Mechanistic Studies of the Flavoprotein Tryptophan 2-Monooxygenase. 1. Kinetic Mechanism†

John J. Emanuele‡,§ and Paul F. Fitzpatrick*,‡,∥,⊥

Departments of Biochemistry and Biophysics and Chemistry, Texas A&M University, College Station, Texas 77843-2128

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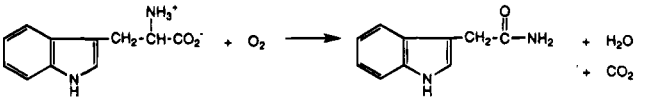
ABSTRACT: The flavoprotein tryptophan 2-monooxygenase catalyzes the oxidative decarboxylation of tryptophan to indole-3-acetamide, carbon dioxide, and water. The kinetic mechanism of the enzyme has been determined with tryptophan as substrate at pH 8.3. Initial velocity patterns, when both amino acid and oxygen concentrations are varied, are sequential with tryptophan and ping-pong with phenylalanine and methionine. Reduction by tryptophan in the absence of oxygen is biphasic. The rate of the rapid phase varies with the tryptophan concentration, with a limiting rate of 139 s^{-1} and an apparent K_d value of 0.11 mM. There is a primary deuterium kinetic isotope effect on the limiting rate of reduction of 2.4. The rapid phase is followed by a slow, concentration and isotope-independent phase that is much slower than turnover; this is ascribed to dissociation of a reduced enzyme−imino acid complex. In the absence of oxygen, tryptophan is converted to indolepyruvate imine. The rate of this reaction is the same as that of the rapid phase in the reduction. Reaction of the reduced enzyme−imino acid complex with oxygen to form oxidized flavin is monophasic, with a rate constant of 196 mM^{-1} s^{-1}; no intermediates are detectable. The rate of formation of indole-3-acetamide agrees with the rate of reaction with oxygen. This is followed by slow product dissociation.

The flavoprotein tryptophan 2-monooxygenase from *Pseudomonas savastanoi* catalyzes the oxidative decarboxylation of tryptophan to indole-3-acetamide, carbon dioxide, and water (Scheme 1) (Comai & Kosuge, 1982). This is the first step in the biosynthesis of the plant growth hormone indoleacetic acid by a number of plant pathogens (Comai & Kosuge, 1980; Klee et al., 1984). As a consequence of the infection of plants by the bacteria and subsequent production of high levels of indoleacetic acid, plant tumors known as galls form at the infection site (Magie et al., 1963). Deletion of the gene for tryptophan 2-monooxygenase from the bacteria does not prevent infection, but results in no galls being formed (Comai & Kosuge, 1980).

Tryptophan 2-monooxygenase contains one tightly bound FAD per monomer. It is one of several flavoproteins which oxidatively decarboxylate amino acids to amides. Others are lysine monooxygenase (Takeda et al., 1969) and phenylalanine oxidase (Koyama, 1982). The steady-state kinetics of lysine monooxygenase have been studied by Flashner and Massey (1974a,b); no mechanistic or structural analyses have been reported for phenylalanine oxidase or tryptophan monooxygenase. Tryptophan 2-monooxygenase is the only member of this group for which a primary sequence is available; outside of the putative FAD-binding fold, there is no sequence similarity to known structures. The lack of study of this group of flavoenzymes is in contrast to the situation with D-amino acid oxidase, which similarly contains FAD but oxidizes substrates without subsequent decarboxylation (Curti et al., 1991) and the FMN-containing hydroxy acid oxidases such as glyoxylate oxidase and lactate oxidase, which form a mechanistically and structurally homologous group of proteins (Giegel et al., 1990; Diêp Lê & Lederer, 1991; Ghisla & Massey, 1991). The differences in structure and the divergence between oxidation and oxidative decarboxylation suggest that tryptophan 2-monooxygenase and related enzymes evolved separately from other flavoprotein oxidases. Thus, the study of the mechanism and structure of tryptophan 2-monooxygenase has the potential to provide insight into general principles of substrate oxidation by flavoproteins in general. We have recently expressed tryptophan 2-monooxygenase from *P. savastanoi* at high levels in *Escherichia coli* (Emanuele et al., 1995), so that significant amounts of pure enzyme are readily available. As a first step in mechanistic analysis, the kinetic mechanism has been determined. The results of that study are reported here. The accompanying manuscript (Emanuele & Fitzpatrick, 1995) describes the use of pH and kinetic isotope effects to analyze the catalytic mechanism.

