BBA 66684

MECHANISM OF THE SALICYLATE HYDROXYLASE REACTION

V. KINETIC ANALYSES*

SHIGEKI TAKEMORI[a], MASAHIKO NAKAMURA[a,**] KENZI SUZUKI[a], MASAYUKI KATAGIRI[a] AND TAKAO NAKAMURA[b]

[a]*Department of Chemistry, Faculty of Science, Kanazawa University, Kanazawa 920 and* [b]*Department of Biology, Faculty of Science, Osaka University, Toyonaka 560 (Japan)*

(Received March 10th, 1972)
(Revised manuscript received May 24th, 1972)

SUMMARY

The reaction mechanism of salicylate hydroxylase (salicylate, NADH:oxygen oxidoreductase(1-hydroxylating, 1-decarboxylating)) from *Pseudomonas putida* has been analyzed kinetically by flow rapid reaction techniques. The rate of binding of salicylate to the enzyme in the oxidized state is much faster than that to the enzyme in the reduced state. The kinetic behavior of the flavin moiety of salicylate hydroxylase is influenced by the presence of the substrate to be hydroxylated. In the presence of salicylate, both the NADH reduction of the enzyme and the reoxidation of the reduced enzyme with molecular oxygen are most simply interpreted as being two-step processes, in which one step obeys first-order kinetics. On the other hand, the same reactions in the absence of salicylate obey second-order kinetics and are most simply interpreted as one-step processes. From the comparison with the kinetic parameters in the presence and absence of salicylate, it is suggested that the enzyme–salicylate complex is a much more efficient system than the free enzyme for accepting electrons from NADH. A substrate analogue, benzoate, also facilitates the reduction of the enzyme-bound flavin by NADH.

Based on these kinetic analyses, a mechanism of the salicylate hydroxylase reaction is discussed.

INTRODUCTION

During recent years, the novel functions of flavins as "co-oxygenases" have received considerable attention in the study of biological oxidation, and a number of flavo-oxygenases have so far been obtained in pure states for the elucidation of mecha-

* A part of these results was presented at the meeting of the Third International Symposium on Flavins and Flavoproteins in Durham, 1969[1]; a preliminary report has been published[2].

** Present address: Institute for Protein Research, Osaka University, Suita, Japan.

nisms of the oxygenase reaction[3–5]. As described in previous papers of this series[6–9], salicylate hydroxylase(salicylate, NADH:oxygen oxidoreductase (1-hydroxylating, 1-decarboxylating)), one of the flavo-oxygenases purified from *Pseudomonas putida*, combines specifically with salicylate to form a new enzyme–substrate complex in which the ratio of apoenzyme, FAD and salicylate is 1:1:1. The complex is converted by the addition of an electron donor, NADH, to a two-electron reduced intermediate capable of supporting the aerobic hydroxylation of the salicylate bound to the enzyme. These facts indicate that the enzyme-bound $FADH_2$ formed by NADH serves not only as an ultimate electron donor in the hydroxylation reaction, but also functions for the activation of molecular oxygen to yield the oxygenated product.

This postulated mechanism must be substantiated by a more complete description of the reaction mechanism *via* rapid and sensitive kinetic measurements. By utilizing a flow apparatus, we have been able to clarify some aspects concerning the reaction mechanism of salicylate hydroxylase.

MATERIALS AND METHODS

A purified preparation of salicylate hydroxylase was obtained as previously described from *Ps. putida*, S-1, grown with salicylate as a carbon and energy source[7]. The concentration of the enzyme was expressed with respect to the bound FAD[7].

Salicylic acid and benzoic acid were obtained from Nakarai Chemicals, Kyoto. NADH was obtained from either Boehringer, Mannheim, or Sigma, St. Louis.

The rate of the overall reaction was determined by measuring the consumption of molecular oxygen dissolved in the reaction medium using a Clark oxygen electrode (type 4004) from Yellow Springs Instruments Co., Yellow Springs. The reaction was initiated by introducing 0.1 ml of an enzyme solution into 3.4 ml of a buffer containing known amounts of salicylate, NADH and oxygen.

The fluorimetric measurements were performed in a Hitachi MPF 2A spectrofluorimeter fitted with a xenon arc lamp as the exciting source. Quartz cuvettes of 10- and 2-mm light paths were used.

Flow experiments were performed using a temperature-controlled flow system[10] which was essentially the same as that designed by Chance and Legallais[11]. The semi-tangential 4-jet mixing chamber used in the flow system was a modification of that used by Millikan[12]. The length of the light path of the observation chamber used in the continuous-flow and the stopped-flow experiments was 2 and 10 mm, respectively. The reaction was recorded on a Sanei FR 301 visigraph.

All measurements were performed in 33 mM potassium phosphate buffer, pH 7.0, at 25 °C.

