BBA 65946

MECHANISM OF THE SALICYLATE HYDROXYLASE REACTION*

II. THE ENZYME–SUBSTRATE COMPLEX

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(Received April 1st, 1969)

SUMMARY

1. Salicylate hydroxylase (salicylate, NADH:oxygen oxidoreductase (1-hydroxylating, 1-decarboxylating)) from *Pseudomonas putida* forms an enzyme–substrate complex with salicylate.

2. The complex could be detected by a new absorption maximum around 480 nm. By spectrophotometric titration, it was found that a molar ratio of apoenzyme, FAD and salicylate in the complex was 1:1:1.

3. The complex was more stable than the holoenzyme under any tested conditions, *i.e.*, heat, acid and proteinase treatments.

4. The FAD moiety of the complex was reduced with NADH under anaerobic conditions, and the reoxidation of the reduced complex with air resulted in product formation. The stoichiometric relation in each reaction was demonstrated by using substrate level amounts of the enzyme. A mechanism for salicylate hydroxylation reaction is proposed.

INTRODUCTION

It has already been established that salicylate hydroxylase (salicylate, NADH: oxygen oxidoreductase (1-hydroxylating, 1-decarboxylating)) from a pseudomonad†, which catalyzes the conversion of salicylate to catechol with the stoichiometric consumption of O_2 and NADH, is a flavoprotein with FAD as the prosthetic group[4–6]. In 1957, HAYAISHI AND SUTTON[7] demonstrated that lactate oxidative decarboxylase from *Mycobacterium phlei* catalyzes the incorporation of O_2 into substrate and requires

* The preceding paper in this series is ref. 1. A part of the results was presented at the meeting of the Japan–U.S.A. Symposium on Oxygenases in Kyoto, 1966 (ref. 2).

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† Kindly identified by Dr. G. Hegeman of Department of Bacteriology, University of California, Berkely as a new pseudomonad which is quite similar to *Pseudomonas putida*[3].

FMN as a coenzyme. Since then, a number of flavin(FAD or FMN)-requiring monooxygenases have been studied. However, there has been little experimental evidence, except for several speculations, clarifying the function of the flavin in the monooxygenase reaction. Salicylate hydroxylase with molecular weight of 57 000 provides an especially favorable system for investigating the function of flavin in a monooxygenase reaction since it shows unusual alteration of absorption spectrum in the presence of the substrate[1,2]. Upon addition of salicylate, the absorption peaks are shifted to longer wavelengths with a marked shoulder around 480 nm. These spectral changes suggest the formation of an enzyme–substrate complex. Since the new shoulder around 480 nm is characteristic of the complex, we can directly demonstrate the ratio of apoenzyme, FAD and salicylate in the complex by spectrophotometric titration.

This paper describes spectrophotometric experiments which indicate that salicylate hydroxylase combines specifically with the substrate to form a complex in which the ratio of apoenzyme, FAD and salicylate is 1:1:1. Furthermore, we report preliminary experiments designed to show that this ternary complex is an actual intermediate involved in the overall reaction of salicylate hydroxylase.

MATERIALS

Reagents

FAD (approx. 90% purity) was supplied by Dr. E. Ohmura, Takeda Research Laboratories, Osaka. NADH was obtained from Calbiochem. Co.; DEAE-cellulose from Brown Co.; Sephadex G-25, G-100 and DEAE-Sephadex A-50 from Pharmacia; salicylic acid from Nakarai Chemical, Kyoto; benzoic acid and catechol from Merck Co.; 2,3-, 2,4-, 2,5- and 3,4-dihydroxybenzoic acids, *p*-aminosalicylic acid, 1-hydroxy-2-naphthoic acid, 3-methylsalicylic acid, *m*- and *p*-hydroxybenzoic acids from Tokyo Kasei, Tokyo. Catechol, *m*- and *p*-hydroxybenzoic acids were recrystallized from toluene. 1-Hydroxy-2-naphthoic acid and 2,3-, 2,4- and 3,4-dihydroxybenzoic acids were recrystallized from water. All of the acids used were neutralized with NaOH. Bacterial proteinase, "Nagarse", was donated by Dr. K. Kusai of Nagase and Co., Amagasaki.

Preparation of salicylate hydroxylase

A purified preparation of salicylate hydroxylase was prepared according to a modification of the method reported previously[5]. Cells were cultivated essentially under the same conditions as described previously[4], except that 2 mg of $FeSO_4$ were further supplied to 1 l of the medium.

