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MECHANISM OF THE SALICYLATE HYDROXYLASE REACTION*

III. CHARACTERIZATION AND REACTIVITY OF CHEMICALLY OR PHOTOCHEMICALLY REDUCED ENZYME–FLAVIN

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SUMMARY

1. The spectral natures of chemically- and photochemically-reduced salicylate hydroxylase (salicylate, NADH:oxygen oxidoreductase (1-hydroxylating, 1-decarboxylating)) from *Pseudomonas putida* have been investigated.

2. When salicylate hydroxylase in the presence of substrate was titrated with a limited amount of dithionite or was illuminated in the presence of EDTA, a new species with a weak absorption band at a long wavelength appeared, whereas an entirely different species which was typical for the "red flavoprotein radical" was detected in the absence of substrate. The latter was converted to the former upon anaerobic addition of substrate.

3. Reoxidation of the reduced enzyme–substrate complex with air formed the product in an amount stoichiometric with the reduced flavin.

INTRODUCTION

Salicylate hydroxylase (salicylate, NADH:oxygen oxidoreductase (1-hydroxylating, 1-decarboxylating)) combines specifically with the substrate to form a new enzyme–substrate complex in which the ratio of apoenzyme, FAD and substrate is 1:1:1 (refs. 3 and 4). The enzyme–substrate complex is converted by the anaerobic addition of an external electron donor, NADH, to the stable two-electron reduction intermediate in which the stoichiometric relation between added NADH and formed $FADH_2$ has been demonstrated. This reduced species of the enzyme has been shown to be an active intermediate capable of supporting the aerobic hydroxylation of the substrate bound to the enzyme[3,4]. These results suggest that the enzyme-bound

* A part of the results was presented at the meeting of the 7th International Congress of Biochemistry in Tokyo, 1967 (ref. 1), and a preliminary report has been presented[2].

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$FADH_2$ formed by NADH is an essential electron donor in the hydroxylation reaction. The claim could be greatly strengthened if independent methods (especially redox systems most frequently used in studies with hydrogenating flavoproteins) other than reduction with NADH would lead to the same conclusions.

This paper presents studies indicating that the complex reduced either with sodium dithionite or by illumination in the presence of EDTA does react with O_2 to convert enzyme-bound salicylate into catechol.

MATERIALS AND METHODS

Sodium dithionite, FMN and EDTA were obtained from Wako Pure Chemical Co., Osaka. Other reagents used in this work have been described in a preceding paper[4]. Purified salicylate hydroxylase was prepared from *Pseudomonas putida*, S-1, grown in salicylate according to the method described in a preceding paper[4]. Crystalline metapyrocatechase was prepared from *Ps. putida*, T-2, essentially as described by NOZAKI *et al.*[5].

Optical assays under anaerobic conditions were performed in a Thunberg-type cuvette according to the procedure described in a preceding paper[4]. Spectra were recorded with a Hitachi model EPR-3 recording spectrophotometer and with a Perkin–Elmer Model 202 recording spectrophotometer.

Sodium dithionite solution of a known concentration was prepared in a Thunberg tube with a side arm and an open mouth fitted tightly with a vaccine cup. After placing 33 mM potassium phosphate buffer adjusted to pH 7.0 and the appropriate amount of solid sodium dithionite in the main vessel and in the side arm, respectively, the container was immediately closed and freed from O_2 by at least three cycles of alternate evacuation and flushing with O_2-free N_2. After the tube had been made anaerobic, the solid powder of dithionite in the side arm was introduced into the buffer solution. The concentration of dithionite was determined by titrating FMN under anaerobic conditions. Anaerobic titrations were carried out with the use of a microsyringe through the vaccine cup. The dithionite solution was stable during the experiment under these conditions if it was kept anaerobically in an ice bath.

The EDTA-photochemical reduction was carried out by the method of MASSEY AND PALMER[6]. The reaction mixture for illumination contained EDTA at the concentrations described in the legends for the appropriate figures and tables. The enzyme in the anaerobic cuvette was irradiated using a conventional slide projector through a glass water bath with 10-cm light path containing 100 g $CuSO_4$ per l as a filter.

Experiments for electron spin resonance (ESR) were carried out with a Varian V-4500 spectrometer using 100-kcycles magnetic field modulation, and spectra were measured by the use of an anaerobic tube as described by BEINERT AND SANDS[7].

