Intermediate and mechanism of hydroxylation of o-iodophenol by salicylate hydroxylase

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Intermediate and Mechanism of Hydroxylation of *o*-Iodophenol by Salicylate Hydroxylase[1]

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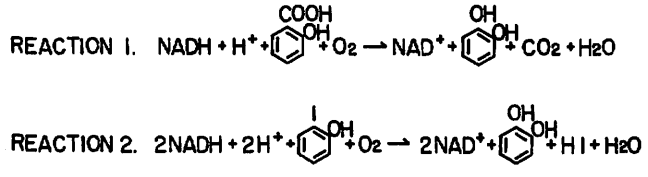
Salicylate hydroxylase [EC 1.14.13.1] from *Pseudomonas putida* catalyzes the hydroxylation of salicylate, and also *o*-aminophenol, *o*-nitrophenol, and *o*-halogenophenols, to catechol. The reactions with these *o*-substituted phenols comprise oxygenative deamination, denitration, and dehalogenation, respectively. The reaction stoichiometry, as to NADH oxidized, oxygen consumed, and catechol formed, is 2 : 1 : 1, respectively. The mechanisms for the deiodination and oxygenation of *o*-iodophenol were investigated in detail by the use of I^+-trapping reagents such as DL-methionine, 2-chlorodimedone, and L-tyrosine. The addition of the traps did not change the molar ratio of catechol formed to NADH oxidized, nor iodinated traps produced were in the incubation mixture. The results suggest that I^+ was not produced on the deiodination in the hydroxylation of *o*-iodophenol. On the other hand, L-ascorbate, L-epinephrine, and phenylhydrazine increased the molar ratio. *o*-Phenylenediamine decreased it, being converted to phenazine. This suggests that *o*-benzoquinone is formed in the oxidation of *o*-iodophenol as a nascent product. The quinone was detected spectrophotometrically by means of the stopped-flow method. Kinetic analysis of the reactions revealed that *o*-benzoquinone is reduced nonenzymatically to catechol by a second molecule of NADH. A mechanism of elimination for the ortho-substituted groups of substrate phenols by the enzyme is proposed and discussed.

Salicylate hydroxylase [salicylate, NADH, oxygen, oxidoreductase (1-hydroxylating, decarboxylating) EC 1.14.13.1] from *Pseudomonas putida*, S-1, catalyzes the decarboxylative hydroxylation of salicylate to catechol and CO_2, with a 1 : 1 : 1 stoichiometry, as to NADH oxidized, oxygen consumed, and catechol formed, respectively (Scheme 1, Reaction 1) (*1-3*). As shown in Reaction 2 (hydroxylation of *o*-iodophenol) in the scheme, the enzyme also oxidizes substrate analogs in which the carboxyl group is substituted with a nitro, amino, or halogeno group at the ortho position, with the consumption of 2 mol NADH per mol substrate (*4*). These reactions were proved to be of a monooxygenase type by that the incorporation of an oxygen atom into the products was demonstrated in ^{18}O-experiments (*4*). The first NADH molecule reduces the flavin of the enzyme·substrate complex, but the role of the second one is unknown. There are two possibilities as to the latter. In the hydroxylation of *o*-iodophenol, either the I^+-*ion or o*-benzoquinone, which may be formed as a labile and transient species, seems to be the oxidant for NADH. Husain *et al.* demonstrated the stoichiometry of 2 : 1 :1, as to NADPH, O_2, and product, respectively, on the hydroxylation of fluoro-derivatives of *p*-hydroxybenzoate catalyzed by *p*-hydroxybenzoate hydroxylase and the formation of *o*-benzoquinone as the reaction intermediate (*5*).



Scheme 1. **Reactions of salicylate hydroxylase.**

To elucidate the reaction mechanism for *o*-iodophenol hydroxylation by the salicylate hydroxylase, at first we examined the use of trapping reagents to chemically detect the I^+-ion, which may be released during the hydroxylation of *o*-iodophenol. Secondly, kinetic and spectrophotometric studies were performed on the formation of intermediates in the reaction, and it was revealed that *o*-benzoquinone reacts non-enzymatically with a second NADH molecule. The mechanisms for hydroxylation of *o*-halogenophenol and *o*-nitrophenol are compared to that for salicylate, and the differences in the elimination reactions for substituted groups are discussed.

MATERIALS AND METHODS

Materials—ICl was purchased from Wako Pure Chemicals and dimedone from Nacalai Tesque. Salicylate hydroxylase was purified from *P. putida*, S-1 (*1-3*). Other reagents and enzymes were obtained as described in the previous paper (*4*). Potassium phosphate buffer is abbreviated to "phosphate buffer," and the pH and concentration are shown in the legends to the figures and tables. *o*-Benzoquinone was synthesized from catechol (*6*) and stored in chloroform at 0°C. It was extracted from the

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chloroform solution with 10 mM sulfuric acid before use. Its concentration was determined spectrophotometrically in chloroform using the molar absorption coefficient of 2.23×10^3 $M^{-1}\cdot cm^{-1}$ at 375 nm (*7*). Its concentration was also determined by means of the metapyrocatechase reaction. *o*-Benzoquinone in 30 mM phosphate buffer (pH 7.0) was reduced with excess NADH and then the catechol produced was determined as to the oxidation with metapyrocatechase. The synthesized benzoquinone was used without further purification, because it is unstable in an aqueous solution. Experiments were carried out as soon as possible after the addition of *o*-benzoquinone to the assay system to avoid its decomposition. The decay constants determined from the decrease in its absorption at 388 nm were 3.5×10^{-4} and 4.5×10^{-4} s^{-1} in 100 mM phosphate buffer (pH 7.0) at 5 and 17°C, respectively.