RESULTS

Salicylate binding with oxidized and reduced enzymes

On the basis of stoichiometric analyses and spectrophotometric investigations, it was proposed that the primary reaction in the salicylate hydroxylation was the coupling of salicylate to the enzyme[6,7]. The flow method was further used to evaluate whether the coupling reaction would occur prior to the reduction of the enzyme by NADH.

If, in the absence of the electron donor, the reaction between salicylate hydroxylase (E) and salicylate (S) was regarded as a simple bimolecular reaction such as

$$E + S \underset{k_s^{-1}}{\overset{k_s^{+1}}{\rightleftharpoons}} ES \tag{1}$$

the net rate of formation of the enzyme–salicylate complex (ES) will be

$$\frac{d[ES]}{dt} = k_s^{+1}[E]\,[S] - k_s^{-1}[ES] \tag{2}$$

On integration, assuming that the initial salicylate concentration $[S]_0$ is much higher than the total enzyme concentration, Eqn 2 becomes

$$\ln \frac{[ES]_{eq}}{[ES]_{eq} - [ES]_t} = (k_s^{+1}[S]_0 + k_s^{-1})t \tag{3}$$

where $[ES]_{eq}$ and $[ES]_t$ denote the concentration of the ES complex in the equilibrium state and at reaction time after mixing, t. According to Eqn 3, the plots of $\ln [ES]_{eq}/([ES]_{eq} - [ES]_t)$ against t should be linear. Thus the values of k_s^{+1} and k_s^{-1} are estimated from the values of both the slope of Eqn 3, $(k_s^{+1}[S]_0 + k_s^{-1})$, and the dissociation constant of the ES complex, $(K_S = k_s^{-1}/k_s^{+1})$.

As the complex formation was manifested by the absorption increase around 480 nm, the reaction of the enzyme with salicylate was pursued by following the increase in absorbance at 480 nm as shown in Fig. 1. When the enzyme was mixed aerobically with a sufficient amount of salicylate, as indicated by the downward deflection of the flow trace, a rapid increase in the absorbance occurred during the flow and the change was terminated at the moment of stopping the flow. A series of experiments similar to this were repeated at different mixing times. Fig. 2 shows the plots of $\ln [ES]_{eq}/([ES]_{eq} - [ES]_t)$ against t. The value for the slope of a straight line was estimated to be 588 s^{-1}. Combining the value thus obtained and the dissociation constant of 3.5 μM, which was derived from the spectrophotometric and fluorimetric titrations of the enzyme with salicylate[7,9], the rate of binding of salicylate to the enzyme, k_s^{+1}, was calculated to be $1.8 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ and the rate of the reverse reaction, k_s^{-1}, 62 s^{-1}.

Kinetic experiments on salicylate binding to the enzyme in the reduced state were also performed. The anaerobic addition of salicylate to the reduced enzyme caused no change in the visible spectrum. However, a rapid partial quenching of fluorescence of salicylate with a maximum at 405 nm caused by the addition of the reduced enzyme was detectable under anaerobic conditions. A fluorescence-quenching experiment with 30 μM salicylate gave a rate of 0.29 s^{-1}. From the value of k_s^{+1}, the rate of salicylate binding to the enzyme in the oxidized state was calculated to be 540 s^{-1} at the same concentration of salicylate. Thus, the extent of the rate of salicylate binding to the enzyme was about 1900 times faster in the oxidized state than in the reduced state. As will be discussed in detail later, these results lead to the conclusion that salicylate is bound to the enzyme prior to reduction of the enzyme by NADH. From the titration curve in fluorescence-quenching experiments, an apparent dissociation constant for salicylate bound to the reduced enzyme was calculated to be 17 μM, which was considerably greater than the value for the oxidized enzyme (3.5 μM).

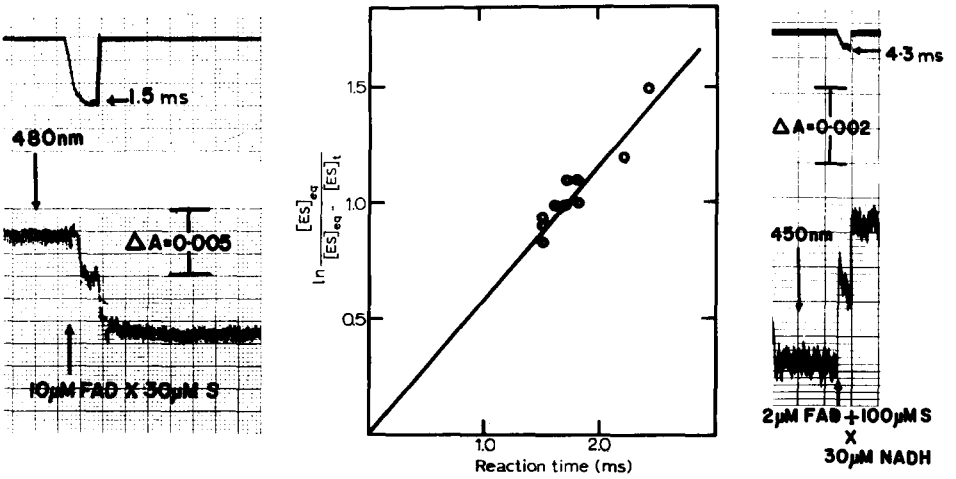

Fig. 1. Time course of absorbance change at 480 nm following rapid mixing of the oxidized enzyme with salicylate. S stands for salicylate. The arrow indicates the time of mixing. The top trace represents the flow velocity. The concentrations indicated in diagram are those after mixing.

