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EXTRADIOL CLEAVAGE OF o-AMINOPHENOL BY PYROCATECHASE

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SUMMARY

o-Aminophenol is cleaved by the intradiol dioxygenase, pyrocatechase, in an extradiol manner to give picolinic acid as the major product. Inhibition of o-aminophenol cleavage with various reagents was comparable to that observed for catechol cleavage, indicating that both reactions are catalyzed by the same enzyme. Though other substrate analogues have been shown to yield some extradiol cleavage products, this is the first case wherein >95% of the products characterized derived solely from the extradiol cleavage of the ring.

INTRODUCTION

Pyrocatechase (catechol 1,2-oxidoreductase (decyclizing) EC 1.13.11.1) from Psuedomonas arvilla C-1 catalyzes the intradiol cleavage of catechol to form cis, cis-muconic acid (1). This enzyme has a broad specificity, catalyzing the cleavage of many substituted catechols (2,3). Fujiwara, et al. (3), recently reported that when 3-methyl- and 3-methoxycatechol were used as substrates, both the intradiol and extradiol cleavage products were observed. Although the extradiol product was the minor component of the reaction mixture (4% for 3-methylcatechol and 16% for 3-methoxycatechol), it was quite surprising that extradiol cleavage occurred at all, since this reaction is catalyzed by enzymes with rather different physicochemical properties (1). Hou, et al. (4), have surveyed other pyrocatechases from several bacterial species and observed similar products.

This report discusses the action of pyrocatechase on o-aminophenol which results in the extradiol cleavage of the aromatic ring adjacent to the hydroxy group in >95% yield.

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MATERIALS AND METHODS

Pyrocatechase was prepared from Psuedomonas arvilla C-1 (ATCC 23974) grown with benzoate as sole carbon source according to the procedure of Fujiwara, et al. (3). o-Aminophenol was obtained by the dithionite reduction of o-nitrophenol in alkaline solution (5). o-Nitrophenol, picolinic acid, thionyl chloride and Tiron were obtained from the Aldrich Chemical Company and used without further purification.

Optical spectra were recorded on a Cary 219 spectrophotometer. Oxygen uptake rates and inhibition data were measured on a Gilson Oxygraph. All measurements were done in 50 mM potassium phosphate buffer pH 7.5 (25°C). GC/MS data was obtained on a Finnigan Corporation Model 3300 instrument, twin stacked EI/CI with Data System 150 from System Industries, run by the Mass Spectrometer Facility at Cornell.

RESULTS AND DISCUSSION

Figure 1 shows the progress of the reaction of o-aminophenol with pyrocatechase in phosphate buffer (pH 7.5, 25°C) as monitored by UV-visible spectroscopy. o-Aminophenol exhibits an absorbance maximum at 281 nm (ϵ 2700 M^{-1} cm^{-1}) in aqueous solution. As the reaction proceeds, this peak diminishes concomitant with the growth of peaks around 264 and 375 nm. The peaks near 264 nm correspond exactly to the spectrum of picolinic acid in phosphate buffer (ϵ_{264} 4000 M^{-1} cm^{-1}). Upon acidification with 0.1 volume of concentrated HCl, these peaks double in intensity (ϵ_{264} 8000 M^{-1} cm^{-1}) as does the picolinic acid.

The product picolinic acid was further characterized as follows: After ultrafiltration through a PM-10 membrane to remove enzyme, the reaction mixture was evaporated to dryness and dissolved in methanol. Addition of an excess of thionyl chloride at 0°C and subsequent warmup to 40°C for 2 hours (6) yielded the methyl ester. The electron impact mass spectrum showed a molecular ion peak at m/e 137, corresponding to a molecular formula $C_7H_7NO_2$. Major fragment ion peaks were observed at m/e 107 ($M^+ - CH_2O$), 106 ($M^+ - OCH_3$), 79 ($M^+ - COOCH_2$) and 78 ($M^+ - COOCH_3$). These features corresponded with the published spectrum of picolinic acid, methyl ester (7).

