

## Interaction of 2-Methyl-3-hydroxypyridine-5-carboxylic Acid Oxygenase with FAD, Substrates, and Analogues

SPECTRAL AND FLUORESCENCE INVESTIGATIONS\*

(Received for publication, August 25, 1980, and in revised form, December 30, 1980)

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Homogeneous preparations of 2-methyl-3-hydroxypyridine-5-carboxylic acid (Cpd I) oxygenase (EC 1.14.12.4) were resolved by treatment with acid ammonium sulfate into apooxygenase and FAD. Gel electrophoresis showed that holoenzyme ( $M_r = 166,000$ ) and apoenzyme ( $M_r = 83,000$ ) correspond to tetrameric and dimeric forms, respectively, of a single 42,000-dalton subunit found on denaturation with sodium dodecyl sulfate. Two FAD molecules/tetramer were found by direct measurement, and by spectrophotometric and fluorescent titration of apoenzyme with FAD. The latter measurements both gave  $K_d$  of  $0.45 \mu\text{M}$  for FAD, in good agreement with the value of  $0.41 \mu\text{M}$  obtained by activity measurements.

Interaction of Cpd I with holoxygenase perturbs the ultraviolet and visible absorption spectra of both enzyme and substrate, and increases the fluorescence due to enzyme-bound FAD. From these changes, the ratio of Cpd I to FAD in the complex appeared to be 1:1, and the  $K_d$  for Cpd I (pH 7.0,  $18^\circ\text{C}$ ) to be  $15.5 \mu\text{M}$ , in agreement with the kinetically determined value of  $22 \mu\text{M}$ . Cpd I in the complex is located close to FAD, as evidenced by energy-transfer measurements. 5-Pyridoxic acid competitively antagonizes formation of the Cpd I-holoenzyme complex, with a  $K_i$  value ( $23 \mu\text{M}$ ) identical with that found in kinetic studies.

NADH has no effect on the visible or fluorescent spectrum of FAD in the holoenzyme, but does interact with the holoenzyme in a slow, catalytically irrelevant manner as evidenced by a reduction in its own fluorescence. The derived  $K_d$  value,  $240 \mu\text{M}$ , is far higher than that ( $60 \mu\text{M}$ ) obtained by kinetic measurements. The results support the kinetic mechanism for oxygenase action described by Kishore and Snell (Kishore, G. M., and Snell, E. E. (1981) *J. Biol. Chem.* 255, 4228-4233) which showed that catalytically effective binding of NADH occurs only to the Cpd I-holoenzyme complex.

In the presence of NADH and  $\text{O}_2$ , 2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase from *Pseudomonas* sp. MA-1 (ATCC 33286) catalyzes the FAD-dependent oxygenation of Cpd I<sup>1</sup> to yield  $\alpha$ -N-(acetylaminomethylene)succinic acid and  $\text{NAD}^+$  as reaction products (1). The overall reaction is the sum of two partial reactions: (a) a Cpd I-dependent electron

transfer from NADH to FAD of the oxygenase and (b) reoxidation of reduced FAD with concomitant oxygenation and reduction of Cpd I to yield Cpd A (1, 2). The reductive phase of the oxygenase-catalyzed reaction follows an ordered mechanism with the binding of Cpd I preceding that of NADH (2).

No change in the absorption spectrum of the enzyme following addition of Cpd I was observed by techniques used in a previous study (1). However, by using larger amounts of enzyme and more sensitive techniques, we found that Cpd I perturbs both absorption and fluorescence spectra of the enzyme, in a manner similar to that reported (3) for other flavoproteins. We report here the dissociation constants determined from these perturbations, which support the previously reported kinetic mechanism for the oxygenase reaction (2), together with some molecular properties of the holo- and apooxygenase.

### EXPERIMENTAL PROCEDURES

#### Materials

Homogeneous Cpd I oxygenase (EC 1.14.12.4) and Cpd I were prepared as previously described (1, 2). 1-Deaza-FAD and 5-deaza-FAD were gifts of Dr. C. T. Walsh; other chemicals were from commercial sources.

#### Methods

Cpd I oxygenase was assayed by either oxygen uptake (2) or dual wavelength absorption measurements (4). Protein concentrations were determined by the Lowry method (5) and compared with values obtained from flavin analysis ( $E_{452} = 13.11 \text{ mM}^{-1} \text{ cm}^{-1}$ ) and  $A_{280}^{1 \text{ cm}}$  measurements (1.7 for 1 mg/ml).

Absorption spectra were recorded in a Cary 118-C double beam spectrophotometer equipped with thermostatted cuvette chambers and interfaced to a microcomputer system which stored spectra on magnetic discs and permitted difference spectra to be generated by subtraction of one spectrum from another.

Fluorescence spectra were recorded (in the ratio mode whenever possible) by use of a temperature-controlled Aminco SPF-500 spectrofluorometer equipped with a 250-watt xenon lamp. All spectra are uncorrected for phototube response. The absorbance of the sample was maintained below 0.05 to minimize light losses due to internal absorption and consequent quenching.