Fig. 2. The plot of ln $[ES]_{eq}/[ES]_{eq} - [ES]_t$ against t. Conditions were the same as in Fig. 1 except that experiments were repeated at a different mixing time.

Fig. 3. Time course of reduction of the enzyme with NADH in the presence of salicylate.

Reduction of the enzyme–salicylate complex by NADH and its reoxidation by molecular oxygen

A solution of the enzyme in the presence of excess amounts of salicylate was aerobically mixed with various concentrations of NADH, and the change in absorbance at 450 nm was recorded. Upon mixing, as indicated by an upward deflection of the trace in Fig. 3, rapid reduction occurred during continuous flow. At the moment the flow was stopped, the absorbance change reached a maximum value which corresponded to full reduction of the enzyme. Subsequently the amplitude of this change decreased very slowly. Under these conditions, the reduction of the flavin moiety by NADH was so fast as compared with the reoxidation reaction that experiments could be carried out without strictly anaerobic treatment.

The rate of reduction of the flavin moiety was computed from the amounts of the reduced enzyme formed during the time of flow using the following formula semi-empirically:

$$\ln \frac{a}{a - x} = k_{red} t \qquad (4)$$

where k_{red} stands for the rate of reduction of the flavin moiety at a given concentration of NADH. The value of a represents the initial concentration of the oxidized enzyme–substrate complex, and x the amount of the reduced complex at reaction time after mixing, t. The measurement of k_{red} was made in the presence of various concentrations of NADH. The reciprocal values of k_{red} thus obtained were plotted against those of NADH concentration. The results are presented in Fig. 4A, which shows a linear relationship between $1/k_{red}$ and 1/[NADH]; the intercept on the ordi-

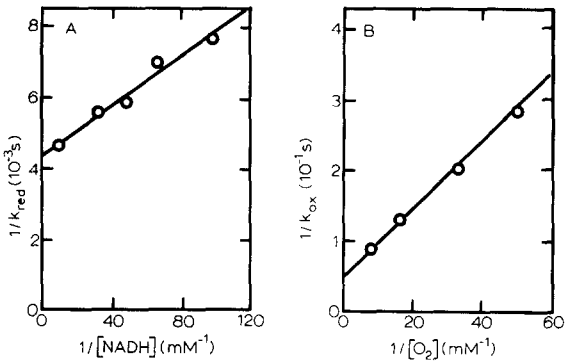

Fig. 4. Flow kinetic data of the enzyme obtained in the presence of salicylate. (A) Plot of reciprocals of k_{red} values against those of NADH concentrations. (B) Plot of reciprocals of k_{ox} values against those of O_2 concentrations. Conditions were the same as in Figs 3 and 5 except that varying concentrations of NADH and O_2 were used.

nate is not zero. The reciprocal of the intercept represents the maximum velocity of the reduction of the oxidized enzyme–salicylate complex, k_{red}^{max}, which was estimated to be 230 s^{-1}.

A similar kinetic analysis was performed on the oxidation of the reduced enzyme–salicylate complex with molecular oxygen. A solution of the enzyme reduced anaerobically with a stoichiometric amount of NADH in the presence of a large excess of salicylate was mixed with an equal volume of the buffer containing various amounts of oxygen. The increase of the absorbance at 450 nm showing the oxidation of the flavin moiety occurred after mixing. A typical record is presented in Fig. 5.

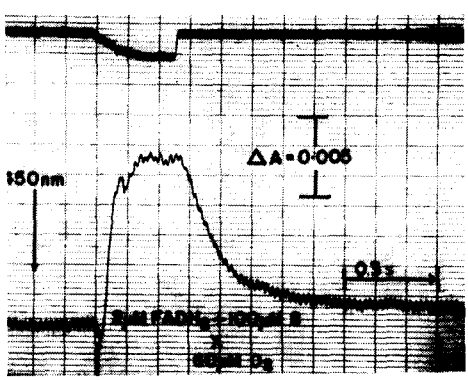

Fig. 5. Time course of reoxidation of the reduced enzyme with O_2 in the presence of salicylate.

