Mechanism of the Salicylate Hydroxylase Reaction*

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SUMMARY

1. A stable ternary complex of salicylate hydroxylase apoenzyme, flavin adenine dinucleotide, and salicylate was detected by a change of the absorption spectrum and by titration of the holoenzyme with salicylate.

2. The complex was enzymatically active, since the stoichiometric formation of the product, catechol, was observed upon introduction of air into a solution of reduced form of the complex.

3. On the basis of the results, the following scheme was postulated for the over-all reaction of salicylate hydroxylase.

\[ E-FAD + \text{salicylate} \rightarrow E-FAD-\text{salicylate} \]

\[ E-FAD-\text{salicylate} + NADH \rightarrow E-FADH_2-\text{salicylate} + NAD \]

\[ E-FADH_2-\text{salicylate} + O_2 \rightarrow E-FAD + \text{catechol} + CO_2 + H_2O \]

Here, \( E \) denotes the protein moiety of the enzyme.

In earlier studies (1-3) salicylate hydroxylase, a flavoprotein from a pseudomonad, was shown to catalyze the conversion of salicylate to catechol with the stoichiometric consumption of molecular oxygen and reduced nicotinamide adenine dinucleotide. Moreover, experiments with purified enzyme indicated that the hydroxylase contains 1 mole of flavin adenine dinucleotide per unit of 57,000 molecular weight, and that reduction of FAD with NADH and hydroxylation of salicylate are tightly coupled. Likewise, evidence has been gradually accumulated that a flavin (FAD or FMN) is specifically required for various monooxygenase reactions (4-7) although the precise role of the coenzyme in the reaction process is not yet clear.

A 1:1:1 ternary complex of the enzyme with FAD and salicylate, the "actual" substrate, has now been demonstrated. This communication reports the direct demonstration of enzyme-bound intermediates in an effort to elucidate the reaction mechanism of mono-oxygenase.

To obtain a completely resolved apoenzyme of salicylate hydroxylase under milder conditions, a modification of the previously described method (2) was used. The ammonium sulfate fraction was dialyzed against 0.005 M K_2HPO_4 and then applied to the column (2.5 x 23 cm) of TEAE-cellulose (Serva Entwicklungslabor) equilibrated with the same solvent. After washing the column with 0.02 M K_2HPO_4, the enzyme fractions were eluted with 0.05 M K_2HPO_4. The eluates containing enzymatic activity were collected and dialyzed against 0.01 M K_2HPO_4 for 3 hours. The dialyzed material was applied to a DEAE-Sephadex A-50 column (2.0 x 23 cm) equilibrated with 0.01 M K_2HPO_4, and the column was immediately washed with the same solvent. Enzyme was eluted with a linear gradient of K_2HPO_4 (0.03 to 0.2 M). The eluates containing enzymatic activity were collected, and enzyme was then precipitated by the addition of solid ammonium sulfate (0.5 g per ml of eluate). The precipitate was dissolved in 0.033 M potassium phosphate buffer (pH 7.0), and the ammonium sulfate was removed by dialysis against the same buffer. The term "apoenzyme" will be used for the enzyme preparation obtained in this way, since this preparation had no absorption band in the visible region and was completely inactive unless FAD was added. For reconstitution of the holoenzyme, the apoenzyme was dissolved in buffer and slight excess FAD was added to the solution. The mixture was carefully layered on the top of a Sephadex G-25 column (2.0 x 20 cm) equilibrated with 0.033 M phosphate buffer (pH 7.0). The column was subsequently developed with the same buffer. During the development, the yellow zone was rapidly separated into two fractions, and the protein fractions were completely isolated from free FAD. The holoenzyme thus obtained was used throughout the experiments.

The absorption spectrum of the holoenzyme exhibited maxima at 450 m\( \mu \) and 375 m\( \mu \), and a minimum at 404 m\( \mu \) (Fig. 1, Curve I). Spectra presented in this paper were observed only in the visible region. Assuming the molar extinction of the bound FAD in holoenzyme to be the same as that of free FAD (11,300), it was calculated that approximately 0.9 molecule of FAD was bound to 1 molecule of enzyme protein. This was in good agreement with the enzyme-FAD ratio obtained by equilibrium dialysis, and the result confirmed that the molar ratio of FAD to apoenzyme was 1:1.

