THE KINETICS OF SALICYLATE HYDROXYLASE REACTION*

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Received 16 December 1969

1. Introduction

Salicylate hydroxylase from *Pseudomonas putida* is an FAD-containing flavoprotein which catalyzes the conversion of salicylate to catechol with stoichiometric consumption of molecular oxygen and NADH [1–3]. Recently, it was shown that the primary reaction in the sequence leading to hydroxylation of substrate is the formation of a substrate-enzyme complex in which the ratio of apoenzyme, FAD and salicylate is 1:1:1. This complex then reacts with NADH and oxygen to give the hydroxylated product [4–8]. In the absence of substrate, the enzyme was also reduced by NADH. When the reduced enzyme reacted with salicylate and oxygen, stoichiometric formation of catechol was observed. Based on these results, the hydroxylation reaction has been depicted as in Scheme 1.

As discussed previously [4–8], the reaction sequence *a*, *b* and *c* was postulated as the overall catalytic process of salicylate hydroxylation. In the present study, the rate constant of each stage of salicylate hydroxylation has been determined by use of a flow technique. The results described fully confirm our earlier conclusions about the hydroxylation reaction.

* A part of the results was presented at Third International Symposium on Flavins and Flavoproteins held at Durham, U.S.A., October 1969.

2. Materials and methods

Salicylate hydroxylase from *Pseudomonas putida* was prepared as previously described [6]. Flow experiments were performed using a temperature-controlled flow system [9] which was essentially the same as that designed by Chance and Legallais [10]. The semitangential 4-jet mixing chamber used in the flow system was a modification of that used by Millikan [11].

3. Results and discussion

If it is assumed that the reaction between salicylate hydroxylase E and salicylate S is a simple bimolecular reaction, it can be given by the expression

$$E + S \underset{k_s^{-1}}{\overset{k_s^{+1}}{\rightleftharpoons}} S \qquad (1)$$

Thus the net rate of formation of the enzyme-substrate complex ES will be

$$\frac{d[ES]}{dt} = k_s^{+1}[E][S] - k_s^{-1}[ES] \qquad (2)$$

When the initial salicylate concentration $[S]_0$ is much larger than that of total enzyme, the salicylate concentration during the reaction will stay constant. Thus intergrating, we get

$$2.3 \log \frac{[ES]_{eq}}{[ES]_{eq}-[ES]_t} = (k_s^{+1}\,[S]_0 + k_s^{-1})\,t \qquad (3)$$

where $[ES]_{eq}$ and $[ES]_t$ represent the concentrations of the ES complex at an equilibrium and at reaction time t, respectively. According to eq. 3, a plot of

$$2.3 \log \frac{[ES]_{eq}}{[ES]_{eq}-[ES]_t}$$

against t is straight line. We can estimate the values of k_s^{+1} and k_s^{-1} from values of both the slope of this line and the dissociation constant, $K_s = k_s^{-1}/k_s^{+1}$ of the ES complex. In order to estimate the rate constant in reaction *a*, a time course curve of the salicylate with the enzyme was analyzed by following the increase in absorbance at 480 nm, since the formation of the salicylate-enzyme complex was characterized by an absorbance increase at 480 nm [4–6]. A typical result of the change in absorbance at 480 nm on mixing the enzyme and salicylate is indicated in fig. 1-A; the time between mixing and observation was 1.5 msec at maximum flow velocity. Combining the value of the slope obtained in eqn. 3 and the dissociation constant of 3.5 μM [6], the k_s^{+1} value of reaction *a* was calculated to be 1.8×10^7 sec^{-1}, and k_s^{-1}, 62 sec^{-1}.

The reduction of the enzyme-salicylate complex by NADH (reaction *b*) was analyzed as shown in fig. 1-B; NADH was aerobically mixed with the enzyme in the presence of a sufficient amount of salicylate. As indicated by an upward deflection of the trace, rapid reduction occurred during continuous flow. At the moment the flow was stopped, the amplitude of this change corresponded to full enzyme reduction and a subsequent slower reaction completed the oxidation. The rate of reduction k_{red} was calculated from the amount of the reduced enzyme formed during the time of flow. When reciprocals of k_{red} were plotted against those of NADH concentration, a linear relationship existed and the ordinate intercept was not zero (fig. 2). The value of k_{red}, when extrapolated to infinite NADH concentration was 200 sec^{-1}. A mechanism accounting for the experimental data is as follows:

$$\text{E.FAD.SA} + \text{NADH} + \text{H}^+ \longrightarrow \text{X}$$
$$\text{X} \xrightarrow{200\ sec^{-1}} \text{E.FADH}_2\text{.SA} + \text{NAD}^+.$$

In this formula, the reaction process involves intermediate X and the reaction via X is expected to be the rate limiting step following first order kinetics.