Cpd I holoxygenase was converted to apoenzyme as follows: 1 ml of the oxygenase solution ( $\geq 3 \text{ mg}$  of protein/ml) in 1 mM dithiothreitol, 0.025 M K-phosphate buffer, pH 7.0, was mixed with 2 ml of a saturated ammonium sulfate, 1 mM dithiothreitol solution, adjusted with  $\text{H}_2\text{SO}_4$  to pH 3.0 just before use and immediately centrifuged. The pellet was washed once with 1 ml of saturated ammonium sulfate, 1 mM dithiothreitol, pH 7.0, then redissolved in the K-phosphate buffer. After a second acid-ammonium sulfate treatment, followed by washing with neutral ammonium sulfate, the protein was redissolved in 1 ml of 0.025 M K-phosphate, 10 mM dithiothreitol buffer, pH 7.0, and desalted over a Sephadex G-26 column ( $2.5 \times 20 \text{ cm}$ ) equilibrated with the same buffer. Insoluble protein was removed by centrifugation and the supernatant solution was stored at  $4^\circ\text{C}$  until use. The apooxygenase was unstable, losing half of its activity (as measured

\* These studies were supported by grants from the Robert A. Welch Foundation (Grant F-714) and the United States Public Health Service (Grants AM 19898 and AI 13940). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> The abbreviations used are: Cpd I, 2-methyl-3-hydroxypyridine-5-carboxylate; Cpd A,  $\alpha$ -N-(acetylaminomethylene)succinic acid.

after reconstitution with FAD) in about 12 h. For most studies it was prepared just before use.

## RESULTS

### Subunit Composition of Cpd I Oxygenase

Polyacrylamide gel electrophoresis of the denatured dioxygenase in sodium dodecyl sulfate under reducing conditions yields a single band (Fig. 1A) of  $M_r = 43,000$  (Fig. 1B). Since sedimentation equilibrium studies (1) and electrophoresis of the native enzyme in acrylamide gels gave  $M_r = 160,000$  to 166,000, the holoprotein appears to contain four subunits.

At pH 8.3 the apooxygenase has a higher electrophoretic mobility than either the FAD-reconstituted apooxygenase or unresolved holoenzyme (Fig. 2). This increased mobility reflects a decreased molecular size, since gel filtration yields a  $M_r = 83,000$  for the apoenzyme (Fig. 3), corresponding to a dimer of the protomers obtained by denaturation in sodium dodecyl sulfate.

### Binding of FAD to Apoenzyme

Previous determinations yielded a value of 1.84 FAD/166,000 daltons of the holoxygenase (1). Because of the presence of four subunits in the native enzyme, we redetermined this stoichiometry as follows.

**Kinetic Studies**—After two acid-ammonium sulfate treatments (see "Methods"), the apoenzyme retained about 2 to 5% of its original activity; addition of FAD at this point regenerated about 90% of the original activity (Table I). Further treatments with acid-ammonium sulfate led to complete inactivation of the oxygenase. The extent of coupling of NADH oxidation to oxygenation of Cpd I was not changed by resolution and reconstitution of the enzyme.

**Determination of oxygenase activity as a function of FAD concentration** gave a value of 412 nm for the kinetic  $K_m$  for FAD (Fig. 4). There was no evidence of cooperativity during reconstitution of oxygenase activity, although the molecular weight doubles during this process.

**Fluorescence Studies**—On complex formation with apooxygenase the fluorescence emission maximum of FAD at 530 nm is shifted to 520 nm; quenching of flavin fluorescence is observed at all excitation wavelengths in the range of 360 to 485 nm. The time course of the fluorescence change is almost identical with that in activity ( $t_{0.5} = 13.5$  s, inset, Fig. 5),

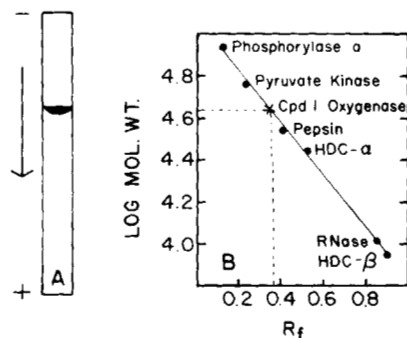


FIG. 1. Gel electrophoresis of denatured Cpd I oxygenase. A, holoxygenase (5  $\mu$ g), denatured in 1% sodium dodecyl sulfate and 100 mM 2-mercaptoethanol, was subjected to electrophoresis for 5 h at room temperature in a 10% polyacrylamide gel using Tris/glycine buffer (6), pH 8.8, and constant current of 20 mA. The gel was stained with Coomassie blue R-250 (7). B, molecular weight of the denatured subunit of Cpd I oxygenase. The electrophoretic mobilities ( $R_f$ ) of standard proteins and Cpd I oxygenase were determined after denaturation with sodium dodecyl sulfate, as described by Laemmli (6). Abbreviations: RNase, ribonuclease; HDC- $\alpha$  and  $\beta$ , the  $\alpha$  and  $\beta$  subunits of bacterial histidine decarboxylase (8).