All subsequent procedures were carried out at below 5°. The buffer used was K_2HPO_4–KH_2PO_4 (pH 7.0), except when otherwise stated. Centrifugations were carried out at 13 000 × *g* for 20 min. A typical protocol is presented in Table I.

Step 1: Crude extract. The cells (100 g, wet wt.) were ground for 45 min in a prechilled mechanical mortar (20.5 cm diameter) with 2 times their weight of aluminum oxide (Wako W, 800) and were mixed with 500 ml of 33 mM buffer. The resultant slurry was centrifuged off. The precipitate together with alumina was resuspended in 300 ml of the same buffer and was centrifuged. The supernatant fluids from the two centrifugation steps were combined to yield a crude extract.

Step 2: $(NH_4)_2SO_4$ precipitation. To 500 ml of the crude extract, 113 g of solid

TABLE I

SUMMARY OF PURIFICATION OF SALICYLATE HYDROXYLASE

Step	*Total vol. (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*	*Yield (%)*
Crude extract	715	9290	7090	0.76	100
$(NH_4)_2SO_4$ fractionation	172	4610	5020	1.09	71
DEAE-cellulose	193	3330	4940	1.49	70
Sephadex G-100	87	714	4160	5.83	59
DEAE-Sephadex A-50	30	384	3840	10.0	54

$(NH_4)_2SO_4$ were added with mechanical stirring. After standing for more than 15 min, the mixture was centrifuged, and 75 g of $(NH_4)_2SO_4$ were further added per 500 ml of supernatant fluid. The precipitate was collected by centrifugation, was dissolved in about 150 ml of 10 mM buffer and was then dialyzed for 3 h against 3 l of the same buffer with two changes.

Step 3: DEAE-cellulose treatment. The dialysate was passed through a column (4.0 cm × 20 cm) of DEAE-cellulose equilibrated with 10 mM buffer, under which the enzyme was not adsorbed on the column. The enzyme fraction was rapidly eluted with the same buffer and was immediately applied to a gel-filtration column.

Step 4: Sephadex G-100 gel filtration. The eluted enzyme solution (193 ml) was applied to a column (9.0 cm × 50 cm) of Sephadex G-100 equilibrated with 10 mM buffer, and the column was developed with the same buffer at the flow rate of 60 ml per h. To the eluate containing the enzymatic activity (450 ml), 225 g of $(NH_4)_2SO_4$ were added. The precipitate was removed by centrifugation, was dissolved in 10 mM K_2HPO_4 solution freshly prepared to give about 50 ml of the enzyme solution and was dialyzed for 3 h against 10 mM K_2HPO_4.

Step 5: DEAE-Sephadex chromatography. The dialyzed material was applied to a DEAE-Sephadex A-50 column (2.5 cm × 30 cm) equilibrated with a fresh solution of 10 mM K_2HPO_4. The enzyme was eluted with a linear gradient established between 300 ml of 50 mM K_2HPO_4 (mixing vessel) and 300 ml of 0.2 M K_2HPO_4 (reservoir). The enzyme fractions (250 ml) were combined, 125 g of $(NH_4)_2SO_4$ were added and the resulting precipitate was removed by centrifugation. The preparation, dissolved in a minimum amount of 33 mM buffer, was dialyzed for 3 h against the same buffer.

Step 6: Reconstitution of holoenzyme. Since the flavin was partially leached from the protein moiety of the enzyme during the purification procedure, 2 mg of FAD per 100 mg protein were added to the dialyzed enzyme solution at the final step. The mixture was then applied to a Sephadex G-25 column (2.5 cm × 25 cm) equilibrated with 33 mM buffer. The column was subsequently developed with the same buffer. The holoenzyme thus obtained was completely free of unbound FAD and was used as the source of enzyme, except when stated otherwise. The enzyme was quite stable for a few weeks when stored at −20°. Disc electrophoresis of this preparation by the method of DAVIS[8] showed a single band, and the enzyme had a specific activity of 10.