Oxidation of the reduced enzyme-salicylate (reaction *c*) can also be examined by using NADH as an electron donor. The values of the apparent rate constant for enzyme oxidation were calculated from the kinetic curve in fig. 1-C according to a formula proposed by Chance [12]

$$k_{ox} = [NADH]_0/P_m \cdot t_{1/2\ off}$$

where P_m is maximum concentration of the reduced form of the FAD moiety and $t_{1/2\ off}$ is the time interval from maximal formation of the reduced form until its concentration has fallen to half maximal value. $[NADH]_0$ represents the initial concentration of NADH. The k_{ox} values were essentially constant (22 sec^{-1}) at various O_2 concentrations (table 1). These results suggest that oxidation of the reduced enzyme-salicylate complex also involves the reaction intermediate and the reaction after the intermediate is the rate limiting step following first order kinetics with a rate constant of 22 sec^{-1}. This interpretation is the same as that described in reaction *b*. Although we assume a reaction intermediate X in reactions *b* and *c*, no spectrophotometrically detectable intermediate was observed.

Reduction of the FAD moeity of the enzyme by NADH in the absence of salicylate (reaction *b*) was analyzed by mixing a small amount of oxygen with the enzyme reduced by prior addition of a sufficient amount of NADH**. As shown in fig. 1-D, the enzyme was at first oxidized by O_2 and then returned to the reduced state when the oxygen was used up. The rate k'_{red} was estimated from the kinetic curve by using the Chance's formula. The k'_{red} value increased proportionally with NADH concentration and the second order rate constant of the reaction was 2.5×10^3 M^{-1} sec^{-1} (table 2).

When kinetics of reduction of the FAD moeity of both the enzyme and the enzyme-salicylate complex are compared, the k_{red} values of the enzyme and the complex are 0.25 and 190 sec^{-1}, respectively, with 100 μM NADH as electron donor. Thus the rate of

** It was technically difficult to estimate k'_{red} under anaerobic conditions, because significant denaturation of the enzyme occurred by N_2-bubbling treatment necessary for anaerobiosis.