2-Chlorodimedone was synthesized from dimedone (*8*) and recrystallized from an ethanol solution. It had a melting point of 160°C, as compared to m.p. 161°C in the literature. Its concentration was determined at pH 2.75 using the molecular absorption coefficient of 1.22×10^4 $M^{-1}\cdot cm^{-1}$ at 292 nm (*8*).

Iodine monochloride (ICl) was used soon after removal of I_3Cl by agitating an aqueous solution with soluble starch and its concentration was determined by titration with NADH.

Stopped-Flow Experiments—Stopped-flow experiments were carried out with a Union Giken RA-1100 stopped-flow spectrophotometer with a light path of 2 mm, with operation in either the absorbance or fluorescence mode, according to the procedures described previously (*9, 10*). The signals from the logarithmic photometer were processed with a Union Giken RA-108S digital memory, and recorded with a synchroscope and an X-Y recorder. The experiments were carried out at 5°C and the sample compartment of the instrument was purged with nitrogen gas to avoid fogging. The light emission due to NADH was filtered out with a cut filter (V-Y 42).

Spectral experiments under anaerobic conditions were carried out in Thunberg type cuvettes as described previously (*4*).

Preparation of the Reduced Enzyme-o-Iodophenol Complex—In a Thunberg type cuvette, 50 μM salicylate hydroxylase and 1.5 mM *o*-iodophenol in 30 mM phosphate buffer (pH 7.0) were in the main compartment and 50 μM NADH (stoichiometric to the enzyme) was in the side arm. The concentrations of the enzyme, substrates and NADH were varied, as indicated in legends of the tables and figures. The gas phase was repeatedly evacuated and N_2 introduced. The enzyme-*o*-iodophenol complex was reduced by mixing the solutions at room temperature and formation of the reduced complex was confirmed by a spectral method.

Enzyme Assay and Other Determinations—Salicylate hydroxylase activity was determined by the method described (*4*). Determination of catechol was performed with the use of metapyrocatechase (*4*). Phenazine was detected on a thin layer chromatography sheet, Eastman Chromagram Sheet No. 6060 (Eastman Kodak), with the following solvent systems (v/v/v) : benzene/dioxane/acetic acid, 90 : 25 : 4; benzene/ethanol/acetic acid, 48 : 8 : 4; and benzene/ethanol, 95 : 4. Monoiodotyrosine was analyzed by thin layer chromatography on a cellulose plate (*11*) and determined by the spectrophotometric method (*12*). Adrenochrome was determined using the molar absorption coefficient of 4.02×10^3 $M^{-1}\cdot cm^{-1}$ at 480 nm (*13*). Protein was determined by the biuret method (*14*), with bovine serum albumin (Fraction V, Wako) as the standard.

RESULTS

Deiodination of o-Iodophenol—To disclose the mechanism for deiodination of *o*-iodophenol by salicylate hydroxylase, an attempt was made to detect the I^+-ion as the nascent product of the reaction. The reactive and labile I^+-ion oxidizes the reduced enzyme, NADH, and other reagents, acting as a cation trapper (Table I). 2-Chlorodimedone, DL-methionine, and L-tyrosine were converted by ICl to 2-chloro-2-iododimedone, methionine sulfoxide, and monoiodotyrosine, respectively, with large rate constants, in 30 mM phosphate buffer (pH 7.0) at 5°C. These values are so large that they can be expected to trap the I^+-ion during the hydroxylation of *o*-iodophenol by the enzyme. These trapping reagents did not react with the oxidized or reduced enzyme. When they were added to the routine assay system for *o*-iodophenol oxidation by salicylate hydroxylase, the molar ratio of catechol produced and NADH oxidized was not affected. Furthermore, iodinated

TABLE I. **Rate constants of the reactions of ICl with several reagents.** ICl and the reagents were dissolved in 30 mM phosphate buffer (pH 7.0), and the reactions were performed at 5°C in a stopped-flow spectrophotometer. In Exp. A, B, and C, each reagent at the indicated concentration and ICl at various concentrations between 20 and 400 μM were used. In Exp. D and E, 50 μM ICl and various concentrations (100-1,000 μM) of each reagent were used. In Exp. F, G, and H, 100 μM ICl was reacted with various concentrations (100 to 1,000 μM) of each reagent. EFH_2 denotes the NADH-reduced enzyme.