Crystallization of salicylate hydroxylase

The $(NH_4)_2SO_4$ precipitate obtained at Step 5 was used as the starting material for crystallization. The precipitate was redissolved in a minimum amount of 50 mM

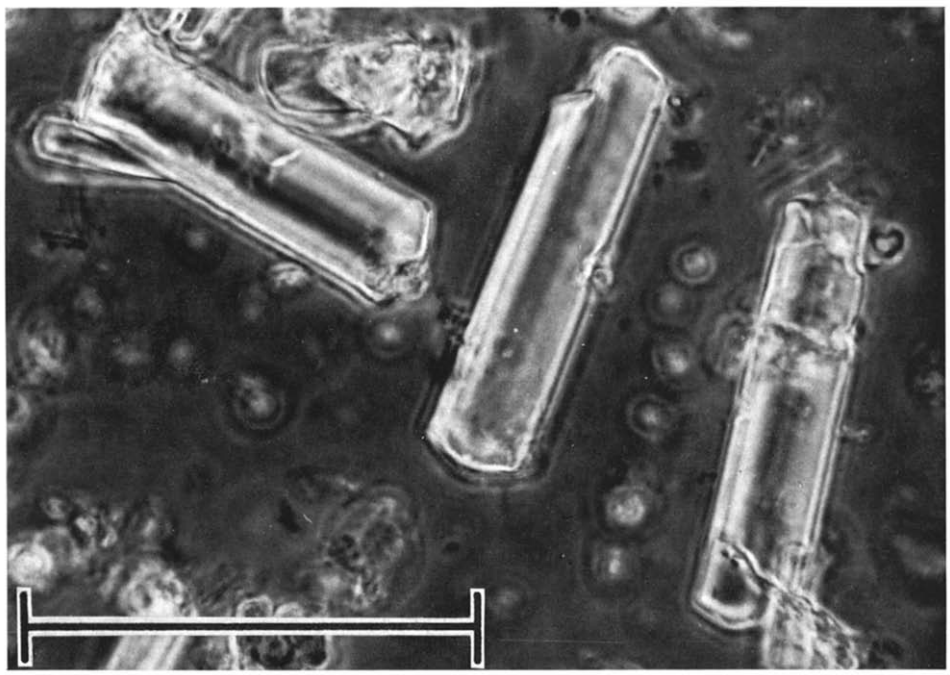


Fig. 1. A phase-contrast photomicrograph of crystalline salicylate hydroxylase. The indicated scale represents 0.1 mm.

Tris–HCl buffer (pH 8.0) containing a few mg of FAD, and solid $(NH_4)_2SO_4$ was added slowly to the solution until a slight turbidity appeared. The pH of the solution was adjusted to 8.0 with aqueous ammonia. The solution was left to stand at 5° for a few hours, and the precipitate was then removed by centrifugation. This procedure was repeated until the first traces of yellow precipitate started to settle. This solution was left to crystallize at 5°. Yellow crystals were slowly formed, and over a period of several days the crystal size increased (Fig. 1). The specific activity was not increased by this procedure. Therefore, crystallization was not used for purification of the enzyme.

Preparation of apo-salicylate hydroxylase

The apoenzyme was prepared by the acid–$(NH_4)_2SO_4$ method in the same way described previously[4]. This preparation of apoenzyme was stable for several days when stored at −20°.

Metapyrocatechase

This was prepared from *Pseudomonas putida*, T-2, essentially as described by NOZAKI *et al.*[9]. The enzyme was stored as crystals suspended in 50 mM potassium phosphate buffer (pH 7.5) containing 10% acetone.

METHODS

Estimation of enzymatic activities

The units of salicylate hydroxylase activity and the assay procedures have been described elsewhere[5]. The amount of enzyme protein was estimated by the method of

LOWRY *et al.*[10], using bovine serum albumin (Sigma Chemical Co.) as a protein standard. Concentrations of the holoenzyme were determined spectrophotometrically with the use of the molar extinction coefficient of 11.2 $mM^{-1} \cdot cm^{-1}$ at 450 nm*.

Measurement of absorption spectrum

A Hitachi model EPR 3 recording spectrophotometer and a Perkin–Elmer Model 202 recording spectrophotometer were used with a 1-cm light path cuvette.

Determination of catechol or 3-methylcatechol

The amounts of catechol or 3-methylcatechol were estimated enzymatically with the use of a crystalline metapyrocatechase. To a sample containing catechol or 3-methylcatechol in 3 ml of 40 mM potassium phosphate buffer (pH 7.2), 70 ng of metapyrocatechase in 50 μl were added by means of a micropipette. After the mixture was kept at room temperature for more than 3 min, the amounts of catechol or 3-methylcatechol were determined from the changes of the absorbance at 375 nm (ε (375 nm) = $3.6 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$) or at 389 nm (ε (389 nm) = $9.6 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$), respectively. Under the assay conditions, the absorbances at these wavelengths were found to be proportional to the concentrations of catechol or 3-methylcatechol, respectively.

