

# Kinetic Investigations on a Flavoprotein Oxygenase, 2-Methyl-3-hydroxypyridine-5-carboxylic Acid Oxygenase\*

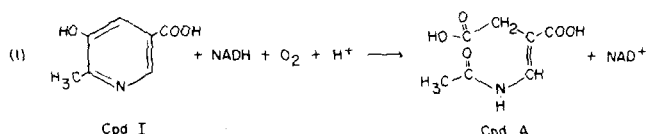
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In the presence of NADH and O<sub>2</sub>, 2-methyl-3-hydroxypyridine-5-carboxylate oxygenase (EC 1.14.12.4) from *Pseudomonas* sp. MA-1 catalyzes reductive oxygenation of 2-methyl-3-hydroxypyridine-5-carboxylate (Cpd I) to yield  $\alpha$ -N-(acetylaminomethylene)succinic acid (Cpd A). Steady state kinetic data and studies with alternate substrates are consistent only with an ordered mechanism in which Cpd I binds first, followed by NADH; the first product, NAD<sup>+</sup>, is then released. This event is followed by oxygen binding, and finally release of the oxygenated and reduced cleavage product, Cpd A. This kinetic mechanism was confirmed by studying inhibition by NAD<sup>+</sup>, which binds competitively with oxygen, but not with NADH. The kinetic mechanism of this reaction resembles that proposed for bacterial flavin monooxygenases that catalyze hydroxylation of aromatic homocyclic compounds.

2-Methyl-3-hydroxypyridine-5-carboxylic acid oxygenase catalyzes the reaction shown in Equation 1 (1). This oxygenase



is one of several inducible enzymes formed when *Pseudomonas* sp. MA-1 is grown with pyridoxine as a sole source of carbon and nitrogen. It was purified to homogeneity by Sparrow *et al.* (1), who demonstrated that it was an FAD-protein,  $M_r = 166,000$ . Some of the interesting features of this oxygenase-catalyzed reaction are: (a) Cpd I<sup>1</sup> regulates the rate of NADH oxidation, (b) oxidation of NADH is coupled to oxygenation of Cpd I, (c) oxygenation of Cpd I appears to be a dioxygenase reaction, but unlike previously studied dioxygenase reactions, oxygenation is accompanied by reduction of the substrate. Although it was shown (1) that ternary complexes were involved in the reaction and that NADH probably served only to reduce the FAD prosthetic group of the enzyme, the order of addition of substrates and desorption of products was not established. The kinetic investigations described below demonstrate that Cpd I and then NADH add to the enzyme, followed by the sequential release of NAD<sup>+</sup>, addition of oxygen, and release of Cpd A.

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

## EXPERIMENTAL PROCEDURES

**Materials**—*Pseudomonas* sp. MA-1 (ATCC No. 33286) was grown on a synthetic medium supplemented with 0.2% pyridoxine (2). Cpd I oxygenase was purified from cells harvested in the late log phase. The procedure of Sparrow *et al.* (1) was followed except that 10 mM dithiothreitol was used in place of 2-mercaptoethanol throughout the purification scheme. Cpd I and 5-pyridoxic acid were isolated and purified from culture filtrates of *Pseudomonas* sp. MA-1 and *Pseudomonas* sp. 1A, respectively, by procedures described elsewhere (3, 4). NADH and NAD<sup>+</sup> (Boehringer) and NMNH (Sigma) were from commercial sources.

**Assay**—Cpd I oxygenase was assayed either spectrophotometrically by absorbance measurements at 340 nm or by use of a Gilson oxygraph equipped with a Clark electrode (ox 15259). For spectrophotometric assays, the standard reaction mixture contained 0.2 mM NADH, 40  $\mu$ M FAD, and approximately 10  $\mu$ g of enzyme in a total volume of 1.0 ml of 0.05 M potassium phosphate buffer, pH 8.0. After 2 min at 25 °C, the reaction was initiated by adding 0.2  $\mu$ mol of Cpd I. Absorbance changes at 340 nm result from disappearance of both Cpd I and NADH; this complication could be circumvented either by dual wavelength measurements (5) or by assays in the oxygraph. For oxygraph assays, the standard reaction mixture contained 0.5 mM NADH and 0.5 mM Cpd I in a total volume of 2 ml of the buffer; other additions, pH, and temperature were identical with those described for spectrophotometric assay.

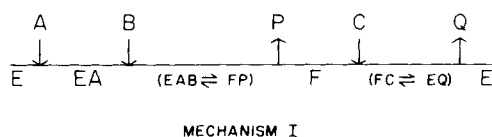
**Data Processing**—Double reciprocal plots of initial velocities versus substrate concentrations were analyzed by Cleland's method (6) using a BASIC Program written for this purpose by Dr. W. L. Lopatin. Eadie plots with alternate substrates were drawn by eye. The nomenclature used in this paper is that of Cleland (7, 8).

