UNCOUPLING OF ELECTRON TRANSPORT FROM OXYGENATION IN THE MONO-OXYGENASE, ORCINOL HYDROXYLASE

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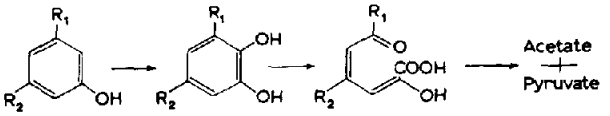
1. Introduction

During the investigation of the pathway of catabolism of orcinol (3,5-dihydroxytoluene) by *Pseudomonas putida* it was observed that orcinol grown cells were also adapted to oxidize resorcinol (1,3-dihydroxybenzene) [1, 2]. It was concluded on the basis of work with cell-free extracts that resorcinol, and also *m*-cresol, were substrate analogues for orcinol hydroxylase, and yielded the analogous catechols (hydroxyquinol and 3-methyl-catechol) which were known to be substrates for the second enzyme in the pathway, a ring cleavage enzyme (see scheme 1).



orcinol $R_1 = CH_3, R_2 = OH$
resorcinol $R_1 = H, R_2 = OH$
m-cresol $R_1 = CH_3, R_2 = H$

Scheme 1

Thus it seemed possible that this series of reactions leading to pyruvate represented another example of non-specific sequences of enzymes that could completely catabolize a variety of substrates [4–7].

We have recently crystallized orcinol hydroxylase from *Pseudomonas putida* [3]. It is a typical mono-oxygenase, catalyzing the oxygenation of orcinol to 2,3,5-trihydroxytoluene with concomitant transfer of two electrons from NADH to oxygen:

$$\text{orcinol} + O_2 + NADH_2 \rightarrow \text{2,3,5-trihydroxytoluene} + NAD + H_2O$$

Electron transport from NADH to O_2 is mediated by FAD, the only known prosthetic group for the enzyme [3].

The product of orcinol hydroxylation, 2,3,5-trihydroxytoluene, is easily oxidized non-enzymically to a quinone. This complicated the determination of the stoicheiometric relations of the reaction. Furthermore $NADH_2$, the best electron donor known for orcinol hydroxylation, could non-enzymically reduce the quinone formed. Thus accurate determinations of both O_2 and NADH consumption were impossible. This led us to investigate the stoicheiometry of the reaction with substrate analogues. The results reported here show that several suspected substrate analogues of orcinol hydroxylase allow a transfer of electrons from NADH to O_2, but that hydroxylation does not always occur and that hydrogen peroxide is formed in amounts dependent upon the substrate analogue used.

2. Materials and methods

Twice-crystallized orcinol hydroxylase was used for these experiments [3]. It had a specific activity of 14.1 μmoles/min/mg of protein at 30° and pH 6.8 Polarographic and spectrophotometric methods of assay have been described previously [3]. Details of individual experiments appear in the legends to the figures.

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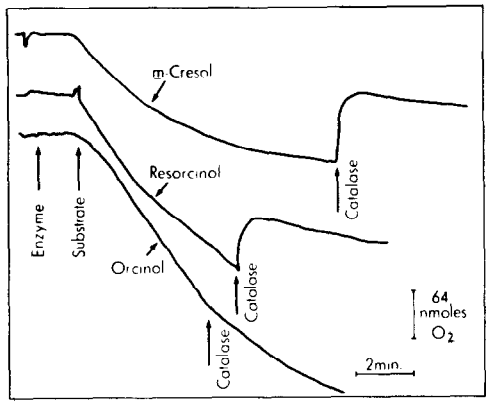


Fig. 1. Hydrogen peroxide formation during oxidations catalyzed by orcinol hydroxylase. Polarographic trase of O_2 were made in reaction mixtures containing: 100 mM phosphate buffer, pH 6.8 (3.0 ml) and 25 mM NADH (20 μl). Crystalline orcinol hydroxylase solution (10 μl for orcinol trace; 50 μl for the others) and 25 mM aromatic substrate (20 μl) were added as indicated . Catalase (Sigma 50 μl) was added as indicated. Temperature, 30°.