RESULTS

Reduction of salicylate hydroxylase with sodium dithionite

Upon anaerobic titration of salicylate hydroxylase with a limited amount of dithionite in the presence of substrate, the absorption over the whole spectral range diminished instantaneously, and a new absorption band extending beyond 500 nm was formed (Fig. 1A). The intensity at long wavelength was maximal with 0.5 mole

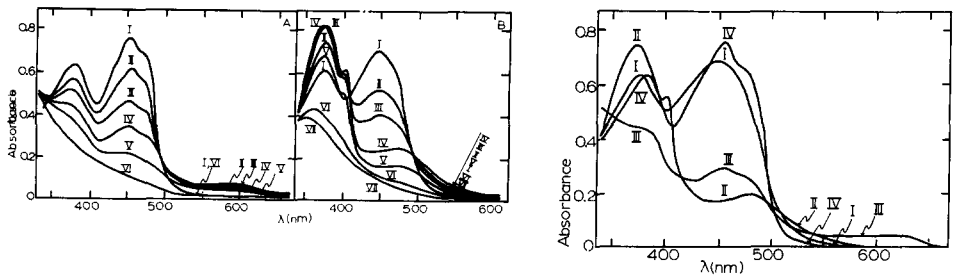


Fig. 1. Sodium dithionite titration of salicylate hydroxylase in the presence (A) and absence (B) of 5 μmoles salicylate. Assay conditions: 147 nmoles of enzyme, 100 μmoles of potassium phosphate buffer (pH 7.0) and $Na_2S_2O_4$ as indicated in a total volume of 3 ml. A. Curve I, oxidized enzyme; Curves II–VI, after addition of 0.29, 0.52, 0.73, 0.95 and 1.39 moles of $Na_2S_2O_4$ per mole of FAD, respectively. B. Curve I, oxidized enzyme; Curves II–VII, after addition of 0.23, 0.47, 0.70, 0.94, 1.07 and 1.40 moles of $Na_2S_2O_4$ per mole of FAD, respectively.

Fig. 2. Conversion of the red intermediate to the species with a long wavelength absorption band by the addition of salicylate. Curve I, oxidized enzyme (147 nmoles); Curve II, after the addition of 0.7 mole of $Na_2S_2O_4$ per mole of FAD; Curve III, after the subsequent addition of 5 μmoles salicylate; Curve IV, after admitting air into the reaction mixture. Other conditions were the same as in Fig. 1.

dithionite per mole flavin. The resulting spectra were not similar to those which had been found on titration of the enzyme with an external electron donor, NADH[3,4]. The flat long wavelength band disappeared completely after full reduction was obtained with 1 mole of dithionite per mole of the enzyme-bound flavin. Similar absorption spectra were also produced in the presence of other substrates for the hydroxylation reaction, namely, 2,5-dihydroxybenzoate and 3-methylsalicylate.

When the titration was carried out in the absence of substrate, the reddish color appeared indicating the possible formation of a semiquinoid intermediate (Fig. 1B). Its spectrum exhibited maxima at 375, 400 and 480 nm. The intensity at 520 nm was maximal with 0.5 mole dithionite per mole flavin. The complete reduction of the enzyme was obtained with 1 mole of dithionite per mole flavin. The conversion between two types of intermediates in the presence and absence of substrate, respectively, was obtained from the experiment depicted in Fig. 2. The enzyme was first reduced anaerobically with dithionite to produce a red intermediate (Curve II). Salicylate in the side arm was then mixed anaerobically with the red intermediate in the main tube. As expected, the color changed from red to yellow. The resulting intermediate was found to have the same spectral properties as an intermediate reduced in the presence of salicylate (Curve III). The addition of 2,5-dihydroxybenzoate or 3-methylsalicylate to the red intermediate resulted in similar changes in the spectrum.

Photoreduction of salicylate hydroxylase

Spectral changes very similar to those observed upon dithionite titrations were also observed when salicylate hydroxylase was photoreduced. Fig. 3A shows the changes in spectrum produced on light irradiation in the presence of EDTA. A rapid development of a flat long wavelength band very similar to that which appeared during the anaerobic dithionite titration was observed. Prolonged light irradiation resulted in a gradual disappearance of the long wavelength band and a concomitant appearance of the spectrum of the fully reduced enzyme. Upon admitting air into the

cuvette, the original oxidized spectrum was completely restored. A similar species could be obtained when the enzyme was irradiated with light in the presence of 2,5-dihydroxybenzoate or 3-methylsalicylate.