Scheme 1



EXPERIMENTAL PROCEDURES

Materials. Tryptophan and methionine were from United States Biochemical Co., Cleveland, OH. Alanine, phenylalanine, tyrosine, D,L-*N*-methylphenylalanine, phenylpyruvate, indole-3-acetamide, pyridoxal phosphate, hog renal acylase I, pyridoxal hydrochloride, and ninhydrin were from Sigma

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* Address correspondence to this author at the Department of Biochemistry and Biophysics; Phone: 409-845-5487; Fax: 409-845-9274.

‡ Department of Biochemistry and Biophysics.

§ Present address: Bristol-Myers Squibb Pharmaceutical Research Institute, P.O. Box 4000, Princeton, NJ 08543.

∥ Established Investigator of the American Heart Association.

⊥ Department of Chemistry.

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Table 1: Steady-State Kinetic Parameters for Tryptophan 2-Monooxygenase at pH 8.3, 25 °C

substrate	K_a (mM)	V/K_a (mM^{-1} s^{-1})	K_{O_2} (mM)	V/K_{O_2} (mM^{-1} s^{-1})	V_{max} (s^{-1})	rate eq
tryptophan	0.040 ± 0.005	360 ± 37	0.09 ± 0.01	140 ± 18	13.2 ± 0.7	3
phenylalanine	2.4 ± 0.7	4.5 ± 1.3	0.25 ± 0.01	43 ± 1.3	11 ± 0.2	2
methionine	22 ± 2	0.25 ± 0.017	0.09 ± 0.01	61 ± 4.7	5.6 ± 0.2	2

Chemical Co., St. Louis, MO. Indolepyruvate and *S*-methyl-L-cysteine were from Aldrich Chemical Co., Milwaukee, WI. Indolepyruate was purified by octadecyl silica gel chromatography before use; the column was loaded in water and eluted with methanol. Deuterium oxide was from Cambridge Isotope Laboratory, Woburn, MA. [5-^{3}H]-L-Tryptophan was from American Radiolabeled Chemicals Inc., St. Louis, MO. [α-^{2}H]-L-Tryptophan was synthesized by the method of Kiick and Phillips (1988). Catalase was from Boehringer Mannheim, Indianapolis, IN. Tryptophan indole-lyase was a generous gift from Dr. Robert S. Phillips, University of Georgia. Tryptophan 2-monooxygenase from *P. savastanoi* was purified from *E. coli* by the method of Emanuele et al. (1995).

The concentration of tryptophan 2-monooxygenase was determined using an ϵ_{466} value of 11.4 M^{-1} cm^{-1} (Emanuele et al., 1995). The concentrations of tryptophan and phenylalanine in stock solutions were determined from their absorbances in 0.1 N HCl (Dawson et al., 1986). The concentration of methionine was estimated using a colorimetric assay (Greenstein & Winitz, 1961).

Methods. UV−visible spectra were taken on a Hewlett Packard Model 8452A diode array spectrophotometer. The HPLC apparatus consisted of Waters 501 pumps with a waters automated gradient maker, a Waters 470 scanning fluorescence detector, a Waters Lamda Max Model 481 LC spectrophotometer, and a Waters 740 data module. Rapid reaction kinetic measurements were performed using an Applied Photophysics Limited Model DX.17MV stopped-flow spectrophotometer interfaced with an Acorn Archimedes 420/1 computer and thermostated with a Lauda RM6 water bath. Rapid quench experiments were carried out using a BioLogic QFM 5 apparatus linked to a BioLogic MPS-51 computer interface; this system was under the control of an IBM PS-2 computer running software supplied by BioLogic. Oxygen was removed from the stopped-flow spectrophotometer and rapid quench apparatus by equilibration with oxygen-free nitrogen as described by Denu and Fitzpatrick (1992). Oxygen was removed from the nitrogen by passage over BASF catalyst at 120 °C. NMR spectra were taken on a Varian 200 MHz spectrometer.