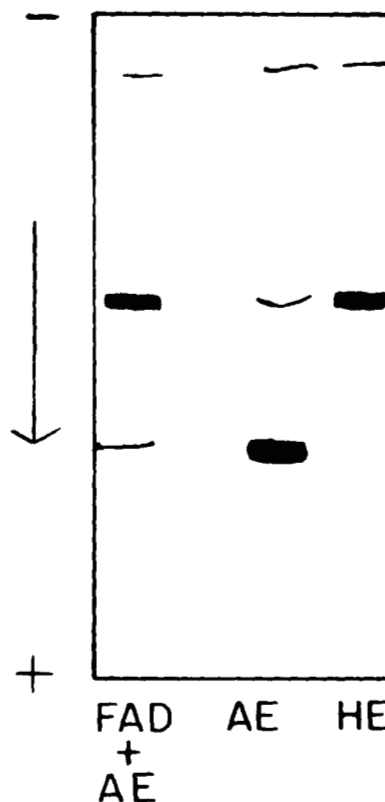


FIG. 2. Electrophoresis of unresolved Cpd I holoxygenase (HE), apooxygenase (AE), and FAD-reconstituted apoenzyme (FAD + AE). Electrophoresis was performed in 7.5% polyacrylamide gels in Tris/glycine buffer (pH 8.3) at a constant current of 10 mA. About 10  $\mu$ g of protein was used for electrophoresis and the proteins were stained with Coomassie blue.

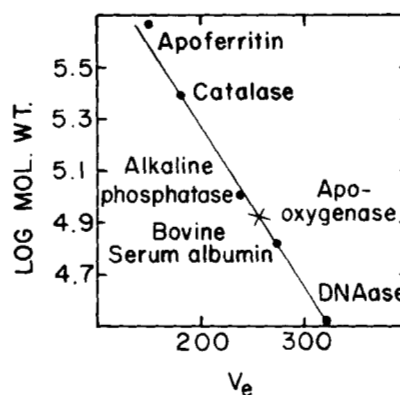


FIG. 3. Molecular weight of Cpd I apooxygenase. A column of Sephacryl S-300 superfine (2.5  $\times$  71.3 cm) was equilibrated with 0.05 M K-phosphate/10 mM dithiothreitol buffer, pH 7.0, at 4  $^{\circ}$ C. Two mg of each protein in 5 ml of the equilibration buffer was layered on top of the column and 30-drop fractions were collected at a rate of 12 ml/h. Elution volumes and protein identity were determined by optical density, enzymatic activity, or both.

indicating that the quench-complex represents the holoenzyme. The  $K_d$  for FAD (468 nm) calculated from Fig. 5 agrees well with that derived from kinetic studies (412 nm). Similar results (not shown) were obtained when the quenching of protein fluorescence was followed.

**Absorption Spectral Studies**—The major differences between the visible absorption spectrum of Cpd I holoxygenase and that of free FAD include the appearance of a shoulder near 485 nm and a shift in the absorption maxima of FAD at 452 and 372 nm to 455 and 387 nm, respectively, in the

TABLE I  
Resolution of Cpd I holooxygenase by acid-ammonium sulfate treatment

System <sup>a</sup>	Activity <sup>a</sup>	Coupling ratio <sup>b</sup>
1) Holooxygenase + Cpd I + NADH	41.66	1.17
2) Holooxygenase + NADH	0.00	
3) First acid-ammonium sulfate-treated enzyme + NADH + Cpd I	9.11	1.18
4) Second acid-ammonium sulfate-treated enzyme + NADH + Cpd I	1.90	1.21
5) Second acid-ammonium sulfate-treated enzyme + NADH + Cpd I + FAD	36.82	1.19
6) Second acid-ammonium sulfate-treated enzyme + NADH	1.10	

<sup>a</sup> Activity is expressed as nmol of oxygen consumed/min by 6  $\mu$ g of protein in 1 ml of 0.05 M K-phosphate buffer, pH 7.0 and 25 °C. NADH, Cpd I, and FAD were added where indicated at 200, 200 and 5  $\mu$ M, respectively.

<sup>b</sup> The molar ratio of NADH oxidized to Cpd A formed.

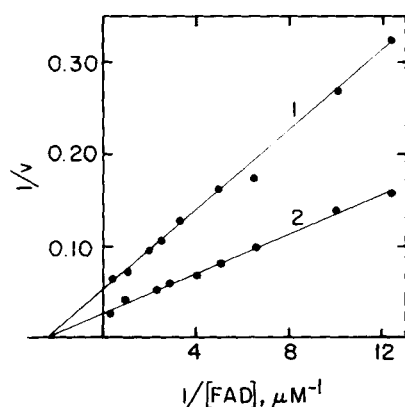


FIG. 4. Relation of activity of Cpd I apooxygenase to FAD concentration. Assays were performed in air-saturated, 0.1 M K-phosphate buffer, pH 7.0, as described under "Methods." NADH and Cpd I were both 0.2 mM; apoprotein concentrations were 3 and 6  $\mu$ g/ml in Curves 1 and 2, respectively. Velocity is expressed as nmol of oxygen consumed/min/ml.