In the absence of substrate, as shown in Fig. 3B, illumination resulted in the formation of a red intermediate which exhibited the same characteristic features as those found in dithionite reduction, the peaks being situated at 375, 400 and 480 nm. Photoreduction of the enzyme at an alkaline pH produced more typical semiquinoid spectrum than at neutral pH.

The addition of substrate to a red intermediate resulted in the conversion of it to a species with a long wavelength band (Fig. 4). Reoxidation of the reduced enzyme with air was rapid, and the original oxidized spectrum was regained.

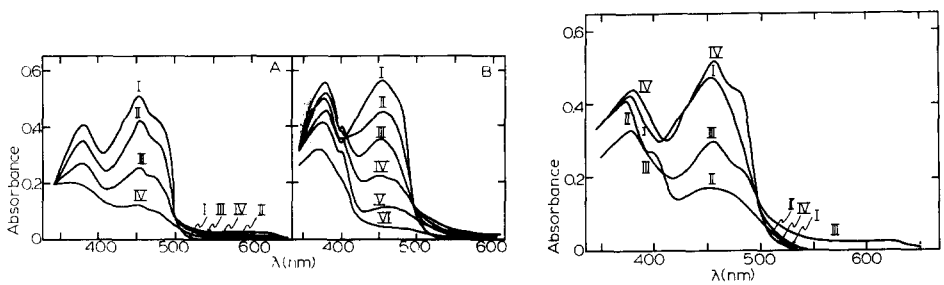
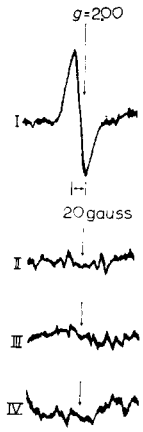


Fig. 3. Comparison of the spectra of salicylate hydroxylase during photoreduction in the presence (A) and absence (B) of 5 μmoles salicylate. Assay conditions: 100 μmoles of potassium phosphate buffer (pH 7.0) and 150 μmoles of EDTA in a total volume of 3 ml. Illumination was carried out with 100 W (A) or 1 kW (B) tungsten lamp for the times shown at 20°. A. Curve I, oxidized enzyme (110 nmoles); Curves II–IV, after illumination for 70, 180 and 570 min, respectively. B. Curve I, oxidized enzyme (121 nmoles); Curves II–VI, after illumination for 20, 40, 60, 100 and 400 sec, respectively.

Fig. 4. Conversion of the red intermediate to the species with a long wavelength absorption band by the addition of salicylate. Curve I, oxidized enzyme (111 nmoles); Curve II, after illumination for 138 min with 100-W tungsten lamp; Curve III, after the subsequent addition of 5 μmoles salicylate; Curve IV, after admitting air into the reaction mixture. Other conditions were the same as in Fig. 3.

Spectral changes of the red intermediate described above were correlated with the appearance of a free radical signal as determined by ESR spectrometer. As shown in Curve I of Fig. 5, a typical signal was observed with the red intermediate. The signal converged toward 20 gauss peak to peak width, the g value being 2.00. However, this signal gradually disappeared as the illumination was continued for prolonged times. The absorption spectrum and ESR signal of the red intermediate of salicylate hydroxylase represent a semiquinoid form of the enzyme-bound flavin. No ESR signal was demonstrable when the enzyme was illuminated anaerobically in the presence of the substrate such as salicylate, 3-methylsalicylate or 2,5-dihydroxybenzoate (Curves II–IV in Fig. 5).

The substrate has a marked effect on the rate of photoreduction of salicylate hydroxylase. As shown in Fig. 6, the holoenzyme was completely reduced in 20 min, while in the presence of the substrate, the rate became slow. The maximum photoreduction required 180 min with salicylate or 120 min with 3-methylsalicylate, respectively. A similar effect was observed in the presence of *o*-phenolsulfonate, a



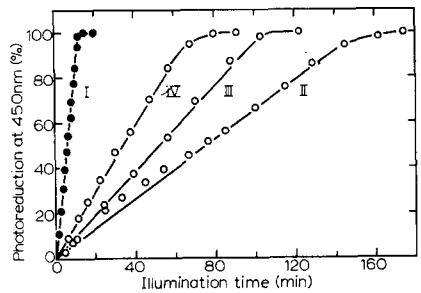


Fig. 5. Comparison of ESR spectra of salicylate hydroxylase as photoreduced in the presence and absence of substrate. The assay system (0.3 ml) contained: 27 nmoles of enzyme, 4.7 μmoles of EDTA, 10 μmoles of potassium phosphate buffer (pH 7.0) and 1.3 μmoles of substrate indicated. The assay mixture was illuminated for the time required to half reduce the flavin with 750-W tungsten lamp. Curve I, without substrate; Curve II, with salicylate; Curve III, with 2,5-dihydroxybenzoate; Curve IV, with 3-methylsalicylate.