The rate of oxidation, k_{ox}, was calculated from the half-time of the flow trace. Fig. 4B gives the plot of the reciprocal values of k_{ox} against those of oxygen concentration. The intercept of the line on the ordinate was not zero. The k_{ox}^{max} value was estimated to be 21 s^{-1}.

All of these data may be rationalized in terms of a mechanism involving two-

step processes. The following reaction may be proposed as the simplest possible mechanism which fits all the data:

$$EF{\cdot}S + NADH + H^+ \rightarrow X_1 \xrightarrow{k_{red} = 230\ s^{-1}} EFH_2{\cdot}S + NAD^+$$

$$EFH_2{\cdot}S + O_2 \rightarrow X_2 \xrightarrow{k_{ox} = 21\ s^{-1}} EF + \text{catechol} + CO_2 + H_2O$$

where $EF{\cdot}S$ and $EFH_2{\cdot}S$ denote the oxidized and reduced enzyme–salicylate complexes, respectively, and X_1 and X_2 kinetically required intermediates. This indicates that the process *via* intermediate X_1 or X_2 is rate determining, but only the transition stage from the oxidized to the fully reduced form was observed spectrophotometrically.

Reduction and reoxidation of the enzyme in the absence of salicylate

The reduction of the FAD moiety of the enzyme in the absence of salicylate was analyzed by mixing NADH anaerobically with the oxidized enzyme. As shown in Fig. 6, an upward deflection of the trace, upon stopping the flow, indicates the reduction of the FAD moiety. A series of similar experiments were repeated by mixing the enzyme with various concentrations of NADH. The rate of the FAD reduction, k'_{red}, was estimated from the time course of reduction applying Eqn 4.

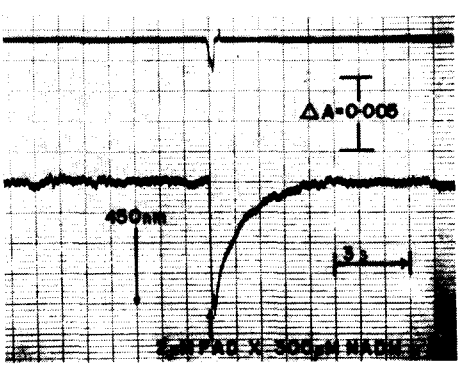

Fig. 6. Time course of reduction of the enzyme with NADH in the absence of salicylate.

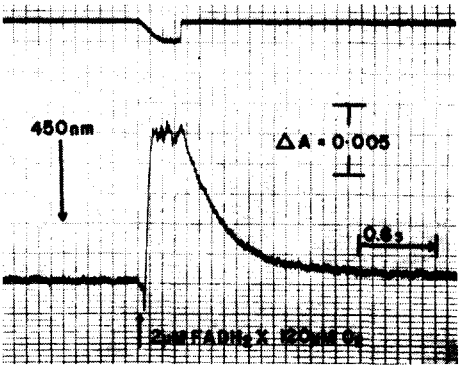

Fig. 7. Time course of reoxidation of the reduced enzyme with O_2 in the absence of salicylate.

The reoxidation of the $FADH_2$ moiety of the enzyme in the absence of salicylate was analyzed by mixing molecular oxygen with the enzyme which had been reduced anaerobically by prior addition of a stoichiometric amount of NADH. As shown in Fig. 7, upon stopping the flow, a downward deflection of the trace represents the reoxidation of $FADH_2$. The rate of oxidation of the $FADH_2$ moiety, k'_{ox}, was computed from the time course of reoxidation by application of Eqn 4. The measurement of k'_{ox} was made in the presence of various concentrations of oxygen. As shown in Fig. 8, the reaction rates thus obtained were plotted as a function of NADH or O_2 concentrations, respectively. Both plots were expressed by straight lines and the intercepts on the ordinate were zero. Apparently, the values of both k'_{red} and k'_{ox} depend on the concentrations of NADH and O_2, respectively. The second-order rate constants of the reaction were calculated from the slope of the line shown in Fig. 8 to

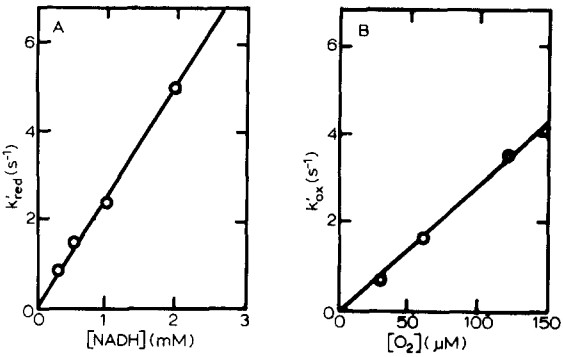

Fig. 8. Flow kinetic data of the enzyme obtained in the absence of salicylate. (A) Variation of k'_{red} as a function of NADH concentration. (B) Variation of k'_{ox} as a function of O_2 concentration. Conditions were the same as in Figs 6 and 7 except that varying concentrations of NADH and O_2 were used.

be $k'_{red} = 2.5 \cdot 10^3\ M^{-1} \cdot s^{-1}$, and $k'_{ox} = 2.9 \cdot 10^4\ M^{-1} \cdot s^{-1}$, showing that the reduction–reoxidation reactions of the enzyme in the absence of salicylate followed second-order kinetics and are most simply interpreted as one-step processes.