Titration of enzyme-bound FAD with NADH

The experiments were performed in a Thunberg-type cuvette (1-cm light path) equipped with a side arm and a vaccine cup. The cuvette was evacuated at room temperature for 15 min and was then flushed with N_2 gas, alternately twice. O_2-free N_2 was prepared with the treatment of alkaline pyrogallol. After placing it in an ice bath for 1 h, the same procedure was repeated once more. Finally the cuvette was flushed with N_2. The solution of NADH was anaerobically prepared in a Thunberg tube in the same way as above. Titration of the enzyme with NADH was performed by stepwise additions of small portions (10–20 μl) of the latter into the former solution by means of a 50-μl microsyringe.

RESULTS

Absorption spectrum of the enzyme–substrate complex

As illustrated in Fig. 2, the holoenzyme showed a slight difference in spectrum compared with free FAD. When FAD was mixed with an equimolar amount of the apoenzyme, the absorption intensity of FAD was slightly lowered, while the peaks at 450 and 375 nm were not shifted. When salicylate was added to the holoenzyme, the peaks at 450 and 375 nm were shifted to 455 and 385 nm, respectively, and a marked shoulder appeared around 480 nm (Fig. 3). Similar spectral shifts were also produced by a number of other substrates for the hydroxylase reaction, namely 2,3-, 2,4- and 2,5-dihydroxybenzoates, *p*-aminosalicylate, 1-hydroxy-2-naphthoate and 3-methylsalicylate. Some representative spectra in the absence and presence of these compounds are shown in Fig. 4. However, compounds such as benzoate, *m*- and *p*-hydroxybenzoates and 3,4-dihydroxybenzoate which were not substrates of the enzyme had little or no effect on spectral shift at 375 and 450 nm or in forming a shoulder at 480 nm. Free FAD did not replace the holoenzyme. *o*-Phenolsulfonate, a compe-

* Calculated on the basis of direct analysis of FAD released from the protein moiety by heat and acid treatments.

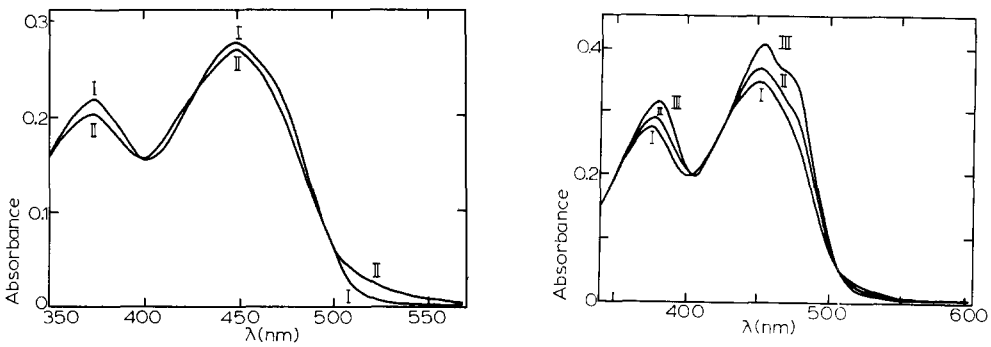


Fig. 2. Comparison of absorption spectra of FAD and salicylate hydroxylase in 33 mM potassium phosphate buffer (pH 7.0) (3.0 ml). Curve I, FAD (74 nmoles); Curve II, FAD + apoenzyme (74 nmoles).

Fig. 3. Effect of salicylate on the spectrum of salicylate hydroxylase in 33 mM potassium phosphate buffer (pH 7.0) (3.0 ml). Curve I, holoenzyme (92 nmoles); Curve II, holoenzyme + salicylate (50 nmoles); Curve III, holoenzyme + salicylate (1 μmole).

titive inhibitor for the enzyme[6], gave another type of spectral change (Fig. 4D). This compound differed in effect from salicylate, producing only a slight shift in the maximum at 375 nm to shorter wavelengths. The peaks around 450 nm were not essentially affected, and no shoulder at 480 nm was observed.

Stoichiometric relation of apoenzyme, FAD and salicylate in the enzyme–substrate complex

Since the complex formation was observed by the absorption increase at the shoulder at 480 nm, stoichiometry was determined by titration of the holoenzyme with salicylate. The enzyme-bound FAD (153 nmoles) was titrated precisely with varying amounts of salicylate (Fig. 5, Curve I). No significant difference was observed when