## RESULTS

Lineweaver-Burk plots in which concentrations of Cpd I and NADH are varied and that of O<sub>2</sub> is fixed are shown in Fig. 1. The lines converge to a common point on the horizontal coordinate at  $-20.2 \text{ mM}^{-1}$ , when Cpd I is the variable substrate and NADH is the fixed variable substrate (Fig. 1A). When NADH is the variable substrate and Cpd I is the fixed variable substrate, the plots intersect at a common point above the x-coordinate at a value of  $-9.6 \text{ mM}^{-1}$  (Fig. 1B). Such plots are indicative of a sequential addition of the two substrates (7, 8), in this case Cpd I and NADH. The intercept replot of each of these plots is shown as an inset in the respective figures. Such plots will be referred to as secondary plots and Lineweaver-Burk plots as primary plots.

Primary plots in which the concentrations of O<sub>2</sub> and Cpd I were varied with NADH fixed (Fig. 2A) and the concentrations of NADH and O<sub>2</sub> were varied at a fixed concentration of Cpd I (Fig. 2B) are also shown. Both plots yielded a series of parallel lines, consistent with a mechanism involving two partial reactions. The secondary plots are shown as insets in the respective figures.

Of several possibilities, mechanism I, designated Bi Uni Uni Ping Pong by Cleland (7, 8), fits these plots best, where C is O<sub>2</sub>, A and B are reactants yet to be assigned, and P and Q are products. Two substrates, NADH and Cpd I, interact



sequentially with the enzyme to generate a complex which first releases *P* and then interacts with the third substrate,  $O_2$ , in a distinct partial reaction.

The initial rate equations, 2–5 (see Supplement),<sup>2</sup> for Mechanism I were derived by the King-Altman procedure (9) for the interconversion pattern shown in Fig. 3. To determine the values of the kinetic constants, it is necessary to identify *A* and *B*. This was done as follows:

(a) The rate of oxidation of NADH (and reduction of enzyme-bound FAD) in the absence of Cpd I is negligible (<0.5%) compared to that observed in the presence of Cpd I (1). It is likely, therefore, that NADH is not the first substrate to interact with the enzyme. However, the possibility that NADH is bound in the absence of Cpd I but cannot reduce the flavin efficiently under these conditions is not excluded by this observation. (b) Cpd I perturbs the absorption spectrum of enzyme-bound flavin in the absence of NADH under both aerobic and anaerobic conditions (10), thus demonstrating that Cpd I can interact with the free enzyme in the absence of both NADH and  $O_2$ . NADH does not perturb the spectrum of the oxygenase in the absence of Cpd I, but this does not rule out binding of NADH by the free enzyme. (c) The order of binding of substrates can be determined kinetically by the use of alternate substrates (11–13). Such experiments, described below, proved that Cpd I was the first substrate to interact with the oxidized enzyme under steady state conditions.

#### Alternate Substrate Studies

**5-Pyridoxic Acid and Cpd I**—In the presence of  $O_2$ , 5-pyridoxic acid stimulated NADH oxidation by the oxygenase in a manner similar to Cpd I (1). Little or no hydrogen peroxide (assayed by the method of White-Stevens and Kamin (14)) appeared in such reaction mixtures and since the spectral changes observed in the presence of a NADH regenerating system showed a steady decrease in absorption at 323 nm and an increase at 260 nm similar to those observed with Cpd I as a substrate, we concluded that 5-pyridoxic acid was a true (but sluggish) analogue substrate for the oxygenase.

Cpd I and 5-pyridoxic acid are closely similar compounds, as are also their reaction products, and we could not devise any sensitive procedures to follow the rate of the oxygenase reaction with one substrate in the presence of the other. We therefore measured the combined rate with the two substrates and derived the rate equation 6 for the case where *A* is the variable substrate on the assumption that Cpd I (*A*, Fig. 4)<sup>2</sup> and 5-pyridoxic acid (*A'*, Fig. 4) are the first substrates to interact with the enzyme, and do so independently and by the same mechanism, as shown in Fig. 4.

Equation 6 predicts that both double reciprocal and Eadie plots (which are reported (15) to reveal the linear or nonlinear nature of the velocity curves better than Lineweaver-Burk plots) of rate *versus* Cpd I concentration should be nonlinear if Cpd I (or 5-pyridoxic acid) is the first substrate, *A*, (or *A'*), to interact with the enzyme. Such plots at two different

<sup>2</sup> All of the kinetic rate equations and Figs. 4–12 are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20014. Request Document No. 80M-1801, cite author(s), and include a check or money order for \$4.40 per set of photocopies. Full sized photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