Exp.	Reagent	k, $M^{-1}\cdot s^{-1}$
A.	EFH_2 (10 μM)	1.7×10^5
B.	EFH_2 (10 μM)+iodophenol (1 mM)	4.5×10^5
C.	Chlorodimedone (20 μM)	$>8\times10^7$
D.	Chlorodimedone	$>8\times10^7$
E.	DL-Methionine	3.0×10^4
F.	L-Tyrosine	1.4×10^3
G.	NADH	$>10^7$
H.	L-Ascorbate	$>10^7$
I.	*o*-Iodophenol	2.2×10^3

TABLE II. **Effects of I^+-traps on reoxidation of the reduced salicylate hydroxylase·*o*-iodophenol complex by oxygen.** The reaction mixture comprised 50 μM salicylate hydroxylase and 1.5 mM *o*-iodophenol in 30 mM phosphate buffer (pH 7.0), with the indicated concentrations of DL-methionine or chlorodimedone in a Thunberg-type cuvette. The gas phase comprised N_2. After reduction with 50 μM NADH, the enzyme was reoxidized by air-bubbling and then the catechol formed was determined. The data represent means±SD for five and three determinations in the cases of DL-methionine and chlorodimedone, respectively.

I^+-traps	Concentration (mM)	Catechol formed/EFH_2S oxidized (%)
DL-Methionine	0	39.0±2.9
	1.5	38.4±2.0
	3.0	36.8±2.5
2-Chlorodimedone	0	37.9±2.0
	1.0	35.1±1.0
	1.5	29.9±2.1

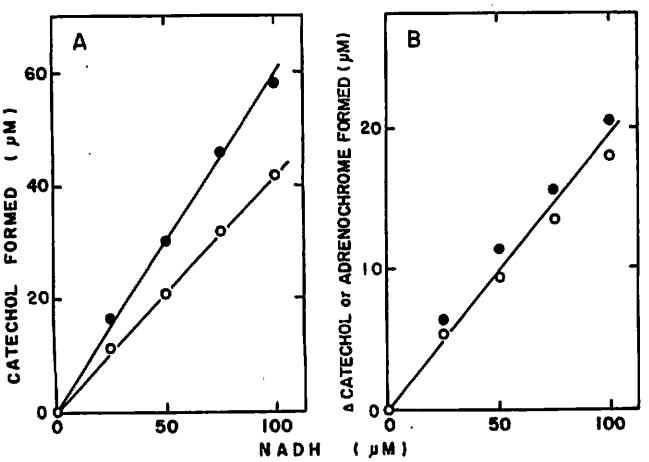


Fig. 1. **Effect of L-epinephrine on the formation of catechol through the hydroxylation of *o*-iodophenol by salicylate hydroxylase.** (A) The reaction mixture comprised 7 μM FAD, 1.5 mM *o*-iodophenol and 5.1 μM enzyme in 1 ml of 30 mM phosphate buffer (pH 8.0), with (●) or without (○) 0.52 mM L-epinephrine. The reactions were started by the addition of various amounts of NADH at room temperature and at the end of the oxidation, the amounts of catechol formed were determined by the metapyrocatechase method. (B) Relationship of adrenochrome production and the increase in catechol formation. During the incubation with L-epinephrine in (A), the increase in the absorbance at 480 nm was monitored for the formation of adrenochrome. The amounts of adrenochrome formed (●) were plotted against the differences in catechol formation between in the presence and absence of L-epinephrine (○) in (A).

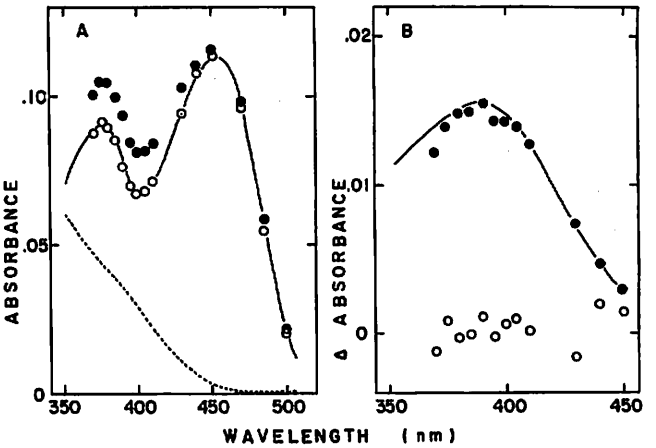


Fig. 2. **Absorption spectra of the intermediates formed during oxidation of the reduced salicylate hydroxylase·*o*-iodophenol complex by oxygen.** The experiments were carried out with using a stopped-flow spectrophotometer at 5°C, as described under "MATERIALS AND METHODS." An anaerobic solution containing 20 μM reduced salicylate hydroxylase and 1 mM *o*-iodophenol in 30 mM phosphate buffer (pH 7.0) was mixed with the same buffer containing 1 mM *o*-iodophenol and 1.9 mM oxygen in the instrument, and then the changes in absorbance at wavelengths from 370 to 500 nm were monitored. (A) Spectral species observed at 5 s after the mixing (●) and that in the presence of 2 mM ascorbate (○). In the figure, the spectra of 10 μM salicylate hydroxylase complexed with *o*-iodophenol (——) and the reduced form (- - -), obtained separately, are also presented. (B) The difference spectrum (●) between the spectrum of the 5 s species in (A) and that of the oxidized enzyme·*o*-iodophenol complex, and the difference spectrum (○) between the spectrum of the 5 s species with ascorbate in (A) and that of the oxidized enzyme·substrate complex. The spectrum in the case of 6.5 μM *o*-benzoquinone (——) is also shown.