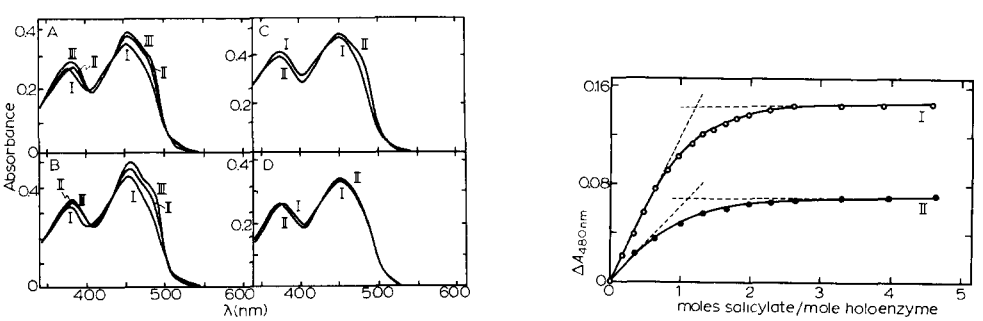


Fig. 4. Effect of other substrates and an inhibitor on the spectrum of salicylate hydroxylase in 33 mM potassium phosphate buffer (pH 7.0) (3.0 ml). A. Curve I, holoenzyme (92 nmoles); Curve II, holoenzyme + 2,5-dihydroxybenzoate (5 μmoles); Curve III, holoenzyme + *p*-aminosalicylate (5 μmoles). B. Curve I, holoenzyme (110 nmoles); Curve II, holoenzyme + 2,3-dihydroxybenzoate (5 μmoles); Curve III, holoenzyme + 2,4-dihydroxybenzoate (5 μmoles). C. Curve I, holoenzyme (129 nmoles); Curve II, holoenzyme + 3-methylsalicylate (5 μmoles). D. Curve I, holoenzyme (92 nmoles); Curve II, holoenzyme + *o*-phenolsulfonate (5 μmoles).

Fig. 5. A spectrophotometric titration of salicylate hydroxylase with salicylate. The reaction mixture contained 153 nmoles (Curve I) or 76 nmoles (Curve II) of the holoenzyme and indicated amounts of salicylate in 33 mM potassium phosphate buffer (pH 7.0) in 3.0 ml system.

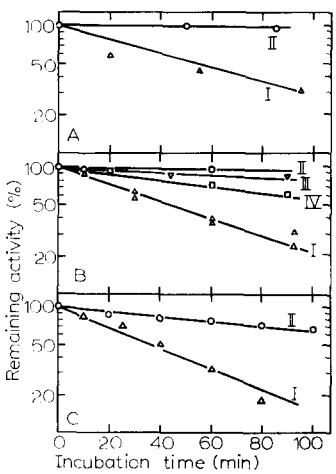
the titration was carried out in the presence of excess free FAD. A similar titration was performed with salicylate in the presence of 76 nmoles holoenzyme (Fig. 5, Curve II). In both cases, the initial slope of the titration curve intercepted the maximum value observed at 1 mole salicylate per mole enzyme-bound flavin.

The fact that the molar ratio of FAD to the apoenzyme in salicylate hydroxylase is 1:1 has been confirmed by equilibrium dialysis[5] and by fluorometric analysis[11]. From combination of these results, it is now possible to conclude that a molar stoichiometry of apoenzyme, FAD and salicylate in the complex is 1:1:1.

Since the increases in absorption at 480 nm upon addition of salicylate were assumed to be proportional to the amounts of the complex, it was possible to estimate the dissociation constant from data of the titration curve shown in Fig. 5. A mean value of the dissociation constant of salicylate in the complex was calculated to be 3.5 μM.

Stability of the enzyme–substrate complex

Experiments demonstrated in Figs. 6A and 6B indicate that the holoenzyme is rapidly inactivated at 41° or at pH 4.2. However, the complex which was formed in the presence of salicylate was found to be completely stable under these conditions. The protective effect of salicylate in a proteinase treatment was also similar to that