Rate of overall reaction

The overall reaction was measured by oxygen uptake at various concentrations of NADH and oxygen in the presence of a large excess of salicylate. On the basis of the Lineweaver–Burk plot for NADH concentrations at various fixed concentrations of oxygen, the maximum values of the rate extrapolated to infinite NADH concentrations were obtained at known concentrations of oxygen. The reciprocal values of the rates thus obtained were replotted against those of oxygen concentrations (Fig. 9A). From the intercept on the ordinate the maximum value of the molecular activity to be obtained at infinite NADH and oxygen concentrations was estimated to be 21 s^{-1} at 25 °C.

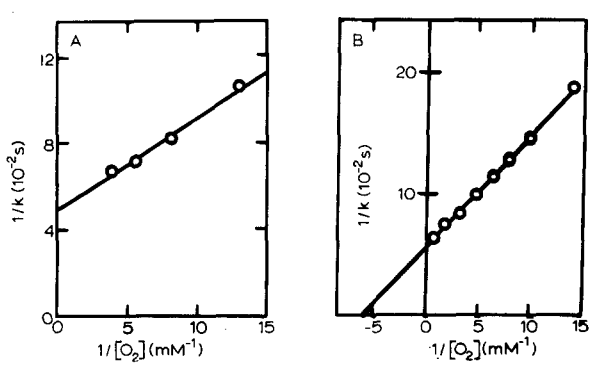

Fig. 9. The plot of $1/k$ against $1/[O_2]$. (A) The value of k stands for the maximum rate obtained by extrapolating to infinite NADH concentration at various fixed concentrations of oxygen. Measured in the presence of 100 μM salicylate. (B) The value of k stands for the maximum rate obtained by extrapolating to infinite salicylate concentration at various fixed concentrations of oxygen. Measured in the presence of 150 μM NADH.

Michaelis constants for NADH and molecular oxygen of the enzyme in the presence and absence of salicylate

NADH at relatively low concentrations was not oxidized to an appreciable extent in the absence of salicylate. When the concentration of NADH was raised, salicylate hydroxylase showed NADH oxidase activity even in the absence of salicylate. However, the rate of enzymic oxidation of NADH in the absence of salicylate was extremely low compared to that in the presence of salicylate[13]. Therefore, it seemed worthwhile to study the K_m values for NADH and molecular oxygen of the enzyme in the presence and absence of salicylate. The kinetic analyses of these mechanisms were carried out polarographically by measuring the rate of oxygen uptake in the presence and absence of salicylate.

In order to estimate the K_m value of the enzyme for oxygen in the presence of salicylate, the rate of oxygen uptake was measured at various concentrations of salicylate and oxygen in the presence of a large excess of NADH. On the basis of the Lineweaver–Burk plot for salicylate concentrations at various fixed concentrations of oxygen, the maximum values of the rate extrapolated to infinite salicylate concentration were obtained. As shown in Fig. 9B, the reciprocal values of the rates thus obtained were replotted against those of oxygen concentrations. From the intercept on the abscissa, the K_m value for molecular oxygen at sufficient concentrations of both NADH and salicylate was estimated to be $1.7 \cdot 10^{-4}$ M. As reported previously[13], the K_m value for NADH in the salicylate hydroxylase reaction is determined to be $2.6 \cdot 10^{-6}$ M. The data shown in Fig. 4 gave Michaelis constants in both the NADH reduction of the enzyme–salicylate complex (Step b in Fig. 12) and the reoxidation of the reduced complex with molecular oxygen (Step c). From the data in Fig. 4A and B the K_m value of the enzyme for NADH was estimated to be $8.2 \cdot 10^{-6}$ M, and that for molecular oxygen $1.1 \cdot 10^{-4}$ M, showing that the values obtained from flow experiments coincide well with those in the overall hydroxylase reaction.

The K_m values of the enzyme for NADH and molecular oxygen in the absence of salicylate were estimated by plotting the reciprocals of the rate of oxygen uptake against those of NADH or oxygen concentrations (Fig. 10A and B). From these data, the K_m value for NADH was estimated to be $3.2 \cdot 10^{-3}$ M and that for molecular oxygen $1.8 \cdot 10^{-3}$ M.

These results indicate that the K_m value of the enzyme for NADH is approximately 1200 times greater in the absence of salicylate than in its presence. However, more precise studies are required to determine whether the effect of salicylate on the Michaelis constant for NADH of the enzyme is due to a change in the dissociation constant of NADH toward the enzyme.

