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ENZYMATIC OXIDATION OF *p*-NITROPHENOL

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SUMMARY

A *Moraxella* sp. capable of growth with *p*-nitrophenol was isolated from activated sludge. Differential centrifugation of crude cell extracts gave a membrane preparation that oxidized *p*-nitrophenol to hydroquinone and nitrite. Enzymatic activity was dependent on the presence of oxygen and reduced pyridine nucleotides and was stimulated by the addition of flavin adenine dinucleotide. Experiments with $^{18}O_2$ showed that the incoming hydroxyl group was derived from molecular oxygen. The soluble fraction prepared from crude cell extracts oxidized hydroquinone to a product whose spectral properties were identical to those reported for γ-hydroxymuconic semialdehyde.

INTRODUCTION

The oxidative release of nitrite from *p*-nitrophenol by a soil pseudomonad was first described by Simpson and Evans (1). Since that time several different microorganisms have been reported to catalyze the release of nitrite from various nitroaromatic compounds (2,3,4,5,6,7,8,9). In some instances the nitro group was shown to be replaced by a hydroxyl group prior to fission of the aromatic nucleus. However, little is known concerning the biochemical mechanism of nitrite release from aromatic substrates. Previous investigations have been hindered by the lack of active cell-free preparations. We now report the preliminary characterization of a bacterial enzyme that catalyzes the oxidative release of nitrite from *p*-nitrophenol.

MATERIALS AND METHODS

Organism and growth conditions. A gram-negative bacterium was isolated from activated sludge by selective enrichment with *p*-nitrophenol.

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The organism was an aerobic, nonmotile, oxidase-positive coccobacillus and was tentatively identified as a *Moraxella* species based on the classification scheme outlined in Bergey's Manual of Determinative Bacteriology (10). Cultures were grown in minimal salts medium with *p*-nitrophenol (0.015%) and NH_4Cl (0.05%) as the carbon and nitrogen sources, respectively. The nitrogen requirement was not satisfied by *p*-nitrophenol. The medium was supplemented with yeast extract (0.1%) when increased cell yields were required.

Preparation of cell extracts. Washed cells were resuspended in 0.02 M Na_2HPO_4, KH_2PO_4 buffer, pH 7.0 (1.0 g cells per 3.0 ml of buffer) and broken by two passages through a French Pressure Cell at 20,000 lbs./sq. in. The resulting viscous liquid was treated with deoxyribonuclease (0.02 mg/ml) and centrifuged at 12,000 x g for 15 min. The supernatant liquid was separated into soluble and particulate fractions by centrifugation at 177,000 x g for one hour. Particulate fractions were resuspended in phosphate buffer and washed by an additional high-speed centrifugation.

Enzyme assays. *p*-Nitrophenol oxidation was assayed spectrophotometrically by following the decrease in absorbance at 420 nm. Reaction mixtures contained: 0.05 mM *p*-nitrophenol; 0.3 mM NADPH; 0.03 mM FAD; 0.05-0.20 mg of protein and 20 mM phosphate buffer (pH 7.0) in a final volume of 1.0 ml. Reactions were initiated by the addition of NADPH. An alternative assay procedure involved the polarographic measurement of oxygen uptake in the presence of *p*-nitrophenol. Reaction mixtures were the same as those described for the spectrophotometric assay.

1,4-Benzoquinone reduction was measured either by following the rate of quinone disappearance at 244 nm or the oxidation of NADPH at 340 nm. Reaction mixtures contained: 0.2 mM 1,4-benzoquinone; 0.02 mM NADPH; 20 mM phosphate buffer (pH 7.0); and 0.1-0.3 mg of protein in a final volume of 1.0 ml. Reactions were initiated by the addition of cell extract.

Analytical methods. Protein was estimated by the biuret procedure (11). Nitrite was determined colorimetrically by the Griess-Ilosvay reac-

tion (12). Oxygen uptake was measured polarographically with a Clark-type electrode from Yellow Springs Instrument Company. Mass spectral analyses were performed with a Bell and Howell 21-491 mass spectrometer. Thin-layer chromatography was performed on precoated silica gel plates (Brinkmann Instrument Company). The solvent used for development was chloroform:acetone (8:2). Compounds were detected by fluorescence quenching when viewed under ultraviolet light. Radioactive compounds were located on thin-layer chromatograms by autoradiography on Kodak NS54T X-ray film developed with Kodak D-19 developer. Radioactivity was measured in a Beckman LS 100C liquid scintillation counter.

Chemicals. Hydroquinone, NADPH, NADH and FAD were from Sigma Chemical Company. *p*-Nitrophenol and 1,4-benzoquinone were from Eastman Organic Chemicals. [2,6-^{14}C]-*p*-Nitrophenol (30 mCi/mmol) was from ICN Chemicals and Radioisotopes Division. [^{18}O]-Oxygen gas (99%) was from Stohler Isotope Chemicals.