3. Results

When resorcinol is used as a substrate for orcinol hydroxylase, the product hydroxyquinol is not formed in sufficient amounts to account for the amounts of oxygen and NADH consumed. Addition of catalase to such reaction mixtures, showed that hydrogen peroxide had also been formed, and that this could possibly account for the O_2 and NADH consumption that occurred in excess of the resorcinol supplied.

Fig. 1 is a composite of the progress of three of the reactions catalyzed by orcinol hydroxylase. When orcinol is substrate, it is completely consumed with formation of the quinone of its hydroxylated product (lower curve); catalase does not affect the rate of O_2 consumption. During resorcinol stimulated O_2 consumption, some product, hydroxyquinol, was detected, but hydrogen peroxide was also produced. Subsequent O_2 consumption that occurred after the addition of catalase was much slower (middle curve fig. 1). When *m*-cresol was the aromatic effector for the stimulation of NADH respiration, we were unable to detect the formation of any product, 3-methylcatechol (top curve fig. 1). Furthermore, the addition of catalase to this reaction mixture showed that one mole of H_2O_2 was formed for each mole of O_2 previously consumed.

Fig. 2 shows the effect of catalase upon the O_2 consumption rates catalysed by orcinol hydroxylase when orcinol, resorcinol or *m*-cresol are provided as the aromatic substrate (or effector). Catalase does not appreciably inhibit the "orcinol respiration," but it inhibits "resorcinol respiration" and "*m*-cresol respiration" by 40 percent and 50 percent respectively. The latter value suggests that a complete uncoupling of electron transfer from hydroxylation has occurred; this is also consistent with our failure to detect 3-methylcatechol as a product, and the stoicheiometric formation of H_2O_2 from NADH and O_2 (fig.1).

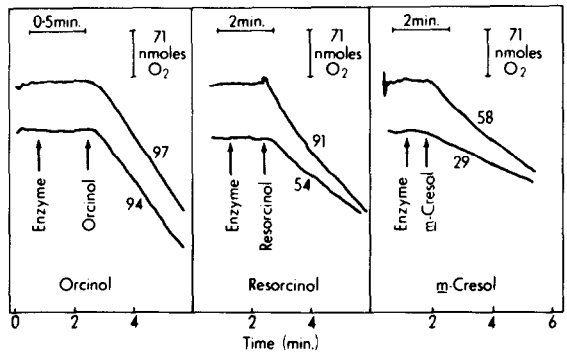


Fig. 2. Effect of catalase upon the rates of O_2 consumption catalyzed by orcinol hydroxylase in the presence of orcinol, recinol or *m*-cresol as effector. The reaction mixtures were the same as for fig. 1 expect that the additions were made in different orders. The top traces were obtained in the absence of catalase; the bottom traces were obtained from reaction mixtures containing catalase. The figures appearing by the lines represent the O_2 consumption rates as moles O_2/min. Temperature, 30°.

Fig. 3 demonstrates that the addition of catalase to reaction mixtures oxidizing orcinol affects neither O_2 consumption nor electron transfer from NADH. When, however, *m*-cresol is used as substrate analogue, addition of catalase causes an immediate release of O_2 equivalent to one half of that previously consumed; O_2 consumption then resumes at less than 50 percent of the initial rate. Catalase does not, however, affect the rate of NADH oxidation.

Earlier results [3] suggested that electrons are transferred from NADH to FAD on the enzyme only when the aromatic substrate orcinol was present, i.e., FAD reduction by NADH can be only demonstrated in the presence of orcinol and absence of O_2. Fig. 4 shows that the electron transport from NADH to O_2, when stimulated by *m*-cresol, is also probably medi-

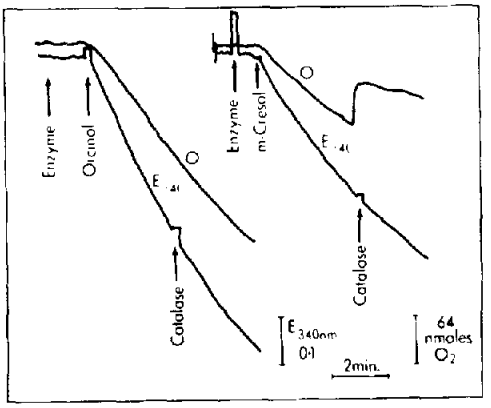


Fig. 3. The effect of catalase on the oxidation of NADH and consumption of O_2 when orcinol and *m*-cresol are used as effectors of orcinol hydroxylase. Simultaneously assays of NADH oxidation and O_2 consumption were made as described previously [3]. Reaction mixtures were similar to those used in fig. 1. The discontinuities seen in the A_{340} traces when catalase was added are due to the automatic switching of the voltage from the photomultiplier tubes when the cuvette housing is exposed for additions. Temperature, 30°.

ated by the FAD of the enzyme. However, hydroxylation does not occur but electron transfer NADH to O_2 occurs with H_2O_2 formation.

