CRYSTALLIZATION OF ORCINOL HYDROXYLASE FROM *PSEUDOMONAS PUTIDA*

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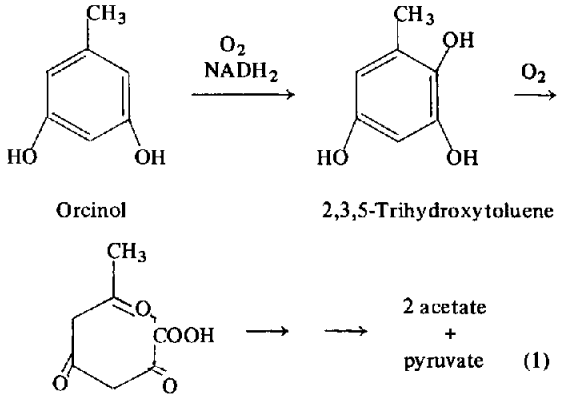
1. Introduction

The microbial metabolism of resorcinylic compounds has received little attention even though they are amongst the most common phenolic constituents in plant residues. The information available suggests that their metabolism occurs by introduction of a third hydroxyl group into the benzenoid nucleus followed by ring cleavage to yield oxoacids [1, 2]. Our own studies on the metabolism of orcinol by *Pseudomonas putida* indicated that orcinol is hydroxylated to 2,3,5-trihydroxytoluene, followed by a *meta*-ring cleavage between carbon atoms 1 and 2, according to scheme 1.



Orcinol

2,3,5-Trihydroxytoluene

2,4,6-Trioxoheptanoate

Scheme 1

When extracts of orcinol grown *Ps. putida* are supplemented with $NADH_2$ (or $NADPH_2$), orcinol is oxidized to acetate and pyruvate [2]. We recently separated from each other the enzymes catalyzing the first two reactions of the sequence, i.e., orcinol hydroxylase and 2,3,5-trihydroxytoluene oxygenase [3]. This article describes the further purification, and crystallization, of orcinol hydroxylase.

2. Materials and methods

Ps. putida 01 was isolated in 1963 from enrichment cultures provided with orcinol as sole source of carbon. The bacteria were grown in 10 l stirred fermenters on the mineral media described earlier [4] containing orcinol (0.1%). Two more similar quantities of orcinol were added after it had been utilized. Cells were harvested with an air turbine Sharples Super centrifuge and disrupted within 24 hr by exposure of suspensions (1 g wet weight cells in 2 ml of 20 mM KH_2PO_4–NaOH, pH 6.8) to ultrasound, and centrifuged at 27,000 *g* for 20 min. The supernatants were treated with protamine and the clarified supernatant was applied directly to a DEAE-cellulose column in the presence of 2-mercaptoethanol. Elution of the proteins was effected with a linear gradient of KCl (0.1–0.4 M) which gave a complete separation of orcinol hydroxylase from the ring cleavage enzyme (fig. 2).

The fractions containing orcinol hydroxylase were concentrated by $(NH_4)_2SO_4$ precipitation and the redissolved protein was applied to a Sephadex G-100 column. The hydroxylase was again precipitated with

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Table 1
Purification of orcinol hydroxylase from *Ps. putida.*

Fraction	Total protein (mg)	Specific activity (μmoles/min/mg protein)	Recovery (%)
Crude extract	5120	0.39	100
Protamine sulphate	2660	0.65	86
DEAE-cellulose	673	1.44	48
Sephadex G-100, G-75	36	12.1	20
Second DEAE-cellulose	28	12.3	13
Crystallization	25	12.9	11

$(NH_4)_2SO_4$ and applied to Sephadex G-75. The eluted enzyme was absorbed onto DEAE-cellulose in 20 mM phosphate and a linear KCl gradient 0.1–0.4 M used to elute the hydroxylase. The enzyme was crystallized in 2–3 days by addition of $(NH_4)_2SO_4$ until the solution was just turbid. All steps after the protamine sulphate treatment were performed in the presence of 2-mercaptoethanol. Details of the purification of orcinol hydroxylase are summarized in table 1.

Orcinol hydroxylase was routinely assayed by polarographic measurement of O_2 consumption with a Clark oxygen electrode. The assay mixture contained: 100 mM KH_2PO_4–NaOH buffer pH 6.8 (2.8 ml); enzyme solution (as required): 25 mM NADH (20 μl); and 25 mM orcinol (20 μl). Simultaneous measurements of oxygen and absorbance were made as described earlier [5]. Details of individual experiments appear in the legends to the figures.