holoenzyme, with a resultant change in the  $A_{387}/A_{452}$  ratio from 0.825 for FAD to 1.07 in the holoenzyme. From the difference spectra generated by addition of various amounts of FAD to a solution of apoprotein (Fig. 6), the molar ratio of FAD to holoenzyme ( $M_r = 166,000$ ) was established as 2:1 (inset, Fig. 6) and the  $K_d$  for FAD as 480 nM. The fluorescent quenching measurements shown in Fig. 5, although less reliable because of the gradual curvature of Curve 1, also indicate that 1 mol of FAD interacts with one mol of the dimeric apoenzyme ( $M_r = 83,000$ ). These and other spectral changes emphasize that significant changes in the environment or ionization state of FAD occur during conversion of apoenzyme to holooxygenase. Following this conversion, excess FAD was easily removed by either dialysis against 0.025 M K-phosphate, 10 mM dithiothreitol buffer, pH 7.8, or filtration on a Sephadex G-25 column equilibrated with the same buffer. Treatment of the resulting holoenzyme with 1% trichloroacetic acid, removal of the denatured protein by centrifugation, and fluorometric estimation of the FAD released confirm the stoichiometry of 2 FAD per 166,000 daltons of holooxygenase.

#### Binding of Cpd I

**Absorption Spectral Studies**—Addition of Cpd I perturbs the absorption spectrum of the holooxygenase in both the visible (Fig. 7) and ultraviolet regions (data not shown). A plot of the change in absorbance at 487 nm as a function of concentration of Cpd I indicates (with some uncertainty due

to lack of a sharp end point in the titration) (inset, Fig. 7) that 2 mol of Cpd I probably bind per mol ( $166,000 \times g$ ) of protein. No breaks were detected in either spectral titrations or kinetic experiments, indicating that there is only one kind of binding region for Cpd I. Thus, each flavin-containing active site titrates independently with Cpd I and the sites appear to be equivalent.

From the limiting regions of the binding curve (inset, Fig. 7), the  $K_d$  for Cpd I was determined to be 8 to 17  $\mu$ M and 16  $\mu$ M from a similar curve (not shown) relating  $\Delta A_{330}$  to Cpd I concentration. These values compare well with the kinetic dissociation constant for Cpd I of 22  $\mu$ M (2) indicating that the spectral complex represents a catalytically significant intermediate generated during turnover of the oxygenase.

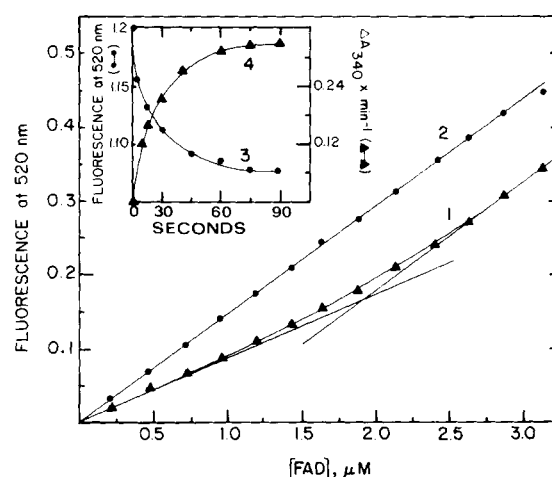


FIG. 5. Dissociation constant for interaction of apooxygenase with FAD during holoenzyme formation. Apoenzyme (1.89  $\mu$ M) in 1.5 ml of 0.05 M K-phosphate buffer (pH 7.0) was titrated with FAD and the fluorescence emission intensity at 520 nm (Curve 1) was measured after 2 min, using 452 nm as excitation wavelength. Fluorescence intensity of equal concentrations of free FAD (Curve 2) was determined under identical conditions. The inset shows the time course of changes in (a) flavin fluorescence upon addition of 19.2  $\mu$ M apoenzyme to 8.90  $\mu$ M FAD (Curve 3) and (b) activity (Curve 4) following addition of 8  $\mu$ M FAD to 4.8  $\mu$ g of apoprotein, 200 nmol of Cpd I, and 200 nmol of NADH in 1 ml of 0.05 M K-phosphate buffer, pH 7.0.

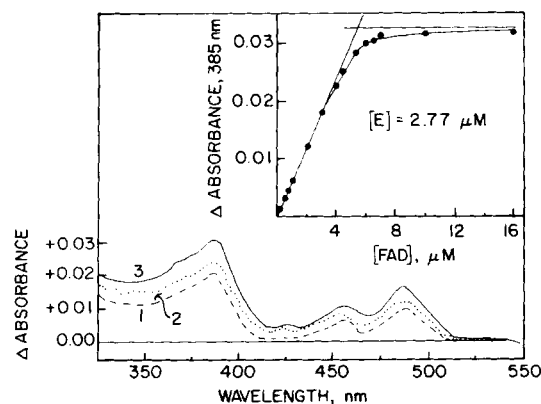


FIG. 6. Difference spectra generated by treatment of apooxygenase with FAD. To 460  $\mu$ g of apoenzyme in 1 ml of 0.05 M K-phosphate buffer, pH 7.0 and 18 °C, were added sufficient FAD to bring its final concentration to 3.5  $\mu$ M (Curve 1), 4.4  $\mu$ M (Curve 2), or 7  $\mu$ M (Curve 3). The absorption spectra of these samples were recorded against identical solutions of FAD. These spectra were then subtracted from the spectrum of apoenzyme stored in the computer. The inset shows absorbance change at 385 nm versus FAD concentration under these same conditions.

