Oxidative Ring-Cleavage of Catechol in *meta*-Position by Superoxide

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Meta-Cleavage, Catechol, Superoxide

Superoxide reacts with catechol in dimethylsulfoxide to form a yellow product. The structure of this product was established by different methods. UV-spectra of the yellow compound corresponded under all circumstances to those of enzymatically produced 2-hydroxymuconic acid semialdehyde. In the presence of ammonia the product was converted to picolinic acid. The UV-spectra, the retention times in GC and HPLC and the mass spectra after methylation corresponded to those of authentic picolinic acid. Thus, the yellow reaction product was unequivocally identified as 2-hydroxymuconic acid semialdehyde. The formation of the *meta*-cleavage product was only observed at low catechol concentrations (< 2 mM), at higher concentrations the product was not formed. The reaction was complete within 10 min at room temperature. Spectroscopic data suggest a first intermediate in the reaction with λ_{max} = 804 nm, 772 nm and 660 nm.

Introduction

In the degradation of aromatic compounds by bacteria the oxidative ring-cleavage is an important step. Several enzymes, that cleave various dihydroxy-aromatic compounds, have been described [1]. This cleavage can proceed in two different ways. Either the ring is cleaved between the hydroxygroups (*ortho*-cleavage) or adjacent to the hydroxygroups (*meta*-cleavage). All of these enzymes except one [2] contain iron as sole cofactor. Surprisingly all of the *ortho*-cleaving enzymes contain iron in the trivalent state, whereas the *meta*-cleaving enzymes contain divalent iron. Due to spectroscopic data there is a general agreement, that in *ortho*-cleaving enzymes the substrate binds to the iron [3]. In the case of the *meta*-cleaving enzymes, however, the situation is different. Since these enzymes show neither a color nor an ESR-signal, the analysis of the enzyme-substrate complex has not been possible. In contrast to the *ortho*-cleavage, where several model reactions have been described [4–10], for the *meta*-cleavage no chemical model reaction is known, that could give some hints on the reaction mechanism. Only in one case the formation of both, *ortho* and *meta* product, has been described in the oxidation of 3,5-di-*tert*-butylcatechol by a (bipyridine) (pyridine)iron(III) complex [11].

In the present paper we describe in detail a model reaction, in which the *meta* product is formed without the involvement of any metal, and which might help to understand more of the enzymatic *meta*-cleavage reaction

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Materials and Methods

Chemicals

Potassiumsuperoxide was purchased from Fluka AG, Buchs, Switzerland. Dimethylsulfoxide (dry min 99.5%) was from E. Merck, Darmstadt, Germany. These and all other chemicals, analytical grade, were used without further purification.

Chromatographic methods

For HPLC-analysis a column (0.8 × 25 cm) of Lichrosorb RP 18 (7 μm) (E. Merck, Darmstadt, Germany) with 1% acetic acid as solvent was used. In thin-layer chromatography precoated plates (silicagel with fluorescent indicator from E. Merck, Darmstadt, Germany) were developed with toluene, methanol, acetic acid (8, 2, 1/v, v, v) as solvent. GC-MS-analysis was performed by G. Schwinger, Institute of Organic Chemistry, Universität Hohenheim, on a mass spectrometer Varian 3700 (Varian, Bremen, Germany) equipped with a glass capillary column (25 m, SE30) at 150 °C (injector 240 °C).

Determination of the superoxide concentration

In order to estimate the superoxide concentration in the dimethylsulfoxide, we diluted 20 ml of the superoxide saturated dimethylsulfoxide solution ten-

fold with water and titrated the resulting KOH with 2 mM oxalic acid.

Standard method for the meta-cleavage of catechol by superoxide

To 100 ml dimethylsulfoxide 50–100 mg of KO_2 were added in a mortar. The KO_2 was ground, till a fine suspension was obtained. This was filtered, and the clear filtrate was used as superoxide saturated dimethylsulfoxide. For analytical purpose to 1 ml of this solution in 1 cm quartz cuvettes 50 µl of a solution of catechol (1 mg/ml) in dimethylsulfoxide was added, and the reaction was followed spectrophotometrically. For the preparation of the *meta*-cleavage product, 25 ml of the same catechol solution were added to 500 ml of superoxide solution under vigorous stirring. After ten minutes the reaction was stopped by the addition of 1 l of water, and the product was isolated as described in results.

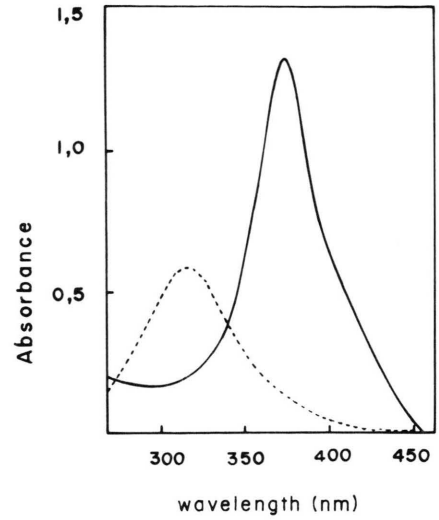

Fig. 1. Absorption spectrum of the yellow compound obtained in the reaction of superoxide with catechol after separation on a DEAE-cellulose column. The spectra were recorded in 1.0 M NaOH and in 0.5 N HCl (dotted line).