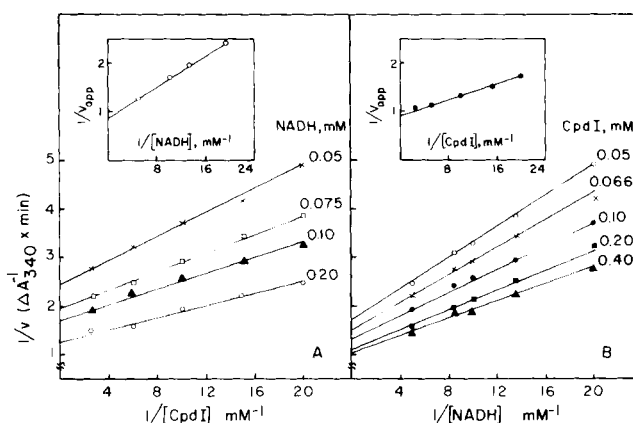


FIG. 1. Cpd I oxygenase activity as a function of Cpd I concentration at fixed concentrations of NADH (*A*) or of NADH concentration at several fixed concentrations of Cpd I (*B*). *A* 1.5-ml cuvette contained: 40  $\mu$ M FAD, 10  $\mu$ l of enzyme (approximately 10  $\mu$ g), variable substrate as indicated, and fixed substrate at concentrations indicated on each curve in a total volume of 1.0 ml of 0.05 M K-phosphate buffer, pH 8.0 at 25 °C. Reactions were initiated by addition of Cpd I and velocity (*v*) was measured immediately thereafter for 3 to 4 min and expressed as absorbance change at 340 nm per min. Each point represents the average of two separate determinations, in which the duplicate reaction was run using  $\frac{1}{10}$  the above concentration of the enzyme. The variations in reaction rates obtained in two such independent assays was always within  $\pm 0.004$  A unit. The insets show secondary plots of  $1/V_{app}$  versus the reciprocal of the fixed substrate concentration.

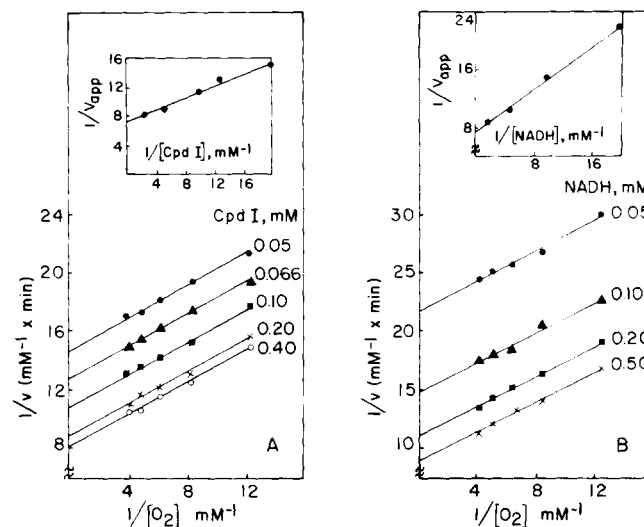


FIG. 2. Oxygenase activity as a function of oxygen concentration at various fixed concentrations of Cpd I or of NADH. Oxygen concentration in the oxygraph reaction vessel was varied by mixing appropriate amounts of buffers saturated with nitrogen or air to yield a final volume of 2 ml. The concentrations of enzyme and FAD were the same as those listed in Fig. 1. The concentration of NADH in *A* and Cpd I in *B* was 1 mM and 0.5 mM, respectively. Concentrations indicated above the lines correspond to those of Cpd I in Fig. 2*A* and of NADH in Fig. 2*B*. Duplicate reactions were run in which the reactions were alternately initiated with Cpd I or NADH; each point represents the average of three independent determinations. The insets are double reciprocal replots of apparent maximum velocities *versus* concentrations of Cpd I or NADH.

concentrations of 5-pyridoxic acid and fixed concentrations of NADH and  $O_2$  are clearly nonlinear (Fig. 5*A*).

Equation 6 can be linearized to yield equation 7. In accordance with predictions of equation 7, plots of  $\Delta v$  *versus*  $\Delta v/[A]$  are linear (Fig. 5*B*) and thus justify the assumptions made in deriving equations 6 and 7.

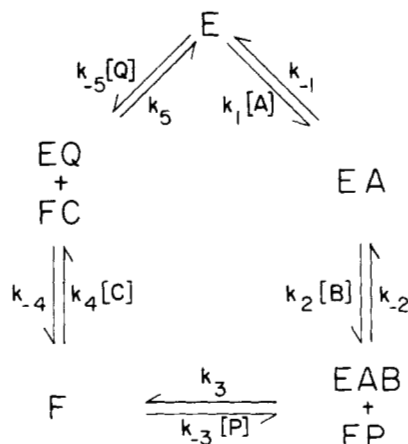


FIG. 3. King-Altman representation of mechanism I (see text) for Cpd I oxygenase. E and F are the two enzyme forms, A, B, and C are substrates and P and Q are the products.  $k_{-5}$  through  $k_5$  are rate constants for the designated steps.

A similar analysis can be made when NADH is the variable substrate, B, in the presence of 5-pyridoxic acid. In this case, the rate equation for the mechanism of Fig. 4 takes the form shown in equation 8. If the assumptions made in deriving equation 8 are correct, then an Eadie plot relating rate to NADH concentration should be linear at high ratios of Cpd I to 5-pyridoxic acid (where NADH would react only with E-Cpd I), nonlinear at lower ratios of Cpd I to 5-pyridoxic acid (where NADH would react at different rates with the two species, E-Cpd I and E-5-pyridoxic acid) and linear at very high ratios of 5-pyridoxic acid to Cpd I (where NADH would again react only with one species, E-5-pyridoxic acid). Plots of data obtained under these conditions (Fig. 6A) conform to these patterns.