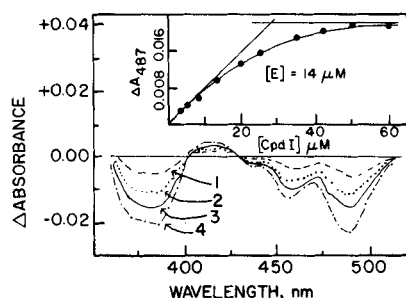


FIG. 7. Difference spectra obtained for Cpd I holooxygenase (2.32 mg in 1 ml of 0.05 M K-phosphate buffer, pH 7.0) in the presence of 6.75, 13.5, 23, or 60  $\mu\text{M}$  Cpd I (Curves 1 to 4, respectively). Spectra were recorded against buffer solutions containing identical concentrations of Cpd I. Each difference spectrum was derived by computer subtraction of these spectra from the spectrum of free holooxygenase in the same buffer. The straight line corresponding to zero absorbance changes was also generated by computer subtraction of the holooxygenase spectrum from itself. The inset shows a plot of the absorbance change at 487 nm versus concentration of Cpd I.

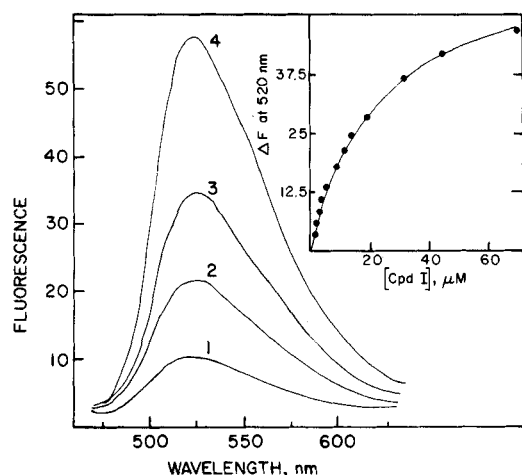


FIG. 8. Effect of Cpd I on the fluorescence emission spectrum of Cpd I oxygenase. The holooxygenase solution ( $100 \mu\text{g ml}^{-1}$  in 0.05 M K-phosphate buffer, pH 7.0) contained 0, 4.4, 14, and 72  $\mu\text{M}$  Cpd I for Curves 1 to 4, respectively. The excitation wavelength was 452 nm; measurements were made at 18  $^{\circ}\text{C}$ . The inset shows changes in flavin fluorescence emission intensity ( $\Delta F$  at 520 nm) following addition of Cpd I to the oxygenase solution.

**Fluorescence Studies**—The position of the fluorescence emission maximum of Cpd I oxygenase is altered only slightly by addition of Cpd I (Fig. 8), but its intensity at pH 7.0 increases to 60 to 70% of that observed upon release of FAD by denaturation. However, this increase is not due to dissociation of the bound coenzyme since dialysis or gel filtration and ammonium sulfate precipitation in the presence of Cpd I fails to release any FAD.

From the fluorescence titration curve (inset, Fig. 8), a  $K_d$  for Cpd I of  $15.5 \mu\text{M}$  was calculated, showing that this fluorescence derives from the same complex detected kinetically and by measurements of absorption spectra. 5-Pyridoxic acid, which is an inefficient substrate of Cpd I oxygenase (1, 2), does not increase flavin fluorescence significantly upon binding to the oxygenase, but does competitively inhibit the increase elicited by Cpd I. The  $K_i$  for this interaction ( $23 \mu\text{M}$ ) is identical within experimental error with that ( $27 \mu\text{M}$ ) found for this substrate in the oxygenase reaction (2). Since the rate of reduction of enzyme-bound flavin by NADH in the presence of 5-pyridoxic acid is only 4 to 6% of that observed with Cpd I, while the reoxidation of the reduced complex by  $\text{O}_2$  is very rapid (1), the conformational changes accompanying the in-

crease in flavin fluorescence appear to be involved in bringing about the enhanced affinity and reactivity of the oxygenase with NADH that occur (1) upon addition of Cpd I.

A comparison of the fluorescence excitation spectra of holooxygenase alone (Curve 1, Fig. 9), of holooxygenase-Cpd I complex (Curve 2), and of a mixture of FAD and Cpd I (Curve 3) reveals that only in the complex is light absorbed at 320 nm efficiently channeled for fluorescence emission at 520 nm. Since Cpd I is primarily responsible for absorption at 320 nm and FAD for emission at 520 nm, the energy must be transferred from Cpd I to FAD, indicating that the distance between them in the complex is less than about 80  $\text{\AA}$  (9).