Steady-state and rapid reaction kinetic measurements were carried out in 50 mM Tris-HCl, 1 mM EDTA, 0.5 mM dithiothreitol, pH 8.3 at 25 °C. All steady-state activity assays were performed in 2- or 3-mL volumes by following oxygen consumption with a Yellow Springs Instrument Model 5300 biological oxygen monitor. Assays not run in air-saturated buffer were equilibrated in the appropriate mixture of O_2/N_2 by bubbling the gas through the assay mixture for 10 min.

Indolepyruvate Formation. To detect indolepyruvate formation, a stock solution of tryptophan 2-monooxygenase (0.65 mL) was transferred to an anaerobic cuvette. One side arm of the cuvette contained 20 μL of amino acid; a second side arm contained 0.6 N HCl. The cuvette was made anaerobic by alternately evacuating and flushing with oxygen-free nitrogen a total of 10 times. The reaction was initiated by mixing 26 μM tryptophan 2-monooxygenase with tryptophan to give a final concentration of 100 μM tryptophan; the spectrum was monitored at 466 nm to determine when the enzyme was reduced. After 10 min, 0.6 N HCl was added to a final concentration of 0.2 N to quench the reaction. The reaction was opened to air and centrifuged at 14 000 rpm at room temperature for 10 min. The pellet was discarded, and the supernatant was mixed 1:1 with 1 M sodium acetate, pH 6.0; centrifuged; and analyzed by HPLC.

For rapid quench experiments, 120 μL of 49.2 μM tryptophan 2-monooxygenase and 120 μL of 2.13 mM tryptophan containing 20 μCi mL^{-1} of [5-^{3}H]tryptophan were mixed under anaerobic conditions. The reaction was allowed to age for 7−70 ms and quenched by mixing with 120 μL of anaerobic 0.6 N HCl. Samples were frozen and stored overnight at −20 °C. Frozen samples were thawed and diluted 1:1 with 1 mM sodium acetate, pH 6.0, and centrifuged at 14 000 rpm for 10 min. The pellet was discarded, and the supernatant was again centrifuged before analysis by HPLC. Tryptophan, indole-3-acetamide, and indolepyruvate were separated using a Waters 130-mm μBondapak C_{18} reversed-phase column equilibrated with 0.1% trifluoroacetic acid, pH 4.0, and 5% acetonitrile, at a flow rate of 1.5 mL min^{-1}. The elution profile was determined by monitoring fluorescence, with excitation at 278 nm and emission at 350 nm. Individual 1.5-mL fractions were collected, mixed with 5 mL of scintillation fluid, and counted for 5 min. The rate of indole-3-acetamide formation was determined in a similar manner: the enzyme was mixed with tryptophan to give final concentrations of 6.7 μM tryptophan 2-monooxygenase, 1 mM tryptophan, and 0.4 mM oxygen. The reaction was quenched by mixing with 1 M HCl. The amount of indole-3-acetamide formed was determined by HPLC with fluorescence detection.

Data Analysis. Steady-state rate data were fit to eqs 1−3 (Cleland, 1979) using the KinetAsyst software from IntelliKinetics, State College, PA. Data were fit to eq 1 when only one substrate was varied in both steady-state and stopped-flow experiments. When both substrates were varied, the data were fit to eqs 2 and 3. Data collected on the stopped-flow spectrophotometer were fit to eqs 4 and 5 using a Marquardt algorithm with software provided by Applied Photophysics. Equation 4 describes a single first-order reaction. Equation 5 describes a biphasic process; λ_1 and λ_2 are first-order rates, t is time, and A_a, A_b, and A_c are the absorbances of species *A*, *B*, and *C* (Alcock et al., 1970). These rates were fit to eqs 6 and 7 using NonLin for Macintosh (Robelko Software, Carbondale IL), a Macintosh-compatible version of software developed by Johnson and Frasier (1985). The confidence limits of the optimized parameters were set at 67%. Simulation of kinetic results was performed using the program KINSIM (Barshop et al., 1983).

