AROMATIC RING CLEAVAGE BY THE WHITE-ROT FUNGUS *SPOROTRICHUM PULVERULENTUM*

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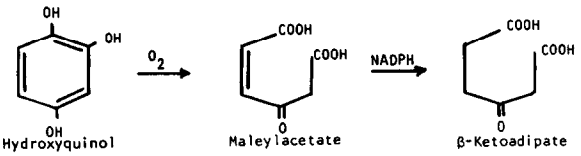
1. Introduction

Compared to other microbial groups relatively little is known about the modes of aromatic ring cleavage in the lignin-degrading white-rot fungi [1–3]. In an investigation of vanillic acid catabolism by *Sporotrichum pulverulentum* we have demonstrated an enzyme which catalyses the oxidative decarboxylation of vanillate to methoxyhydroquinone [4]. However, no further conversion of methoxyhydroquinone or cleavage of the benzene nucleus was observed using cell-free systems even though $^{14}CO_2$ is released from ring-labelled vanillate in growing cultures [5]. Similarly, two potential substrates for ring-fission dioxygenases, protocatechuic acid and gentisic acid, were not affected by another white-rot fungus, *Polyporus dichrous*, which also converts vanillate to methoxyhydroquinone [6]. We now report a dioxygenase from *S. pulverulentum* which catalyses the intradiol ring cleavage of hydroxyquinol to maleylacetate which is then reduced to β-ketoadipate according to the scheme 1.



2. Materials and methods

Conditions for the cultivation of *S. pulverulentum* and the preparation of mycelial extracts have been described previously [4]. Where dioxygenase levels were compared in extracts of mycelia grown in the presence of vanillate or methoxyhydroquinone the fungus was cultured initially on glucose and sterile solutions of the aromatic compound (1 mM final concentration) were added after 24 h. Mycelial pellets were harvested after a further 18 h cultivation.

Dioxygenase activity was detected spectrophotometrically and assayed by measuring oxygen consumption with a Clark oxygen electrode. Maleylacetate reductase was determined by measuring the rate of decrease in absorbance at 243 nm due to maleylacetate disappearance using NADPH as coenzyme [7], or by the rate of NADPH consumption at 340 nm. Maleylacetate was prepared enzymically in situ using crude mycelial extracts without NADPH. Extracts were previously passed through a Sephadex G-25 column to remove endogenous metabolites which interfered with the assay of NADPH oxidation. Details of individual assays are described in table legends.

β-Ketoadipate was detected by the Rothera reaction [8] and determined manometrically by catalytic decarboxylation using 4-aminoantipyrine [9]. Pyruvate was determined using lactate dehydrogenase (Sigma Chemical Co.). 2,4-Dinitrophenylhydrazone derivatives were identified by thin-layer chromatography on Eastman silica gel layers (No. 13181) developed in the solvent systems described by Dagley and Gibson [10]. Thin-layer chromatography of *trans*-acetylacrylate was carried out using benzene-ethyl alcohol–acetic acid (80 : 14 : 7) as solvent system [11].

3. Results

Mycelial extracts of *S. pulverulentum*, grown in the presence of vanillic acid, oxidatively cleaved the aromatic ring of hydroxyquinol (1,2,4-trihydroxybenzene) with the consumption of 1 mol of O_2/mol of substrate (table 1). During enzymic oxidation spectral changes were observed which have been previously described when *ortho*-fission of hydroxyquinol to maleylacetate was catalysed by dioxygenases from *Pseudomonas putida* [7] and the yeast *Trichosporon cutaneum* [11]. Hydroxyquinol undergoes rapid autooxidation in slightly acidic and basic aqueous solutions which is accompanied by the appearance of an absorption maximum at 260 nm [7]. However, when hydroxyquinol was added to reaction mixtures containing crude extracts of *S. pulverulentum* the peak at 260 nm due to autooxidation was absent and a new peak appeared at 243 nm which disappeared when the reaction mixture was acidified to pH 3. Dioxygenase activity was also found in extracts of mycelia grown on glucose as the sole carbon source, but enzyme levels were increased 3-fold with vanillate present (table 2). Pyruvate was not formed from hydroxyquinol indicating the absence of any *meta*-fission [7]. Ring-fission dioxygenases for protocatechuate, gentisate, catechol and pyrogallol were not detected.

Maleylacetate was established as the product of *ortho*-cleavage by the isolation of *trans*-acetylacrylic acid from reaction mixtures containing crude mycelial extract and hydroxyquinol. Attempts to isolate maleylacetate lead to its decarboxylation and isomerization to *trans*-acetylacrylate [7,11]. After crystallization from ether–petroleum ether the isolated material, obtained following continuous ether-extraction of acidified reaction mixtures, was identified by m.p. (122–124°C; 120–122°C on admixture with authentic *trans*-acetylacrylate), u.v. spectrometry (λ_{max} = 220 nm in neutral and acidic solutions) and co-chromatography with authentic *trans*-acetylacrylate.