**Effect of pH on the Affinity of Holooxygenase for Cpd I**—Throughout the range 5.5 to 9.0, where Cpd I oxygenase is stable, addition of Cpd I increased the flavin fluorescence at 550 nm. This fluorescence changes significantly between pH 6.0 and 7.5 (Fig. 10A), possibly due to ionization of a cysteine or histidine residue near the flavin in the *E*-Cpd I complex, but changes only slightly above and below this range. Although points on Curve 1, Fig. 10B are scattered below pH 7.0 they indicate that  $K_d$  for Cpd I is essentially constant between pH 5.5 and 7.0, but decreases linearly above pH 8.5. Extrapolation of these linear portions of the curve yields lines that intersect at about pH 8.1, which we interpret as the pK value of a group important for interaction of Cpd I with the holooxygenase. This conclusion is supported by the reduced activity observed for the enzyme above pH 8.0 (Curve 2, Fig. 10B). The latter decrease must reflect a decreased affinity of enzyme for Cpd I since  $V_{\text{max}}$  is fairly constant up to pH 8.7.

Spectral titrations of Cpd I showed pK values at 8.25 and 5.4 which, by analogy with those of other 3-hydroxypyridines (10), can be assigned to the pyridinium nitrogen and the phenolic oxygen, respectively. Neither of these groups can be responsible for the pH-dependent changes in the  $K_d$  of the Cpd I-holooxygenase complex, since the competitive antagonist, 6-methylnicotinic acid, whose pyridinium nitrogen has a pK of about 5.5, shows similarly decreased affinity for the oxygenase in the same pH range ( $K_i = 290, 332, 600$ , and  $>1000 \mu\text{M}$  at pH values of 6, 7, 8, and 8.4, respectively). The decreased affinity of the oxygenase for Cpd I that becomes

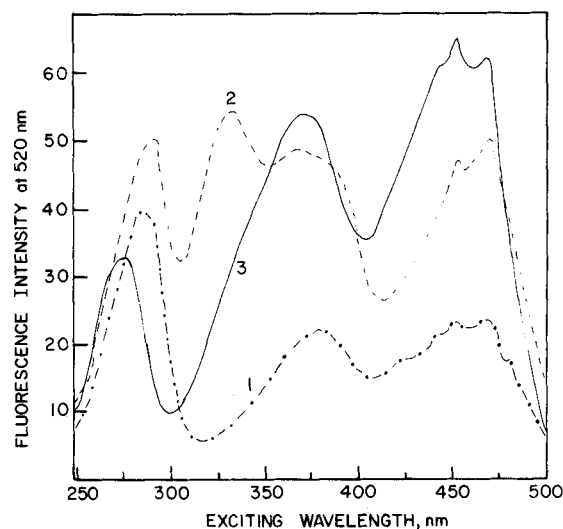


FIG. 9. Effect of Cpd I on the fluorescence activation spectra of Cpd I holooxygenase. Curve 1, 200  $\mu\text{g}$  of oxygenase/ml of 0.05 M K-phosphate buffer, pH 7.0 and 18  $^{\circ}\text{C}$ ; Curve 2, 540  $\mu\text{g}$  of oxygenase/ml of buffer (as in 1; bound FAD =  $6.5 \mu\text{M}$ ) plus 45  $\mu\text{M}$  Cpd I; Curve 3, 6.5  $\mu\text{M}$  FAD plus 48  $\mu\text{M}$  Cpd I under the same conditions as in 1. Fluorescence emission was monitored at 520 nm and 18  $^{\circ}\text{C}$ . The relative fluorescence intensities of the three spectra cannot be compared since they were recorded under different instrument settings.

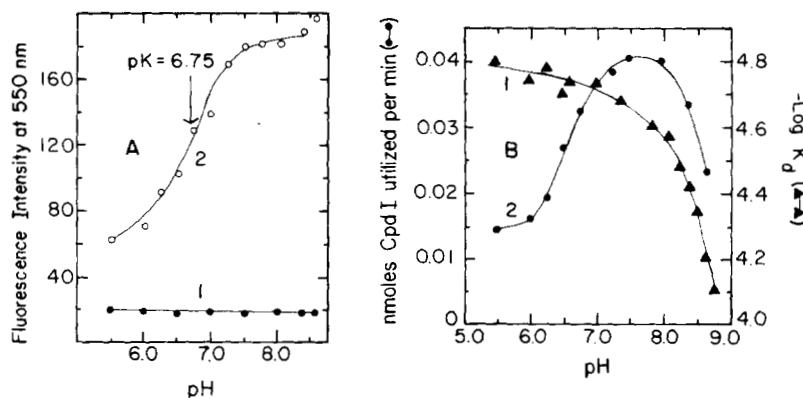


FIG. 10. Effect of pH on the fluorescence of Cpd I oxygenase and on  $K_d$  for Cpd I. A, the fluorescence intensity of Cpd I oxygenase (200  $\mu\text{g/ml}$ ) in the absence (Curve 1) or presence (Curve 2) of excess Cpd I. The values plotted in Curve 2 are theoretical maxima obtained by extrapolation of titration curves such as that shown in the inset of Fig. 8 to infinite Cpd I concentrations at each pH. The excitation wavelength was 452 nm and emission was measured at 550 nm. B, effect of pH on the  $K_d$  for dissociation of Cpd I from the Cpd I-