Results

Isolation and characterization of the reaction product formed during the reaction of catechol with superoxide

When we added to a saturated solution of potassium-superoxide in dimethylsulfoxide a solution of catechol in dimethylsulfoxide under vigorous stirring immediately a blue color developed. Within 10 min the color changed from blue *via* green to yellow. In order to isolate the yellow compound, we stopped the reaction after ten minutes by the addition of water. The obtained solution was first extracted 3 times with equal volumes of methylenechloride to remove some of the dimethylsulfoxide, then it was acidified with conc. HCl and extracted 5 times with CH_2Cl_2. The organic layer was extracted with 0.1 N NaOH. This solution was diluted tenfold with water and applied to a column of DEAE-cellulose. The column was rinsed with water and then a linear gradient from 0 to 7.5% NaCl was applied. The spectra of the yellow fractions were identical with those obtained on enzymatic *meta*-cleavage of catechol [12] (Fig. 1). The brown oily compound, which was obtained after evaporation of the solvent, gave no reasonable mass spectra. Silylation as well as methylation with diazomethane yielded black tars, and in HPLC several broad overlapping peaks appeared, indicating a decomposition of the substance on the column. Therefore, the substance was converted by the addition of NH_3 according to the method of Fujiwara *et al.* [13]. As expected, after 4 days the yellow color had disappeared and instead the spectrum of picolinic acid ($\lambda_{max} = 263$ nm) was obtained (Fig. 2). The substance obtained comigrated with au-

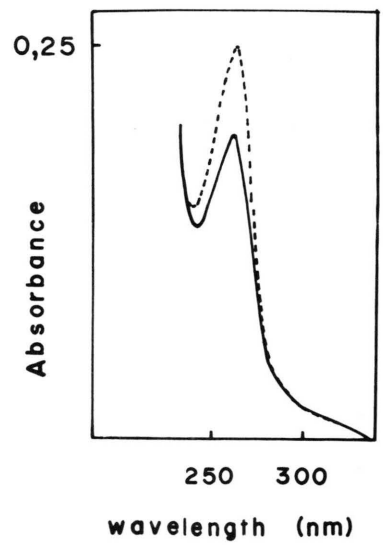

Fig. 2. Absorption spectrum of the reaction product of superoxide with catechol four days after the addition of ammonia and of authentic picolinic acid (dotted line).

thentic picolinic acid in thin layer chromatography as well as in HPLC (Fig. 3). Finally the picolinic acid was methylated according to Klosa [14], and in GC-MS a peak with retention time and mass spectrum identical to that of picolinic acid methylester was obtained (Fig. 4). Thus, the above obtained yellow compound was unequivocally identified as 2-hydroxymuconic acid semialdehyde, the *meta*-cleavage product of catechol (Fig. 5). The yield was 29% assuming an $\varepsilon = 44000\ M^{-1}\ cm^{-1}$ for the product in 0.1 N NaOH.

Concentration dependence of the reaction

Fig. 6 shows the dependence of the reaction on the concentration of catechol. It is surprising, that at

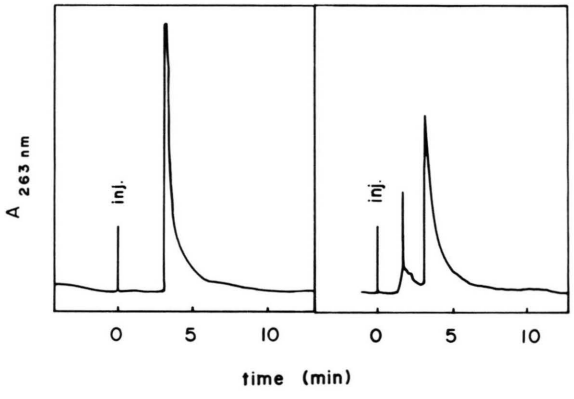

Fig. 3. HPLC-elution pattern of the ammonia treated reaction product of superoxide with catechol (right) and of authentic picolinic acid (left).

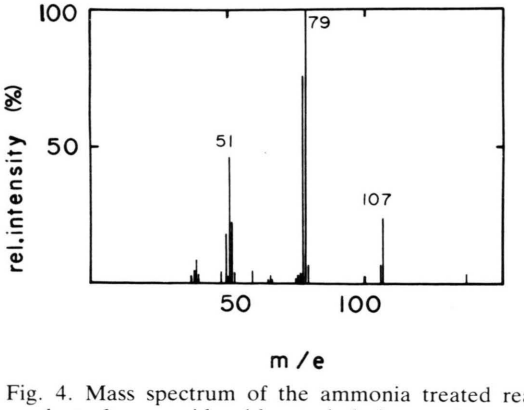

Fig. 4. Mass spectrum of the ammonia treated reaction product of superoxide with catechol after methylation with diazomethane.