RESULTS

Crude extracts prepared from *p*-nitrophenol-grown cells catalyzed a pyridine nucleotide-dependent oxidation of *p*-nitrophenol with a concomitant stoichiometric release of nitrite. All of the enzymatic activity was found in the particulate fraction that was obtained by centrifugation at 177,000 x g (Table 1). Enzyme activity was routinely measured spectrophotometrically by following the decrease in absorbance at 420 nm due to the disappearance of *p*-nitrophenol.

The effects of various cofactors on the rate of *p*-nitrophenol oxidation by washed membrane preparations are shown in Table 2. Negligible activity was observed in the absence of reduced pyridine nucleotides. NADH could serve as an electron donor for the reaction but was less effective than NADPH. Omission of FAD from the assay mixture produced a significant reduction in enzyme activity. FMN was not an effective substitute for FAD.

Table 1. Oxidation of *p*-nitrophenol by different fractions of a crude cell extract[1].

Fraction	Volume (ml)	Protein (mg)	Activity (units[2])	Specific Activity (units/mg protein)
12,000 x g Supernatant	50	2150	110	0.05
177,000 x g Supernatant	48	1575	0	0.00
177,000 x g Pellet (second wash)	20	320	83	0.26

[1]Enzymatic activity was determined by following the disappearance of *p*-nitrophenol at 420 nm as described in Materials and Methods.

[2]One unit of enzyme catalyzes the disappearance of 1.0 μmole of *p*-nitrophenol per minute.

Table 2. Cofactor requirements for *p*-nitrophenol oxidation by washed membrane preparations.[1]

Reaction Mixture	Activity (percent)[2]
Complete	100
-NADPH	3
-NADPH, +NADH	80
-FAD	56
-FAD, +FMN	61

[1]Enzymatic activity was determined spectrophotometrically in the standard assay described in Materials and Methods.

[2]The rate of enzymatic activity observed with the complete reaction mixture was assigned a value of 100%.

In order to identify the product of the reaction a particulate preparation (39.5 mg of protein) was incubated with 0.2 mM [^{14}C]-*p*-nitrophenol (20 μCi); 0.5 mM NADPH; 0.03 mM FAD, and 20 mM phosphate buffer (pH 7.0) in a final volume of 20 ml. After seven minutes the reaction mixture was

acidified with 5N H_2SO_4 and extracted with ethyl acetate. The organic layer was dried over Na_2SO_4 and evaporated to dryness under reduced pressure. The residue was taken up in a small volume of ethyl acetate and analyzed by thin-layer chromatography. An autoradiogram, prepared from the resulting thin-layer chromatogram, revealed the presence of a small amount of *p*-nitrophenol and a product that cochromatographed with hydroquinone (R_F, 0.22). Of the radioactivity initially added as *p*-nitrophenol 82% was recovered as hydroquinone. The identity of the reaction product was firmly established by mixing the radioactive compound with authentic unlabelled hydroquinone and recrystallizing to constant specific activity.

The incorporation of molecular oxygen into *p*-nitrophenol to form hydorquinone was investigated with [^{18}O]-oxygen. A washed, particulate preparation (345 mg of protein) was incubated with 0.3 mM NADPH; 0.04 mM FAD, and 0.13 mM *p*-nitrophenol, in 715 ml of 20 mM phosphate buffer (pH 7.0). The reaction vessel was alternatively evacuated and flushed with nitrogen several times before being allowed to equilibrate at atmospheric pressure with a gas mixture containing $^{18}O_2$, 35 ml; $^{16}O_2$, 35 ml and N_2, 265 ml. After 8 minutes the pH of the reaction mixture was adjusted to 2.5 with concentrated HCl. The hydroquinone produced during the reaction was extracted with ethyl acetate, purified by thin-layer chromatography and analyzed by mass spectrometry. During the reaction a sample of the gas phase was removed from the reaction vessel for subsequent mass spectral analysis. The results (Fig. 1) show that hydroquinone formed in the presence of air has a parent ion at m/e 110 with a small P + 1 peak at m/e 111. The spectrum was identical to that of authentic hydroquinone. The mass spectrum of hydroquinone produced in the presence of $^{18}O_2$ shows a new parent ion at m/e 112 indicating the incorporation of one atom of molecular oxygen per molecule of hydroquinone. The results show a 43.2% incorporation of ^{18}O which correlates well with the 43.9% $^{18}O_2$ measured in the gas phase of the reaction vessel.

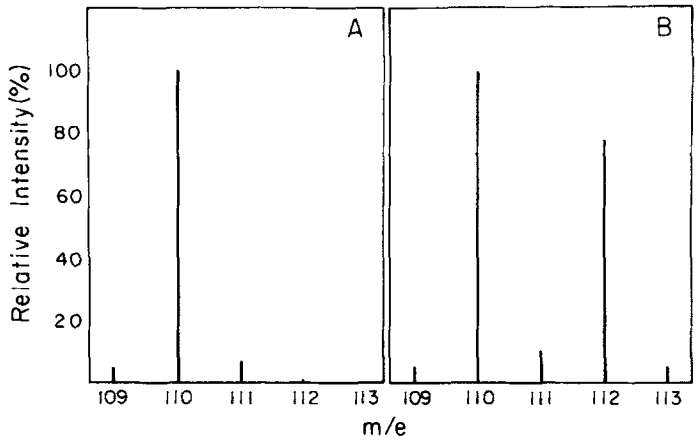

Fig. 1. Mass spectra of hydroquinone produced from *p*-nitrophenol in the presence of air (A) or a mixture of $^{16}O_2$ and $^{18}O_2$ (B). Experimental details are described in the text.

