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MECHANISM OF THE SALICYLATE HYDROXYLASE REACTION*

IV. FLUOROMETRIC ANALYSIS OF THE COMPLEX FORMATION

KENZI SUZUKI, SHIGEKI TAKEMORI AND MASAYUKI KATAGIRI
Department of Chemistry, Faculty of Science, Kanazawa University, Kanazawa 920 (Japan)

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SUMMARY

1. Binary and ternary complexes between apoenzyme of salicylate hydroxylase (salicylate, NADH:oxygen oxidoreductase (1-hydroxylating, 1-decarboxylating)) and components such as FAD, substrate and NADH were fluorometrically studied to determine dissociation constant and stoichiometric ratio of each component. This was based on the changes in fluorescence intensity when the apoenzyme was mixed with these components.

2. A protein-denaturing agent or an alkali treatment was shown to depress the fluorescence emission of the apoenzyme with the red shift of the maximum.

3. The addition of FAD to the apoenzyme resulted in a marked decrease in both FAD and protein fluorescences.

4. Either substrate or NADH formed 1:1 complex with the apoenzyme. This was indicated by both quenching of protein fluorescence and enhancement of substrate or NADH fluorescence.

5. The holoenzyme combined specifically with substrate to form a fluorescent ternary complex in which the ratio of apoenzyme, FAD and substrate was 1:1:1.

6. Upon formation of these complexes, the blue shift occurred in the fluorescence spectrum of protein, salicylate, NADH or FAD.

INTRODUCTION

To elucidate the mechanism of enzymatic catalysis, it is essential to study the intermediate complexes of an enzyme and a substrate. Salicylate hydroxylase (salicylate, NADH:oxygen oxidoreductase (1-hydroxylating, 1-decarboxylating)) is a particularly appropriate enzyme for such a study. The formation of the complex between salicylate hydroxylase and substrate is characterized by the appearance of a new shoulder at 480 nm in the absorption spectrum[2,3]. This change in spectrum has been used to determine the stoichiometry and dissociation constant of the complex as described in the preceding paper[3].

* A preliminary report of this work has been presented[1].

This report presents the results of fluorometric experiments which indicate that binary and ternary complexes are formed between salicylate hydroxylase apoenzyme and components such as FAD, substrate and NADH. These measurements have also made it possible to determine the stoichiometries and dissociation constants of the various complexes.

MATERIALS AND METHODS

Holo- and apoenzymes of salicylate hydroxylase were prepared by the methods described in the preceding paper[3]. Guanidine·HCl, urea, 2-chloroethanol, dioxane and riboflavin were obtained from Wako Pure Chemical Co., Osaka. NAD^+, NADH, $NADP^+$ and NADPH were purchased from Calbiochem. Co. Other chemicals were from the same sources as described in the preceding papers[3,4]. FAD, FMN and riboflavin were further purified by gel filtration on a column (1.5 cm × 60 cm) of Sephadex G-15 (Pharmacia) employing 10 mM Tris–HCl buffer (pH 8.0).

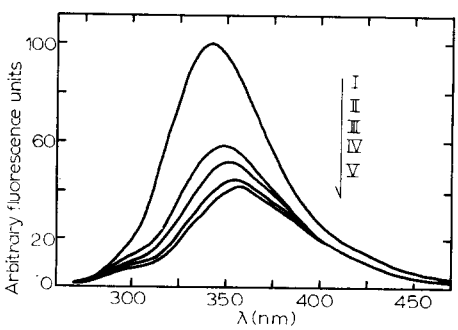
The fluorometric measurements were performed in a Farrand spectrofluorometer, in which a xenon arc lamp (150 W) was used as the exciting source and the slit width was 5–20 nm. All wavelengths reported were uncorrected. All measurements were performed in 10 mM Tris–HCl buffer (pH 8.0) at 20° with the use of quartz cuvettes of 10- and 2-mm light paths.