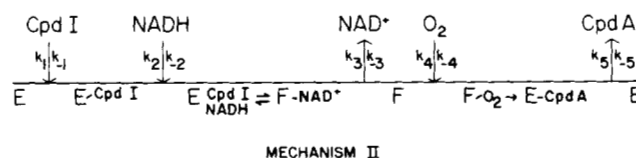
When  $O_2$  is the variable substrate, C, the rate equation in the presence of 5-pyridoxic acid has the form shown in equation 9. Plots of the data at several oxygen concentrations (Fig. 6B) are linear as predicted by equation 9 suggesting the basic validity of the assumptions made in deriving these equations.

**NMNH As a Substitute for NADH**—NMNH effectively replaces NADH as a substrate for Cpd I oxygenase (equation 1) and, since there was no significant uncoupling of its oxidation from Cpd I oxygenation, the mechanism of the oxygenase reaction was assumed to be identical with either NADH or NMNH as substrates (see Fig. 7). If Cpd I is the first substrate to interact with the oxidized enzyme, and is also the variable substrate, rate equation 10 is obtained. The experimental data for the case where NADH, NMNH, or both are present all give linear plots (Fig. 8A), as predicted by this equation, thus confirming that during steady state turnover Cpd I interacts with the free enzyme.

If NADH is the variable substrate, B, and NADH and NMNH are assumed to interact only with the E-Cpd I complex and not with the free enzyme, equation 11 is obtained. This equation predicts that plots of  $v$  versus  $v/[NADH]$  will be nonlinear when NMNH is present, in accord with the experimental data (Fig. 8B, curves 1 and 2). Equation 11 is similar to equation 6 and can be linearized in the same way. The linearized form predicts that a plot of  $\Delta v$  versus  $\Delta v/[B]$ , where  $\Delta v = v - b/d$  and  $b/d$  is the observed velocity when only NMNH is present, should be linear, again in accord with experimental observation (Fig. 8B, curves 3 and 4). If NADH were bound by both free and Cpd I-bound enzyme, the rate equations (not shown) would be nonlinear even after transformation.

With  $O_2$  as a variable substrate, C, the rate equation takes

the linear form shown in equation 12. Eadie plots of velocity versus oxygen concentration (Fig. 9) are linear and nearly parallel to each other, emphasizing the basic correctness of the general kinetic Mechanism I and the more specific Mechanism II for the action of Cpd I oxygenase.



No evidence has yet been presented to show that  $NAD^+$  release occurs prior to  $O_2$  binding. Since for *p*-hydroxybenzoate hydroxylase from *P. fluorescens*,  $NADP^+$  release is reportedly independent of  $O_2$  binding (16), this point was clarified by studies of product inhibition.

#### Product Inhibition of Cpd I Oxygenase

At concentrations up to 10 mM, Cpd A did not inhibit the oxygenase reaction, whereas added  $NAD^+$  (>1 mM) did inhibit. Mechanism II predicts that if  $NAD^+$  release precedes and is essential for  $O_2$  binding,  $NAD^+$  and  $O_2$  should interact competitively with the enzyme. Data of Fig. 10 show this to be the case. Variations in concentrations of Cpd I and NADH did not affect the nature of the inhibition by  $NAD^+$  with respect to  $O_2$ . This too is in accord with rate equation 13 which relates  $v$  to  $[NAD^+]$  and  $[O_2]$ .  $NAD^+$  affects only the term that includes  $K_c$ , and hence should act as a competitive inhibitor with respect to  $O_2$ .  $NAD^+$  is a noncompetitive inhibitor with respect to NADH at all concentrations of Cpd I and unsaturating concentration of  $O_2$  (Fig. 11A). This finding also supports mechanism II, for which rate equation 14 relates  $NAD^+$  inhibition to  $[NADH]$ . Replots of intercepts and slopes of curves in Fig. 11A against  $[NAD^+]$  should be and are linear (Figs. 11B and 11C). The negative x-intercept of these plots is related to kinetic constants as shown in equations 15 and 16. From these relationships, the ratios of rate constants,  $k_{-2}/k_2$  and  $k_3/k_{-3}$  can be obtained, provided the values of the kinetic constants,  $K_a$ ,  $K_b$ , and  $K_c$  are known. These constants were determined as described below.