Maleylacetate was further metabolized by a NADPH-dependent maleylacetate reductase present in extracts of mycelia grown in the presence of vanillate (table 2). Maleylacetate reduction (measured from the rate of decrease in absorbance at 243 nm) was accompanied by an oxidation of NADPH. When 100 to 200 nmol of hydroxyquinol were used to generate maleylacetate under the conditions given in table 2 (NADPH omitted from control cuvette), 0.91 mol of NADPH was oxidized for every mol of maleylacetate reduced. Maleylacetate reductase activity was barely detectable when NADH was substituted as coenzyme. Extracts of *Trichosporon*

Table 1
Stoicheiometry of hydroxyquinol oxidation

Hydroxyquinol supplied (nmol)	O_2 consumed (nmol)	O_2 consumed/hydroxyquinol (nmol/nmol)
200	185	0.93
400	357	0.89

Reaction mixtures contained in 3 ml, 280 μmol phosphate buffer (pH 6.5), 1.94 mg cell extract protein and hydroxyquinol as indicated. Temp. 30°C

Table 2
Hydroxyquinol 1,2-dioxygenase and maleylacetate reductase in extracts of *S. pulverulentum*

Growth substrate	Hydroxyquinol 1,2-dioxygenase[a]	Maleylacetate reductase[b]
Glucose	11.9	N.D.
Glucose + 3 mM vanillate	35.0	4.5

[a] Oxygen consumption was measured polarographically in reaction mixtures (3 ml) containing: 250 μmol phosphate buffer, pH 6.5; 1.24–1.95 mg cell extract protein; 0.5 μmol hydroxyquinol (prepared immediately before use). Temp. = 30°C. Enzyme activity is expressed as nmol O_2 consumed/min/mg protein.

[b] Maleylacetate reductase was measured spectrophotometrically at 22°C in reaction mixtures containing: 250 μmol phosphate buffer, pH 6.5, and 0.83–0.98 mg cell extract protein. Hydroxyquinol (0.5 μmol) was added and maleylacetate production observed from the increase in absorbance at 243 nm. After no further increase occurred, 0.5 μmol NADPH was added to both control and sample cuvettes and the rate of decrease in absorbance at 243 nm measured. Enzymic activity is expressed as nmol maleylacetate reduced/min/mg protein assuming complete conversion of hydroxyquinol to maleylacetate. N.D. = not detected

cutaneum reduced maleylacetate with either NADH or NADPH [11], while a requirement for NADH was reported for the reductase from *P. putida* [7]. Formation of β-ketoadipate from maleylacetate reduction by NADPH was indicated by a positive Rothera reaction [8], and catalytic decarboxylation with 4-aminoantipyrine [9], although these methods for estimating β-ketoadipate are not specific [11]. However, the formation of β-ketoadipate was confirmed by thin-layer chromatography of its 2,4-dinitrophenylhydrazone. For this, a reaction mixture contained in 3 ml of 0.1 M phosphate buffer (pH 7.4), 5 μmol hydroxyquinol, 4.9 mg of cell extract protein and a NADPH-generating system consisting of 1.0 μmol NADPH, 10 μmol glucose-6-phosphate and 17.5 units glucose-6-phosphate dehydrogenase. After incubation at 28°C for 60 min the 2,4-dinitrophenylhydrazone derivative was prepared and extracted as previously described [10]. Manometric estimation of CO_2 evolution in similar reaction mixtures of crude extract, hydroxyquinol (3 to 5 μmol) and a NADPH-generating system showed that 0.82 μmol CO_2 was evolved per μmol of hydroxyquinol supplied. Although alternative routes for maleylacetate metabolism cannot be excluded the low recoveries probably depend on spontaneous side reactions of hydroxyquinol [11].

4. Discussion

Microbial degradation of benzenoid compounds is well documented and in most cases reported so far two hydroxy groups, located either *ortho* or *para* to each other, must be attached to the benzene nucleus for enzymic ring cleavage to occur [12]. Where two existing hydroxy substituents are *meta* oriented a third hydroxyl is introduced to facilitate ring-fission [7,11,13–15]. More recently, it was shown that 3,4-dihydroxyphenylacetic acid, an intermediate in the degradation of 4-hydroxyphenylacetate by *T. cutaneum*, underwent a further hydroxylation at the C-6 position before the aromatic ring was cleaved [16].

The role of a tri-hydroxylated intermediate in vanillate catabolism by *S. pulverulentum* is not yet clear. NAD(P)H-dependent oxidative decarboxylation of vanillate to methoxyhydroquinone by partially purified mycelial extracts has been reported [4]. However, we are unable to demonstrate hydroxylation or demethylation of the reaction product although extracts of mycelia grown in the presence of methoxyhydroquinone contain low levels of induced hydroxyquinol 1,2-dioxygenase. It is conceivable that vanillate itself is hydroxylated prior to the oxidative decarboxylation step which subsequently provides the third hydroxyl necessary for ring cleavage. Several compounds with a hydroxyl group *para* to a carboxyl substituent serve as substrates for the decarboxylating enzyme [4].

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