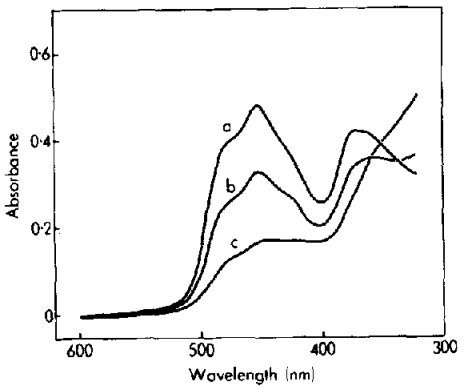
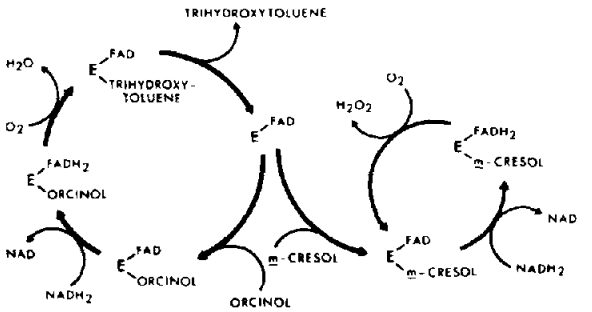


Fig. 4. Reduction of the flavin of orcinol hydroxylase by NADH in the presence of *m*-cresol as the aromatic effector. The cuvette contained orcinol hydroxylase in N_2 saturated 20 mM phosphate buffer, pH 6.8 (1 ml; 3 mg of protein) and 25 mM *m*-cresol (10 μl). The cuvette was sealed with a serum cap and flushed with N_2 (spectrum (a)). 25 mM NADH (4 μl) was added and spectrum (b) was obtained. A further 3μl of 25 mM NADH was added and spectrum (c) was obtained. Temperature, 26°. Admission of oxygen to the cuvette restored spectrum (a).

4. Discussion

The earlier conclusion that resorcinol and *m*-cresol were alternative aromatic substrates for the orcinol hydroxylase must now be modified. Orcinol is the only known substrate that is quantitatively hydroxylated with the expected consumption of O_2 and NADH for a mono-oxygenase. Hydrogen peroxide is not a product. Resorcinol and *m*-cresol, however, allow the transfer of electrons to O_2 with formation of hydrogen peroxide. The amount of hydrogen peroxide formed with each aromatic substrate is consistent with the extent of hydroxylation of the aromatic substrate that occurs.

Since transfer of electrons from NADH to O_2 does not occur at appreciable rates (nor to the flavin of the enzyme) in the absence of certain aromatic compounds, we suggested [3] that the sequence of substrate binding to orcinol hydroxylase was: aromatic substrate, $NADH_2$ and finally O_2. When orcinol is the aromatic substrate, one of the oxygen atoms is transferred to the benzenoid nucleus. Resorcinol is not such a good substrate for hydroxylation but allows electrons to flow, albeit at slower rates, to O_2 without cleavage of this diatomic molecule. The sequence of reactions is indicated in scheme 2.



Scheme 2. Electron transfer and hydroxylation reactions catalyzed by orcinol hydroxylase.

This uncoupling of electron transport from hydroxylation was first reported by White-Stevens and Kamen for salicylate hydroxylase from *Ps. fluorescens* when benzoate was provided as the substrate analogue. Our data for orcinol hydroxylase provide another illustration of this phenomenon but also includes an example of a substrate analogue, resorcinol, that is hydroxyl-

ated to a limited extent but allows most of the oxygen to be reduced by NADH to H_2O_2.

References

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