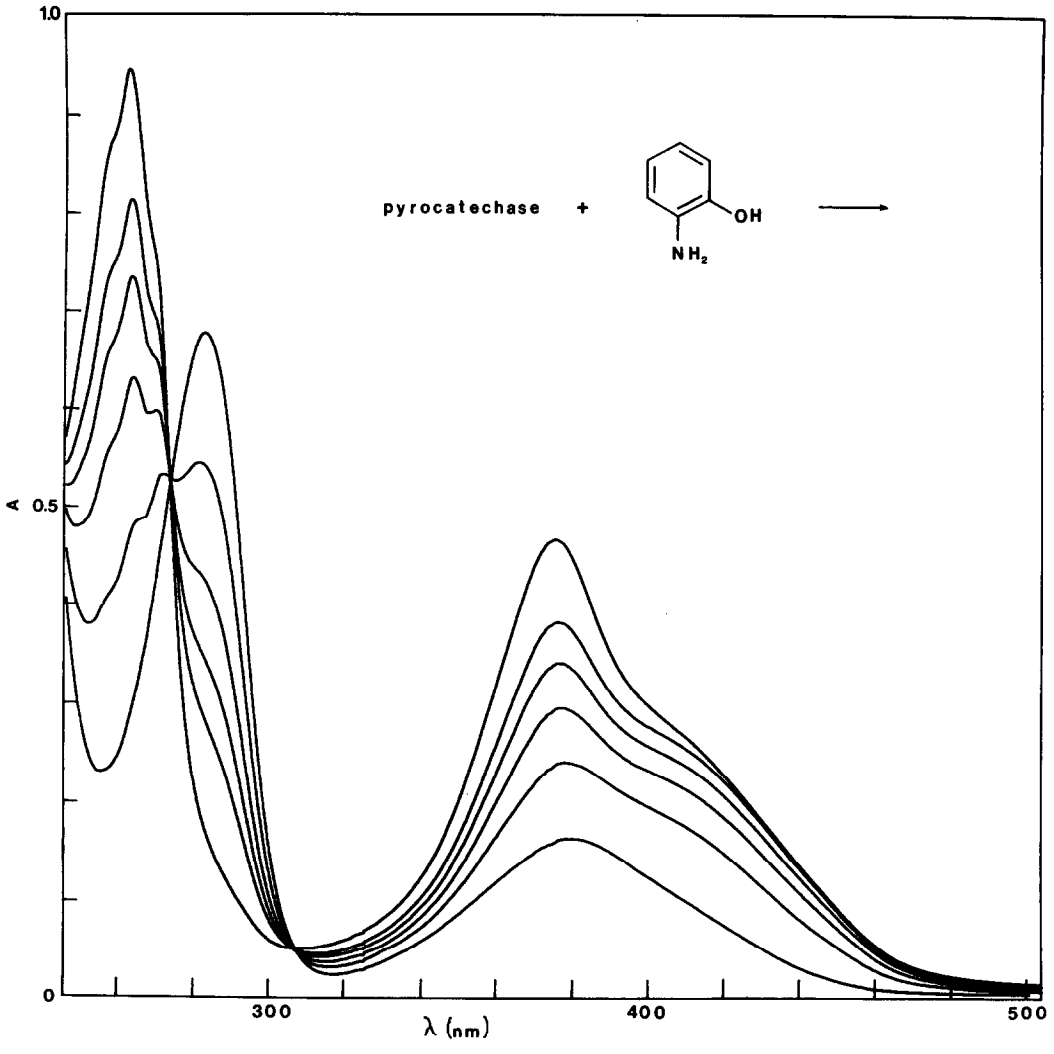


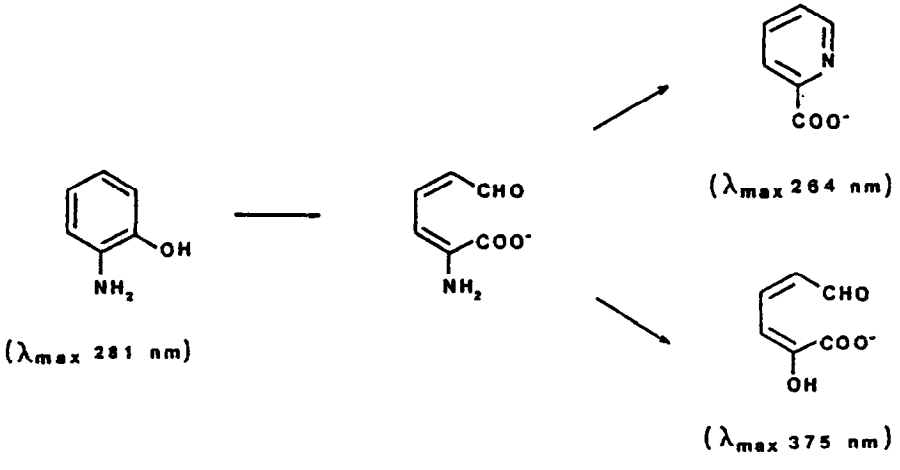
Figure 1. Reaction of pyrocatechase with o-aminophenol. 0.5 mg pyrocatechase and 0.25 μmole o-aminophenol in 1 mL 50 mM phosphate buffer, pH 7.5, 25°C. Spectra were recorded at the following times after mixing: 0.2, 12, 30, 50, 80 and 240 minutes. The absorbances near 260 nm and 380 nm increase with time while the peak near 280 decreases with time. Quantitation of products: picolonic acid, 0.235 μmole; α-hydroxymuconic semialdehyde, 0.014 μmole.