holoenzyme complex (Curve 1) and on activity of the enzyme (Curve 2).  $K_d$  was determined by fluorescence titrations conducted as in Fig. 8 at each pH. From a plot of reciprocal fluorescence change versus Cpd I concentration, the apparent dissociation constants could be determined by dividing the slope by the intercept. Activity was measured spectrophotometrically in air-saturated, 0.05 M K-phosphate buffers 0.02 mM in NADH and Cpd I and containing 6  $\mu\text{g}$  of holoxygenase per ml.

obvious above pH 7.0 must therefore result from titration of a group on the protein with a  $pK$  of approximately 8.1.

#### Binding of NADH

**Absorption and Fluorescence Spectra**—Addition of NADH to a solution of holoxygenase did not lead to any spectral changes in the visible region or to changes in the fluorescence of enzyme-bound flavin, and there was no oxidation of NADH. However, the fluorescence of NADH itself was enhanced slowly (several minutes were required to achieve maximal values) with a slight blue shift from 460 nm to 445 nm. From the extent and concentration dependence of this increase, the  $K_d$  for the  $E$ -NADH complex was determined to be 240  $\mu\text{M}$ . The kinetically determined value for  $K_{\text{NADH}}$  under similar conditions is 60  $\mu\text{M}$ . Since the latter value reflects the affinity of  $E$ -Cpd I complex for NADH, it is evident that association with Cpd I increases the affinity of the holoxygenase for NADH. Because of its slow formation and high  $K_d$ , it is unlikely that the NADH-holoxygenase complex plays any catalytic role. No energy transfer from NADH to FAD could be detected in this complex.

#### Binding of FAD Analogs to the Apooxygenase

The fluorescence of riboflavin, FMN, and 5-deaza-FAD was not affected by addition of apooxygenase. Of the several analogues tested, only 1-deaza-FAD competitively inhibited ( $K_I = 460$  nm) the quenching of FAD fluorescence that occurs upon addition of apoenzyme. This value is similar to the  $K_d$  for FAD in the binding reaction. The N(1) of FAD is therefore not an important site for complex formation with the apoenzyme.

Under aerobic conditions, the 1-deaza-FAD-apoprotein complex did not catalyze oxidation of NADH, either in the presence or absence of Cpd I, and it was not reduced under anaerobic conditions by NADH. It was reduced anaerobically by dithionite, but addition of Cpd I and oxygen to the reduced mixture did not lead to Cpd A formation. These findings suggest either that N(1) of FAD participates in the catalytic events leading to the oxidation of NADH and oxygenation of Cpd I, or is required for conformational purposes in the generation of an enzymatically active FAD-apoenzyme complex.

Reconstitution of the oxygenase activity of apoprotein by FAD was also competitively inhibited ( $K_I = 460$  nm) by 1-

TABLE II  
Effect of various flavins and adenine nucleotides on the activity of Cpd I apooxygenase and its reactivation by FAD

Complete system <sup>a</sup> plus	Oxygen uptake $\text{nmol min}^{-1}$
FAD <sup>b</sup>	37.0
FMN	2.23
FAD and FMN	38.4
1-d-FAD and FAD	18.4
5-d-FAD and FAD	37.1
FMN and AMP	2.10
FMN and ADP	2.10
ADP and FAD	34.0
No addition	2.10

<sup>a</sup> Complete refers to 0.2  $\mu\text{mol}$  of Cpd I, 0.2  $\mu\text{mol}$  of NADH, and 6  $\mu\text{g}$  of apo-oxygenase in 1 ml of 0.05 M K-phosphate buffer, pH 7.0.

<sup>b</sup> All compounds were added at 5  $\mu\text{M}$  concentration.

deaza-FAD but not by 5-deaza-FAD (Table II). Adenine nucleotides, alone or in combination with FMN, neither inhibited the reconstitution reaction with FAD nor reconstituted activity.

#### DISCUSSION

Limited acid-ammonium sulfate treatment of the holoenzyme yielded satisfactory preparations of apooxygenase. Treatment with KBr or urea (11) led to extensive inactivation of the apoenzyme. Reconstitution of holoxygenase from FAD and apooxygenase requires several seconds, and is very slow compared, *e.g.*, to complex formation between holoenzyme and Cpd I. Similarly slow reconstitution has been observed with several flavoproteins. Within the time scale used here, the fluorescence intensity changes in the apoprotein and in FAD during reconstitution of holoxygenase run exactly parallel indicating that conformational changes leading to alterations of protein fluorescence neither precede nor follow flavin binding significantly. Binding of FAD to the apooxygenase under pseudo-first order conditions is a monophasic reaction, unlike the situations observed with glucose oxidase or D-amino acid oxidase (12, 13).