TABLE III. **Effects of *o*-benzoquinone-trapping reagents on the catechol formation in (A) the routine assay system and (B) the oxidation system for the reduced enzyme·*o*-iodophenol complex.** (A) The reaction mixture comprised 1.6 μM salicylate hydroxylase, 7 μM FAD, 1 mM *o*-iodophenol, and the reagent, at the indicated concentrations, in 1.0 ml of 30 mM phosphate buffer (pH 7.0). The reaction was initiated by the addition of 44 μM NADH. The catechol formed was determined by the metapyrocatechase method after complete oxidation of NADH. (B) As described in Table II, 40 μM enzyme in 30 mM phosphate buffer (pH 7.0) containing 1 mM *o*-iodophenol and the indicated reagent was reduced anaerobically with 40 μM NADH, and then the reduced enzyme·*o*-iodophenol complex was oxidized by air-bubbling. The oxidized enzyme and catechol formed were determined. EFH_2S denotes the reduced enzyme·*o*-iodophenol complex.

(A) Reaction with the routine assay system.

Additions		Catechol formed/NADH oxidized (mol/mol)
None		0.41
L-Ascorbate	(1 mM)	0.65
L-Epinephrine	(1 mM)	0.58
Phenylhydrazine	(1 mM)	0.68
o-Phenylenediamine	(3 mM)	0.13

(B) Oxidation of the reduced enzyme·substrate complex.

Additions		EFH_2S oxidized (nmol)	Catechol formed (nmol)	Catechol formed/EFH_2S oxidized (mol/mol)
None		115	49.5	0.43
L-Ascorbate	(1.5 mM)	172	151	0.88
L-Epinephrine	(1 mM)	138	81.6	0.59
Phenylhydrazine	(1 mM)	152	154	1.01
o-Phenylenediamine	(3 mM)	129	12.9	0.10

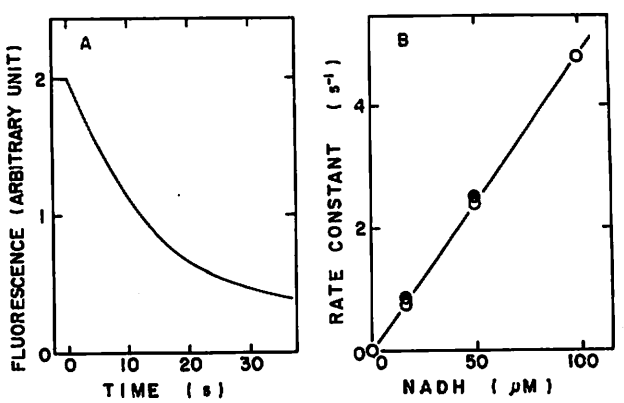


Fig. 3. **Effect of salicylate hydroxylase on the reduction of *o*-benzoquinone with NADH.** (A) Changes in the fluorescence of NADH at 420 nm after mixing 600 μM *o*-benzoquinone in 100 mM phosphate buffer (pH 7.0) and 100 μM NADH in the same buffer at 5°C in a stopped-flow apparatus. The cuvette contents were excited at 330 nm. (B) Plots of the apparent first order rate constant of NADH oxidation against its concentrations in the absence (○) and presence (○) of 10 μM salicylate hydroxylase. The slope represents an estimate of the second order reaction rate constant between NADH and *o*-benzoquinone ($8.0\times10^3\ M^{-1}\cdot s^{-1}$).

derivatives of tyrosine were not detected in the incubation mixture after hydroxylation of *o*-iodophenol in the presence of 1 mM L-tyrosine. Chlorodimedone and DL-

TABLE IV. **Transient kinetic parameters for the hydroxylation of *o*-iodophenol by salicylate hydroxylase.** The rate constant for each partial reaction denoted in the following equation was estimated from the plots shown in Figs. 4 and 5. The rate constants at pH 7.0 were also estimated by the same method. The partial reactions for *o*-iodophenol hydroxylation can be written as follows:

$$EF+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EFS \overset{NADH,}{\rightleftharpoons} X_1 \xrightarrow{k_2} EFH_2S \overset{O_2}{\rightleftharpoons} X_2 \xrightarrow{k_3} EF+Q$$

where EF is salicylate hydroxylase, S is *o*-iodophenol, EFS is the enzyme·substrate complex and EFH_2S is its reduced form, X_1 and X_2 are kinetical intermediates, and Q is *o*-benzoquinone.

Rate constant	At pH 7.0	At pH 8.0
k_1 ($M^{-1}\cdot s^{-1}$)	0.95×10^6	1.3×10^6
k_{-1} (s^{-1})	230	180
k_2 (s^{-1})	~50	~50
k_3 (s^{-1})	24	6.8

