear optimum between pH 7.2 and 7.5, and dropped sharply at higher pH (not shown). Although *D. inopinatus* is a brackish water organism that grows optimally with 1% NaCl in the medium, HHQ reductase activity was inhibited by the presence of NaCl in the assay, with 50% inhibition at 120 mM NaCl (not shown). KCl had a similar, $(\text{NH}_4)_2\text{SO}_4$ a lower inhibitory effect. HHQ reductase activity in cell-free extracts was comparably stable under air at pH 7.0 or 6.0, but was destroyed nearly completely during storage at pH 2.7. There was no significant difference in stability in the presence or absence of air oxygen. Surprisingly, the activity was less stable during storage on ice

Fig. 4A,B Conversion of HHQ in cell-free extract of *D. inopinatus* and absorbance spectrum of one product. **A** Time course of the reaction showing the decrease of HHQ (○) and NADH (□) and the delayed formation of acetate (▲) and an unknown product (▼) eluting from the ion exchange column at 31 min retention time. **B** UV absorption spectrum of the unknown product. Fractions containing this compound were collected from the ion exchange column, lyophilized, and redissolved in 10 mM acetic acid

than at room temperature; however, activity losses after cold incubation could be largely restored upon re-exposure to room temperature (24 °C; Fig. 5).

Fig. 5 Stability of HHQ reductase of *D. inopinatus*. Effect of temperature during incubation in 100 mM potassium phosphate buffer, pH 7.0, at different conditions. Cell-free extract was freshly prepared and kept anoxic at room temperature (24°C) (■), anoxic on ice (□), or under air on ice (○). The latter was set to room temperature after 24 h of incubation

HHQ-reductase was fairly stable to thermal denaturation and did not lose activity during 10 min incubation at 50°C. At 60°C, 10 min incubation caused an activity loss of 50%, and 5 min incubation at 95°C destroyed the activity entirely.

The enzyme activity was comparably stable towards inhibitors tested with other reductase enzymes (Table 1). There was little sensitivity towards *p*-chloromercuribenzoate and copper chloride, and iodoacetamide had no effect. EDTA caused a rather strong inactivation, with 50% activity loss after 5 min of incubation with 0.2 mM EDTA; *o*-phenanthroline acted in a similar manner. This inactivation pattern differs substantially from that of phloroglucinol reductase of *E. oxidoreducens* (Table 1).

Discussion

In the present communication, we report for the first time on an enzyme that destabilizes HHQ by a reduction reaction. This enzyme plays a key role in anaerobic HHQ degradation by *D. inopinatus*. Either NADH or NADPH could serve as electron donors in this reaction. Several fermenting bacteria employ NADPH as electron donor in reductive destabilization of the trihydroxybenzene isomer phloroglucinol (Samain et al. 1986; Haddock and Ferry 1989; Brune and Schink 1992). Although the two reactions initially appear to be similar, HHQ reductase differs substantially from phloroglucinol reductases, of which the enzyme of *E. oxidoreducens* has been described best (Haddock and Ferry 1989). Both enzymes are very specific for their respective substrate. They differ substantially with respect to inactivation by various compounds (see Table 1). Whereas HHQ reductase shows little sensitivity towards sulfhydryl reagents such as *p*-chloromercuribenzoate and copper chloride, phloroglucinol reductase is inactivated very strongly by these compounds. On the other hand, HHQ reductase is sensitive to EDTA and *o*-phenanthroline, which does not apply for phloroglucinol reductase. These results indicate that divalent cations such as Fe²⁺ may play an essential role in the reaction of HHQ reductase, whereas sulfhydryl groups appear not to be involved, and the opposite appears to be true for phloroglucinol reductase. Thus, although the catalyzed reactions appear to be similar, the reaction mechanisms might be substantially different.

