# NAPHTHALENE METABOLISM BY PSEUDOMONADS: THE OXIDATION OF 1,2-DIHYDROXYNAPHTHALENE TO 2-HYDROXYCHROMENE-2-CARBOXYLIC ACID AND THE FORMATION OF 2'-HYDROXYBENZALPYRUVATE

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SUMMARY: In pseudomonads metabolizing naphthalene the oxidation of 1,2-dihydroxynaphthalene yields 2-hydroxychromene-2-carboxylic acid, and the conversion of this compound to 2'-hydroxybenzalpyruvate is then catalyzed by another inducible enzyme. Evidence for the nature of the oxidation is given, and the separation of some of the enzymic activities is described.

## INTRODUCTION

It has been reported that the enzymic oxidation of 1,2-dihydroxynaphthalene in pseudomonads metabolizing naphthalene yields 2'-hydroxybenzalpyruvate (C, Fig. 1), and that 2-hydroxychromene-2-carboxylic acid (B) is an

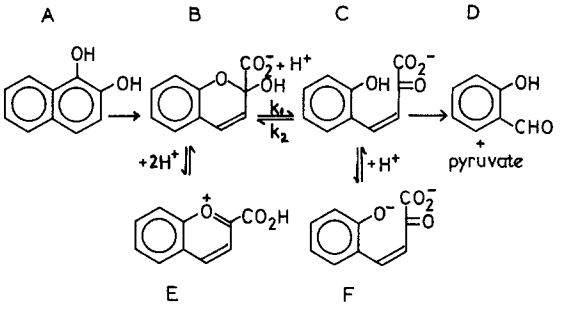


Fig. 1. Proposed pathway for the metabolism of 1,2-dihydroxynaphthalene (A) in pseudomonads. The metabolites are 2-hydroxychromene-2-carboxylic acid (B), cis-2'-hydroxybenzalpyruvate (C), and salicylaldehyde (D). The compounds E and F are derived from B and C respectively by ionization.

artefact produced during the isolation of the pyrilium compound E (1). An examination of the properties of the oxidation product of 1,2-dihydroxy-naphthalene shows, however, that it lacks a phenolic hydroxyl group but slowly isomerizes to a phenol. This isomerization is catalyzed by cell extracts, and the reaction product is cis-2'-hydroxybenzalpyruvate. The properties of the oxidation product are consistent with its identification as 2-hydroxychromene-2-carboxylic acid.

## MATERIALS AND METHODS

Bacterial cultures and cell extracts. Organisms and methods were described previously (2), but for the preparation of 2-hydroxychromene-2-carboxylic acid and for the separation of enzymes cell extracts were made from suspensions (0.5 g wet wt cells/ml) in 5 mM $KH_2PO_4$ - NaOH buffer, and for the measurement of $O_2$ uptake and $H^+$ release during the oxidation of 1,2-dihydroxy-naphthalene they were made in 5 mM acetic acid - NaOH (pH 5.5).

Measurement of enzymic activities. 2-Hydroxychromene-2-carboxylic acid isomerase (which catalyzes the interconversion of B and C) was measured by the rate of increase in absorbance at 420 nm when 0.05 μmoles of 2-hydroxy-chrome-2-carboxylic acid were added to a solution containing cell extract, 0.1 M tetrasodium pyrophosphate - HCl buffer (pH 10.0) and 0.5 mM glutathione (final volume 1.0 ml). The extinction coefficient was 10.5 $mM^{-1}cm^{-1}$. The $K_m$ for the substrate was about 0.2 mM; the optimum pH was 10.0. The enzyme could be frozen and thawed, but in either state lost its activity in a few days. Other thiol reagents could replace glutathione in the crude preparations used. They all reacted, however, with the product, 2'-hydroxybenzalpyruvate, causing some decrease in absorbance at 420 nm. No correction for this was applied to the extinction coefficient used to calculate reaction rates.

2'-Hydroxybenzalpyruvate aldolase was measured by the rate of decrease in absorbance at 296 nm when cell extract was added to solution (final volume 3.0 ml) containing 0.1 M $KH_2PO_4$ - NaOH buffer (pH 7.0) and 0.03 μmoles cis-2'-hydroxybenzalpyruvate. The $K_m$ was very low and could not be measured accurately. The activity was independent of pH between pH 5.5 and 8.0, but above pH 8.0 activity declined rapidly. The enzyme was very stable in buffer at pH 7, stored either at 0° or frozen. Reaction rates were calculated with an extinction coefficient of 12.8 $mM^{-1}cm^{-1}$, the difference between that of cis-2'-hydroxybenzalpyruvate (14.0) and the product salicylaldehyde (1.2) at 296 nm and pH 7.

1,2-Dihydroxynaphthalene oxygenase was measured by a published method (3). One unit of enzymic activity causes a change of 1 μmole $min^{-1}$. Specific activities were based on protein concentrations determined by the method of Lowry et al. (4).