$$v = \frac{VA}{K_a + A} \quad (1)$$

$$v = \frac{VAB}{K_aB + K_bA + AB} \quad (2)$$

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (3)$$

$$A_{total} = A_a e^{-\lambda_1 t} + A_b \quad (4)$$

$$A_{total} = (A_a - A_c)e^{\lambda_1 t} + \frac{\lambda_1(A_b - A_c)}{(\lambda_1 - \lambda_2)}(e^{-\lambda_2 t} - e^{\lambda_1 t}) + A_c \quad (5)$$

$$k_{obs} = \frac{k_2 S}{K_1 + S} \quad (6)$$

$$k_{obs} = \frac{k_2 S}{K_1 + S} + k_{-2} \quad (7)$$

RESULTS

Steady-State Kinetics. While tryptophan is the best substrate for tryptophan 2-monooxygenase, methionine and phenylalanine are also reasonably good substrates (Emanuele et al., 1995). Steady-state initial rates were determined with these three substrates by varying both amino acid and oxygen concentrations at pH 8.3, where the enzyme is most stable (Emanuele et al., 1995). With phenylalanine and methionine, the data fit best to eq 2, which describes a parallel line pattern in double reciprocal plots. In contrast, the data with tryptophan fit best to eq 3, which describes an intersecting line pattern, with a $K_{ia}K_b$ value of 0.0027 ± 0.0012 mM^2. The values of V/K_{O_2} changed with the amino acid, consistent with oxygen binding as the second substrate. The kinetic parameters determined from these experiments are summarized in Table 1.

Spectral Changes Accompanying the Reductive Half-Reaction. When tryptophan 2-monooxygenase was mixed with tryptophan at pH 8.3 in the absence of oxygen, the enzyme's spectrum changed dramatically (Figure 1). The absorbance between 350 and 520 nm rapidly decreased; above 520 nm there was a rapid increase in absorbance. These changes were followed by a slow decrease in absorbance at all wavelengths until the spectrum of the fully reduced enzyme was obtained. After reduction was complete, the sample was exposed to air overnight at 4 °C. The spectrum returned to one similar to that observed before the anaerobic reaction (results not shown). When this experiment was repeated with phenylalanine or methionine, the observed spectral changes were similar to those observed with tryptophan. The largest change in the 500−600 nm region of the spectrum was observed with tryptophan, followed in order by phenylalanine and methionine. The first-order rate constants for conversion of the rapidly formed intermediate to the fully reduced enzyme were 0.005, 0.012, and 0.003 s^{-1} for tryptophan, phenylalanine, and methionine, respectively.

Indolepyruvate Formation. The only product formed during aerobic turnover when tryptophan is the substrate is indole-3-acetamide (Hutcheson & Kosuge, 1985). When tryptophan 2-monooxygenase was allowed to react with tryptophan under anaerobic conditions and the reaction was quenched with acid, 0.78 ± 0.05 molecule of indolepyruvate was produced per monomer of tryptophan 2-monooxygenase. The formation of a keto acid product under anaerobic conditions is consistent with the initial formation of an imino acid bound to reduced enzyme prior to the reaction with oxygen.