Reduction and oxidation kinetics of the enzyme–benzoate complex

NADH oxidase activity of the enzyme in the absence of salicylate was almost negligible under the assay conditions. However, a substrate analogue with a free carboxyl group such as benzoate, in high concentration, was effective in the stimulation of the enzyme-catalyzed NADH oxidation[14–16]. Under these conditions benzoate remained unchanged, while a stoichiometric amount of H_2O_2 to NADH oxidation was concomitantly generated. Addition of a large excess amount of benzoate to salicylate hydroxylase was accompanied by spectral shifts similar to those observed on binding of salicylate. In order to analyze the reduction and reoxidation of the FAD

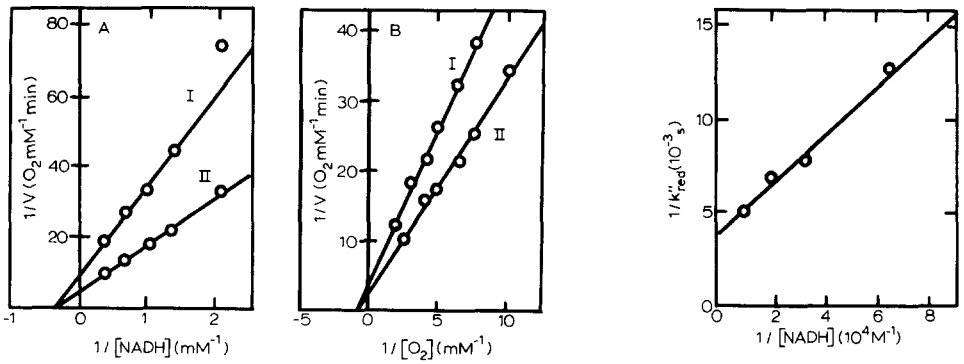

Fig. 10. Determination of the K_m values of the enzyme in the absence of salicylate for NADH (A) and for O_2 (B). (A) Measured at different enzyme concentrations: 330 nM (Line I); 650 nM (Line II). Initial O_2 concentration: 260 μM. (B) Measured at different NADH concentrations: 6.0 mM (Line I); 6.5 mM (Line II). Enzyme concentration: 330 nM.

Fig. 11. The plot of $1/k''_{red}$ against 1/[NADH] in the enzyme–benzoate complex. The enzyme (2 μM) in the presence of 10 mM benzoate was aerobically mixed with varying concentrations of NADH.

moiety of the enzyme–benzoate complex, kinetic experiments were carried out by the use of a flow technique.

When NADH was aerobically mixed with the enzyme in the presence of an excess of benzoate, the time course of the reduction of the FAD moiety was very similar to that described in the kinetics of NADH reduction of the enzyme–salicylate complex. The rate of reduction, k''_{red}, was calculated from the amount of the reduced enzyme formed during the time of flow. A double reciprocal plot of k''_{red} values thus obtained against NADH concentration was linear; the intercept on the ordinate was not zero (Fig. 11). From the intercept, the maximum rate of the reduction of the FAD moiety, k''^{max}_{red}, was estimated to be 260 s^{-1}. These results coincide well with those of the enzyme–salicylate complex.

The reoxidation of the reduced enzyme–benzoate complex with oxygen was analyzed by mixing molecular oxygen with the enzyme–benzoate complex, which had been reduced by prior addition of a sufficient amount of NADH. From the kinetic curve by application of the formula of Chance[17], the rate of oxidation of the $FADH_2$ moiety, k''_{ox}, was estimated to be $4.5 \cdot 10^4$ $M^{-1} \cdot s^{-1}$. The reoxidation of the enzyme-bound $FADH_2$ was found to follow simple second-order kinetics.

DISCUSSION

Since salicylate hydroxylase interacts with three kinds of substrates, *i.e.* salicylate, NADH and molecular oxygen, there are *a priori* a total of six possible sequences by which hydroxylation can be accomplished through supposed intermediates. The hypothetical reaction mechanism is illustrated in Fig. 12, in which intermediate complexes conceivably produced during the reactions are included.

When the reduction rate of the flavin moiety of the enzyme was compared with that of the enzyme–salicylate complex, the respective values for the free enzyme and the complex were 0.08 and 180 s^{-1} with NADH at 30 μM, the standard concentration