The enzyme activity of salicylate hydroxylase was determined according to the method described previously[5].

RESULTS

Fluorescence characteristics of the apoenzyme

As shown in Fig. 1, the fluorescent emission spectrum of the apoenzyme was formed with maximum at 342 nm when activated by light of 292 nm. Upon the addition of guanidine to a solution of the apoenzyme, the fluorescence was quenched with the red shift of the maximum to 360 nm (Curves II–V in Fig. 1). In the presence of 7.5 M



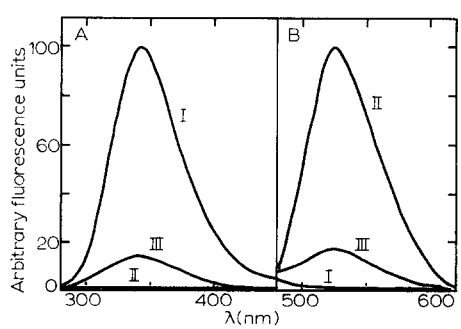


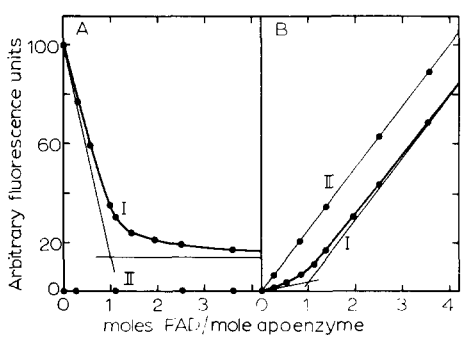
Fig. 1. The effect of guanidine on the emission spectrum of the apoenzyme activated at 292 nm. 4 μM apoenzyme and guanidine were mixed in 10 mM Tris–HCl buffer (pH 8.0) at 20°. The concentrations of guanidine used were as follows: I, 0; II, 1.5 M; III, 2.0 M; IV, 4.0 M; V, 7.5 M.

Fig. 2. Emission spectra of the apoenzyme, FAD and their mixture activated at 292 nm (A) and at 450 nm (B). I, 7.0 μM apoenzyme; II, 7.4 μM FAD; III, apoenzyme + FAD.

guanidine, the fluorescence of the apoenzyme decreased to about 40% of the original intensity. These spectral changes proceeded instantly at room temperature, and further incubation showed no appreciable effect on the fluorescence intensity. Similar effects were observed with protein-denaturing agents such as urea, 2-chloroethanol and dioxane. However, these spectral changes were not induced by increasing an ionic strength in the solvent; no appreciable effects were observed even with 4 M KCl. The fluorescence intensity at 342 nm of the apoenzyme was quenched at alkaline pH values above 9. At pH 13.8, it decreased to about 20% of the original value with the red shift of the maximum to 350 nm.

Holoenzyme formation

Fig. 2 illustrates the fluorescence behavior of the complex between the apoenzyme and FAD. When FAD was added to the apoenzyme solution, the protein fluorescence at 342 nm markedly decreased to 14% of the original intensity (Fig. 2A). The FAD solution emitted fluorescence light at 525 nm when activated at a wavelength of 450 nm. This fluorescence was also quenched by the apoenzyme to 17% of the original intensity (Fig. 2B). The blue shift of 2–3 nm occurred in the fluorescence maximum of either the apoenzyme or FAD. These results indicate that the apoenzyme combines with FAD to form the holoenzyme. Since the formation of the holoenzyme was indicated by quenching of both protein and FAD fluorescences, the stoichiometry and dissociation constant of the holoenzyme could be determined by fluorometric titration. Upon the addition of appropriate amounts of FAD to a solution of the apoenzyme (10–0.1 μM), the fluorescence at 342 nm decreased as indicated by the titration curve in Fig. 3A, and an inflection occurred at the equivalent point. When a similar titration was performed at 525-nm emission band, a clearly defined inflection occurred that indicated 1 mole of FAD was bound to 1 mole of the apoenzyme (Fig. 3B). This