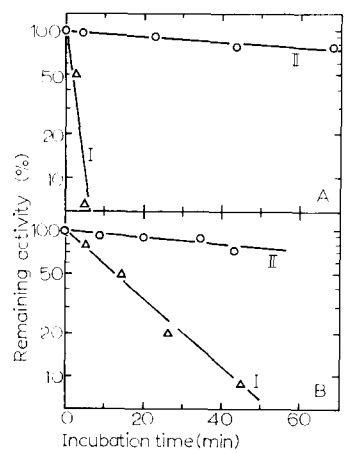


Fig. 6. Effect of substrate on acid (A), heat (B) and Nagarse (C) inactivations of salicylate hydroxylase. A. The reaction mixture (2.0 ml) containing 5.7 nmoles of the holoenzyme, 60 μmoles of acetate buffer (pH 4.2) and 800 nmoles of salicylate was incubated at 20°. Samples were withdrawn at time intervals, and assayed for activity. B. The reaction mixture (2.0 ml) containing 5.7 nmoles of the holoenzyme and 60 μmoles of potassium phosphate buffer (pH 7.0) was supplemented with 800 nmoles of the substrates and incubated at 41°. C. The reaction mixture (3.0 ml) containing 5.7 nmoles of the holoenzyme, 48 μmoles of Tris–HCl buffer (pH 8.0), 10 μg of Nagarse and 800 nmoles of salicylate was incubated at 29°. Curve I, without substrate; Curve II, with salicylate; Curve III, with 2,5-dihydroxybenzoate; Curve IV, with *p*-aminosalicylate.

Fig. 7. Effect of substrate on the inactivation of the apoenzyme by heat (A) and Nagarse (B). A. The reaction mixture (2.0 ml) containing 1.5 nmoles of the apoenzyme, 60 μmoles of potassium phosphate buffer (pH 7.0) and 800 nmoles of salicylate, was incubated at 41°. B. The reaction mixture was as described in A, except that 10 μg of Nagarse were further added. The reaction mixture was incubated at 20°. Curve I, without salicylate; Curve II, with salicylate.

observed in the heat and acid inactivation experiments (Fig. 6C). Under the conditions in which the holoenzyme lost 80% of its initial activity, the complex lost only 30%.

To determine whether the addition of other substrates related to salicylate would also affect the stability of the enzyme, the holoenzyme was incubated with such compounds at 41°. The data collected in Fig. 6B show that the stability is significantly increased by incubation with such compounds, while other substances such as catechol or benzoate afforded scarcely any significant protection.

These results provide an additional evidence that the holoenzyme combines with salicylate to form a complex having a more rigid structure than the holoenzyme.

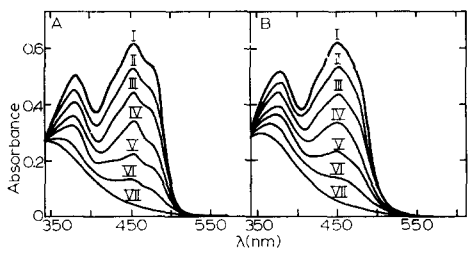
As shown in Fig. 7A, the apoenzyme was also spontaneously destroyed at 41°. Enzymatic activity was completely lost in 10 min. However, an appreciable protective effect was observed in the presence of salicylate. When the apoenzyme was incubated with Nagarse, its activity decreased considerably. This inactivation was depressed by the addition of salicylate, as shown in Fig. 7B. Similar protective effects were observed with substances related to salicylate but not with substances which were not substrates for the enzyme.

Spectrophotometric and stoichiometric demonstration that the enzyme–substrate complex is involved in the catalysis

Anaerobic titration of the enzyme–substrate complex with NADH. To elucidate the catalytic mechanism of FAD in the complex, the effect of NADH on the spectrum of the complex was investigated. Fig. 8A shows the results of anaerobic titration of the complex with NADH. Curve I represents oxidized form. Upon addition of appropriate amounts of NADH to a solution of the complex, the absorption over the whole spectral range diminished instantaneously (Curves II–VI), and no increase in absorption at a wavelength greater than 530 nm, which could be attributed to a stable one-electron reduced form of the enzyme–flavin, was observed. The resulting spectra were very similar to those of the fully reduced form of flavin, and the amount of the change in absorbance at 450 nm was proportional to the amount of added NADH. After addition of 1 mole of NADH per mole of the complex, the spectrum of the reaction mixture showed full reduction (Curve VII).

Anaerobic titration of the holoenzyme with NADH. In the absence of salicylate, the holoenzyme was also reduced with NADH, and the situation was quite similar to the case in its substrate complex. As shown in Fig. 8B, a fully reduced form appeared throughout the titration, and the full reduction was observed on the addition of 1 mole of NADH per mole of enzyme-bound FAD.