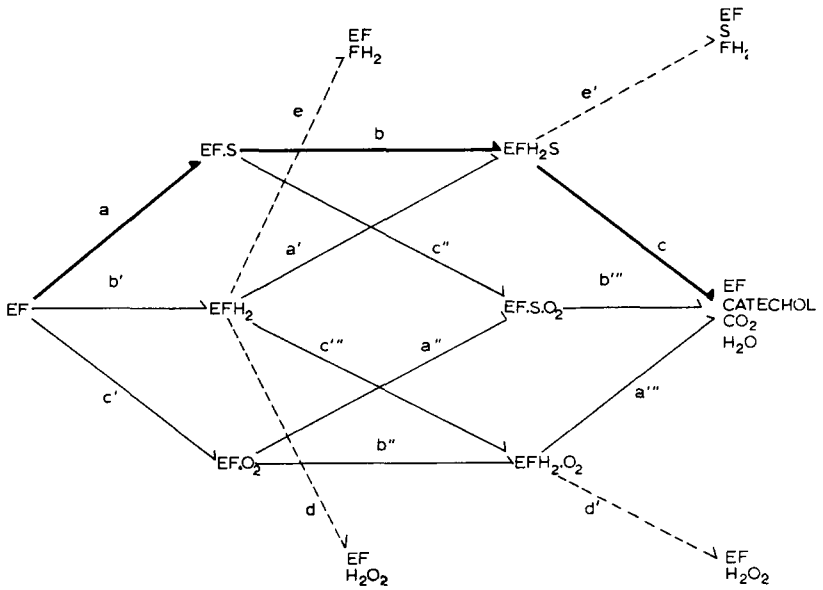

Fig. 12. Representation of possible intermediates during salicylate hydroxylase reaction. EF and S denote salicylate hydroxylase and salicylate, respectively. EF·S and EFH_2·S denote the oxidized and reduced enzyme–salicylate complexes, respectively. Other complexes are also defined in the similar way. Steps d, d′ and e, e′ represent a side reaction, either "oxidase reaction" or "diaphorase reaction" in the presence of externally added FAD.

in the enzyme assay system. Thus the rate of reduction of the flavin moiety is more than 2000 times greater in the presence of salicylate than in its absence. The rates of salicylate binding by the enzyme in the oxidized and reduced forms, respectively, were estimated to be 540 and 0.29 s^{-1} when the concentration of salicylate was 30 μM. Since the molecular activity for salicylate hydroxylase reaction is 21 s^{-1}, the results described here lead to the conclusion that the oxidation process of the reduced enzyme–salicylate complex by molecular oxygen (Step c) with $k_{ox} = 21\ s^{-1}$ is the rate-limiting step in the overall reaction and the hydroxylation reaction does not normally proceed *via* Steps b′ (0.08 s^{-1}) and a′ (0.29 s^{-1}). The other alternatives are sequences involving Steps c′, c″ or c‴. The rates of binding of salicylate to the enzyme and the reduction of flavin moiety of the enzyme–salicylate complex were essentially the same as those either in the presence of oxygen or in its absence (M. Nakamura, unpublished results). These observations exclude the necessity of involvement of these steps in the hydroxylation reaction. The experiments presented in this paper illustrate that the reaction sequence *via* Steps a, b and c should be predominant in the overall catalytic process of the salicylate hydroxylase reaction. These kinetic results are in good agreement with the previously proposed hypothetical reaction mechanism based on the results of spectroscopic investigations and the stoichiometric analyses demonstrated by using substrate level amounts of the enzyme[6,7]. In this proposed mechanism, it should be noted that the coupling of salicylate to the enzyme induces the reactivity of the enzyme toward NADH. Salicylate might serve to put the enzyme into a conformation that facilitates the increase. Recent data strongly suggest that the coupling of salicylate to the enzyme induces an allosteric change in the protein

conformation; the circular dichroism spectrum of salicylate hydroxylase differed somewhat from that of the enzyme–substrate complex (K. Suzuki, unpublished work); the visible absorption and fluorescent spectra of the enzyme–substrate complex were different from those of the free enzyme[7,9]; and the stability of the enzyme was remarkably enhanced by the complex formation[7].

White-Stevens and Kamin[14,15] reported that, at high concentrations, benzoate stimulated NADH oxidase activity of a salicylate hydroxylase. Similar effects of substrate analogues with a free carboxyl group were also observed in our salicylate hydroxylase[16]. These analogues were not hydroxylated during the reoxidation of the enzyme by oxygen, but enhanced the reactivity of NADH towards the enzyme. As described in this paper, the rate of reduction of the FAD moiety by NADH was also remarkably increased by the presence of benzoate: the extent of the rate of the NADH reduction of the FAD moiety was approximately the same as that of the enzyme–salicylate complex. Also, the enzyme–benzoate complex was detectable by a change of the absorption spectrum in the presence of the excess amounts of benzoate. These phenomena, however, were not initiated by substances such as methylsalicylate and phenol which do not have an intramolecular free carboxyl group, suggesting that the free carboxyl group of the substrate molecule is a functional group which may initiate the activation of the enzyme.