#### Kinetic Parameters of the Oxygenase Reaction

Rate equations 3, 4, and 5 show that both the intercept and slope of the secondary plots of Figs. 1 and 2 contain terms that include  $K_m$  and  $V$ . For this reason, it is not possible to evaluate these kinetic constants accurately from such plots alone. To circumvent this problem, experiments were performed in which Cpd I was the variable substrate, and NADH and  $O_2$  were "fixed" variable substrates added at concentrations such that  $[NADH]/[O_2] = 1$ . Under these conditions,

TABLE I  
Comparative kinetic constants (pH 8.0, 25 °C) obtained for Cpd I oxygenase with different substrate combinations

Kinetic constant	Substrate combination		
	Cpd I (A), NADH (B), $O_2$ (C)	Cpd I (A), NMNH (B), $O_2$ (C)	5-Pyridoxic acid (A), NADH (B), $O_2$ (C)
	$\mu M$	$\mu M$	$\mu M$
$K_a$	$42.5 \pm 7.5^b$	9.0	50.0
$K_{ia}$	$40.5 \pm 9.5$	50.0	75.0
$K_b$	$80.0 \pm 5.0$	90.0	100
$K_c$	$87.5 \pm 7.5$	80.0	90.0
$V^a$	2407	480	132

<sup>a</sup> Turnover number ( $\mu mol \min^{-1} \mu mol^{-1}$  of enzyme).

<sup>b</sup> The variation is that obtained in five independent experiments.

TABLE II

Accessible rate constants for the Cpd I oxygenase reaction (pH 8.0, 25 °C) determined by initial velocity and product inhibition studies

Rate constant(s)	Value
$k_1$	$6.9 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$
$k_{-1}$	$2.2 \times 10^3 \text{ min}^{-1}$
$k_{-2}/k_2$	$6.36 \times 10^{-5} \text{ M}$
$k_3/k_{-3}$	$6.6 \times 10^{-3} \text{ M}$

rate equation 17 is obtained.

A secondary plot of the apparent maximum velocities,  $V_{app}$ , against  $1/[O_2]$  on extrapolation (Fig. 12) yields true values of  $1/V$ , as shown by equation 18, from which values for  $K_a$ ,  $K_b$ , and  $K_c$  (Table I) can be calculated. Rate constants  $k_1$  and  $k_{-1}$  can be calculated by equations 19 and 20, respectively. Ratios  $k_{-2}/k_2$  and  $k_3/k_{-3}$  can be calculated from  $NAD^+$  inhibition data (equations 15 and 16). These values are presented in Table II.

#### DISCUSSION

All of the data presented in this paper support Mechanism II for the turnover of the dioxygenase. This mechanism involves two different ternary complexes ( $E_{ox}$ -Cpd I-NADH and  $E_{red}$ -Cpd I- $O_2$ ) for the Ter Bi reaction catalyzed by the oxygenase. Closely related mechanisms have been proposed for several flavoprotein monooxygenases (17-20).

The fact that Cpd I participates in both ternary complexes may be important from the physiological standpoint of the organism. If NADH were to reduce the enzyme in the absence of Cpd I, it could lead to the formation of  $H_2O_2$ , since reduced enzyme reacts with  $O_2$  in the absence of Cpd I to form  $H_2O_2$ . The extremely slow rate of reduction of the FAD-enzyme by NADH in the absence of Cpd I ensures that such wasteful pathways do not operate. The rate of reoxidation of  $FADH_2$ -enzyme is also stimulated by Cpd I (1). Cpd I thus has the capacity to interact with both oxidized and reduced forms of the enzyme and efficiently channel electrons toward oxygen activation for the oxygenation reaction.

In interpreting the data with alternate substrates, only the linear or nonlinear nature of the plots was used as evidence for mechanism II. Cooperativity in binding of any of the substrates could not be detected in initial velocity studies with either physiological or alternate substrates. A random mechanism for binding of Cpd I and NADH was excluded, since such a mechanism would yield nonlinear plots in both Fig. 5B and Fig. 8.

Since NADH does not form a catalytically active complex with free oxygenase during steady state turnover, binding of Cpd I must increase both the affinity for NADH and its reactivity in the ternary complex. The anaerobic rate of reduction of  $E$ -FAD by NADH in the presence of 5-pyridoxic acid is only 3.8% of that observed with Cpd I (1), and this low rate probably explains the low rate of the overall reaction with the former substrate. Reoxidation of the reduced enzyme by oxygen is extremely rapid in the presence of either substrate.<sup>3</sup>

Few studies of oxygenase inhibition by products have been described. Inhibition by protocatechuate was used as an aid in elucidating the mechanism of action of *p*-hydroxybenzoate hydroxylase (16).  $NAD^+$  did not inhibit the latter enzyme, a result interpreted to mean that addition of  $O_2$  to the reduced enzyme is independent of  $NAD^+$  release (16). However, if (as in the case studied here) inhibition by  $NAD^+$  were competitive with  $O_2$ , inhibition by  $NAD^+$  might not be observed at saturating concentrations of  $O_2$ .  $NADP^+$  is a product inhibitor of mammalian microsomal amine oxidase (23), but the kinetic

mechanism of this reaction is ordered Ter Bi with NADPH being the first substrate to complex with the free enzyme and  $NADP^+$  the last product to leave the active site. In contrast to bacterial enzymes so far described, the flavin-oxygen adduct is formed prior to the binding of the amine substrate.