Preparation of 2-hydroxychromene-2-carboxylic acid. To 5.0 ml of 5 mM acetic acid - triethylamine (pH 5.7) were added bacterial extract containing 5 units of 1,2-dihydroxynaphthalene oxygenase and then 50 μl of 20 mM 1,2-dihydroxynaphthalene in tetrahydrofuran. After 2 min at 25° the reaction mixture was applied to a column of Sephadex G25 (bed volume 50 ml), and buffer was pumped through the column at 5°. The low molecular weight fraction was

frozen in liquid $N_2$, freeze dried and stored at -25°. The elapsed time between the start of the reaction and freezing was 30 min. The absorption spectra of the low molecular weight fraction and of the redissolved freeze dried product were identical, and 1,2-naphthoquinone and low molecular weight material from cell extracts did not contribute significantly to the spectra.

Preparation 2'-hydroxybenzalpyruvate. The pH of an aqueous solution of 2-hydroxychromene-2-carboxylic acid was raised to 11 with NaOH. When the absorbance at 420 nm had reached a maximum the pH was lowered to 10 and the solution was frozen at -25°.

Partial purification of 2-hydroxychromene-2-carboxylic acid isomerase and 2'-hydroxybenzalpyruvate aldolase. Cell extract (7.5 ml, 60 mg protein) was applied to a column of DEAE-cellulose (bed volume 50 ml) equilibrated with 0.05 M trishydroxymethylaminomethane - HCl (pH 7.5 at 25°). The column was eluted with 65 ml of the same buffer, and then with 500 ml of buffer containing a gradient of NaCl increasing linearly from 0 to 0.5 M. The maximum activities of aldolase and isomerase appeared at eluate volumes of 310 and 410 respectively, and represented 43 and 20 fold purifications respectively.

Measurement of oxygen consumption and acid production in the enzymic oxidation of 1,2-dihydroxynaphthalene. Oxygen concentration was measured polarographically, assuming a saturated solution in equilibrium with air at 25° is 0.265 mM. Cell extract containing 2 units of 1,2-dihydroxynaphthalene oxygenase was added to 1.85 ml 0.05 M acetic acid - NaOH buffer (pH 5.5), and then the oxygen uptake caused by the addition of 20 mM 1,2-dihydroxynaphthalene in tetrahydrofuran (volumes from 5 to 20 µl) was measured. $H^+$ production was measured by the decrease in absorbance at 616 nm when the reaction was carried out in the presence of bromocresol green. Extract containing 3 units of 1,2-dihydroxynaphthalene oxygenase was added to 3.0 ml of 2 mM acetic acid - NaOH (pH 6.10) containing 0.02 mM indicator, and a few µl of 0.06 M HCl were added to return the absorbance to the original value. A measured volume (between 10 and 30 µl) of 20 mM 1,2-dihydroxynaphthalene in tetrahydrofuran was then added and the decrease in absorbance measured. The final pH of reaction mixtures lay between 5.5 and 5.75. The concentration of $H^+$ released by the oxidation was determined from a calibration made by measuring the absorbance decrease when standard volumes of 0.06 M HCl were added to 3.0 ml samples of indicator solution.

Measurement of salicylaldehyde and pyruvate produced in the cleavage of cis-2'-hydroxybenzalpyruvate. A predetermined volume of M-$KH_2PO_4$ was added to 3 ml of 0.18 mM cis-2'-hydroxybenzalpyruvate to reduce the pH to 7.0. Aldolase was immediately added and the solution was incubated at 25° until the change in the absorption spectrum ceased (5 min). To a 1.0 ml sample 0.2 ml 2 N NaOH was added, the absorption at 375 nm due to salicylaldehyde was immediately measured, and the concentration calculated using an extinction coefficient of 6.40 $mM^{-1}cm^{-1}$. To another 1.0 ml sample 0.05 ml 5 mM NADH was added, followed by lactic dehydrogenase. The decrease in absorbance at 340 nm was measured and the pyruvate concentration calculated using an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$.

## RESULTS AND DISCUSSION

The enzymic oxidation of 1,2-hydroxynaphthalene (A) at pH 5.5 was accompanied by the uptake of 0.99 $\pm$ 0.02 mole proportions of $O_2$ and the release

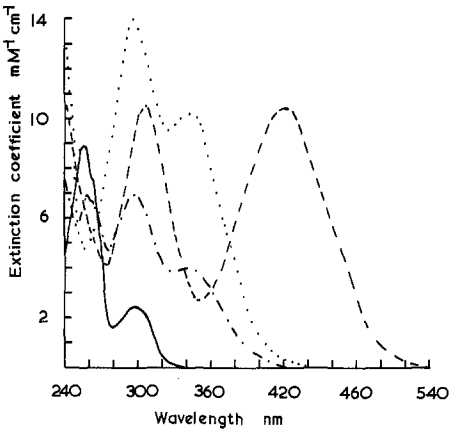


Fig. 2. Absorption spectra of 2-hydroxychromene-2-carboxylic acid and its reaction products. I, ——— , 2-hydroxychromene-2-carboxylic acid (B) prepared from A as described in the text with extracts of Pseudomonas putida NCIB 9816; II, — — —, cis-2'-hydroxybenzalpyruvate at pH 10 (F) prepared from B at pH 11; III, · · · · · ·, cis-2'-hydroxybenzalpyruvate at pH 5.5 (C) prepared from F; IV, — · — · —, equilibrium mixture of B and C at pH 5.5.