When salicylate was added to the holoenzyme, the peak at 375 m\( \mu \) was shifted to the red. In the position of the peak at 450 m\( \mu \), its absorbance increased slightly and a marked shoulder appeared around 480 m\( \mu \) (Fig. 1, Curve II). A quite similar spectrum was observed when salicylate was replaced in the above system by any one of the other substrates for the hydroxylase reaction, namely 2,5-dihydroxybenzoate, p-aminosalicylate, or 1-hydroxy-2-naphthoate. However, benzoate, 3,4-dihydroxybenzoate, catechol and o-phenolsulfonate, which were not substrates of the enzyme (2, 3), did not affect the original spectrum of the holoenzyme. Free FAD could not replace the holoenzyme...
The whole system contained, in 3 ml, 141 μmoles of the holoenzyme (measured by the method of Lowry et al. (8) with crystalline egg albumin as a standard), 5.0 μmoles of salicylate, 81.5 μmoles of potassium phosphate buffer at pH 7.0. Curve I, without salicylate and NADH; Curve II, without NADH; and Curve III, NADH placed in the side arm of a Thunberg type cuvette was tipped into the main vessel containing holoenzyme and salicylate in the buffer under anaerobic conditions as described in Table I.

Since no spectral change in FAD was observed under these conditions. These results indicate that the holoenzyme combines specifically with the substrate to form a new ternary complex.

When 0.5 eq of NADH was added under anaerobic conditions to the cuvette containing the holoenzyme and salicylate, the absorption over the whole spectral range diminished instantaneously (Fig. 1, Curve III), indicating the stoichiometric formation of the fully reduced form of the holoenzyme-salicylate complex.

Since the formation of the ternary complex was indicated by the increase in absorption at 480 μm, the stoichiometry of the components of the complex could be determined spectrophotometrically by titration of the holoenzyme with salicylate. As shown in Fig. 2, when the holoenzyme was titrated with salicylate, the initial slope of the titration curve intercepted the maximum value at a concentration of 1.0 mole/l mole of holoenzyme. These results indicate that 1 mole of salicylate is bound to 1 mole of holoenzyme, and so the ratio of apoecyme, FAD, and salicylate in the complex is 1:1:1.

Evidence that the ternary complex is an actual intermediate involved in the over-all reaction is provided by an experiment under anaerobic conditions as shown in Table I. When a limited amount of NADH was added to the solution containing the holoenzyme and salicylate, the decrease in absorbance at 450 μm corresponded to the full reduction of 1 mole of FAD moity in the complex with 1 mole of NADH added. On admitting air to the cuvette, the reduced complex was rapidly reoxidized, and the absorption in the visible region increased to the same level as that of the untreated preparation. Under these conditions 1 mole of catechol was shown to be produced.

![Table I](http://www.jbc.org/)

<table>
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<tr>
<th>Experiment</th>
<th>Holoenzyme</th>
<th>NADH added</th>
<th>Enzyme-FAD reduced</th>
<th>Catechol formed</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>173</td>
<td>79</td>
<td>89</td>
<td>54</td>
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<td>95</td>
</tr>
<tr>
<td>II</td>
<td>165</td>
<td>118</td>
<td>115</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Fig. 1. The effect of salicylate and NADH on the spectrum of salicylate hydroxylase. Hitachi model EPR-3 recording spectrophotometer was used with 1-cm light path cuvettes.

![Fig. 1](http://www.jbc.org/)

![Fig. 2](http://www.jbc.org/)

Scheme 1 Schematic representation of intermediates during salicylate hydroxylation. E and SA denote protein moiety of the enzyme and salicylate, respectively.
from 1 mole of the reduced complex, while in the absence of oxygen, the formation of catechol was not observed.

These results demonstrate that NADH is oxidized anaerobically with concomitant formation of enzyme-bound FADH$_2$ (III) from II as shown in Scheme 1 where the intermediate complexes with the sequence of reactions in the conversion of salicylate to catechol are summarized. Upon introducing oxygen into the solution, Complex III reacts with molecular oxygen to produce products such as catechol, CO$_2$, and water, by the reaction sequence a, b, and c shown in the scheme.

In the absence of salicylate, the stoichiometric reduction of the holoenzyme with NADH was also observed anaerobically. When holoenzyme which had been reduced with NADH (Complex III') was mixed with salicylate, and then air was introduced into the reaction mixture, the stoichiometric formation of catechol could again be observed. These results suggest the possible presence of the alternative sequence b', a', and c for salicylate hydroxylation. However, Reaction b' is not an efficient one for salicylate hydroxylation since the $K_m$ for NADH determined by the decrease of absorbance at 340 nm is about 400 times larger in the absence of salicylate than in its presence (3), while the affinity of the holoenzyme for salicylate is not influenced by the presence of NADH as cited above.

Our previous observations (3) have shown that the anaerobic reduction of free FAD with NADH in the presence of a catalytic amount of the enzyme indicates the presence of the over-all Reactions a, b and c, and b' and c'' in Scheme 1, respectively. However, free FADH$_2$ produced under anaerobic conditions did not serve as an efficient electron donor in the hydroxylase reaction (3). These results suggest that Reactions c' and c'' may be side reactions in the hydroxylase reaction.

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REFERENCES

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