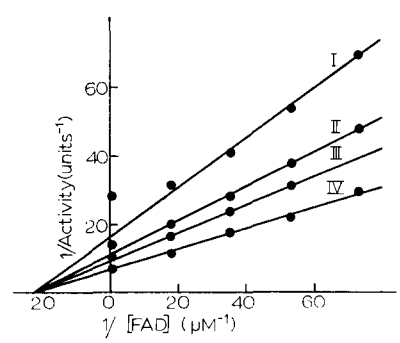


Fig. 3. Fluorometric titration of the apoenzyme with FAD. Fluorescence activated at 292 nm was measured at 342 nm (A) and at 525 nm (B), respectively. Titrations were carried out with 0.5 μM apoenzyme (closed circles in Curve I). The thick line in Curve I was calculated theoretically by using the equation: [apoenzyme] [FAD]/[holoenzyme] = 45 nM. The equivalent point is indicated by the asymptotic lines. Curve II shows the changes of fluorescence caused by FAD alone.

Fig. 4. Determination of Michaelis constant for FAD by a Lineweaver–Burk plot. The apoenzyme was incubated with varying amounts of FAD in the standard assay mixture without NADH. After 5 min preincubation at 20°, the enzymatic assay was performed by the addition of NADH. The concentrations of the apoenzyme used were as follows: I, 48 nM; II, 100 nM; III, 135 nM; IV, 241 nM.

TABLE I

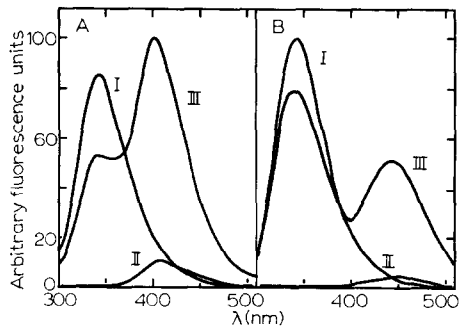
DISSOCIATION CONSTANTS AS MEASURED BY THE STERN–VOLMER EQUATION

Compound	K_d (μM)
Salicylate	1.8
3-Methylsalicylate	6.6
2,3-Dihydroxybenzoate	19
2,4-Dihydroxybenzoate	19
2,5-Dihydroxybenzoate	5.7
2,6-Dihydroxybenzoate	3.6
p-Aminosalicylate	6.8
p-Hydroxybenzoate	660
m-Hydroxybenzoate	840
o-Phenolsulfonate	23
Catechol	440
Benzoate	1160
NADH	11
NADPH	15
NAD^+	180
$NADP^+$	200
FMN	24

value is in accordance with results of equilibrium dialysis[5] or of the direct analysis of the holoenzyme[2]. The dissociation constant of FAD in the holoenzyme could be obtained from these curves. It was calculated to be 45 nM. In order to confirm this value, the Michaelis constant for FAD was determined from changes of hydroxylase activity with variation of FAD and enzyme concentration in the assay system. Fig. 4 illustrates a typical Lineweaver–Burk plot for FAD at different concentrations of the apoenzyme. The K_m value for FAD was found to be 45 nM, which agreed with the dissociation constant obtained from the fluorometric titrations. When FMN at 10 μM level, which was ineffective as a coenzyme of salicylate hydroxylase, under the standard assay conditions was added to the apoenzyme (0.5 μM), the protein fluorescence was also quenched. The dissociation constant of FMN was found to be 24 μM as shown in Table I. These results indicate that the apoenzyme may bind FMN although much less tightly than FAD.