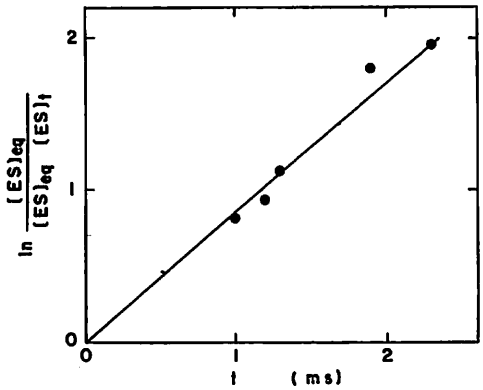


Fig. 4. **Determination of the formation and dissociation constants of the salicylate hydroxylase·*o*-iodophenol complex.** Formation of the enzyme·*o*-iodophenol complex (ES) was monitored as to the increase in the absorbance at 485 nm in a stopped-flow apparatus at different times (t), from 1 to 2.3 ms, after mixing 20 μM salicylate hydroxylase with an equal volume of 2 mM *o*-iodophenol in 30 mM phosphate buffer (pH 8) at 5°C. The equation, $\ln[ES]_{eq}/([ES]_{eq}-[ES]_t) = (k_1[S]_0 + k_{-1})t$, was reported for analysis of the reaction in the presence of a large excess concentration of substrate (*12*). In the equation, $[ES]_{eq}$, $[ES]_t$, $[S]_0$, K_1 and k_{-1} denote the concentrations of the enzyme·*o*-iodophenol complex at the equilibration time and the time, t, the initial concentration of *o*-iodophenol, and the formation and the dissociation rate constants of the complex, respectively. The rate constants were calculated from the slope of the plotted line.

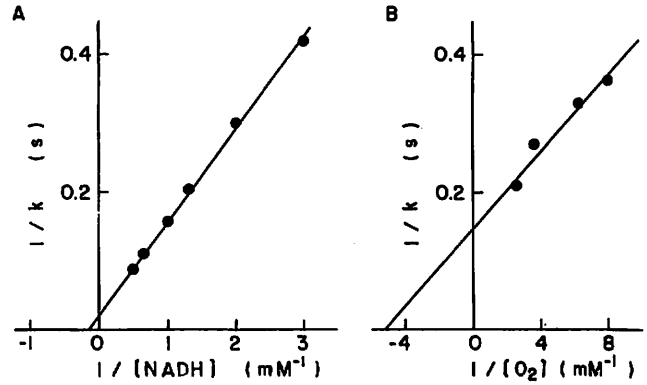


Fig. 5. **Reduction and reoxidation constants of the salicylate hydroxylase·*o*-iodophenol complex.** (A) Determination of the reduction rate constant. 30 μM salicylate hydroxylase in the presence of 2 mM *o*-iodophenol and 0.67 to 4 mM NADH in 30 mM phosphate buffer (pH 8.0) were mixed, in an equal volume, anaerobically in a stopped-flow apparatus at 5°C, and then the decreases in the absorbance at 450 nm were recorded and subjected to semi-logarithmic plotting. (B) Determination of the reoxidation rate constant. 30 μM reduced salicylate hydroxylase prepared anaerobically with NADH in the presence of 2 mM *o*-iodophenol and 30 mM phosphate buffer (pH 8.0) containing 0.25 to 0.8 mM oxygen were mixed, in an equal volume, at 5°C, and then the increases in the absorbance at 450 nm were recorded and subjected to semi-logarithmic plotting. The reciprocal values of the rate constants obtained in each experiment were plotted against those of the concentrations of NADH (A) or oxygen (B).

methionine did not have a significant effect on the amount of catechol formed on oxidation of the reduced enzyme·*o*-iodophenol complex (Table II). The results suggest that deiodination does not include I^+-ion formation and that the I^- ion is the only product of the reaction.

Detection of the Nascent Product of o-Iodophenol Hydroxylation—L-Epinephrine increased the formation of catechol on *o*-iodophenol oxidation by salicylate hydroxylase and also the molar ratio of catechol formed and NADH oxidized (Fig. 1A). In the reaction, L-epinephrine is oxidized simultaneously to adrenochrome and the net increase in catechol formed on the addition of the catecholamine is the same as the amount of adrenochrome produced (Fig. 1B). Similar effects were observed on the oxidation of *o*-nitrophenol by the enzyme (data not shown), and also on that of *o*-bromophenol, *o*-chlorophenol, and *o*-aminophenol. The formation of adrenochrome was not observed on the oxidation of salicylate, salicylaldehyde, and benzoate. Table IIIA shows that other reducing reagents, such as L-ascorbate and phenylhydrazine, stimulate catechol formation like L-epinephrine in the hydroxylation of *o*-iodophenol. *o*-Phenylenediamine, however, decreases the formation of catechol. This reagent is known to react rapidly with *o*-benzoquinone to produce phenazine (*15*). The reaction mixture was extracted with diethylether and then analyzed by thin layer chromatography, with three solvent systems, for the detection of phenazine formation. It was identified from its R_f value and color reaction with phenol reagent.

Similar results were obtained for the oxidation of the reduced enzyme·*o*-iodophenol complex (Table IIIB). Some of the reagents profoundly stimulated catechol formation trough the oxidation, as compared with the stimulation observed in the turnover experiments described above, and *o*-phenylenediamine affects the reaction negatively. Since phenylhydrazine can only reduce high potential phenols such as *o*-benzoquinone (*15*), the results also support the formation of *o*-benzoquinone as an intermediate in the hydroxylase reaction.