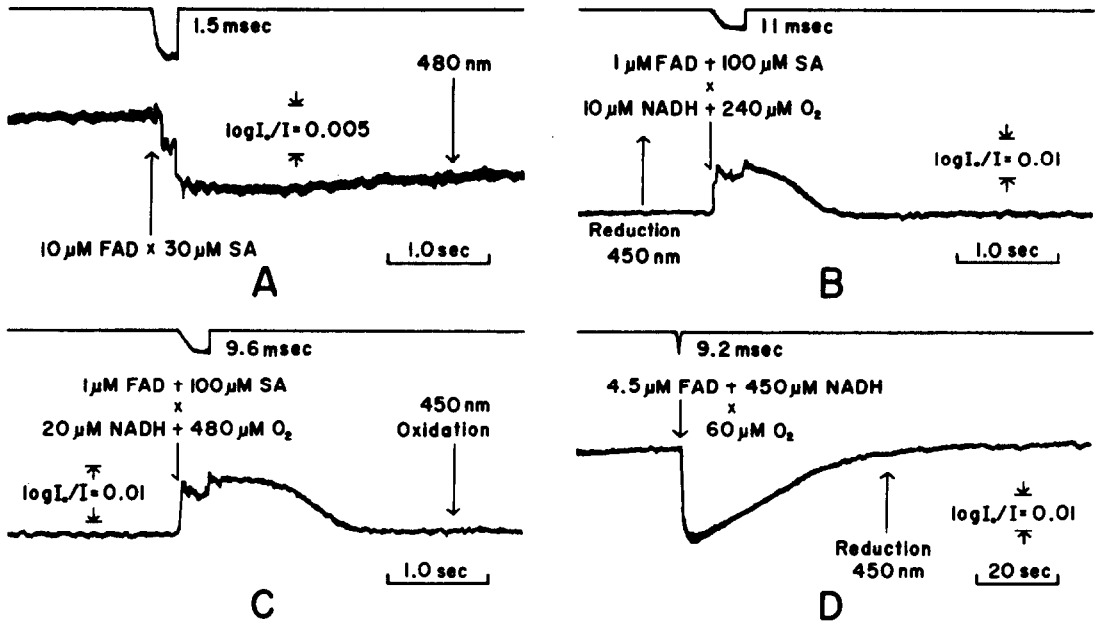


Fig. 1. The flow kinetic measurements of salicylate hydroxylase reaction. The top trace represents the flow velocity. The arrow in the left portion indicates the time of mixing. The concentrations indicated in each diagram are those after mixing. All measurements were performed at 25°C in 33 mM potassium phosphate buffer, pH 7.0.
(A) Time course of change in absorbance at 480 nm during reaction between salicylate hydroxylase and salicylate. The downward deflection of the trace shows the appearance of absorbance at 480 nm.
(B and C) Time course of reduction and reoxidation of the enzyme-salicylate complex. The upward- and downward deflections of trace represent the disappearance and appearance of absorbance at 450 nm, respectively. In curve C, the values of P_m and $t_{1/2}$ off were 1 μM and 1.07 sec, respectively, and $k_{ox} = 19$ sec^{-1}.
(D) The time course of oxidation and reduction of the enzyme in the absence of salicylate. The values of P_m and $t_{1/2}$ off were 2.1 μM and 24 sec, respectively, and $k'_{red} = 2.7 \times 10^3$ M^{-1} sec^{-1}.

Table 1
The values of k_{ox} of the enzyme obtained in the presence of salilylate at different oxygen concentrations.

Concentration of oxygen (μM)	k_{ox}(sec^{-1})*
240	22
480	21
720	22

* Mean values estimated from a series of experiments.

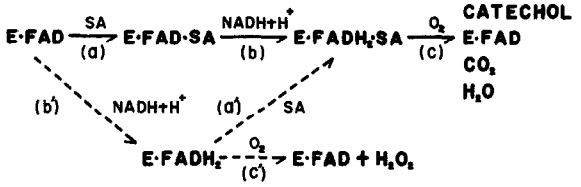
Table 2
The values of k'_{red} of the enzyme obtained in the absence of salicylate at different NADH concentrations.

[NADH] (μM)	k'_{red}(sec^{-1})	k'_{red}/[NADH] (10^{3}M^{-1}sec^{-1})
200	0.40	2.0
300	0.61	2.0
450	1.19	2.7
500	1.26	2.5
800	2.13	2.7
1200	3.59	3.0
	Mean value	2.5

the flavin reduction step is about 800 times larger in the presence of salicylate than in its absence. The rate of reduction of the flavin moiety increases considerably in the presence of salicylate. Since the molecular activity for the salicylate hydroxylase reaction is 17 sec^{-1} [13], the results described here lead to the conclusion that reaction *c* ($k_{ox} = 22$ sec^{-1}) is the rate limiting step in the overall reaction. The alternative

Table 3
The values of the rate constant of enzyme oxidation obtained in the absence of salicylate at different oxygen concentrations.

Concentration of oxygen (μM)	Rate constant (10^4 $M^{-1}sec^{-1}$)
24	2.9
60	2.9
120	3.0



Scheme 1. Proposed mechanism of salicylate hydroxylase reaction. E and SA denote protein moiety of the enzyme and salicylate, respectively.

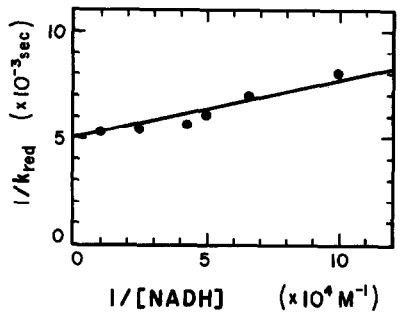


Fig. 2. Variation of rate constants for reduction of the flavin moiety of salicylate hydroxylase as a function of NADH concentration. Conditions were tha same as in fig. 1-B except that varying concentrations of NADH were used.

sequence b' and a' is not essential in the hydroxylation reaction.

Oxidation of the $FADH_2$ moiety of the enzyme in the absence of salicylate (reaction c') was analyzed from reoxidation of NADH-reduced enzyme by O_2 (not shown in the figure). The second order rate constant was 2.9×10^4 $M^{-1}sec^{-1}$ and was independent of initial oxygen concentrations (table 3). Thus reactions b' and c' may be interpreted as a one-step reaction.

Although it has been postulated that the reduced enzyme-salicylate complex ($E.FADH_2.SA$) reacts directly with O_2 [4–7], the process may be more complex. More detailed evidence is required to evaluate the real intermediate in the reaction with molecular oxygen. This problem may be a key to the solution of the mechanism of the oxygenase reaction.

Acknowledgements

We are grateful to Prof. Y.Tonomura, Osaka University for valuable discussion.

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