# THE FLUOROMETRIC DEMONSTRATION OF BINARY AND TERNARY COMPLEXES OF SALICYLATE HYDROXYLASE

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During spectral studies on salicylate hydroxylase we found that the absorption spectrum with peaks at 375 and 450mμ is markedly changed by the addition of salicylate and a new shoulder appears at about 480mμ. These spectral changes have been interpreted as being due to the formation of a ternary complex of apoenzyme, FAD and salicylate ( Katagiri et al., 1966 ). Since the formation of the ternary complex was characterized by a new shoulder at 480mμ, the molar stoichiometry of apoenzyme, FAD and salicylate in the complex could be measured by spectrophotometric titration of the enzyme with salicylate. It was found to be 1 : 1 : 1.

The present paper describes the fluorometric analysis of this ternary complex. Evidence is also presented to show that the apoenzyme of salicylate hydroxylase forms a " binary complex " with salicylate in the absence of FAD.

The holoenzyme was prepared as reported previously ( Katagiri et al., 1966 ) with slight modifications. The flavin-free apoenzyme was prepared by the usual acid-ammonium sulfate method and dialyzed against

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0.01 M Tris-HCl buffer, pH 8.3 to remove contaminating ammonium sulfate completely. This preparation of apoenzyme was stable for several days when stored at below 5°. FAD ( approximately 90 % purity ) was kindly supplied by Dr. E. Ohmura of Takeda Research Laboratory. Fluorescence was measured at room temperature with a Hitachi recording spectrophotofluorometer, model EPF-2A, in which a xenon lamp was used as the exciting source and the slit width was 10mμ x 10mμ. A standard lamp ( NBS. Co. ) was used to correct for variations in intensity.

Fig. 1-A shows the emission spectra of the holoenzyme, salicylate and ternary complex activated at 280mμ. The emission maxima of the holoenzyme ( Curve I ) and salicylate ( Curve II ) were at 325 and 410mμ, respectively. When salicylate and the holoenzyme were mixed at the concentrations used for the measurement of their separate spectra, the emission spectrum produced was not a simple arithmetic sum of the individual absorptions of the holoenzyme and salicylate. The addition of salicylate to the holoenzyme resulted in a marked increase in the fluorescence peak at 410mμ, with a decrease in intensity at 325mμ ( Curve III ). The addition to the holoenzyme of 2,5-dihydroxybenzoate and p-aminosalicylate, which are the other substrates for salicylate hydroxylase, caused similar spectral effects. However, no effect was observed when analogues of the substrate, such as benzoate or m-hydroxybenzoate, were added. These results indicate that the holoenzyme combines specifically with the substrate to form a fluorescent ternary complex. Since the formation of the complex was indicated by an increase in fluorescence at 410mμ, the stoichiometry of apoenzyme, FAD and salicylate in the complex could be determined by titration of the holoenzyme with salicylate. As shown in Fig. 2-A, when a constant amount of holoenzyme was titrated with varying amounts of salicylate, the initial slope of the titration curve

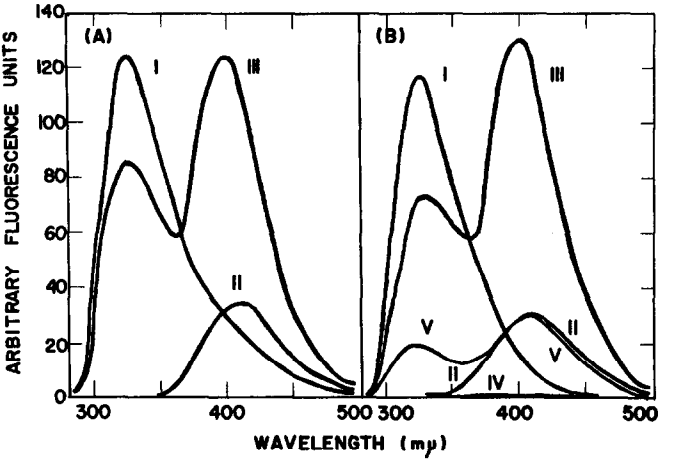


Fig. 1-A and -B. Emission spectra of salicylate, apoenzyme, holoenzyme and their complexes activated at 280mμ. Measurements were carried out with 3.2 μM salicylate, 3.2 μM FAD and 1.45 μM holoenzyme or 0.53 μM apoenzyme in 0.01 M Tris-HCl buffer, pH 8.3. ( A ) : I, holoenzyme; II, salicylate; III, holoenzyme + salicylate. ( B ) : I, apoenzyme; II, salicylate; III, apoenzyme + salicylate; IV, FAD; V, apoenzyme + salicylate + FAD.