In phloroglucinol, all hydroxyl groups are in *meta*-position to each other, which allows a tautomerization to a nonaromatic trioxo isomer and renders a reductive attack easy. Pyrogallol has all hydroxyl groups in *ortho*-position to each other and can be attacked preferentially by an oxygenase reaction, analogous to catechol; for anaerobic degradation of pyrogallol, a transformation to phlorogluci-

Table 1 Inactivation of HHQ reductase activity in cell-free extracts of *Desulfovibrio inopinatus* and of purified phloroglucinol reductase of *Eubacterium oxidoreducens*

Compound	HHQ reductase ^a			Phloroglucinol reductase ^b		
	Concentration (mM)	Incubation time (min)	Remaining activity (%)	Concentration (mM)	Incubation time (min)	Remaining activity (%)
<i>p</i> -Chloro-mercuribenzoate	1	20	69	0.1	5	0
CuCl ₂	0.1	5	87	0.1	0.2	0
	0.1	30	88	0.001	0.2	57
Iodoacetamide	10	1	106	1	25	100
	10	30	104			
EDTA	2	30	21	2	200	100
	0.2	1	79			
	0.2	5	51			
<i>o</i> -Phenanthroline	0.5	5	16	10	200	68

^aOne milligram of cell-free extract was incubated at 24°C in 100 mM potassium phosphate buffer, pH 7.0, for the time indicated prior to activity assay. Enzyme assays contained 2 mM

HHQ, 0.3 mM NADH, and 0.03 mg crude extract. 100% activity equalled 109 mU (mg protein)⁻¹

^bData taken from Haddock and Ferry (1989)

Fig. 6 HHQ reduction by HHQ reductase of *D. inopinatus* and tautomeric forms of dihydrohydroxyhydroquinone. Only one of the possible HHQ tautomers carrying a carbonyl group is shown

nol is required (Schink and Pfennig 1982; Brune and Schink 1990). HHQ carries hydroxyl groups in *meta*-position and in *ortho*-position to each other. The latter allows HHQ to be attacked by an oxygenase (Middelhoven 1993), and the former makes HHQ prone to direct reduction (this article). As a third alternative, HHQ is transformed to phloroglucinol, which is reduced afterwards (Brune et al. 1992). Obviously, the hydroxyl group in *ortho*-position does not prevent a reduction reaction that probably acts on the two hydroxyl groups in *meta*-position. Thus, the different chemistries of the trihydroxybenzene isomers HHQ and phloroglucinol might explain why different reductase enzymes with different reaction mechanisms are required.

The reduction of HHQ probably proceeds via reduction of a carbonylic group, analogous to other NADH-dependent reductase reactions. HHQ tautomers containing at least one carbonylic group can be formulated, and one of these tautomers has been suggested in the reaction scheme given in Fig. 6. The primary product of the HHQ reductase reaction was identified as dihydrohydroxyhydroquinone, a nonaromatic compound for which again several different tautomeric structures can be anticipated (Fig. 6). It is premature to speculate which structure may be prevalent; the form(s) prevailing in aqueous solution may be different from that on which the subsequent enzymatic cleavage reaction acts. The 1,3-dioxo tautomer of this aliphatic ring compound appears to be especially prone to a nucleophilic (thiolytic or hydrolytic) attack on carbon atoms 1 or 3, releasing an acetyl moiety and a four-carbon residue similar to succinate semialdehyde. The reaction product we found, together with acetate, was not identical with succinate semialdehyde, but it can be assumed that this rather unstable intermediate would quickly be further

metabolized. The absorption spectrum of this compound indicates strong absorption maxima that might be attributed to double linkages; however, the identity of this compound is still open at the moment. Nonetheless, cleavage of the HHQ molecule to an acetyl residue and a four-carbon compound that might be metabolized to succinate is in accordance with the overall product pattern of HHQ degradation by *D. inopinatus*, provided that the four-carbon compound is further oxidized to one acetyl moiety, with concomitant transfer of the released electrons to sulfate reduction. The ring cleavage reaction will be subject to further study in our laboratory.

The HHQ reductase activity described in this communication initiates a further pathway of anaerobic HHQ degradation, which differs entirely from that used by the nitrate-reducing bacteria *Azoarcus anaerobius* (Philipp and Schink 1998) and *Thauera aromatica* (Gallus and Schink 1998). Whereas the nitrate reducers prepare HHQ ring cleavage by oxidation, *D. inopinatus* destabilizes the aromatic structure by a reductive step. With this, the strategy of *D. inopinatus* more nearly resembles the pathways of anaerobic degradation of aromatic compounds described so far, which all destabilize the aromatic ring by reduction (Schink et al. 1992; Fuchs et al. 1994; Heider and Fuchs 1997; Schink et al., in press). The new HHQ transformation reactions discovered recently add further details to the picture of anaerobic degradation of aromatic compounds, and broaden our view of the diversity of microbial metabolic capacities in general.

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