Formation of the apoenzyme–substrate complex

Fig. 5A illustrates the emission spectra of the apoenzyme, of salicylate and of their mixture activated at 292 nm. Salicylate quenched the protein fluorescence at 342 nm, while the apoenzyme enhanced the salicylate fluorescence at 405 nm (Curve III in Fig. 5A). The blue shift of 2–3 nm occurred in the fluorescence maximum of either the apoenzyme or salicylate. The maximum of the excitation spectrum of salicylate was at 305 nm, so that these phenomena seem to be due to the energy transfer. Quite similar spectral changes were also observed when salicylate was replaced by any one of the other substrates for the hydroxylase reaction, namely 2,5-dihydroxybenzoate, 3-methylsalicylate and *p*-aminosalicylate or by a competitive inhibitor, *o*-phenolsulfonate. The emission spectra obtained with 2,5-dihydroxybenzoate are shown in Fig. 5B. However, other substances such as benzoate, *m*-hydroxybenzoate and catechol did not affect the original spectrum of the apoenzyme at 10 μM concentration levels. These results indicate that the apoenzyme combines specifically with the substrate or



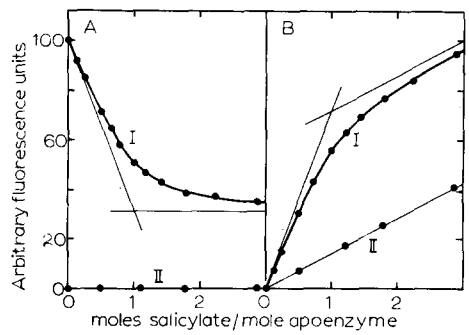


Fig. 5. Emission spectra of the apoenzyme, substrate and their complex activated at 292 nm. A. I, 0.53 μM apoenzyme; II, 3.1 μM salicylate; III, apoenzyme + salicylate. B. I, 0.53 μM apoenzyme; II, 3.1 μM 2,5-dihydroxybenzoate; III, apoenzyme + 2,5-dihydroxybenzoate.

Fig. 6. Fluorometric titration of the apoenzyme with salicylate. Fluorescence activated at 292 nm was measured at 342 nm (A) and at 405 nm (B), respectively. Titrations were carried out with 15 μM apoenzyme (closed circles in Curve I). The thick line in Curve I was calculated theoretically by using the equation: [apoenzyme] [salicylate]/[binary complex] = 1.8 μM. The equivalent point is indicated by the asymptotic lines. Curve II shows the change of fluorescence caused by salicylate alone.

with the inhibitor to form a fluorescent binary complex. This conclusion is consistent with the evidence that the apoenzyme is more resistant to acid and heat inactivations and to proteolytic digestion in the presence of substrate[3].

Since the formation of the complex was indicated by both quenching of the protein fluorescence at 342 nm and enhancement of the salicylate fluorescence at 405 nm, the stoichiometry of the apoenzyme and salicylate in the complex could be determined by titration of the apoenzyme with salicylate. It was graphically derived from the titration curves of Fig. 6 that 1 molecule of salicylate is bound to 1 molecule of the apoenzyme in the fluorescent binary complex. Furthermore, the equilibrium dialysis (K. SUZUKI AND M. KATAGIRI, unpublished work) was adopted to confirm this ratio. The number of molecules of salicylate bound to 1 molecule of the apoenzyme was calculated to be approx. 1 molecule. The dissociation constant of salicylate in the binary complex was calculated to be 1.8 μM when the level of the apoenzyme concentration was 1.0–15 μM. The dissociation constant was also analyzed by using the Stern–Volmer equation[6]:

$$K_d = \frac{Q}{F^\circ/F - 1}$$

where F° and F mean protein fluorescence in the absence or presence of substrate, and Q represents a concentration of substrate. A typical plot in the case of salicylate is illustrated in Fig. 7A. The dissociation constant of salicylate, derived by this equation, was 1.8 μM, which was in accordance with the value obtained by the fluorometric titration. The calculated values of dissociation constant for various substrates are given in Table I.