3. Results

The standard assay for orcinol hydroxylase is shown in fig. 1. As was indicated earlier [2, 3] crude extracts of cells oxidize both orcinol and its hydroxylated product (2,3,5-trihydroxytoluene) with the consumption of 2 moles of O_2 per mole of orcinol. Consequently, the apparent loss in total activity of orcinol hydroxylase is partly due to the complete separation of the ring cleavage enzyme from orcinol hydroxylase (fig. 2; the substrate analogue of the ring cleavage enzyme, 3-methylcatechol, was used to assay the second enzyme of the sequence).

Orcinol hydroxylase crystallized from ammonium sulphate solutions as pale yellow plates with specific activities of about 11–14 μmoles/min/mg protein. Further crystallization did not improve the specific activity of the enzyme. Disc electrophoresis showed the presence of one diffuse protein band and two minor bands. The molecular weight of the orcinol hydroxylase was approximately 60,000 by gel filtration and 70,000 by ultracentrifugation.

Prosthetic group

The crystals of orcinol hydroxylase are pale yellow and dissolve in buffer to give bright yellow solutions. The UV visible absorption spectrum of the enzyme is shown in fig. 3 and is typical of a flavoprotein. The

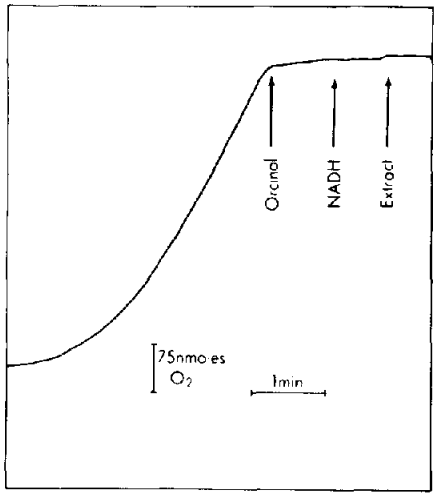

Fig. 1. Polarographic assay of orcinol hydroxylase in crude extracts of *Pseudomonas putida.* The reaction chamber contained: 100 mM KH_2PO_4–NaOH buffer, pH 6.8 (2.8 ml); cell-free extracts (20 μl), 25 mM NADH (20 μl) and 25 mM orcinol (20 μl) were added as indicated. Time sequence right to left. Temperature 30°.

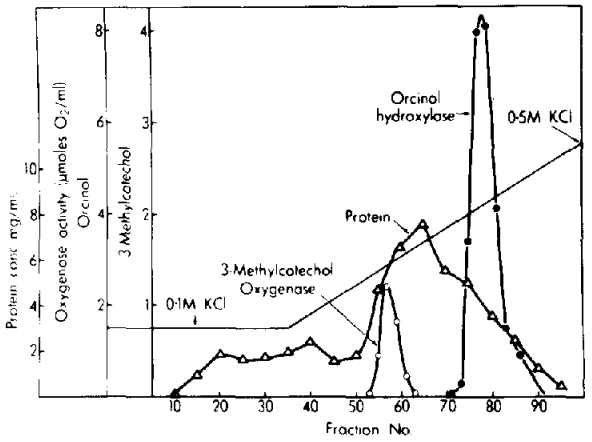

Fig. 2. Separation of orcinol hydroxylase from the ring cleavage enzyme by chromatography on DEAE-cellulose. Details of assays appear in the methods section or fig. 1.

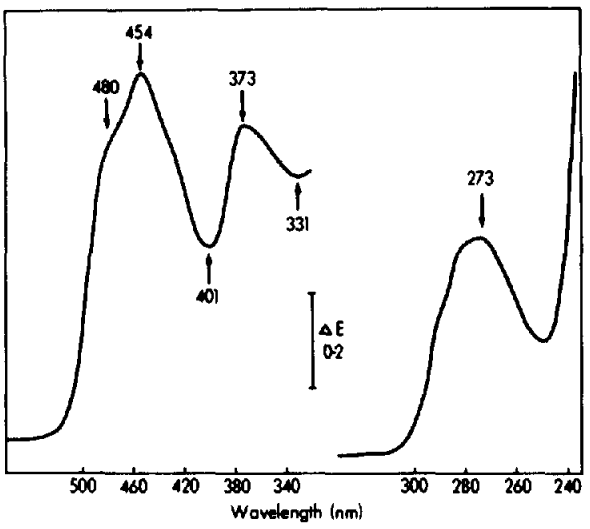

Fig. 3. Absorption spectrum of crystalline orcinol hydroxylase. A solution of 4 mg protein/ml of the crystalline preparation was used for the visible spectrum and 0.8 mg/ml for the UV spectrum.