Similar substrate activation phenomena have recently been observed with other flavo-oxygenases such as *p*-hydroxybenzoate hydroxylase[18,19] and 3-hydroxypyridine ring oxygenase[20]. In these enzymes, the reduction of the flavin moiety by reduced pyridine nucleotide was also remarkably accelerated by the addition of the specific substrate. A situation comparable to that described here for flavo-oxygenases exists for a "hemo-oxygenase", P-450$_{cam}$, which functions as the camphor hydroxylase[21,22]. In this system the coupling of camphor to the cytochrome P-450 is the first step in the hydroxylase reaction. The kinetic analyses of these reactions have been discussed by Gunsalus *et al.*[23].

In summary, since the enzyme–substrate complex is a much more efficient system than the free enzyme for accepting electrons from an electron donor such as reduced pyridine nucleotides or reduced non-heme iron proteins, a substrate activation mechanism of the oxygenase may be common to many flavo- and hemo-oxygenases. The enzyme–substrate complex might also have a regulatory function in the metabolic pathway oxidizing reduced pyridine nucleotides.

ACKNOWLEDGMENTS

This investigation was supported in part by research grants from the Ministry of Education of Japan. We are grateful to Prof. Y. Tonomura, Osaka University for valuable discussion.

REFERENCES

1 S. Takemori, M. Nakamura, M. Katagiri and T. Nakamura, in H. Kamin, *Flavins and Flavoproteins (Proc. 3rd Int. Symp. Flavins and Flavoproteins), Durham, 1969*, University Park Press, Baltimore, 1971, p. 463.
2 S. Takemori, M. Nakamura, K. Suzuki, M. Katagiri and T. Nakamura, *FEBS Lett.*, 6 (1970) 305.

3 T. Nakazawa, S. Yamamoto, Y. Maki, H. Takeda, Y. Kajita, M. Nozaki and O. Hayaishi, in K. Yagi, *Flavins and Flavoproteins*, University of Tokyo Press, Tokyo, 1968, p. 214.
4 M. Katagiri and S. Takemori, in H. Kamin, *Flavins and Flavoproteins*, University Park Press, Baltimore, 1971, p. 447.
5 S. Nakamura, Y. Ogura, K. Yano, N. Higashi and K. Arima, in H. Kamin, *Flavins and Flavoproteins*, University Park Press, Baltimore, 1971, p. 475.
6 M. Katagiri, S. Takemori, K. Suzuki and H. Yasuda, *J. Biol. Chem.*, 241 (1966) 5675.
7 S. Takemori, H. Yasuda, K. Mihara, K. Suzuki and M. Katagiri, *Biochim. Biophys. Acta*, 191 (1969) 58.
8 S. Takemori, H. Yasuda, K. Mihara, K. Suzuki and M. Katagiri, *Biochim. Biophys. Acta*, 191 (1969) 69.
9 K. Suzuki, S. Takemori and M. Katagiri, *Biochim. Biophys. Acta*, 191 (1969) 77.
10 T. Nakamura, *Seikagaku*, 39 (1967) 855.
11 B. Chance and V. Legallais, *Rev. Sci. Instrum.*, 22 (1951) 6727.
12 G. A. Millikan, *Proc. R. Soc. London Ser. A*, 155 (1936) 277.
13 M. Katagiri, H. Maeno, S. Yamamoto, O. Hayaishi, T. Kitao and S. Oae, *J. Biol. Chem.*, 240 (1965) 3414.
14 R. H. White-Stevens and H. Kamin, *Biochem. Biophys. Res. Commun.*, 38 (1970) 882.
15 R. H. White-Stevens, H. Kamin and Q. H. Gibson, preprint presented at the *Wenner-Gen Symp. Structure and Function of Oxidation–Reduction Enzymes, Stockholm, 1970.*
16 K. Suzuki and M. Katagiri, *Seikagaku*, 42 (1970) 563.
17 B. Chance, *J. Biol. Chem.*, 151 (1943) 553.
18 S. Nakamura, Y. Ogura, K. Yano, N. Higashi and K. Arima, *Biochemistry*, 9 (1970) 3235.
19 L. G. Howell and V. Massey, in H. Kamin, *Flavins and Flavoproteins*, University Park Press, Baltimore, 1971, p. 499.
20 L. G. Sparrow, P. P. K. Ho, T. K. Sundaram, D. Zach, E. J. Nyns and E. E. Snell, *J. Biol. Chem.*, 244 (1969) 2590.
21 M. Katagiri, B. N. Ganguli and I. C. Gunsalus, *J. Biol. Chem.*, 243 (1968) 3543.
22 M. Katagiri, S. Takemori, M. Nakamura and T. Nakamura, *Proc. 2nd Int. Symp. Oxidases and Related Redox Systems, Memphis, 1971*, University Park Press, Baltimore, 1972, in the press.
23 I. C. Gunsalus, C. A. Tyson and J. D. Lipscomb, *Proc. 2nd Int. Symp. Oxidases and Related Redox Systems, Memphis, 1971*, University Park Press, Baltimore, 1972, in the press.