Flavin Adenine Dinucleotide Requirement for the Enzymic Hydroxylation and Decarboxylation of Salicylic Acid

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In an attempt to elucidate the mechanism of the enzyme hydroxylation of aromatic compounds, we have isolated and partially purified an aromatic hydroxylase from a soil bacterium. This enzyme, which will be referred to as "salicylate hydroxylase," is soluble and relatively stable, in contrast to most hitherto known aromatic hydroxylases, and catalyzes a stoichiometric conversion of salicylate to catechol in the presence of reduced diphosphopyridine nucleotide and oxygen. After acid ammonium sulfate treatment of the enzyme, its catalytic activity was shown to require the addition of a factor present in boiled juice of rat liver that has now been identified as flavin adenine dinucleotide. A brief description of the experimental evidence and the implication of these findings are reported here.

The cells of a gram-positive coccus, which was isolated from soil by the enrichment technique (1), were grown for approximately 30 hours at 30°, with constant shaking, in a medium containing 0.1% NH\textsubscript{4}NO\textsubscript{3}, 0.1% sodium salicylate, 0.15% K\textsubscript{2}HPO\textsubscript{4}, 0.05% KH\textsubscript{2}PO\textsubscript{4}, 0.02% MgSO\textsubscript{4}-7H\textsubscript{2}O, and 0.3 mg per liter of riboflavin. Cell-free extracts were prepared by subjecting the cell suspension to the action of a Kubota 10-kc sonic oscillator for 15 minutes, followed by centrifugation at 104,000 \times g for 60 minutes.

To 100 ml of the supernatant solution containing usually approximately 10 mg of protein per ml were added 100 ml of a saturated ammonium sulfate solution, and the pH of the mixture was adjusted to 2.7 by the addition of 0.5 N HCl. The precipitate that formed after the mixture had been left overnight in a refrigerator was collected by centrifugation, washed once with approximately 100 ml of a 60% saturated ammonium sulfate solution (pH 2.7), and dissolved in 30 ml of 0.5 M phosphate buffer, pH 8.0. The enzyme solution thus obtained was dialyzed against a large amount of 33 mM phosphate buffer, pH 7.0, to remove ammonium ion, which is inhibitory to the enzyme activity.

As shown in Table I, 1 mole of catechol was produced from 1 mole of salicylate with the consumption of approximately 1 mole of DPNH. TPNH showed approximately 80% of the activity of DPNH under the conditions tested. In the absence of FAD, practically no reaction took place. FMN, riboflavin, tetrahydrofolic acid,\textsuperscript{2} or ferrous sulfate did not replace FAD. Reducing agents such as GSH, ascorbic acid, tetrahydrofolic acid, and ferrous sulfate were ineffective in place of DPNH. A parallel experiment carried out in a Warburg apparatus indicated a stoichiometric consumption of oxygen and formation of CO\textsubscript{2}, 2,3-Dihydroxybenzoate was also metabolized by this enzyme. Phenol, benzoate, and anthranilate, however, were completely inert as substrate.

\textsuperscript{1}The abbreviation used is: FMN, flavin mononucleotide.

\textsuperscript{2}Tetrahydrofolic acid, pyrodoxase, and metapyrodoxase were kindly donated by Dr. Hiroshi Taniuchi of Kyoto University.

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Table I

<table>
<thead>
<tr>
<th>Salicylate</th>
<th>DPNH</th>
<th>Catechol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disappeared</td>
<td>Disappeared</td>
<td>Formed</td>
</tr>
<tr>
<td>System - DPNH</td>
<td>0.72</td>
<td>0.92</td>
</tr>
<tr>
<td>System - salicylate</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>System - FAD</td>
<td>0.12</td>
<td>0.06</td>
</tr>
<tr>
<td>System - FAD, - salicylate</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>System - enzyme</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>System - DPNH, + TPNH</td>
<td>0.53</td>
<td>0.68</td>
</tr>
<tr>
<td>System - DPNH, + TPNH, - salicylate</td>
<td>0.04</td>
<td>0.04</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Catechol was also determined by the use of metapyrocatechase (4). Essentially identical results were obtained.

\textsuperscript{†}TPNH instead of DPNH.