The amounts of NADPH and oxygen consumed during the enzymatic oxidation of *p*-nitrophenol were determined. NADPH oxidation was followed spectrophotometrically by measuring the *p*-nitrophenol-dependent change in absorbance at 340 nm of the standard reaction mixture. Oxygen uptake was determined polarographically. Oxidation of *p*-nitrophenol required one mole of oxygen and two moles of NADPH per mole of substrate. The utilization of one mole of oxygen per mole of substrate is consistent with a monooxygenase reaction. However, the oxidation of two moles of NADPH during the reaction is unusual, and suggested that an intermediate in the conversion of *p*-nitrophenol to hydroquinone might require further reduction to form the final product. *p*-Benzoquinone seemed a likely intermediate and was indeed found to stimulate both the enzymatic and nonenzymatic oxidation of NADPH. Enzymatic, quinone-dependent NADPH oxidation was measured by following the decrease in absorbance at 340 nm of a reaction mixture containing cell extract, NADPH, and 5×10^{-5} M *p*-benzoquinone. Details of the assay procedure are provided in Methods The reduction of one mole of *p*-benzoquinone required one mole of NADPH. The quinone reductase activity in a typical washed particulate fraction from *p*-nitrophenol-grown cells was

0.320 μmoles/min/mg protein. A similar enzyme preparation from glucose-grown cells had a specific activity of 0.016 μmoles/min/mg of protein.

Soluble fractions of cell extracts oxidized hydroquinone with the uptake of one mole of oxygen per mole of substrate. Crude extracts did not require the addition of metal ions. However, after dialysis maximum enzymatic activity was observed only when the cell extracts were incubated with ferrous ions (2.0 mM, $FeSO_4$) for five minutes prior to enzyme assay. Preincubation of cell extracts with 10 mM α,α-dipyridyl, an iron chelator, completely inhibited the oxidation of hydroquinone. The product of hydroquinone oxidation gave an absorption spectrum identical to that reported for γ-hydroxymuconic semialdehyde by Larway and Evans (7). The identification and further metabolism of the ring fission product are subjects of current investigation.

DISCUSSION

The results presented in this manuscript show that the initial reaction in the degradation of *p*-nitrophenol is catalyzed by a novel particulate enzyme. The requirement for an electron donor and the incorporation of one atom of molecular oxygen into the substrate indicate that the enzyme is a monooxygenase (13). Although hydroquinone was identified as a reaction product it is conceivable that 1,4-benzoquinone is the first intermediate in the enzyme-catalyzed reaction. Support for this hypothesis comes from the observation that the formation of hydroquinone is accompanied by the oxidation of two moles of NADPH and the presence of an inducible 1,4-benzoquinone reductase in the crude membrane preparations. These preliminary results show that the bacterial *p*-nitrophenol monooxygenase is considerably different in its properties from the fungal enzymes that catalyze the release of the nitro group from nitroalkanes (14,15,16).

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REFERENCES

1. J.R. Simpson and W.C. Evans, Biochem. J., 55, XXIV (1953).
2. K. Gunderson and H.L. Jensen, Acta. Agr. Scand., 6, 100 (1956).
3. N.J. Cartwright and R.B. Cain, Biochem. J., 71, 248 (1958).
4. V.R. Germanier and K. Whurmann, Path. Microbiol., 26, 569 (1963).
5. H.L. Jensen and G. Lathrup-Larsen, Acta. Agr. Scand., 17, 115 (1967).
6. D.G.M. Raymond and M. Alexander, Pest. Biochem. Phys., 1, 123 (1971).
7. R. Sudhakar-Barik, R. Siddaramappa and N. Sethunathan, Antonie van Leeuwenhoek, 42, 461 (1976).
8. O. Larway and W.C. Evans, Biochem. J., 95, 52P (1965).
9. D.M. Munnecke and D.P.H. Hsieh, Appl. Microbiol., 28, 212 (1974).
10. H. Lautrop, in Bergey's Manual of Determinative Bacteriology, Ed. 8, R.E. Buchanan and N.E. Gibbons, eds. Williams and Wilkins Co., Baltimore, Md., p. 433.
11. A.G. Gornall, C.J. Bardawill and M.M. David, J. Biol. Chem., 177, 751 (1949).
12. H.A.C. Montgomery and J.F. Dymock, Analyst., 86, 414 (1961).
13. O. Hayaishi, Proc. 6th Int. Congr. Biochem., New York, I.U.B. Vol. 33, 31 (1964).
14. T. Kido, T. Yamamoto and K. Soda, J. Bacteriol., 126, 1261 (1976).
15. T. Kido and K. Soda, J. Biol. Chem., 251, 6994 (1976).
16. T. Kido, K. Hashizume and K. Soda, J. Bacteriol., 133, 53 (1978).