Reaction of the reduced enzyme–substrate complex with O_2. To show that NADH-reduced complex is an actual intermediate in the overall hydroxylation reaction, attempts were made to demonstrate that the reduced complex is capable of aerobic hydroxylation of the substrate. Upon introducing air into the reduced form of the complex, the reduced flavin moiety was rapidly reoxidized, and the absorption in the visible region increased to the same level as that of the untreated preparation. Under these conditions, the reaction product, catechol, was stoichiometrically produced in the reaction mixture. As shown in Fig. 9, the stoichiometric relation was apparently maintained until 1 mole of NADH per mole of the enzyme-bound FAD had been added. When the reduced complex was heated before admitting air, no product was observed. This indicated also that anaerobiosis had been completely achieved. When



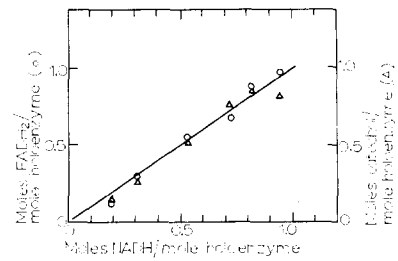


Fig. 8. Anaerobic titration of salicylate hydroxylase with NADH in the presence (A) and absence (B) of 5 μmoles salicylate. Curve I, oxidized enzyme (142 nmoles) in 33 mM potassium phosphate buffer (pH 7.0) (3.0 ml); Curves II–VII, after addition of 0.19, 0.37, 0.55, 0.73, 0.92 and 1.10 moles of NADH per mole of enzyme-bound flavin, respectively.

Fig. 9. A graphic representation of stoichiometry of salicylate hydroxylase reaction. The reaction mixture contained, in the main vessel of a Thunberg-type cuvette with 1-cm light path, 100 μmoles of potassium phosphate buffer (pH 7.0), 5 μmoles of salicylate and 142–165 nmoles of the enzyme in a total volume of 2.9 ml, and indicated amounts of NADH (in 0.1 ml) in the side arm at 20°. The cuvette was evacuated according to the same procedure as described in METHODS. After the addition of NADH into the main vessel, the amount of enzyme-bound $FADH_2$ was calculated by the decrease of the absorbance at 450 nm, taking an extinction coefficient of 10.3 $mM^{-1} \cdot cm^{-1}$. Then air was introduced into the cuvette and the amount of catechol produced in the reaction mixture was estimated with the use of metapyrocatechase.

the holoenzyme was reduced with NADH in the absence of salicylate and then was allowed to react with air after addition of salicylate, a stoichiometric amount of catechol was also detected as observed in the complex.

3-Methylsalicylate–holoenzyme was found to show spectral and enzymatic properties similar to those of salicylate–holoenzyme (Table II). The amount of the reduced complex formed was identical with that of NADH added, and upon admitting air a stoichiometric amount of 3-methylcatechol was produced from the reduced complex. When the reduced holoenzyme was allowed to react with air after addition of 3-methylsalicylate, the product was also detected stoichiometrically.

These results support the conclusion that the holoenzyme–substrate complex interacts with NADH to form an actual reduced intermediate (enzyme–$FADH_2$–substrate), which reacts with O_2 to form the product.

TABLE II

STOICHIOMETRY OF SALICYLATE HYDROXYLASE REACTION WITH 3-METHYLSALICYLATE AS SUBSTRATE

Expts. I and II, the assay conditions were as described in Fig. 9, except that 5 μmoles 3-methylsalicylate were used as a substrate. Expt. III, after the enzyme had been reduced, 5 μmoles substrate were added from the side arm, and the reaction mixture was then exposed to air.

Expt. No.	*Enzyme (nmoles)*	*3-Methylsalicylate (μmoles)*	*NADH (nmoles)*	*Enzyme reduced (nmoles)*	*3-Methylcatechol formed (nmoles)*
I	111	5	58	47	67
II	107	5	107	98	107
III	109	—	110	116	113

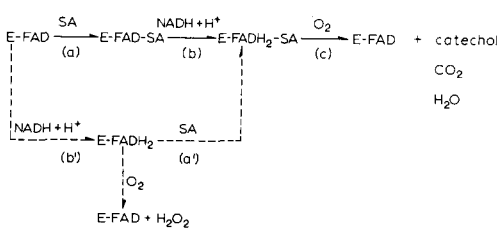
DISCUSSION

The interaction of substrate with salicylate hydroxylase is characterized by an absorption spectrum in which a new shoulder is formed at about 480 nm. The appearance of the shoulder is considered to be due to the formation of an enzyme–substrate complex. The dissociation constant of salicylate in the complex is found to be very small, so that we can now directly demonstrate the stoichiometry between apoenzyme, FAD and salicylate to be 1:1:1 by spectrophotometric titration with salicylate. Recently we have carried out a fluorometric analysis of the enzyme–substrate complex of salicylate hydroxylase and have found that the enzyme combines specifically with the substrate to form a fluorescent complex; the molar ratio of the components in the complex is 1:1:1 (refs. 11 and 12). This is in good accordance with the results of spectrophotometric investigations described here.