O-Phenanthroline at 1 mM showed 50% inhibition. However, preincubation of the inhibitor (5 mM) with the enzyme at 2° for 1 hour, followed by dialysis against buffer restored the full activity and further addition of ferrous sulfate or sodium molybdate at final concentrations of 0.1 mM and 0.01 mM did not affect the activity. \(\alpha,\alpha'-\text{Dipyridyl (1 mM)}\) or \(\alpha\text{-phenanthroline (0.1 mM)}\) did not show appreciable inhibition either by the direct addition of the inhibitors or by preincubation with the enzyme at 17° for 10 minutes before adding salicylate. Na\textsubscript{2} at 10 mM showed 30% inhibition, while KCN (10 mM) or Na\textsubscript{2}S (1 mM) did not. Addition of crystalline beef liver catalase (0.6 mg per ml, corresponding to 340 units per ml) in the presence of ethanol (1 M) to the reaction system did not reduce the rate of decrease of absorbancy either at 340 nm or 295 nm.\textsuperscript{3} A substrate amount of H\textsubscript{2}O\textsubscript{2} (0.2 \textmu mole), which did not destroy the enzyme under these conditions, did not replace DPNH plus FAD in the reaction.

In most hydroxylation reactions, including those catalyzed by salicylic acid oxidase from \textit{Pseudomonas eurupas} (5), TPNH or DPNH appears to be a specific electron donor. With some hydroxylases, such as phenolase or dihydroxyphehylamine-hydroxylating system, ascorbic acid serves as a reductant.

\textsuperscript{3}Except when otherwise stated, the enzyme activity was routinely assayed spectrophotometrically at 17° by measuring the rate of decrease of absorbancies at 340 nm (DPNH) and 265 nm (salicylate). The reaction mixture contained 0.067 mM potassium salicylate, 0.067 mM DPNH, 0.0067 mM FAD, 33 mM potassium phosphate buffer, pH 7.0, and 0.0075 mg of enzyme protein per ml; total volume, 3.0 ml.
while tetrahydropteridine is the immediate electron donor in the phenylalanine hydroxylase system (6). In the latter case, TPNH serves as a reductant indirectly to reduce the oxidized pteridine derivatives. Crystalline lactate oxidative decarboxylase (7) was shown to be an oxygenase (8), containing 2 moles of FMN per mole of the enzyme protein. More recently, the enzymatic lactonization of diketocamphane was reported to require DPNH and FMN (9); it is postulated that DPNH reduces the iron of the lactonizing enzyme to an active "ferrous" state through FMN. Ferrous ion does not seem to be involved in the salicylate hydroxylase system.

The results of nutritional studies on the effect of vitamin B₁₂ on tryptophan metabolism have led to the suggestion that flavin coenzymes might be involved in the hydroxylation of tryptophan metabolites (10). However, the role of flavins in aromatic hydroxylating systems has not been definitely established. The present communication provides experimental evidence for the participation of FAD in such enzyme reactions. FAD could be a new and probably a direct electron donor of the salicylate hydroxylase. Although further experiments are necessary to elucidate the role of flavin coenzymes in aromatic hydroxylation reactions, flavin coenzymes might serve as the immediate electron donor in many other hydroxylation systems.

REFERENCES

Isolation and Some Properties of the Green Heme of Cytochrome Oxidase from Beef Heart Muscle*

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This paper reports the first direct isolation of the green α-type heme, which is associated with cytochrome oxidase of beef heart muscle, by new and mild methods of extraction and chromatography. The heme (an iron (II) compound) was obtained in two molecular species that were interconvertible under conditions suggesting that one is the oxygcnated form of the other. The analytical data indicate that this α heme is possibly in a more native condition than the hemin α (an iron (III) compound) isolated by Warburg and Gevirtz in 1951 (1). The problems involved in the isolation and structure of hemin α have been reviewed by Lemberg (2).

In our isolation procedure beef heart muscle was extracted with pyridine-chloroform solutions. Lipids were grossly removed from the homogenate by extraction and the homogenate was separated and freed of lipids by partition chromatography on Celite. In the pyridine solutions used in the isolation procedure, the green heme remained in the fully reduced or Fe (II) oxidation state throughout, without the addition of reducing agents such as dithionite. The isolation procedure avoided the use of the rather harsh HCl-acetone extraction and iron removal steps used by others (1, 2) and minimized the possibility that the labile substitutnts on the porphyrin ring are modified during isolation.

In a typical isolation, 4 kg of minced beef heart muscle were washed three times with 8 liters of water at 4°C, once with 4 liters of 80% acetone at 4°C, and twice with 7 liters of chloroform, and the washings were discarded. The residue was extracted three times with 2 liters of pyridine-chloroform (1:2) for 60 minutes. The red heme (protoporphyrin) and green α-type hemes were isolated by Warburg and Gevirtz in 1951 (1). The problems involved in the isolation and structure of hemin α have been reviewed by Lemberg (2).

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