Kinetics of Reduction. The absorbance changes at 466 and 560 nm were used to follow the reduction of the flavin by tryptophan in the absence of oxygen using a stopped-flow spectrophotometer. The rates of spectral changes at both wavelengths agreed well. At both wavelengths, there was a rapid change to form the long-wavelength absorbing complex of reduced enzyme and imino acid. The rate of the rapid phase as a function of tryptophan concentration is shown in Figure 2. Similar results obtained with [α-^{2}H]-tryptophan are also shown. The data could be fit to eq 6. With tryptophan, the values of K_1 and k_2 were 0.11 ± 0.015 mM and 139 ± 4.1 s^{-1}, respectively. Fitting the data to eq 7 did not significantly decrease the variance of the fit. With [α-^{2}H]tryptophan, values of 0.078 ± 0.019 mM and 59 ± 2.9 s^{-1} were obtained for K_1 and k_2, respectively. Thus, the primary kinetic isotope effect on k_2 is 2.36 ± 0.06. The rapid phase was followed by a slow absorbance change due to loss of long-wavelength absorbance. The rate of this phase was independent of tryptophan concentration. The rate of decay of the intermediate was 0.0078 ± 0.00042 s^{-1} with tryptophan and 0.0080 ± 0.00026 s^{-1} with [α-^{2}H]tryptophan.

The kinetics of amino acid oxidation during reduction were measured directly by rapid quench methods. Under anaerobic conditions, tryptophan 2-monooxygenase was mixed with tryptophan, the reaction was quenched with acid, and the products were determined by HPLC. The only radioactive product that was detected had a retention time identical to that of indolepyruvate, the expected product from hydrolysis of the imino acid formed by oxidation of tryptophan. The amount of indolepyruvate formed as a function of time could be fit to eq 4 to give a rate of 107 ± 43 s^{-1}. This value, determined with 1.06 mM tryptophan, agrees well with the rate of the reduction by 1 mM tryptophan determined spectrally, 129 ± 8.9 s^{-1}.

These results are consistent with the kinetic mechanism of Scheme 2 for the reductive half-reaction of tryptophan 2-monooxygenase. Binding of substrate is followed by a single kinetic step in which flavin reduction and oxidation of the substrate to an imino acid occurs. In the absence of oxygen, the reduced enzyme−imino acid complex slowly dissociates at a rate that is much slower than turnover.

Oxidative Half-Reaction. Stopped-flow spectrophotometry was also used to determine the rate of reaction of the reduced enzyme−imino acid complex with oxygen. Tryptophan 2-monooxygenase was mixed with tryptophan to form this intermediate, which was then mixed with the oxygenated buffer. The oxygenated buffer contained 1 mM indole-3-acetamide, a competitive inhibitor of tryptophan 2-monooxygenase with a K_i value of 16 μM (Emanuele et al., 1995) to prevent subsequent turnover. The absorbance changes as a function of time could be fit to eq 4. The rates determined at both 460 and 550 nm were in good agreement with each other and varied directly with oxygen concentration (Figure 3). The rate of reoxidation was independent of the aging time over the time scale studied (0.1−1 s) and of the amount