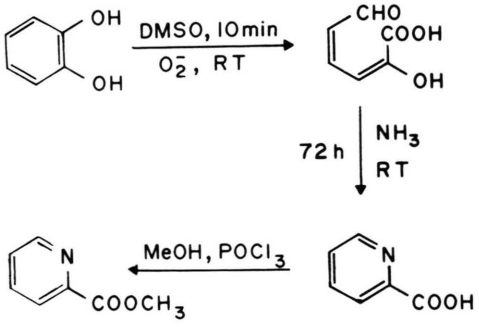

Fig. 5. *Meta*-cleavage of catechol and the conversion of the reaction product to picolinic acid methylester.

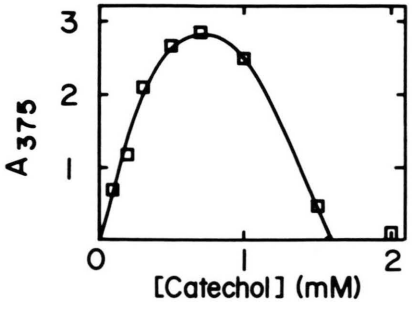

Fig. 6. Concentration dependence of the reaction of superoxide with catechol. The assays contained 1 ml dimethylsulfoxide saturated with potassiumsuperoxide and varying concentrations of catechol. The reactions were followed at 375 nm, and the highest absorption reached (usually after about 10 min) was plotted *versus* the catechol concentration.

higher concentrations of catechol (< 2 mM) no *meta*-cleavage at all is observed. The highest amount of the reaction product was obtained when the concentration of catechol was 0.8 mM. This concentration corresponds to the amount of superoxide dissolved in dimethylsulfoxide.

Time dependence of the reaction

Fig. 7 shows the spectra of the reaction mixture at different times. The increase at 804 nm, 772 nm and 660 nm was very fast. Even in the stopped flow apparatus this increase could not be observed (< 3 msec) (data not shown). This very fast increase may indicate, that the formation of the blue intermediate is a diffusion controlled process. The decrease at the three wavelenghts is absolutely parallel,

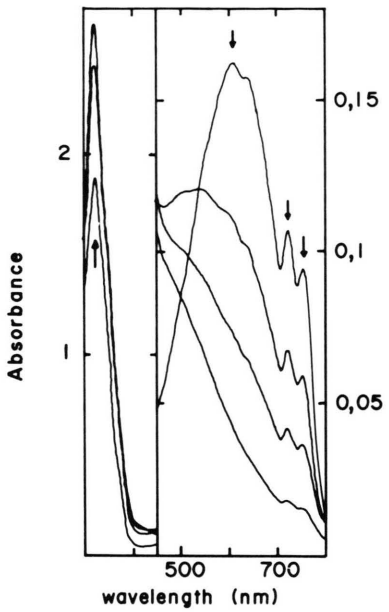

Fig. 7. Time dependence of the reaction of catechol with superoxide. The assay contained 1 ml dimethylsulfoxide saturated with superoxide and 50 µl of catechol (1 mg/ml) in dimethylsulfoxide. The spectra were recorded 1, 4, 7 and 10 min after the addition of catechol.

and is complete, when the formation of the product with $\lambda_{max} = 375$ nm reached its maximum, suggesting, that these peaks are derived from a single substance, which is finally converted to this product. After 10 min at room temperature the peak at 375 nm reached its maximum, then it slowly decreased and after 24 h no more *meta*-cleavage product was found.

Discussion

The reaction of superoxide with catechols unequivocally results in *meta*-cleavage. This is the first time, such a *meta*-cleavage has been achieved without the involvement of an enzyme or a metal ion. In all enzymatic reactions described for the oxidative cleavage of aromatic compounds transition metals, preferably iron, play a key role. In the above described reaction no metal is present. This clearly shows, that the *meta*-cleavage of catechol is possible without the involvement of iron, once superoxide is formed from oxygen. One might speculate, that in *meta*-cleaving enzymes divalent iron is necessary for the formation of superoxide according to the equation:

$$Fe^{2+} + O_2 = (Fe^{2+}O_2) = (Fe^{3+}O_2^-).$$

This assumption would agree with the fact, that in some *meta*-cleaving enzymes a valence change of the iron during the catalytic cycle has been observed by ESR-spectroscopy [15].

In the enzymatic reaction the role of the protein would be to prevent the disproportionation of superoxide in the presence of water and to bind the substrate in the right way for a specific cleavage. This would also explain, why with the enzyme the yield of the product is quantitative, whereas in the chemical reaction described here only one third of the catechol is converted to the *meta*-cleavage product.

The initial reaction of superoxide with catechol could be the abstraction of a hydrogen as described for the reaction of superoxide with catechol in aprotic media [16]. Another possibility would be the formation of a substrate radical as described in the oxidation of substituted benzenes in one electron oxidations of the Fenton type [11]. At the moment we can't distinguish between these two mechanisms. Also we don't know at the moment, which role molecular oxygen plays in this reaction. However, the further characterization of the blue intermediate of the above described reaction might help to get some more insight into this important reaction.

A preliminary abstract of this work has been presented before [17].

Acknowledgements

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