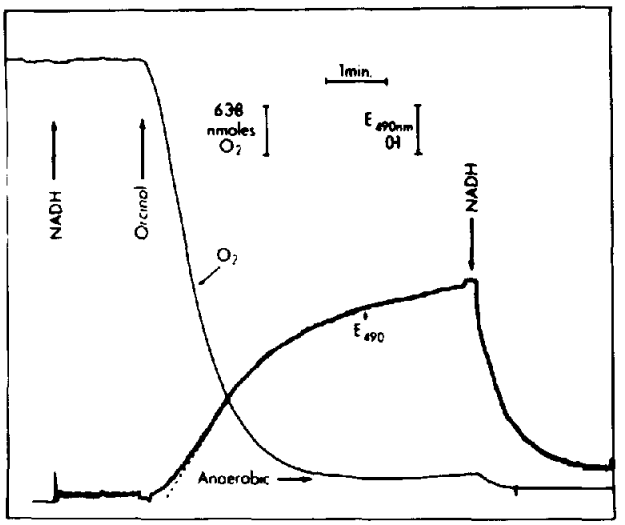

Fig. 4. Polarographic and spectrophotometric assay of crystalline orcinol hydroxylase. The absorbance changes occurring at 490 nm are due to the non-enzymic formation of a quinone from the product 2,3,5-trihydroxytoluene. The spectrophotometric cuvette contained: 100 mM KH_2PO_4–NaOH buffer pH 8.0 (2.5 ml); orcinol hydroxylase (10 μl); 25 mM NADH (20 μl) and 25 mM orcinol (20 μl) as indicated. When the reaction mixture had become anaerobic and the rate of quinone formation became limited by diffusion of air into the reaction mixture, 25 mM NADH (20 μl) was added and immediate reduction of the quinone is seen. The formation of the quinone is a secondary reaction even at pH 8.0 – see dotted line. Oxygen consumption (560 nmoles) is clearly in excess of that required for the hydroxylase reaction before the system became anaerobic (500 nmoles was the theoretical value expected).

yellow colour is bleached on addition of dithionite, and by the addition of NADH and orcinol under anaerobic conditions. The flavin was removed from the protein by heating at pH 6.8 at 100° for 3 min and identified by its absorption spectrum and chromatographic characteristics as FAD [6]. Initial estimates suggest that 1 mole of FAD is bound per mole of protein (molecular weight approx. 70,000).

Stoicheiometry of reaction

Orcinol hydroxylase was presumed to catalyze a typical mono-oxygenase reaction:

orcinol + O_2 + $NADH_2$ →
2,3,5-trihydroxytoluene + NAD + H_2O

However, the stoicheiometry of the reaction is difficult to establish due to the rapid, non-enzymic oxidation of the product to its quinone. Furthermore, the quinone is reduced by any excess NADH present in the reaction mixture, i.e., it serves as a catalytic electron carrier from NADH to O_2 (fig. 4). However, addition of the purified ring cleavage dioxygenase to the standard assay for orcinol hydroxylase showed that orcinol, O_2 and $NADH_2$ were consumed in the ratio 1:2:1 as expected for the sequential catalyses by a mono-oxygenase and dioxygenase.

4. Discussion

The crystallization of orcinol hydroxylase and the demonstration of the prosthetic group as FAD suggests that it is fairly similar to the previously crystallized aromatic hydroxylases, *p*-hydroxybenzoate hydroxylase [7–10] and salicylate hydroxylase [11–13] though its substrate specificity would appear to be broader. Current studies on the order of substrate addition to enzyme indicate further similarities. Thus, the aromatic substrate, orcinol produces a conformational change in the enzyme, which can now be reduced in the absence of oxygen by NADH. Admission of oxygen to the reaction mixture restores the oxidized flavin form with concomitant formation of product. In this respect, orcinol hydroxylase also resembles *p*-hydroxybenzoate and salicylate hydroxylases. We have not examined the protein for other prosthetic groups, however, metal ions do not appear to be involved since several well-known chelators of Fe^{2+}, Fe^{3+} and Cu^{2+} did not inhibit activity.

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