# THE NATURE OF CHEMICALLY OR PHOTOCHEMICALLY REDUCED FLAVIN OF SALICYLATE HYDROXYLASE

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It has already been established that salicylate hydroxylase from a pseudomonad, which catalyzes the conversion of salicylate to catechol with the stoichiometric consumption of molecular oxygen and NADH, is a flavoprotein with FAD as the prosthetic group ( Katagiri et al., 1962, 1965; Yamamoto et al., 1965 ). Recent communications from this laboratory ( Katagiri, Takemori, Suzuki, and Yasuda, 1966, $1966_a$ ) have firmly established that the enzyme combines specifically with the substrate to form a new ternary complex in which the ratio of apoenzyme, FAD, and salicylate is 1 : 1 : 1.* The ternary complex is the actual intermediate involved in the over-all reaction of salicylate hydroxylation and the flavin moiety in the complex undergoes reduction and oxidation during the reaction. These conclusions are based on measurement of the stoichiometry of the reaction

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* Results of fluorometric analysis of the ternary complex are presented in the subsequent paper.

which showed that the enzyme-bound $FADH_2$ formed by NADH is an essential electron donor, and it is oxidized stoichiometrically during the hydroxylation reaction. In this paper evidence is presented to show that the reduced complex, prepared with either the powerful reducing agent, sodium dithionite or light irradiation, can react enzymatically with molecular oxygen to convert salicylate into catechol.

Purified salicylate hydroxylase was prepared from Pseudomonas sp. grown in salicylate according to the method described previously ( Katagiri et al., 1966 ), with slight modifications. Anaerobic spectrophotometric experiments were carried out in Thunberg-type cuvettes fitted with a vaccine cup. The enzyme was placed in the main vessel and anaerobic conditions were obtained in the cuvette as described previously ( Katagiri et al., 1966) except that the final gas phase was $O_2$ free $N_2$. Titration of the enzyme-bound FAD with dithionite was performed by stepwise addition of dithionite using an injection syringe. The absorption spectrum of the partially reduced enzyme thus prepared was measured with a Hitachi, model EPR-3, recording spectrophotometer. The enzyme was irradiated with light under anaerobic conditions in a Thunberg-type cuvette.

Fig. 1-A shows the results of anaerobic titration of salicylate hydroxylase with dithionite in the presence of salicylate. The spectral changes indicate that the reduction by dithionite leads to formation of the reduced complex with long wavelength absorption at above 500mμ. Full reduction with complete elimination of the long wavelength absorption was obtained with one mole of dithionite per mole of flavin. The reduced complex thus obtained was enzymatically " active " just as the reduced complex prepared by NADH. On admitting air into the cuvette, the reduced complex was rapidly reoxidized and the original oxidized spectrum was regained. Under these

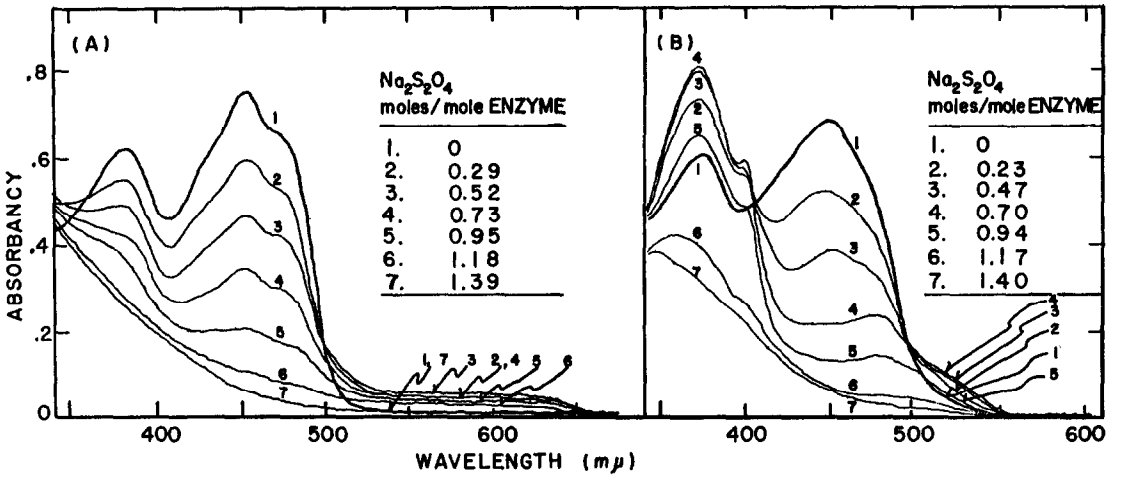


Fig. 1. Anaerobic titration of salicylate hydroxylase with dithionite in the presence (A) and absence (B) of salicylate. The complete system (3 ml) contained: 147 mμmoles, enzyme; 100 μmoles, potassium phosphate buffer, pH 7.0; and dithionite as indicated. Five μmoles of salicylate were also added in (A). Temperature, 20°.

conditions, one mole of the hydroxylated product, catechol was shown to be produced from one mole of reduced complex ( Table I ). In the absence of salicylate, titration of salicylate hydroxylase with dithionite gave a different spectrum from that seen in the presence of salicylate ( Fig. 1-B ). Under these conditions the reddish color appeared as an intermediate with absorption maxima at 375, 400 and 480mμ. The intensity at 520mμ was maximal with 0.5 mole dithionite per mole flavin. The complete reduction of the enzyme was obtained with one mole of dithionite per mole flavin. When salicylate was added to the red intermediate anaerobically, the color changed from red to yellow and the resulting intermediate was found to have the same spectral properties as the intermediate obtained in the presence of salicylate. In Experiment I-C of Table I, salicylate and the reduced enzyme were mixed anaerobically, and the cuvette was then exposed to air. It can be seen that one mole of product was found per mole of flavin. However, no product was form-

ed when the reduced enzyme was exposed to air before the addition of salicylate ( Experiment I-D ). These results indicate that the red intermediate is also enzymatically " active " and capable of hydroxylating the substrate once salicylate is added to the system.