Spectral shifts, similar to those observed with salicylate hydroxylase in the presence of salicylate, have been reported in a study of a benzoate complex of D-amino-acid oxidase[13]. Attempts have been made to explain the spectral shifts seen in the spectrum of D-amino-acid oxidase after the addition of benzoate[13,14]. As demonstrated in this paper, the substrate complex of salicylate hydroxylase is the really "active" form of the enzyme, so that the fine mechanism of the spectral shift in the flavin, induced by the binding of the enzyme and the substrate, may be a key to the solution of the real mechanism involved in the monooxygenase reaction.

The protective effect of substrate against inactivation of salicylate hydroxylase is an additional evidence that the holoenzyme combines with salicylate to firm the substrate complex which has a more rigid structure than the holoenzyme. It is of some interest that the stability characteristics of the apoenzyme in the presence of substrate are very similar to those of the substrate complex of the holoenzyme. The effect of salicylate on the stability of the apoenzyme can be interpreted in terms of stabilization of the protein structure. The fluorometric experiments reported in the subsequent paper[11] show that the apoenzyme forms a binary complex with salicylate at a molar ratio of one to one.

Although more kinetic experimental information is required to establish the mechanism, the data collected in this study form the basis of a tentative hypothesis shown in Scheme I. We suppose that the primary reaction of salicylate hydroxylase involves the interaction of substrate with the holoenzyme as indicated in Reaction a of Scheme I. The enzyme–salicylate complex can now receive electron from NADH (Reaction b) and in the presence of O_2, produces the hydroxylated product, catechol (Reaction c). Another possibility involves the interaction of NADH rather than



Scheme I. Proposed mechanism of salicylate hydroxylase reaction. E and SA denote the apoenzyme and salicylate, respectively.

salicylate, the holoenzyme was found to be reduced with NADH. When the reduced holoenzyme was mixed with salicylate under anaerobic conditions and then O_2 was introduced, the stoichiometric formation of catechol was observed. These results suggest the possible process of the alternative reaction 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 than in the presence of salicylate[6], while the affinity of the holoenzyme for salicylate is not influenced by the presence of NADH as reported here. Kinetic studies by a stopped flow technique, which will be published later, have supported this hypothesis. Thus it is now possible to say that the reaction sequence a, b and c should be predominant in the overall catalytic process of salicylate hydroxylation.

ACKNOWLEDGMENTS

This investigation was supported in part by research grants from the Waksman Foundation of Japan and the Ministry of Education of Japan. We are indebted to Dr. Y. Kishida of the Department of Biology, Kanazawa University for the photomicrograph. Thanks are also due to K. Sei, T. Komiyama and M. Nakamura for their help with some of the experiments.

REFERENCES

1 M. KATAGIRI, S. TAKEMORI, K. SUZUKI AND H. YASUDA, *J. Biol. Chem.*, 241 (1966) 5675.
2 M. KATAGIRI, S. TAKEMORI, K. SUZUKI AND H. YASUDA, in K. BLOCH AND O. HAYAISHI, *Biological and Chemical Aspects of Oxygenases*, Maruzen Co, Tokyo, 1966, p. 315.
3 R. Y. STANIER, N. J. PALLERONI AND M. DOUDOROFF, *J. Gen. Microbiol.*, 43 (1966) 159.
4 M. KATAGIRI, S. YAMAMOTO AND O. HAYAISHI, *J. Biol. Chem.*, 237 (1962) PC 2413.
5 S. YAMAMOTO, M. KATAGIRI, H. MAENO AND O. HAYAISHI, *J. Biol. Chem.*, 240 (1965) 3408.
6 M. KATAGIRI, H. MAENO, S. YAMAMOTO, O. HAYAISHI, T. KITAO AND S. OAE, *J. Biol. Chem.*, 240 (1965) 3414.
7 O. HAYAISHI AND W. B. SUTTON, *J. Am. Chem. Soc.*, 79 (1957) 4809.
8 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
9 M. NOZAKI, H. KAGAMIYAMA AND O. HAYAISHI, *Biochem. Z.*, 338 (1963) 582.
10 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
11 K. SUZUKI, S. TAKEMORI AND M. KATAGIRI, *Biochim. Biophys. Acta*, 191 (1969) 77.
12 K. SUZUKI, H. YASUDA, S. TAKEMORI AND M. KATAGIRI, *Biochem. Biophys. Res. Commun.*, 28 (1967) 129.
13 K. YAGI, *Advan. Enzymol.*, 27 (1965) 1.
14 V. MASSEY AND H. GANTHER, *Biochemistry*, 4 (1965) 1161.