Spectral detection of *o*-benzoquinone during the oxidation was attempted by means of the stopped-flow method. An anaerobic solution of the reduced enzyme in the presence of *o*-iodophenol was mixed with oxygen-saturated buffer (pH 7.0) at 5°C and then the absorbance in the visible region was monitored. Figure 2A shows the spectrum observed at 5 s after mixing the reactants. This spectrum did not change for at least 180 s. A difference spectrum between this one and that of the oxidized enzyme (Fig. 2B) is similar to the spectrum of *o*-benzoquinone, with a maximum at 390 nm (*16*). The spectrum in Fig. 2A might

be composed of those of the oxidized enzyme, *o*-benzoquinone and the complex form. From the spectrum in Fig. 2B, it could be estimated that 6.5 μM benzoquinone is formed from 10 μM enzyme·substrate complex in the reaction. When the experiments were conducted in the presence of 1 mM L-ascorbate, which can reduce *o*-benzoquinone spontaneously, no spectrum was obtained with the difference spectrum method for the quinone.

Reaction Mechanism of o-Iodophenol Hydroxylation— Based on the formation of the *o*-benzoquinone intermediate, two possible mechanisms can be proposed for the second reduction step in *o*-iodophenol hydroxylation, as follows,

$$EF + S \rightleftharpoons EFS$$

$$EFS + NADH + H^+ \longrightarrow EFH_2S + NAD^+$$

$$EFH_2S + O_2 \longrightarrow EFQ$$

Mechanism A.

$$EFQ \rightleftharpoons EF + Q$$

$$Q + NADH + H^+ \longrightarrow \text{catechol} + NAD^+$$

Mechanism B.

$$EFQ + NADH + H^+ \longrightarrow EFH_2Q + NAD^+$$

$$EFH_2Q \longrightarrow EFQH_2 \longrightarrow EF + \text{catechol}$$

where EF is salicylate hydroxylase, EFS is the enzyme·substrate complex and EFH_2S is the reduced enzyme·substrate complex. S is *o*-iodophenol, Q is *o*-benzoquinone, and QH_2 is catechol.

The first molecule of NADH is used for reduction of the EFS complex in both mechanisms, and the second molecule is consumed for nonenzymatic reduction of *o*-benzoquinone to catechol in Mechanism A and for enzymatic reduction in Mechanism B.

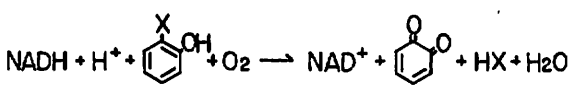
The enzyme forms a 1 : 1 complex with *o*-benzoquinone, which was detected by quenching of the protein fluorescence at 340 nm. Titration of the enzyme with *o*-benzoquinone by the fluorometric method yielded the dissociation constant of 54.1 μM in 100 mM phosphate buffer (pH 7.0) at 5°C. The rate constant of the complex formation was determined by the rapid-mixing method to be 8.0×10^3 $M^{-1} \cdot s^{-1}$. Figure 3A shows the oxidation of NADH by *o*-benzoquinone and the rate constant was estimated to be 8.4×10^3 $M^{-1} \cdot s^{-1}$ (Fig. 3B). The figure also shows the effects of 10 μM salicylate hydroxylase on the oxidation of NADH by *o*-benzoquinone; the addition of the enzyme did not change the rate constant (the filled circles in the figure). The rate of reduction of 10 μM enzyme with 1 mM NADH was the same in the presence and absence of 0.3 mM *o*-benzoquinone (data not shown). In contrast, the presence of salicylate increases the rate of reduction of enzyme-flavin with NADH (*10*). Reduction of *o*-benzoquinone with the reduced enzyme proceeded at the rate of 4.9×10^5 $M^{-1} \cdot s^{-1}$ in 100 mM phosphate buffer (pH 7.0) at 5°C, the constant in the presence of 1 mM *o*-iodophenol being 8.0×10^4 $M^{-1} \cdot s^{-1}$. These values are larger than that of the chemical reaction between NADH and *o*-benzoquinone, but the amount of free reduced enzyme or reduced enzyme·*o*-iodophenol complex during the oxygenation of *o*-iodophenol might be small due to the reason given under "DISCUSSION." Therefore, these results suggest that the reduction of *o*-benzoquinone to catechol with NADH is not stimulated by the enzyme, but that the non-enzymatic reaction is predominant over the reduction, as shown in Mechanism A for *o*-iodophenol hydroxylation.

The rate constant for each step of *o*-iodophenol hydroxylation in Mechanism A was determined by the stopped-flow method at 25°C in 30 mM phosphate buffer (pH 7.0 and 8.0), as summarized in Table IV. Formation of the complex of salicylate hydroxylase with *o*-iodophenol was determined, as shown in Fig. 4. The complex is then reduced with NADH (Fig. 5A). Since the K_m for NADH is large, the obtained rate constant (K_2) for reduction of the enzyme·substrate complex may include some error (Table IV). The data indicate that the rate limiting step is the oxidation of the reduced enzyme·substrate complex. Kinetic analysis of the reduction of the enzyme·*o*-iodophenol complex shown in Fig. 5A suggests the presence of a kinetical intermediate, X_1, which can not be identified spectrophotometrically, in this step. In the reoxidation of the reduced enzyme·substrate complex, a kinetic intermediate, X_2, is also formed, as found on the double reciprocal plot analysis (Fig. 5B). The dissociation constant of the reduced enzyme·substrate complex (EFH_2S) was estimated to be 220 μM. Oxidation of the reduced complex by molecular oxygen was measured in the visible light wavelength range in 30 mM phosphate buffer (pH 7.0) at 5°C. The time course of the absorption change at 450 nm is a little concave and at 375 nm, it has a hyperbolic form. The results suggest the presence of at least one transient intermediate, but we could not analyze them in more detail because two steps in the time course were not well separated under the conditions used.