The kinetic and static dissociation constants for FAD, although in agreement (0.41 to 0.48  $\mu\text{M}$ ), seem high in view of the difficulty in removing the enzyme-bound flavin from the protein. A similar result has been observed with several pyridoxal-5'-P enzymes. For example, tryptophan apocynthase

( $\alpha_2\beta_2$  complex) requires about 1.0  $\mu\text{M}$  pyridoxal-5'-P to achieve half-maximum activity (14), but the holoenzyme is not resolved during precipitation or by extensive dialysis unless "deforming agents" are added (15). This behavior results from conformational changes that follow the initial combination of apoenzyme with pyridoxal-5'-P and prevent the ready loss of the coenzyme (16). In the case of Cpd I oxidase, the experimentally determined affinity constants could indicate low affinity of FAD for the dimeric apoenzyme, as opposed to a much higher affinity for the tetrameric holoenzyme. The fact that Cpd I and NADH stabilize a tetrameric form of the apoenzyme but do not alter its apparent dissociation constant for FAD<sup>2</sup> does not eliminate this possibility since apo- and holo-tetramers may differ in conformation about the coenzyme binding site, as observed for tryptophan synthetase (16). This problem remains unresolved.

The ratio of FAD to protein subunits in Cpd I holoxygenase is 1:2, which suggests the unexplored possibility that the protein subunits, although identical in size, differ in amino acid composition or sequence. 4-Hydroxyisophthalate hydroxylase from *Pseudomonas putida* NCIB 9866 is the only other oxygenase so far found to contain only one flavin per dimeric subunit (17). However, this stoichiometry has been observed for several flavoprotein oxidases (e.g. pyridoxine-5'-phosphate oxidase (18, 19), putrescine oxidase (20)) and other enzymes (e.g. glyoxylate carboxylase (21, 22), dihydrodipicolinate reductase (23)).

The absorption spectrum of Cpd I oxygenase differs significantly from that of free FAD, especially in the  $A_{380}:A_{450}$  ratio which is below 1 for free FAD, but greater than 1 for Cpd I oxygenase. Several other flavoproteins, including dehydrogenases, oxidases, and oxygenases, exhibit this altered ratio, e.g. mammalian pyridoxine-5'-phosphate oxidase (18, 19), thioredoxin reductase from *Escherichia coli* (24), microsomal NADH-cytochrome  $b_5$  reductase (25), oxynitritase (26), and salicylate hydroxylase (27). An increase in ultraviolet absorbance near 275 nm also occurs, probably resulting from a red shift in the ultraviolet absorption band of FAD upon complex formation with apoprotein, in both Cpd I oxygenase and several flavin-containing dehydrogenases (28).

The fluorescence changes that occur upon interaction of Cpd I-apooxygenase with FAD are typical of most flavoproteins (29) with only a few known exceptions (30, 31). Although the relationships between changes in fluorescence and flavin reactivity have not been clarified, such changes are useful in detecting protein-FAD interactions and in determining their stoichiometry.

The failure of 5-deaza-FAD to complex with Cpd I apooxygenase, even after prolonged incubation, is unusual and indicates that N(5) of FAD is important for its interaction with this apoprotein. 5-Deaza-FAD also was not reduced by a mixture of apooxygenase and NADH, under either aerobic or anaerobic conditions. For several other enzymes (e.g. NADPH:flavin oxidoreductase (32), L-lactate oxidase (33), D-amino acid oxidase (34, 35),  $\beta$ -D-glucose oxidase (35, 36), and glycolate oxidase (36), 5-deaza-FAD reconstitutes the apoprotein at rates similar to FAD. In contrast to 5-deaza-FAD, 1-deaza-FAD interacts readily with the apooxygenase, but the reconstituted apooxygenase does not undergo reduction in the presence of NADH and Cpd I, either aerobically or anaerobically. This behavior contrasts with that of orcinol and 4-hydroxybenzoate hydroxylases reconstituted with 1-deaza-FAD, where NADH oxidation is uncoupled from substrate hydroxylation (37, 38).

Recent crystallographic studies indicate that in the ES complex, *p*-hydroxybenzoate lies very close to the FAD of *p*-

hydroxybenzoate hydroxylase, with its phenolic group pointing toward the N(5) position (39). Since energy transfer between Cpd I and the FAD of Cpd I oxygenase occurs, an analogous situation may exist in its ES complex. This complex exhibits a fluorescent emission maximum at 330 nm when activated by light at 290 nm which, upon addition of denaturing agents, shifts to 355 nm with an increase in intensity. Similar effects were observed during denaturation of other proteins by Teale (40). Since tryptophan in polar solvents has an emission maximum at 355 nm which shifts to 335 nm in nonpolar solvents (41) it is likely that the protein fluorescence is due to tryptophan residues located in nonpolar environments. Binding of FAD could lead to a stacking interaction between the indole ring and FAD resulting in the observed decrease in the fluorescence intensity of the holoenzyme.

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