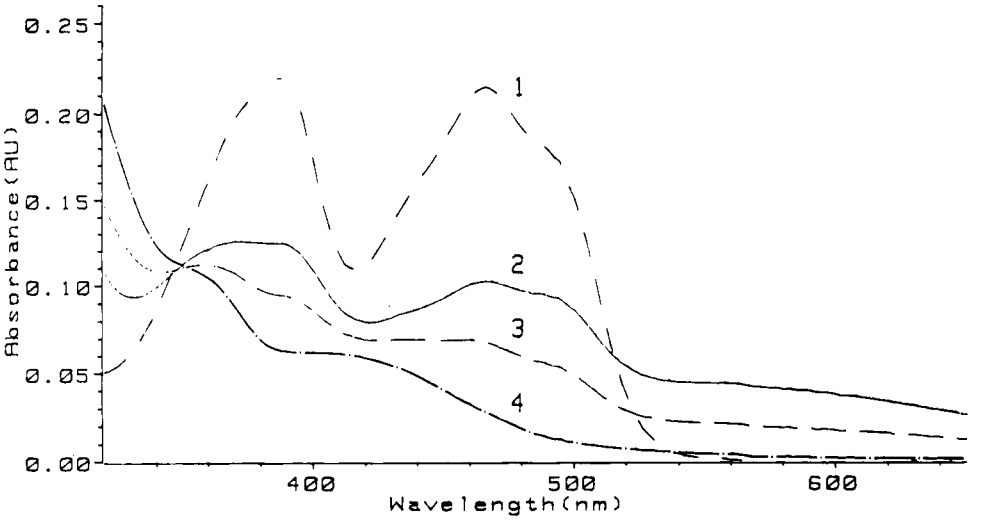


FIGURE 1: Spectral changes upon mixing tryptophan 2-monooxygenase (19 μM) and tryptophan (1 mM) in the absence of oxygen. Spectra were obtained before (1) and 40 s (2), 5 min (3), and 33 min (4) after the addition of tryptophan. The conditions were 50 mM Tris-HCl, 1 mM EDTA, and 0.5 mM dithiothreitol, pH 8.3, 25 °C.

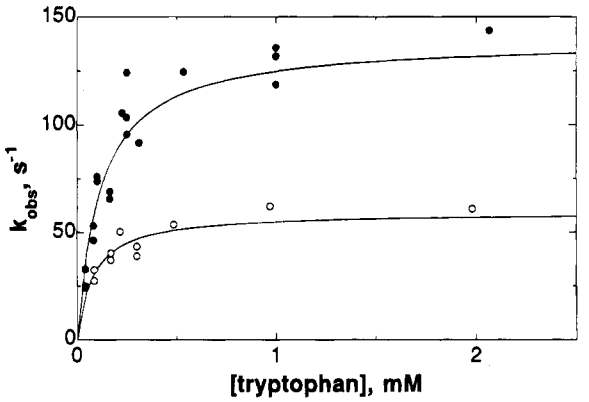


FIGURE 2: Rate of reduction of tryptophan 2-monooxygenase by tryptophan (●) and [α-^{2}H]tryptophan (○) as a function of amino acid concentration. Tryptophan 2-monooxygenase (final concentration 13−29 μM) was mixed anaerobically with the indicated final concentrations of the amino acid. The rate of reduction of the enzyme-bound FAD was determined at 466 and 560 nm. The lines are fits of the data to eq 6.

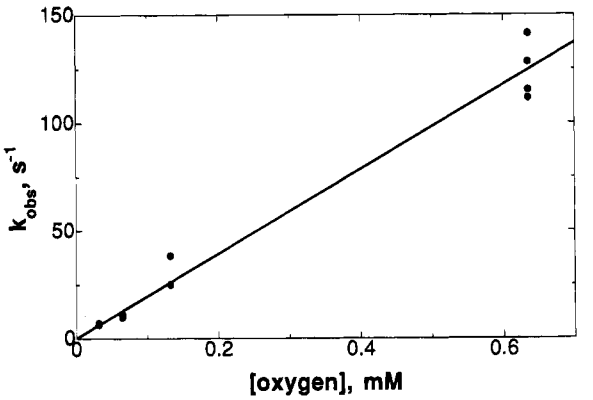


FIGURE 3: Rate of reoxidation of reduced tryptophan 2-monooxygenase as a function of oxygen concentration. Enzyme (25 μM) was mixed anaerobically in the stopped-flow spectrophotometer with an equal volume of 200 μM tryptophan in 50 mM Tris-HCl, 1 mM EDTA, and 0.5 mM DTT, pH 8.3, at 25 °C. The reaction was allowed to age for 0.1−1 s before being mixed with an equal volume of oxygenated buffer containing 1 mM indole-3-acetamide. The reoxidation was monitored at 466 and 560 nm.

Scheme 2

$$EFl_{ox} + S \underset{}{\overset{K_1}{\rightleftharpoons}} EFl_{ox}S \xrightarrow{k_2} EFl_{red}P \xrightarrow{k_3} EFl_{red} + P$$

of indole-3-acetamide used. The data gave a second-order rate constant for the reaction with oxygen of 196 ± 7.0 mM^{-1} s^{-1}.

To determine at which step in the reaction decarboxylation of indolepyruvate imine to indole-3-acetamide occurs, tryptophan 2-monooxygenase was mixed aerobically with saturating (1 mM final) tryptophan, and the reaction was quenched at various times during the first 250 ms. The amount of indole-3-acetamide as a function of time is shown in Figure 4. There was a clear burst of indole-3-acetamide formation, consistent with a slow step occurring after decarboxylation.