of 0.79 ± 0.02 equivalents of $H^+$. The spectrum of the oxidation product at pH 5.5 is shown in Fig. 2 (curve I). It was not greatly changed at pH values up to 10, provided the spectrum was measured with freshly prepared solutions immediately after the change in pH. This last result is not consistent with the view expressed previously (1) that cis-2'-hydroxybenzalpyruvate (C, Fig. 1) is the oxidation product.

A phenolic compound, which is probably cis-2'-hydroxybenzalpyruvate, was obtained quite rapidly from the oxidation product at pH 11, or by treatment of the oxidation product at pH 10 with cell extract or an enzyme (2-hydroxychromene-2-carboxylic acid isomerase) partially purified therefrom. This phenolic compound had spectrum II at pH 10 and spectrum III at pH 5.5. The absorbance at 420 nm changed immediately the pH was adjusted, and the variation with pH indicated the presence of a group with $pK_a$ 8.5. This value, and the high wavelength of the absorption, are consistent with the presence of a phenolic hydroxyl group conjugated with an unsaturated substituent. The phenolic compound, when treated with crude bacterial extracts or an enzyme

(2'-hydroxybenzalpyruvate aldolase) partially purified therefrom, was converted to 0.88 ± 0.02 mole proportions of salicylaldehyde (D) and 0.83 ± 0.03 mole proportions of pyruvate. The identities of the products of this reaction were confirmed by thin layer chromatography of their 2,4-dinitrophenylhydrazones. The cis configuration is assigned because of the difference of the spectrum from that of the synthetic trans isomer (1).

The absorption spectra at pH 5.5 of the enzymic oxidation product of 1,2-dihydroxynaphthalene and of cis-2'-hydroxybenzalpyruvate changed slowly on standing to the same form, IV, which is consistent with the presence together of the oxidation product and the phenol. The kinetics of the interconversion at pH 5.5 were consistent with two first order reactions ($k_1$, 0.113 $h^{-1}$; $k_2$ 0.168 $h^{-1}$; equilibrium constant 0.67). For the enzyme catalyzed interconversion at pH 10, the ionization of 2'-hydroxybenzalpyruvate to F ensures complete conversion of the oxidation product to 2'-hydroxybenzalpyruvate.

The properties of the product of the enzymic oxidation of 1,2-dihydroxynaphthalene are consistent with its identification as 2-hydroxychromene-2-carboxylic acid (B), which was originally viewed as an artefact of isolation (1). The spectrum of the freshly prepared oxidation product was closely similar to that obtained by dissolving the pyrilium perchlorate E (prepared as described in 1) in buffer, pH 5.5, and the dilution of a solution of the oxidation product with an equal volume of concentration HCl gave a spectrum identical with that of the pyrilium salt in 6 N HCl (1).

1,2-Dihydroxynaphthalene oxygenase was not purified significantly. It is very sensitive to oxygen and treatment with $NaBH_4$ (1) gave only transient reactivation. It cannot be ruled out, therefore, that 2-hydroxychrome-2-carboxylic acid is derived in a second reaction from the true oxidation product. In accord with this it may be noted that although it contains an asymmetric C atom, 2-hydroxychrome-2-carboxylic acid was not optically active. On the other hand ionization to give E occurs very rapidly, so that even though the nett production of E is not favoured at pH 5.5, racemization could

occur. Furthermore, B could not be derived from C, because at pH 5.5 the equilibrium conditions require the presence of B and C together whereas only B was initially present. Consequently it does seem most probable that oxidation procedes by oxygen insertion to form B. Oxygen insertion has been observed previously (5), but there may be no close analogy between the two cases. In the oxidation of 1,2-dihydroxynaphthalene insertion appears to be between the 9 and 2 positions of the ring system and not between adjacent carbon atoms, and it seems unlikely the enzyme can be a mixed function oxidase for crude cell extracts do not contain sufficient NADH, or NAD and NAD reducing activity, to support the rates of oxidation observed. It has been shown previously (3, 6) that 1,2-dihydroxynaphthalene oxygenase is a different enzyme from catechol-2,3-dioxygenase, and it appears now that the reactions catalyzed are in fact only superficially similar.

2-Hydroxychromene-2-carboxylic acid isomerase and 2'-hydroxybenzalpyruvate aldolase were found in extracts of Pseudomonas NCIB 9816 at specific activities of 0.13 and 0.27 μmole/min/mg protein respectively, and after induction with salicylate during growth on succinate (2) these activities were increased to 0.39 and 0.93 respectively. Both enzymes are present in other pseudomonads, namely ATCC 17483, ATCC 17484, PpG7 (7) and $P_G$ (8).

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