Formation of the apoenzyme–pyridine nucleotide complex

The emission spectra of the apoenzyme, NADH and their binary complex

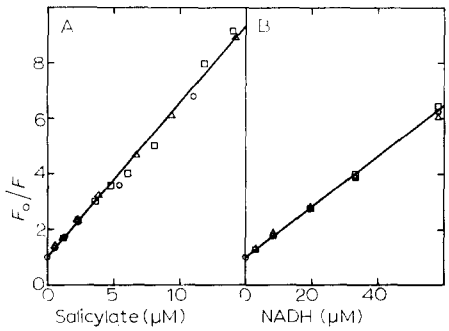
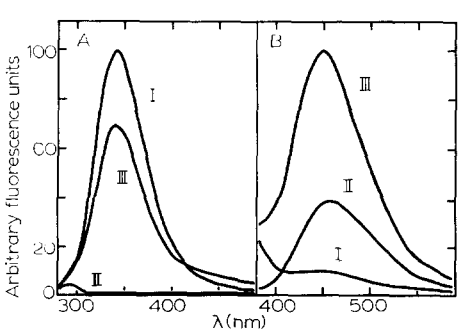


Fig. 7. Determination of dissociation constant of the complex by the Stern–Volmer equation. A. 0.1 μM (○), 0.5 μM (△), and 1 μM (□) apoenzyme were mixed with varying amounts of salicylate as indicated. B. 0.5 μM (○), 1 μM (△) and 4 μM (□) apoenzyme were mixed with varying amounts of NADH as indicated. F° and F are the fluorescence intensities at 342 nm in the absence and presence of salicylate when activated at 292 nm. In the case of A, the fluorescence intensity remaining at the large excess of salicylate was deducted from F° and F.

activated at both the protein exciting light (292 nm) and the NADH exciting light (365 nm) are illustrated in Fig. 8. The protein fluorescence was quenched by NADH (Fig. 8A), while the NADH fluorescence having the maximum at 460 nm was considerably enhanced by the apoenzyme (Fig. 8B). There was the blue shift of 2–3 nm at the emission maximum in both cases.

NADPH, which was less effective than NADH as an electron donor of salicylate hydroxylase[5], gave the similar fluorescence behavior. However, oxidized pyridine nucleotides such as NAD^+ and $NADP^+$ gave no effect on the emission spectrum of the apoenzyme at 10 μM concentration levels.

As shown in Fig. 9, the results from the titration at the concentration level of 1–40 μM apoenzyme indicate that 1 molecule of NADH is bound to 1 molecule of the apoenzyme in the fluorescent binary complex. Because of the high dissociation



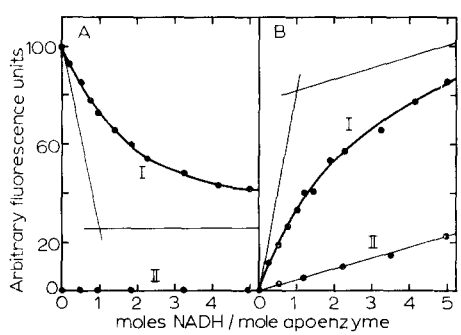


Fig. 8. Emission spectra of the apoenzyme, NADH and their complex activated at 292 nm (A) and at 365 nm (B). I, 4 μM apoenzyme; II, 4 μM NADH; III, apoenzyme + NADH.

Fig. 9. Fluorometric titration of the apoenzyme with NADH. Activation was at 292 nm (A) or at 365 nm (B). Fluorescence intensities were measured at 342 nm (A) or at 452 nm (B), respectively. Titrations were carried out with 10 μM apoenzyme (closed circles in Curve I). The thick line in Curve I was calculated theoretically by using the equation: ([apoenzyme] [NADH])/[binary complex] = 11 μM. The equivalent point is indicated by the asymptotic lines. Curve II shows the change of fluorescence caused by NADH alone.

constant of this complex, the definite inflection could not be observed even at 40 μM apoenzyme, but the titration curves at several concentrations of the apoenzyme agreed with the theoretical curve calculated on the assumption of a 1:1 stoichiometry for the binary complex. The dissociation constant of NADH in the complex was calculated to be 11 μM. This value was further confirmed by using the Stern–Volmer equation (*cf.* Fig. 7B). It was found to be 11 μM at the concentration level of 1–40 μM apoenzyme. However, the affinity of oxidized pyridine nucleotides to the apoenzyme was less than that of NADH and NADPH. The calculated values of dissociation constant are summarized in Table I.