DISCUSSION

The data presented here are consistent with the kinetic mechanism of Scheme 3 for tryptophan 2-monooxygenase. The reaction can be separated into reductive and oxidative half-reactions. In the reductive half-reaction, the amino acid binds to the enzyme. This is followed by a single kinetic step in which the amino acid is oxidized to the imino acid. This process can readily be followed using a stopped-flow spectrophotometer since the complex of reduced FAD and imino acid shows decreased absorbance at 460 nm and increased absorbance above 530 nm. This is consistent with what is observed with other flavoprotein oxidases (Massey & Ghisla, 1974). Oxygen then reacts with the reduced flavin−imino acid complex before the imino acid dissociates. This is demonstrated both by the directly measured rates of the decay of the absorbance of this intermediate, which is far slower than turnover with all three amino acid substrates analyzed, and by the change in the V/K_{O_2} value with a change in the identity of the amino acid substrate.

In the reductive half-reaction, no intermediates were detected between the amino acid and the imino acid. This was true whether the reduction of the flavin or the production of imino acid was monitored. A significant primary kinetic isotope effect was measured for the limiting rate of reduction. This is consistent with carbon−hydrogen bond cleavage being significantly rate-limiting, with subsequent steps much

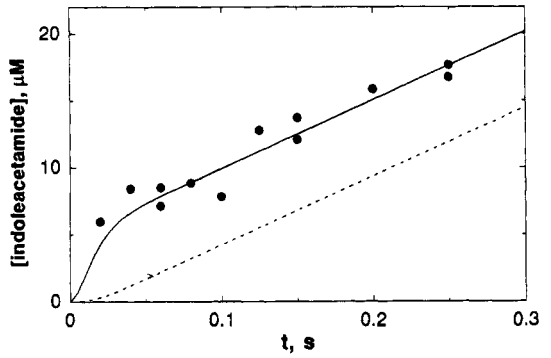
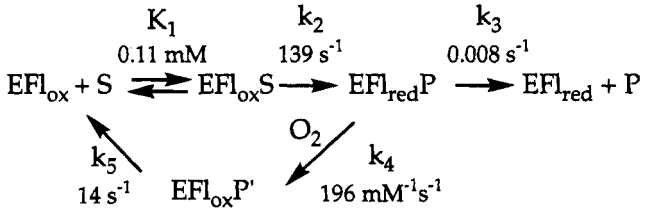


FIGURE 4: Rate of formation of indole-3-acetamide by tryptophan 2-monooxygenase. Enzyme (final concentration 6.7 μM) and tryptophan (final concentration 1 mM) were mixed in the presence of 0.4 mM oxygen in 50 mM Tris-HCl and 0.5 mM EDTA, pH 8.3, at 25 °C. At the indicated times, the reaction was quenched with 1 M HCl. The amount of indole-3-acetamide formed was analyzed by HPLC. The lines are simulations of the reaction using KINSIM and the mechanism of Scheme 3 with the limiting rate of reduction (k_2) set at 150 s^{-1}, the rate of reaction with oxygen (k_4) set at 200 mM^{-1} s^{-1}, and k_5 set at 9 s^{-1}. The solid line is the result of assuming that conversion of indolepyruvate imine to indole-3-acetamide occurs during the conversion of $E_{red}P$ to $E_{ox}P'$. The dashed line is the result of assuming that formation of indole-3-acetamide occurs during the conversion of $E_{ox}P'$ to E_{ox} + P'.

Scheme 3



more rapid. The value of 2.4 measured with tryptophan is significantly smaller than the values of the intrinsic primary deuterium kinetic isotope effects measured with the flavoprotein D-amino acid oxidase. These range from 3.6 with glycine as substrate to 5.7 with D-alanine (Denu & Fitzpatrick, 1994). Similar values of about 5 have also been measured using steady-state kinetic methods for tryptophan 2-monooxygenase with slow substrates (Emanuele & Fitzpatrick, 1995). This suggests either that the transition state for carbon–hydrogen bond cleavage with tryptophan as substrate for tryptophan 2-monooxygenase is asymmetric or that carbon–hydrogen bond cleavage is partially reversible on the enzyme surface, with the rate of subsequent electron transfer to the FAD occurring at a rate comparable to the rate of carbon–hydrogen bond cleavage.