Fig. 6. Comparison of rates of photoreduction of salicylate hydroxylase with EDTA in the presence and absence of substrate. The assay system (2.6 ml) contained: 33 nmoles of enzyme, 86 μmoles of potassium phosphate buffer (pH 7.0), 15 μmoles of EDTA and 5 μmoles of substrate indicated. Illumination was carried out at 20° with 1-kW tungsten lamp for the times shown. Curve I, without substrate; Curve II, with salicylate; Curve III, with 3-methylsalicylate; Curve IV, with *o*-phenolsulfonate.

competitive inhibitor for the enzyme; it required 80 min for the maximum photoreduction. However, no appreciable effect was observed with other nonsubstrate substances such as benzoate.

Stoichiometric demonstration that artificially reduced flavin is "active" as an electron donor

Attempts were made to demonstrate that the reduced enzyme thus obtained is capable of aerobic hydroxylation of the substrate. In the first approach to this problem, sodium dithionite was mixed with the enzyme anaerobically in the presence of salicylate. Air was introduced into the cuvette after the amount of the reduced flavin was estimated. Reoxidation of the reduced flavin with air was rapid, and the original oxidized spectrum was regained. When a limited amount of dithionite was added, it was found that the amount of added dithionite was equivalent to that of the enzyme-bound flavin reduced and the catechol formed, respectively (Expts. IA and IB in Table I). That a stoichiometric relation is apparently maintained between dithionite, flavin and product is strongly supported by these results. The enzyme reduced with dithionite in the absence of salicylate was reacted with air. The addition of salicylate before the introduction of air resulted in the stoichiometric formation of catechol (Expt. IC). However, when the reduced enzyme was mixed with air before addition of salicylate, no product formation was detected (Expt. ID). The results indicate that the reduced enzyme is also capable of hydroxylating the substrate once salicylate is added to the system.

TABLE I

REACTIVITY OF THE ENZYME–FLAVIN REDUCED WITH DITHIONITE AND LIGHT IRRADIATION

Each reaction was carried out in a Thunberg-type cuvette with 3 ml assay system containing 100 μmoles of potassium phosphate buffer (pH 7.0) and the components indicated. Illumination (Expt. II) was performed with the use of 100-W tungsten lamp as in MATERIALS AND METHODS in the presence of 150 μmoles of EDTA. The amount of reduced enzyme-bound FAD was estimated by taking the molecular extinction coefficient as 10.3 $mM^{-1} \cdot cm^{-1}$ at 450 nm. Catechol was determined after air was admitted into the cuvette as described in preceding paper[4].

Expt. No.	*Enzyme (nmoles)*	*Salicylate (μmoles)*	*Reductants*		*Enzyme reduced (nmoles)*	*Catechol formed (nmoles)*
			$Na_2S_2O_4$ (nmoles)	*Light (min)*		
IA	147	5	205		147	174
IB	147	5	76		79	64
IC*	147	—	103		115	107
ID**	147	—	206		147	0
IIA	77	5		181	58	41
IIB	110	5		568	101	98
IIC*	110	—		138	60	48
IID**	110	—		551	105	0

* After the enzyme had been reduced, 5 μmoles of substrate were added from the side arm and the reaction mixture was then exposed to air.

** The reduced enzyme was exposed to air before the addition of substrate.

A stoichiometric relationship quite similar to that observed on dithionite reduction was obtained on light irradiation of salicylate hydroxylase (Expt. II in Table I).

As shown in Table II, the results with 3-methylsalicylate–holoenzyme indicated that a stoichiometric amount of 3-methylcatechol was produced from the reduced complex, prepared either with dithionite or by light irradiation.

TABLE II

STOICHIOMETRY IN THE HYDROXYLATION REACTION WHEN 3-METHYLSALICYLATE WAS USED AS A SUBSTRATE

The assay conditions were the same as those described in the legend of Table I, except that 3-methylsalicylate was used as the substrate, and 1-kW tungsten lamp was used for photoreduction. 3-Methylcatechol produced was estimated as described in preceding paper[4].