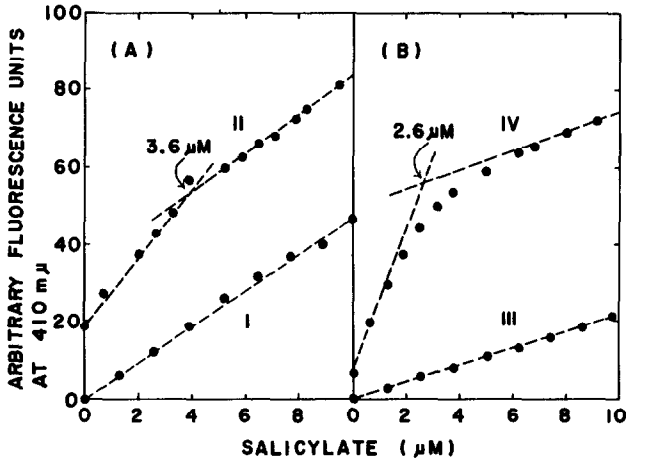


Fig. 2-A and -B. Fluorometric titration of holoenzyme ( A ) or apoenzyme ( B ) with salicylate. Fluorescence, activated at 280mμ, was measured at 410mμ. Titrations were carried out with 3.1 μM holoenzyme ( II ) or 2.5 μM apoenzyme ( IV ) in 0.01 M Tris-HCl buffer, pH 8.3. Curves I and III are calibration curves of salicylate measured in the absence of enzyme.

was enhanced by salicylate and a definite inflection occurred at the equivalent point. Thus 1 mole of salicylate is bound to 1 mole of holoenzyme in the fluorescent complex, and so the ratio of apoenzyme, FAD and salicylate in the complex is 1 : 1 : 1. This is in good agreement with the spectrophotometric results described previously ( Katagiri et al., 1966 ).

When the apoenzyme was excited by light at 280mμ, a fluorescence with a maximum at 325mμ was emitted ( Fig. 1-B, Curve I ). This fluorescence was considerably larger than that given when the holoenzyme was activated by light at the same wavelength. On addition of salicylate to the apoenzyme, spectral changes obtained were closely similar to those observed with the holoenzyme ( Fig. 1-B, Curve III ). Quite similar spectral changes were also observed on addition of 2, 5-dihydroxybenzoate or p-aminosalicylate. However, no substance which was not a substrate for the enzyme affected the original spectrum of the apoenzyme. The appearance of the 410-mμ peak on addition of salicylate to the apoenzyme seems to depend upon the formation of the salicylate-apoenzyme complex. The stoichiometry of the components of the complex could be determined by titration of the apoenzyme with salicylate. As shown in Fig. 2-B, a sharp inflection appeared at the equivalent point. These results indicate that the apoenzyme combines with a molar equivalent of substrate to form a binary complex. This conclusion is in agreement with results of a stability test of the apoenzyme in the presence and absence of salicylate. The apoenzyme in the presence of salicylate was found to be more resistant to acid and heat inactivation and proteolytic digestion ( Takemori , Sei, Yasuda and Katagiri, unpublished work ). This binary complex was enzymatically active and hydroxylated the substrate when FAD was added to the system. When FAD was added to this binary complex, a very marked decrease in the intensity of the fluorescence of the reaction mixture was

observed ( Fig. 2-B, Curve V ). Since the fluorescence emission spectrum of the reaction mixture exhibits a spectrum identical to that of the ternary complex, the decrease in fluorescence of the binary complex on addition of FAD seems to be due to the formation of the ternary complex ( apoenzyme, FAD and salicylate ) from FAD and the binary complex ( apoenzyme and salicylate ). When FAD was added to a solution of the binary complex, the fluorescence decreased in proportion to the amount of FAD added until a constant minimal level, as shown in Fig. 3. The calculations from the value of the break in the titration curve also gave a ratio of FAD to binary complex of 1.

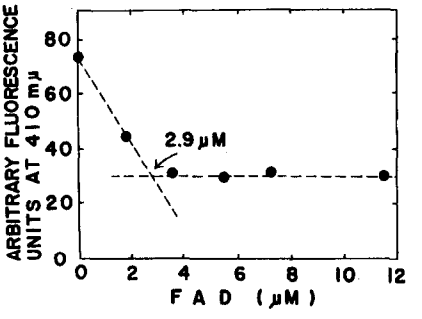


Fig. 3. Fluorometric titration of the binary complex with FAD. Fluorescence, activated at 280mμ, was measured at 410mμ. Titrations were carried out with 3.0 μM apoenzyme in the presence of 29 μM salicylate in 0.01 M Tris-HCl buffer, pH 8.3.

Since fluorometric analysis has proved a useful method for studies on several enzymes ( e. g. Yonetani and Theorell, 1962; Sund and Theorell, 1963; Schwert and Winer, 1963 ), from fluorometric studies on these binary and ternary complexes it may be possible to elucidate the nature and position of the substrate and FAD binding sites of salicylate hydroxylase.

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