The formation of the ternary complex of apoenzyme, FAD and substrate

When the holoenzyme was excited at 292 mμ, the emission spectrum had two maxima, one (at 340 nm) due to the protein fluorescence and the other (at 523 nm) due to the FAD fluorescence. When salicylate was added to the holoenzyme, the spectral changes produced were quite different from those of the binary complex mentioned above. The protein and salicylate fluorescences were quenched to 80 and 50% of the original intensity, respectively (Fig. 10A and 10B), but FAD fluorescence excited at 450 nm was enhanced to 165% of the original intensity (Fig. 10C). The emission maxima at salicylate and FAD fluorescences were blue-shifted to 15 and 2–3 nm, respectively. These changes in fluorescence spectra were measured under the condition that once added, the third component was incorporated into a ternary complex, leaving hardly any excess of the free component.

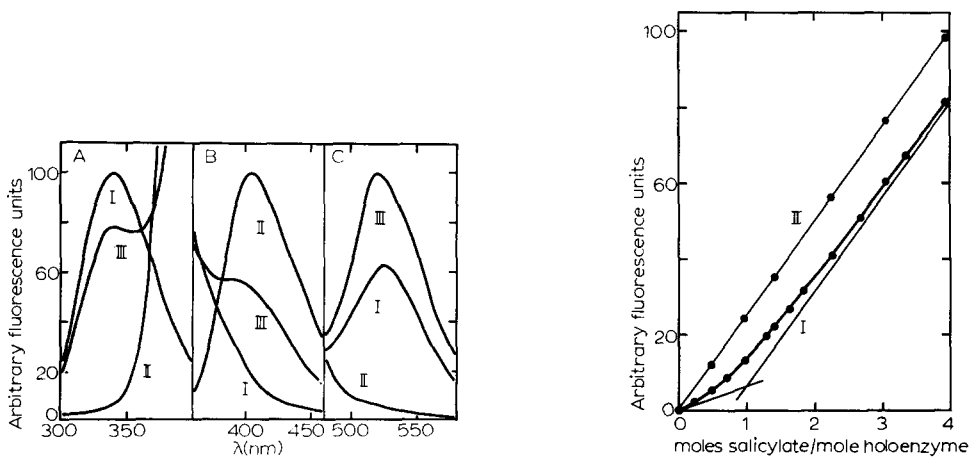


Fig. 10. Emission spectra of the holoenzyme, salicylate and their complex. Activation wavelengths were 292 nm (A and B) and 450 nm (C), respectively. A. I, holoenzyme (7.0 μM apoenzyme + 7.4 μM FAD); II, 20 μM salicylate; III, holoenzyme + salicylate. B. I, holoenzyme (5.6 μM apoenzyme + 7.4 μM FAD); II, 3.9 μM salicylate; III, holoenzyme + salicylate. C. I, holoenzyme (6.1 μM apoenzyme + 5.0 μM FAD); II, 20 μM salicylate; III, holoenzyme + salicylate.

Fig. 11. Fluorometric titration of holoenzyme with salicylate. Fluorescence activated at 292 nm was measured at 405 nm. Titrations were carried out with the mixture of 10 μM apoenzyme and 58 μM FAD (closed circles in Curve I). The thick line in Curve I was calculated theoretically by using the equation: ([holoenzyme] [salicylate])/[ternary complex] = 3.2 μM. The equivalent point is indicated by the asymptotic lines. Curve II shows the change of fluorescence caused by salicylate alone.