Many kinetic investigations on multisubstrate reactions have assumed that  $K_m$  and  $V$  values can be obtained from the  $x$ - and  $y$ -intercepts of secondary plots when the concentrations of only 2 substrates are varied (e.g. 14). This is strictly true only if fixed substrates are not inhibitory and are present at sufficiently high concentrations so that  $K_m/[S]$  is negligible. We have avoided this problem by measuring initial velocities at constant ratios of the fixed substrates. Under these conditions, secondary plots extrapolate to the true value for  $1/V$ . For data reported in Fig. 12,  $V$  is 0.1493 mM/min, which corresponds to a molecular activity of 2407/min at 25 °C and pH 8.0. This value is in the same range as those reported for several flavoprotein monooxygenases (19, 21, 22).

These studies show that Cpd I oxygenase acts by a kinetic mechanism similar to that by which several bacterial flavooxygenases catalyze hydroxylation reactions, i.e. initial binding of the aromatic substrate followed by NADH oxidation and the reaction of reduced flavoenzyme with  $O_2$ , presumably to generate a reduced flavin-oxygen adduct which participates in oxygenation of the bound aromatic substrate (24). Whether the reductive ring-cleavage reaction described here also proceeds through intermediate formation of a dihydroflavin peroxide is not yet known.

In the two instances so far examined, the pyridine ring is cleaved by flavoproteins (1), whereas the benzene nucleus in all of a much larger number of instances is invariably cleaved by iron-containing dioxygenases (25). The basic reasons underlying this difference in the cofactor requirement of what appear superficially to be similar reactions remain to be established.

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## Supplementary Material To

KINETIC INVESTIGATIONS ON A FLAVOPROTEIN OXYGENASE,  
2-METHYL-3-HYDROXYPYRIDINE-5-CARBOXYLIC ACID OXYGENASE

By

Ganesh M. Kishore and Esmond E. Snell

## RATE EQUATIONS

$$2. \quad v/v = [A][B][C]/(K_{ia}K_b[C] + K_a[A][B] + K_b[A][C] + K_a[B][C] + [A][B][C])$$

$$3. \quad v/v = [A]/(K_a(1 + K_bK_c/[B]) + [A](1 + K_b/[B] + K_c/[C]))$$

$$4. \quad v/v = [B]/(K_b(1 + K_a/[A]) + [B](1 + K_a/[A] + K_c/[C]))$$

$$5. \quad v/v = [C]/(K_c(1 + K_a/[A] + K_b/[B] + K_{ia}K_b/[A][B]))$$

In equations 2 to 5  $v$  and  $V$  are the initial and maximum velocities respectively, and the kinetic constants  $K_a$ ,  $K_b$ , etc. are related to the rate constants as in expressions (1) to (14) below.

$$(1) \quad K_a = k_3k_5/k_1(k_3 + k_5)$$

$$(11) \quad K_b = k_5(k_{-2} + k_3)/k_2(k_3 + k_5)$$

$$(111) \quad K_c = k_3(k_{-4} + k_5)/k_4(k_3 + k_5)$$

$$(14) \quad K_{ia} = k_{-1}/k_1$$

6.  $v = (a[A] + b)/(c[A] + d)$ , where  $v$  is the initial velocity,  $[A]$  is the concentration of the first substrate to interact with the enzyme, and  $a$ ,  $b$ ,  $c$  and  $d$  are constants related to rates of turnover of intermediates and to concentrations of fixed substrates. When  $[A] = 0$ ,  $v = b/d = v'$ , the velocity when only the alternate substrate, 5-pyridoxic acid, is present. By subtraction,  $v - v' = \Delta v = (a[A] + b)/(c[A] + d) - b/d$ , or  $\Delta v = ((a(d-bc))/(c[A]d + d^2))$ . Rearranging and dividing by  $[A]$  yields equation 7.

$$7. \quad \Delta v = (-\Delta v/[A])(d/c) + a/c - b/d$$

8.  $v = (a[3]^2 + b[8])/(c[3]^2 + d[B] + e)$ , where  $v$  is the initial velocity, and  $a$ ,  $b$ ,  $c$ ,  $d$  and  $e$  are constants similar to those in Equation 6 and 8 is NADH.

9.  $v = a[C]/(b[C] + d)$ , where  $[C]$  is the concentration of  $O_2$  and  $a$ ,  $b$ , and  $d$  are constants similar to those in Equations 6 and 8.

10.  $v = a[A]/(b[A] + c)$ , where  $[A]$  is the concentration of Cpdl and  $a$ ,  $b$ ,  $c$  are constants.