DISCUSSION

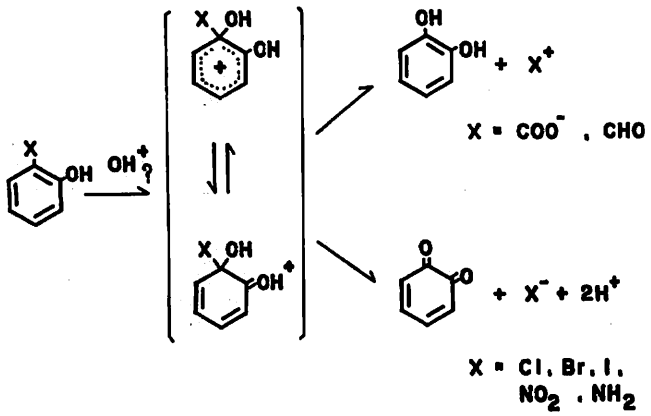
Salicylate hydroxylase oxidizes *o*-iodophenol to catechol and iodide, with the stoichiometry of 2 : 1 : 1, as to NADH oxidation, catechol formation, and oxygen consumption, respectively, and also catalyzes the hydroxylation of *o*-bromophenol, *o*-chlorophenol, *o*-nitrophenol, and *o*-aminophenol in a similar manner (*4*). The consumption of NADH in these reactions is different from that in salicylate hydroxylation (*3*).

The present study on the hydroxylation of *o*-iodophenol by salicylate hydroxylase showed that *o*-benzoquinone and iodide are the primary products of the reaction:



where X denotes either I, Br, Cl, NO_2, or NH_2.

The *o*-benzoquinone formed is rapidly and non-enzymatically converted into catechol with the consumption of a second NADH molecule. Direct evidence of *o*-benzoquinone formation during the reaction was obtained by means of chemical reaction methods. L-Epinephrine, L-ascorbate, or phenylhydrazine increases the molar ratio of catechol formed to the reductant consumed, such as NADH or the reduced enzyme·substrate complex (Table III). These reagents react rapidly with *o*-benzoquinone as a quinone trap. L-Epinephrine was oxidized to adrenochrome with the formation of an equimolar amount of catechol in the reaction (Fig. 1). *o*-Phenylenediamine reacted with *o*-



Scheme 2. **Mechanisms of substituted *o*-phenol hydroxylation by salicylate hydroxylase.**

benzoquinone to produce phenazine, with a decrease in the molar ratio to about 30%. A second approach to identify the quinone formed was direct, involving a spectrophotometric method using a stopped-flow apparatus. When the reduced enzyme·*o*-iodophenol complex was oxidized in the presence of a high concentration of oxygen and at low temperature, a species exhibiting an absorption maximum around 390 nm was yielded, which was not observed in the presence of ascorbate (Fig. 2B). These facts indicate that the transient formation of *o*-benzoquinone occurs and then it is reduced rapidly to catechol by ascorbate. Since the reduction of *o*-benzoquinone by NADH or the reduced enzyme is very fast, it could not be observed under the ordinary assay conditions or in spectral experiments on the oxidation of the reduced enzyme·substrate complex in a Thunberg-type cuvette.

It is probable that oxidation of the reduced enzyme·*o*-iodophenol complex consists of two reactions, as follows:

$$EFH_2S + O_2 \longrightarrow EF + o\text{-benzoquinone} \quad (A)$$

$$EFH_2S + o\text{-benzoquinone} \longrightarrow EFS + \text{catechol} \quad (B)$$

When the rate of Reaction A is high enough, compared with that of Reaction B, *o*-benzoquinone could be detected in the reaction. Therefore, it was necessary to use a large concentration of oxygen in the reaction medium for the detection of *o*-benzoquinone (Fig. 2). Husain *et al.* (*5*) reported the formation of *o*-benzoquinone during the hydroxylation of tetrafluorinated *p*-hydroxybenzoate by *p*-hydroxybenzoate hydroxylase. While they observed a spectrum like that of benzoquinone at 25°C and the oxidation of reduced cytochrome *c* by it, the assignment of the observed spectrum will need more evidences for a final conclusion to be drawn.

Reduction of the enzyme·*o*-iodophenol complex with NADH is slower than that of the salicylate-complex (Table IV). Binding of *o*-benzoquinone to the enzyme did not stimulate the reduction of FAD in the complex and the enzyme did not affect the reaction of the quinone with NADH (Fig. 3B). Thus, the *o*-benzoquinone that dissociates from the complex reacts non-enzymatically with NADH, as shown in Mechanism A. On the other hand, the reduced enzyme·*o*-iodophenol complex could reduce *o*-benzoquinone at a fast rate, but the rate constant of the non-enzymatic reduction of *o*-benzoquinone by 0.2 mM NADH is 1.7 s^{-1} and that by 0.85 μM reduced enzyme·*o*-iodophenol complex is 0.07 s^{-1}, as calculated from the respective second rate constant. Furthermore, only 17% of the enzyme was in the reduced form at the steady state, which appeared between 2 and 10 s after the mixing of 15 μM enzyme with 0.25 mM NADH in the presence of 2 mM *o*-iodophenol. This fact suggests that the rate constant of the enzymatic reduction of *o*-benzoquinone is smaller than the value (0.07 s^{-1}) calculated above, and so there is little possibility of the quinone reduction with the reduced enzyme.