The peak at 375 nm is assigned to α-hydroxymuconic semialdehyde (ε 33,400 M^{-1} cm^{-1}), the product of metapyrocatechase cleavage of catechol (8). Upon acidification, the characteristic yellow color fades and a new band near 320 nm appears. This was indeed observed upon acidification of the reaction mixture.

Furthermore, incubation of the ultrafiltered product solution with 3 volumes of concentrated NH_4OH for 40 hours results in the disappearance of the 375 nm peak and an increase in the absorbance of the picolinic acid bands. In concentrated NH_4OH, α-hydroxymuconic semialdehyde is converted to α-aminomuconic semialdehyde, which then cyclizes to picolinic acid.

Oxygraph measurements of the stoichiometry of the reaction show that one mole of oxygen is consumed per mole of o-aminophenol. Spectral quantitation of the products show (95 ± 2) % picolinic acid and (5 ± 2) % α-hydroxymuconic semialdehyde. There is a long wavelength hump in Figure 1 centered near 400 nm which was not characterized. It was also removed by ultrafiltration through a PM-10 membrane and may result from the autoxidation of the substrate.

The observation of picolinic acid and α-hydroxymuconic semialdehyde as products for the pyrocatechase catalyzed cleavage of o-aminophenol suggests that the cleavage occurs not in an intradiol fashion as with catechol but in an extradiol fashion adjacent to the hydroxy group, i.e:



The resulting α-aminomuconic semialdehyde then cyclizes in a nonenzymatic reaction to picolinic acid or, alternatively, hydrolyzes to yield α-hydroxymuconic semialdehyde. Similar nonenzymatic reactions have been observed for the cleavage

Table I

	catechol	o-aminophenol
V_{max} (relative rates)	100	0.1
K_m	5 μM*	20 μM
K_I-o-nitrophenol	14 μM	18 μM
K_I-o-chlorophenol	3 μM	5 μM
5 mM Tiron inhibition	yes*	yes
10 μM H_2O_2 inactivation	no*	no

*Reference 2

of 3-hydroxyanthranilate by rat liver 3-hydroxyanthranilate 3,4-dioxygenase (9). The transient α-aminomuconic semialdehyde may manifest itself as a peak at 380 nm in the early stages of the reaction, which then gives way to the 375 nm peak as the reaction progresses.

Table I shows a comparison of experimental observations with catechol and o-aminophenol as substrates. The o-aminophenol cleavage is 1000-fold slower than the catechol, though their K_m's are comparable. To show that the cleavage of o-aminophenol is not catalyzed by a trace amount of extradiol cleaving enzyme, several inhibition experiments were performed. o-Nitrophenol (3) and o-chlorophenol are both competitive inhibitors to the pyrocatechase catalyzed cleavage of catechol. The K_I's measured for the inhibition of o-aminophenol cleavage are similar. Tiron (4,5-dihydroxy-m-benzenedisulfonic acid, disodium salt), a ferric chelating agent, inhibits both activities after a two hour incubation with the enzyme, while H_2O_2 has no effect on either enzyme activity. At 10 μM, H_2O_2 completely inactivates the action of metapyrocatechase (3,10), a typical extradiol cleaving enzyme.

Table II*

substrate	relative rates	% extradiol cleavage
catechol	100	0
3-methylcatechol	8	4
3-methoxycatechol	0.8	16
o-aminophenol	0.1	>95
3,5-dicholorcatechol	0.02	0

*Data for substrates other than o-aminophenol were obtained from reference 3.

The data presented here provides evidence for the extradiol cleavage of o-aminophenol by an intradiol cleaving enzyme. Similar observations are found for 2-amino-p-cresol and 6-amino-m-cresol (11). Fujiwara, et al. (3) have reported extradiol cleavage products in the reaction of pyrocatechase with some 3-substituted catechols. These products were minor components (4% for 3-methylcatechol and 16% for 3-methoxycatechol) of the product mixtures. In contrast, the pyrocatechase catalyzed cleavage of o-aminophenol yields >95% extradiol products. Attempts to find intradiol products have thus far been unsuccessful.

It is interesting to note that, in the three cases of pyrocatechase catalyzed extradiol cleavage, the aromatic ring has been modified by electron donating substituents (CH_3, OCH_3, NH_2). Furthermore, as the rates of reaction of these substrate analogues dimish in comparison to catechol, the amount of extradiol cleavage increases (Table II). 3,5-Dichlorocatechol is a case where the ring has been substituted with electron withdrawing substituents and the rate has diminished but no extradiol products were reported (3).

The relative slowness of these reactions indicate that they are probably not biologically significant. These observations, however, suggest an interrelation in the mechanisms of intradiol and extradiol cleavage, despite the markedly different physicochemical properties of these two enzyme groups (1). Spectroscopic investigations are currently in progress to monitor iron site changes upon introduction of o-aminophenol; such studies may provide clues to the interaction of the iron with substrate and shed light on the mechanisms of these enzymes.

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