11.  $v = (a[B] + b)/(c[B] + d)$ , where  $[B]$  is the concentration of NADH, and  $a$ ,  $b$ ,  $c$  and  $d$  are constants.

$$12. \quad v = a[C]/(b[C] + c)$$

$$13. \quad v = \frac{V [O_2]}{K_c \left( 1 + \frac{K_{ib} [NAD^+]}{K_{ip} [NADH]} + \frac{[NAD^+]}{K_{ip}} + \frac{K_{ia} K_{ib} [NAD^+]}{K_{ip} [Cpdl] [NADH]} \right) + [O_2] + \frac{K_a [O_2]}{[Cpdl]} + \frac{K_b [O_2]}{[NADH]} + \frac{K_{ia} K_b [O_2]}{[Cpdl] [NADH]}}$$

where  $K_{ib} = k_{-2}/k_2$  and  $K_{ip} = k_3/k_{-3}$ ; other constants have been previously defined.

$$14. \quad v = \frac{V [NADH]}{K_b \left( 1 + \frac{K_{ia} [NAD^+]}{[Cpdl]} \right) \left( 1 + \frac{K_{ib} K_c [NAD^+]}{K_{ip} [O_2]} \right) + [NADH] \left( 1 + \frac{K_a}{[Cpdl]} + \frac{K_c}{[O_2]} + \frac{K_c [NAD^+]}{K_{ip} [O_2]} \right)}$$

$$15. \quad \frac{K_{NAD^+}}{K_{NADH}} = -K_{ip} \{ 1 + ([O_2]/K_c)(1 + K_a/[Cpdl]) \}$$

$$16. \quad \frac{K_{NAD^+}}{K_{NADH}} = -K_{ip} K_{ib} [O_2]/K_{ip} K_c$$

$$17. \quad \frac{1}{v} = \frac{K_a}{V [Cpdl]} \left( 1 + \frac{K_{ia} K_b}{K_{ip} [O_2]} \right) + \frac{1}{V} \left( 1 + \frac{K_b}{[O_2]} + \frac{K_c}{[O_2]} \right)$$

$$18. \quad \frac{1}{V_{app}} = \frac{K_c}{V [O_2]} \left( 1 + \frac{K_b}{K_c} \right) + \frac{1}{V}$$

$$19. \quad k_1 = V/K_a [E]$$

$$20. \quad k_{-1} = VK_{ia}/K_a [E]$$

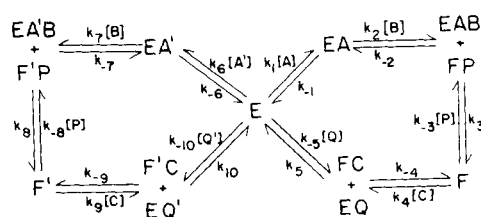


Fig. 4. King-Altman representation of mechanism I in the presence of 5-pyridoxic acid ( $A'$ ) as an alternate substrate for Cpdl ( $A$ ).  $F$  and  $F'$  are reduced enzyme-substrate complexes with Cpdl and 5-pyridoxic acid respectively;  $P$ ,  $Q$ , and  $Q'$  are the products of the reaction.  $k_{-10}$  through  $k_{10}$  are rate constants for designated steps.

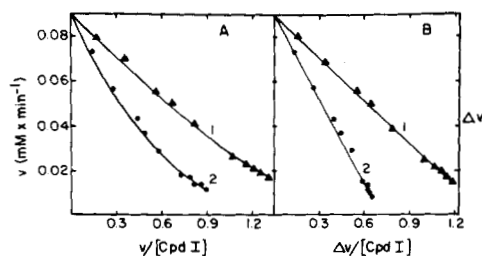


Fig. 5. The effect of 5-pyridoxic acid on the kinetics of the oxygenase reaction. In Part A, the velocity ( $v$ ) was determined in an oxygraph in the presence of 0.025 mM (Curve 1) or 0.1 mM (Curve 2) 5-pyridoxic acid. Concentrations of NADH and oxygen were 0.2 mM and 0.24 mM, respectively. Other conditions were as listed in Fig. 2. In Part B the change in velocity ( $\Delta v$ , see text) is plotted against  $\Delta v/[Cpd I]$ .

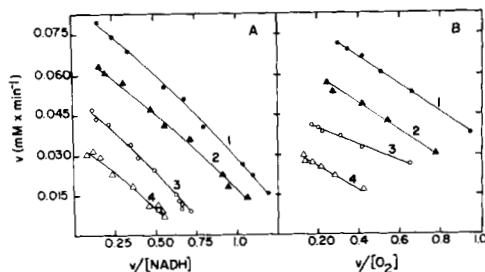


Fig. 6. Effect of 5-pyridoxic acid on the kinetics of the oxygenase reaction when NADH (A) or oxygen (B) is varied. The velocity was determined as in Fig. 5 in the presence of 0.025 mM (Curves 1 and 3) or 0.1 mM (Curves 2 and 4) 5-pyridoxic acid; concentrations of Cpd I were 0.5 mM (Curves 3 and 4), and 0.2 mM (Curves 1 and 2), respectively. Oxygen concentration in Part A was 0.24 mM; NADH concentration in Part B was 0.2 mM.