The I^+-trap experiments disclosed that deiodination proceeds with the elimination of the iodide ion. 2-Chlorodimedone and DL-methionine are sufficiently reactive to trap the I^+-ion, but they could not stimulate the oxygenation of the reduced enzyme·*o*-iodophenol complex. As described in the previous paper (*4*), oxygenation of *o*-nitrophenol does not produce the nitrate but the nitrite ion, and with *o*-aminophenol, the product is not hydroxylamine but ammonia. These results could be explained by the *removal* of the anionic species of the ortho-substituent from the phenolic substrate.

The results presented in this and the previous paper (*4*) are consistent with the hydroxylation mechanisms depicted in Scheme 2. An oxygen-adduct of the substrate is formed after a hypothetical OH^+ ion or oxene produced from the peroxyflavin of the enzyme (*17*) attacks the carbon in the benzene ring linking the substituent X, followed by immediate elimination of the group. The elimination proceeds on the basis of the electron-withdrawing effect of the ortho-substituent. A substrate having a carboxyl or aldehyde group in the ortho-position becomes oxidized to catechol by leaving its electron pair to the benzene ring, while a substrate having a nitro, halogeno, or amino group is converted into *o*-benzoquinone due to the elimination of the nitrite, halide, or ammonium ion, respectively. Similar reactions have been reported for the dehalogenation of halogenated substrates by pteridine-containing phenylalanine hydroxylase (*18*), D-amino acid oxidase (*19*), and lactate oxygenase (*20*), and for the denitrification of 2-nitropropane by 2-nitropropane dioxygenase (*21*). In contrast, halogenation by chloroperoxidase (*8*) and myeloperoxidase (*22*) was reported to involve activated hypohalite ions.

In conclusion, salicylate hydroxylase can catalyze the anionic elimination of the ortho-substituent of a substrate following an attack of the OH^+-species or oxene generated from the peroxyflavin. The unusual stoichiometry of the reaction is due to the non-enzymatic reduction of *o*-benzoquinone by a molecule of NADH.

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REFERENCES

1. Yamamoto, S., Katagiri, M., Maeno, H., and Hayaishi, O. (1965) *J. Biol. Chem.* **240**, 3408-3413
2. Katagiri, M., Maeno, H., Yamamoto, S., Hayaishi, O., Kitao, T., & Oae, S. (1965) *J. Biol. Chem.* **241**, 3414-3417
3. Katagiri, M., Takemori, S., Suzuki, K., & Yasuda, H. (1966) *J. Biol. Chem.* **241**, 5675-5677

4. Suzuki, K., Gomi, T., Kaidoh, T., & Itagaki, E. (1991) *J. Biochem.* **109**, 348-353
5. Husain, M., Entsch, B., Ballou, D.P., Massey, V., & Chapman, Pl. J. (1980) *J. Biol. Chem.* **255**, 4189-4197
6. Brockhaus, R. (1968) *Liebigs Ann. Chem.* **712**, 214-215
7. Goldschmidt, S. & Graef, F. (1928) *Berichte* **61**, 1858-1869
8. Libby, R.D., Thomas, J.A., Kaiser, L.W., & Hager, L. P. (1982) *J. Biol. Chem.* **257**, 5030-5037
9. Suzuki, K. & Ohnishi, K. (1990) *Biochim. Biophys. Acta* **1040**, 327-336
10. Takemori, S., Nakamura, M., Suzuki, K., Katagiri, M., & Nakamura, T. (1972) *Biochim. Biophys. Acta* **284**, 382-393
11. Covelli, I. & Wolff, J. (1966) *Biochemistry* **5**, 860-866
12. Friedman, M.E., Scheraga, H.A., & Goldberger, R. F. (1966) *Biochemistry* **5**, 3770-3778
13. Misra, H. P. & Fridovich, I. (1972) *J. Biol. Chem.* **247**, 188-192
14. Gornall, A.G., Bardawill, C.J., & David, M. M. (1949) *J. Biol. Chem.* **177**, 751-766
15. Horspool, W. M. (1969) *Q. Rev.* **23**, 204-235
16. Mason, H. S. (1949) *J. Biol. Chem.* **181**, 803-812
17. Entsch, B., Ballou, D.P., & Massey, V. (1976) *J. Biol. Chem.* **251**, 2550-2563
18. Storm, C. B. & Kaufman, S. (1968) *Biochem. Biophys. Res. Commun.* **32**, 788-793
19. Walsh, C.T., Schonbrunn, A., & Abeles, R. H. (1971) *J. Biol. Chem.* **246**, 6855-6866
20. Walsh, C., Lockridge, O., Massey, V., & Abeles, R. H. (1973) *J. Biol. Chem.* **248**, 7049-7054
21. Kido, T., Soda, K., & Asada, K. (1978) *J. Biol. Chem.* **253**, 226-232
22. Harrison, J.E., Watson, B.D., & Schultz, J. (1978) *FEBS Lett.* **92**, 327-331