Expt. No.	*Enzyme (nmoles)*	*3-Methylsalicylate (μmoles)*	*Reductants*		*Enzyme reduced (nmoles)*	*3-Methylcatechol formed (nmoles)*
			$Na_2S_2O_4$ (nmoles)	*Light (sec)*		
IA	65	5	72		61	53
IB*	64	—	70		72	74
IC**	117	—	150		118	0
IIA	131	5		300	133	98
IIB*	131	—		60	124	100
IIC**	122	—		170	121	0

* After the enzyme had been reduced, 5 μmoles of substrate were added from the side arm and the reaction mixture was then exposed to air.

** The reduced enzyme was exposed to air before the addition of substrate.

DISCUSSION

In an attempt to gain some insight into the mechanism of the salicylate hydroxylase reaction, efforts have been made to establish a reaction sequence in which NADH, salicylate and O_2 interact with the enzyme. Recent communications from this laboratory[3,4,8,9] have firmly established that salicylate hydroxylase combines specifically with substrate to form a substrate–enzyme complex in which the ratio of apoenzyme, FAD and salicylate is 1:1:1 and its complex is the actual intermediate involved in the overall reaction of salicylate hydroxylation. With the role of flavin, it was suggested that the flavin moiety of the enzyme can participate in the hydroxylation reaction as the sole electron donor. These conclusions were based on the demonstration that enzyme-bound $FADH_2$ produced by NADH is enzymatically reactive as an electron donor and that the hydroxylation reaction was coupled with the electron transfer from the reduced flavin to O_2. The results of the present investigation have confirmed and extended the conclusion that the reduced flavin, which is produced whether the reducing agent is dithionite, EDTA–light or NADH, is enzymatically "active" as an electron donor in the hydroxylation reaction.

The results indicated that the anaerobic reduction of the enzyme proceeds by way of two spectroscopically distinguishable species depending on the substrate. In the absence of substrate, spectral changes considered to be associated with the formation of semiquinoid form of flavoprotein were observed upon dithionite or EDTA–light reduction of the enzyme. The spectrum of the red intermediate seems to be very similar to those shown in photoreduction of D- and L-amino-acid oxidases, oxynitrilase and glucose oxidase[6]. Recently MASSEY AND PALMER[6] demonstrated two different spectral types of flavoprotein semiquinone which they called "red" and "blue". Furthermore they postulated that the red species was the anion radical, the blue species the neutral semiquinone. This was confirmed by EHRENBERG *et al.*[10]. According to this classification, the red intermediate of salicylate hydroxylase belongs to the red flavin radical species (E. G. ERIKSSON, personal communication). The red intermediate formed the species having a long wavelength absorption band, when mixed anaerobically with salicylate. From the ESR results this appeared not to be due to the blue neutral radical, although it had a similar absorption spectrum. Recently, YAGI *et al.*[11] observed an interesting phenomenon in the case of D-amino-acid oxidase. The red radical of D-amino-acid oxidase was easily converted into a blue-colored substance without any change in its paramagnetic nature only by mixing with benzoate under anaerobic conditions. In this case, the blue-colored substance appeared to be identical with the blue radical described by MASSEY AND PALMER[6]. The flat long wavelength band arising when salicylate hydroxylase is reduced in the presence of substrate is somewhat more difficult to interpret. A specific interaction between reduced flavin and substrate, possibly of the charge transfer type is not eliminated. It should be noted that no long wavelength absorption band or semiquinoid form other than steady transition stage from oxidized to the fully reduced form was observed when the enzyme was reduced anaerobically with NADH in the presence or absence of the substrate[3,4]. The rapid reaction techniques will be required to provide further information in this connection. Absorption spectra of the enzyme in the dynamic steady states of the hydroxylation reaction are currently under investigation in this laboratory.

The substrate–holoenzyme complex was more resistant to the photoreduction

than the holoenzyme itself. These results are consistent with the evidences that the enzyme is more resistant to acid and heat inactivations and proteolytic digestion in the presence of substrate[4]. The effect of substrate on photoreduction, therefore, should be attributed to a difference of a protein conformation between holoenzyme and substrate–holoenzyme. MCCORMICK *et al.*[12], who observed that benzoate decreased the rate of photoreduction of D-amino-acid oxidase, have recently reported a quite similar phenomenon with D-amino-acid oxidase.

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