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

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In the presence of NADH and O₂, 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 α-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⁺, 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⁺, 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

![Equation 1](Image)

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, M₉ = 166,000. Some of the interesting features of this oxygenase-catalyzed reaction are: (a) Cpd I regulates the rate of NADH oxidation, (b) oxidation of NADH is coupled to oxygenation of Cpd I, and (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⁺, addition of oxygen, and release of Cpd A.

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The abbreviations used are: Cpd I, 2-methyl-3-hydroxypyridine-5-carboxylate; Cpd A, α-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⁺ (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 μM FAD, and approximately 10 μg 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 μmol 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 O₂ is fixed are shown in Fig. 1. The lines converge to a common point on the horizontal coordinate at −20.2 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 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 replots of each of these plots is shown as an inset in the respective figures. Such plots will be referred to as primary plots and Lineweaver-Burk plots as secondary plots.

Primary plots in which the concentrations of O₂ and Cpd I were varied with NADH fixed (Fig. 2A) and the concentrations of NADH and O₂ 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 Uni Ping Pong by Cleland (7, 8), fits these plots best, where C is O₂, 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), 2 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) 2 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

2 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.

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**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 nM FAD, 10 nM of enzyme (approximately 10 μg), 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 1% of the above concentration of the enzyme. The variations in reaction rates obtained in two such independent assays was always within ±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. 2A and of NADH in Fig. 2B. 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.
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 O₂ 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 ν/[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 Δν versus Δν/[B], where Δν = 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 O₂ 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.

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 O₂ binding, NAD⁺ and O₂ 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 O₂. This too is in accord with rate equation 13 which predicts that curves for [NAD⁺] and [O₂] should be linear and nearly parallel (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⁺/k₂ and k₃/k₅ can be obtained, provided the values of the kinetic constants, Kᵦ, Kᵦ, and Kₕ 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 Kᵦ 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 O₂ were "fixed" variable substrates added at concentrations such that [NADH]/[O₂] = 1. Under these conditions,
rate equation 17 is obtained.

A secondary plot of the apparent maximum velocities, $V_{\text{app}}$, against $1/\text{[O}_2\text{]}$ on extrapolation (Fig. 12) yields true values of $K_a$ and $K_v$ (Table I) can be calculated. Rate constants $k_1$ and $k_{-1}$ can be calculated by equations 19 and 20, respectively. Ratios $k_{-1}/k_1$ and $k_2/k_{-2}$ 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 ($E_{\alpha^*}$-Cpd I-NADH and $E_{\alpha^*}$-Cpd I-O$_2$) for the Ter Bi reaction catalyzed by the dioxygenase. Closely related mechanisms have been proposed for several flavoprotein monoxygenases (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$_2$O$_2$, since reduced enzyme reacts with O$_2$ in the absence of Cpd I to form H$_2$O$_2$. 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.

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-pyridoxal 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.

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 $K_a$ 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 $K_a/\text{[S]}$ 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 that reported for several flavoprotein monoxygenases (19, 21, 22).

These studies show that Cpd I oxygenase acts by a kinetic mechanism similar to that by which several bacterial flavoxygenases 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 Note to

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

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NATURAL EQUATIONS

2. \( v = \frac{k_1 [E][A][B]}{k_2 + [A][B]} \), where [E] is the concentration of the enzyme, and k₁, k₂, and k₃ are the rate constants.
3. \( v = \frac{k_4 [E][A][B]}{k_5 + [A][B]} \), where [E] is the concentration of the enzyme, and k₄, k₅, and k₆ are the rate constants.
4. \( v = \frac{k_7 [E][A][B]}{k_8 + [A][B]} \), where [E] is the concentration of the enzyme, and k₇, k₈, and k₉ are the rate constants.
5. \( v = \frac{k_{10} [E][A][B]}{k_{11} + [A][B]} \), where [E] is the concentration of the enzyme, and k_{10}, k_{11}, and k_{12} are the rate constants.

COMMENTS

24. Entsch, B., Ballou, D. P., and Massey, V. (1976) J. Biol. Chem. 251, 2550-2563
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Fig. 4. Kinetic scheme representation of mechanistic I in the presence of ammonium acid (N⁺). The time-dependent rate constants for each step are given. P, F, and Q are the products of the reaction. E is the enzyme, and F is the substrate.