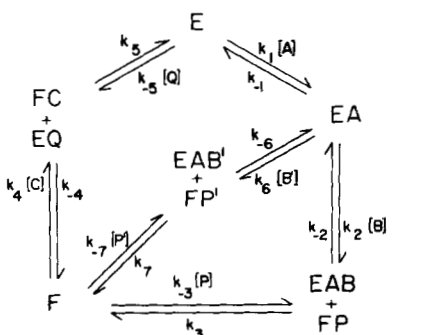


Fig. 7. King-Altman representation of mechanism I in the presence of NADH (B') as an alternate substrate for NADH (B). P, P', and Q are the products of the reaction, while A and C represent the other two substrates for the reaction.

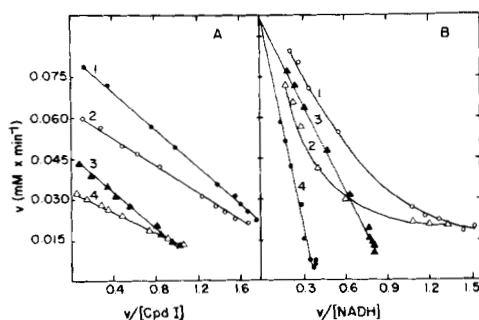


Fig. 8. Kinetics of oxygenase action in the presence of NADH as an alternate substrate and with Cpd I (Part A) or NADH (Part B) as variable substrates. In Part A, NADH was added at 0.05 mM (Curves 1 and 3) or 0.2 mM (Curves 2 and 4) and NADH was either 0.05 mM (Curves 3 and 4) or 0.2 mM (Curves 1 and 2). In Part B, Cpd I was 0.2 mM; curves 1 and 2 represent the velocity determined in the presence of 0.05 mM and 0.2 mM of NADH, respectively, while 3 and 4 are plots of  $\Delta v$  vs.  $\Delta v/[NADH]$  derived from the same data (see text). Oxygen concentration in all experiments was 0.24 mM.

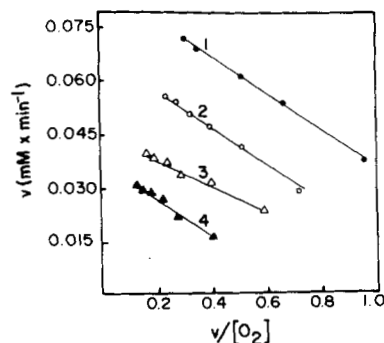


Fig. 9. Oxygenase activity in the presence of 0.05 mM (Curves 1 and 4) or 0.2 mM (Curves 2 and 3) NADH. Concentration of NADH was 0.05 mM (Curves 3 and 4) or 0.2 mM (Curves 1 and 2); Cpd I concentration was 0.2 mM and oxygen was the variable substrate.

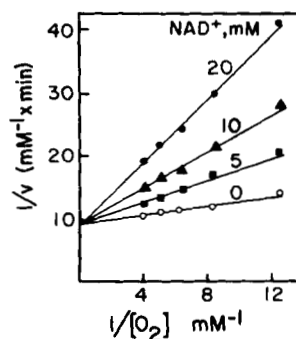


Fig. 10. Competitive relationship between  $NAD^+$  and oxygen in action of Cpd I oxygenase.  $NAD^+$  and NADH and Cpd I were present at 0.8 mM and 0.5 mM, respectively; the concentrations of  $NAD^+$  are indicated above each curve. Oxygen concentrations were varied as described in Fig. 2.

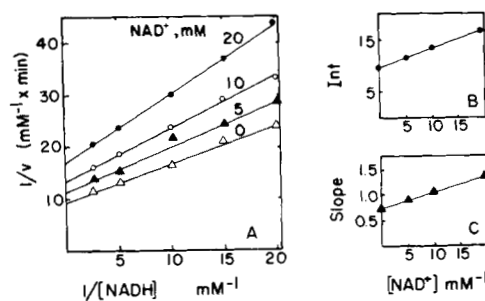


Fig. 11. Non-competitive nature of the relationship between  $NAD^+$  and NADH in action of Cpd I oxygenase. Concentrations of Cpd I and oxygen were 0.5 mM and 0.24 mM, respectively;  $NAD^+$  concentrations are indicated on each curve in Part A. Parts B and C are replots of intercepts and slopes of the curves in Part A vs.  $1/[NAD^+]$ .

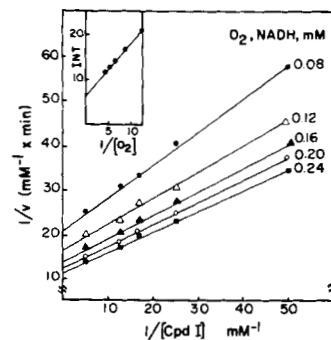


Fig. 12. Determination of true values of  $V$  for Cpd I oxygenase by varying a single substrate at fixed ratios of the remaining two substrates. Cpd I was the variable substrate and both oxygen and NADH were 'fixed' variable substrates, each provided at concentrations indicated on the curves. Velocities were measured in the oxygraph (see Fig. 2). The inset shows a double reciprocal plot of apparent maximum velocity versus oxygen concentration.