Spectral changes closely similar to those observed on dithionite titration were obtained on light irradiation of salicylate hydroxylase. In the presence of salicylate a species with a weak absorption band at a long wave-

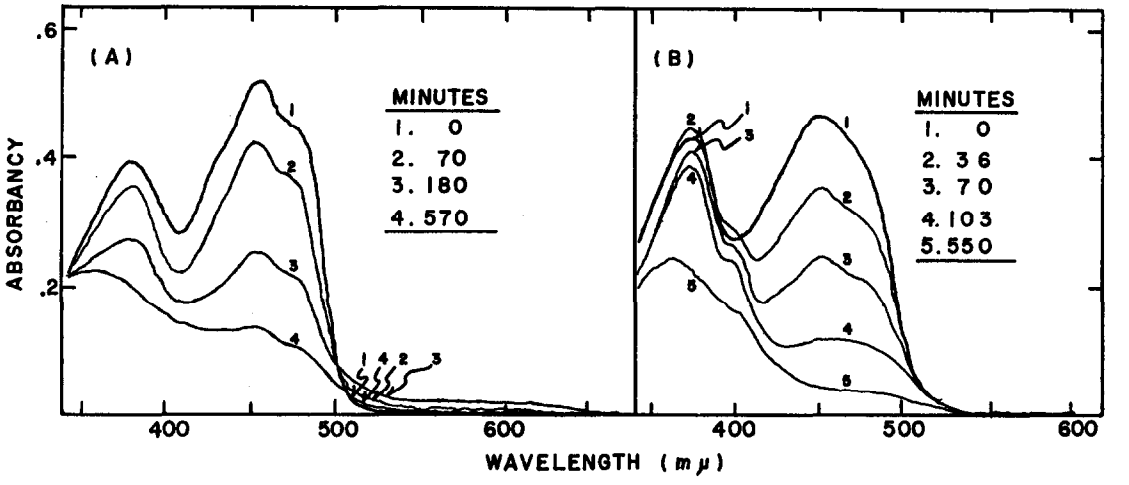


Fig. 2. Comparison of the spectral changes of salicylate hydroxylase induced by light irradiation in the presence (A) and absence (B) of salicylate. The complete system (3 ml) contained: 110 mμmoles, enzyme; 100 μmoles, potassium phosphate buffer, pH 7.0; 150 μmoles, EDTA. Five μmoles of salicylate were also added in (A). Temperature, 20°.

length appeared ( Fig. 2-A ). In the absence of salicylate, a red intermediate was detected which exhibited absorption maxima at 375, 400 and 480mμ ( Fig. 2-B ). When salicylate was added to the latter anaerobically, the intermediate was converted to a species with absorption at a long wavelength. Reoxidation of the reduced complex with air was rapid and the original oxidized spectrum was regained. As shown in Table I, the reduced complex produced by light

Table I Stoichiometry of reactions with dithionite and with light irradiation.

| Experiment | Enzyme | Salicylate | Reductants | Enzyme reduced | Order of additions | Catechol formed |
|---|---|---|---|---|---|---|
| | mμmoles | μmoles | (dithionite) mμmoles | mμmoles | | mμmoles |
| I-A | 147 | 5 | 205 | 147 | Air - | 174 |
| I-B | 147 | 5 | 76 | 79 | Air - | 64 |
| I-C | 147 | - | 103 | 115 | Sal. Air | 107 |
| I-D | 147 | - | 206 | 147 | Air Sal. | 0 |
| | | | (light) minutes | | | |
| II-A | 77 | 5 | 181 | 58 | Air - | 41 |
| II-B | 110 | 5 | 568 | 101 | Air - | 98 |
| II-C | 110 | - | 138 | 60 | Sal. Air | 48 |
| II-D | 110 | - | 551 | 105 | Air Sal. | 0 |

Experiment I: Each reaction was carried out with 3 ml assay system containing 100 μmoles of potassium phosphate buffer, pH 7.0, and the components indicated. In Expts. I-A and I-B, the indicated amount of dithionite was added to the mixture of enzyme and salicylate. The amount of reduced enzyme-bound FAD was calculated by the decrease in absorption at 450mμ, assuming an extinction coefficient of 10.3 $mM^{-1}$. $cm^{-1}$. After air had been introduced into the cuvette, the amount of catechol produced was estimated enzymatically using metapyrocatechase as described previously ( Katagiri et al., 1966 ). In Expt. I-C the enzyme and dithionite were mixed. Five μmoles of salicylate were added from the side arm and the reaction mixture was then exposed to air. In Expt. I-D the reduced enzyme was exposed to air before the addition of salicylate.

Experiment II : Each reaction was carried out with 3 ml assay system containing 100 μmoles of potassium phosphate buffer, pH 7.0, 150 μmoles of EDTA, and the other components indicated. The anaerobic cuvette was placed about 13 cm from a 100-W tungsten lamp, and irradiated for the time indicated at 16°. A glass-plate was placed in front of the lamp to exclude ultraviolet irradiation. Other assay conditions were as in Experiment I.

irradiation was also enzymatically " active " and capable of aerobic hydroxylation of salicylate in the absence of any reducing agent.

These results, together with those related to NADH, clearly demonstrate that chemically or photochemically reduced FAD is fully active as an electron donor in the salicylate hydroxylase reaction.

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