The addition to the holoenzyme of 2,5-dihydroxybenzoate, *p*-aminosalicylate and *o*-phenolsulfonate caused similar spectral changes. However, no effect was observed when substances which were not substrates for the hydroxylation reaction were added. These results indicate that the holoenzyme combines specifically with the substrate to form a fluorescent ternary complex.

As shown in Fig. 11, the initial slope of the titration curve was depressed by salicylate and a definite inflection occurred at the equivalent point. Thus 1 molecule of salicylate is bound to 1 molecule of the holoenzyme, and so the ratio of apoenzyme, FAD and salicylate in the ternary complex is 1:1:1. The dissociation constant of salicylate in the ternary complex was calculated to be 3.2 μM. This value coincides well with that determined by spectrophotometric titration[3].

DISCUSSION

The apoenzyme of salicylate hydroxylase showed the fluorescent emission spectrum with a maximum around 342 nm when activated by light at 292 nm. Since, under these conditions, the emission maxima of tryptophan and tyrosine were 348 and 304 nm, respectively, this observation strongly indicates that the fluorescence behavior of the apoenzyme depends on tryptophan residues of the protein moiety. A red shift of the emission peak with decrease of fluorescence intensity was induced by the addition of denaturing agents for proteins. These effects have been observed in the denaturation of other proteins such as human and bovine serum albumins[7]. On the contrary, the denaturing agents enhanced the protein fluorescence of trypsin and chymotrypsin[7]. These results were interpreted by STEINER AND EDELHOCH[8] as meaning that the intensity of fluorescence of the tryptophan residues is a sensitive function of their environment. Therefore, the fluorescence changes caused by denaturing agents here may be regarded as a reflection of a conformational change of the protein near to the tryptophan residues.

The fluorescence of the apoenzyme at 342 nm was quenched by either FAD, substrate or pyridine nucleotide. There was a detectable blue shift of the emission maximum in the case of FAD or pyridine nucleotide, although the red shift was observed in the presence of the denaturing agents. The fluorescence maximum of tryptophan has been reported to be at 355 nm in an aqueous solution but to be blue-shifted to 335 nm upon increasing the polarity of the solution[7]. Therefore, the above phenomena of the apoenzyme may be interpreted as indicating that the complex formation of the apoenzyme makes the surrounding of tryptophan residues of the protein more nonpolar, while the denaturing agent or high pH makes it more polar. The blue-shift was also observed in the case of salicylate, NADH and FAD fluorescences when the apoenzyme was added to these compounds. These results may indicate the increment of the nonpolarity in the regions of binding sites of these compounds.

Although the rate of the reaction between apoenzyme and salicylate or NADH was rapid, the reaction between apoenzyme and FAD was considerably slower and the reaction required more than a few minutes at room temperature to go to completion at the concentration level of 0.1 μM apoenzyme. In the enzymatic assay of the apoenzyme with excess FAD, it was also observed that maximum regeneration of enzyme activity required prolonged incubation. These phenomena suggest that conformational

changes in the apoenzyme probably occur during the incubation period which generate a structure capable of binding FAD.

Now considering the fluorescence behavior of the apoenzyme, it may be suggested that three different types of sites exist in the apoenzyme. There are (a) the substrate-binding site, (b) the pyridine nucleotide-binding site and (c) a site which appears to be specific for FAD. Titration experiments have shown that the apoenzyme combines with salicylate at a molar ratio of 1 and that the dissociation constant for salicylate is 1.8 μM, somewhat lower than the value of 3.2 μM calculated in the holoenzyme complex. As shown in Table I, the dissociation constants for other substrates were approx. 10–1.0 μM and were somewhat higher than the value obtained with salicylate. On the other hand, the dissociation constants for the substrate analogues, such as benzoate or *m*- and *p*-hydroxybenzoates were in the range of 1.0–0.1 mM. It should be noted that a reaction product, catechol, exhibits a low affinity similar to those described above. These results indicate that there appears to be the relationship between the dissociation constant and the substrate specificity of the hydroxylation reaction.

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