The better fit of the steady-state kinetic data with tryptophan to eq 3 rather than eq 2, indicating a small but significant $K_{ia}K_b/V$ value, requires that the binding of oxygen and amino acid to the enzyme be connected by a reversible step. The rate constant for the formation of $EFl_{ox}P$ from $EFl_{red}P$ in Scheme 3 can be determined by extrapolating the rate of reduction by tryptophan to zero concentration. However, there was no significant improvement when the data in Figure 2 were fit to eq 7, which explicitly includes k_{-2}, the rate constant for the reverse step. This sets an upper limit on the value of k_{-2} of 10 s^{-1} or less. A value of this still magnitude is sufficient to generate a small but significant $K_{ia}K_b$ value with this substrate. An alternative explanation for a nonzero $K_{ia}K_b$ value, formation of a ternary complex of oxygen, tryptophan, and enzyme prior to catalysis, appears unlikely based upon the lack of evidence for oxygen binding in the rapid reaction studies and the catalytic competency of the reduced enzyme–imino acid complex in reacting with oxygen. Such a ternary complex has not been detected with any flavoprotein oxidase to date.

There is also an isotope effect on the K_1 value. This is not expected if this is truly a dissociation constant. However, pH and steady state kinetic isotope effects are consistent with tryptophan having a significant commitment to catalysis (Emanuele & Fitzpatrick, 1995). Consequently, the K_1 value measured with this substrate is not a dissociation constant (k_{-1}/k_1), but instead equals (k_{-1} + k_2)/k_1. Thus, an isotope effect on k_2 will affect both the k_2 and the K_1 values.

Kinetically, the oxidative half-reaction is simpler. The reaction of the reduced flavin imino acid species with oxygen shows no spectral intermediates prior to the formation of the fully oxidized flavin. Instead, the reaction is second order, with no indication of saturation at the highest oxygen concentration readily obtainable. Similar kinetics have been seen with other flavoprotein oxidases (Curti et al., 1991; Ghisla & Massey, 1991). The mechanism of Eberlein and Bruice (1983) for the reaction of reduced flavin with oxygen proposes an initial single electron transfer to form the flavin radical cation and superoxide. This step is rate limiting. The two radicals rapidly react to form oxidized flavin and hydrogen peroxide. The results obtained with tryptophan 2-monooxygenase are consistent with such a mechanism.

To assign the step in which decarboxylation occurs, the program KINSIM was used to simulate the results of the rapid quench analysis of indole-3-acetamide formation (Figure 4). Two limiting mechanisms were simulated using the kinetic mechanism of Scheme 3 and the independently determined rates of reduction and oxidation. In the first model, flavin oxidation and substrate decarboxylation occur in the same step. The results of this simulation are shown by the solid line in Figure 4. In the alternative model, decarboxylation was assigned to a subsequent rate-limiting step. This is shown by the dashed line in Figure 4. The first model shows excellent agreement with the data, establishing that decarboxylation occurs in the same step as flavin oxidation.

Lockridge et al. (1972) have proposed that the mechanism of decarboxylation of pyruvate by lactate oxidase is due to reaction with the hydrogen peroxide formed during flavin oxidation, although the step at which decarboxylation occurred was not established. The results with tryptophan 2-monooxygenase are consistent with hydrogen peroxide formation and amide decarboxylation being much more rapid than the initial one electron reaction between reduced FAD and oxygen, so that no intermediates are detectable between reduced enzyme–imino acid and oxidized enzyme–amide.

There is a final slow step in the reaction after decarboxylation, as shown by the burst of indole-3-acetamide formation and the large difference between V_{max} (13 s^{-1}) and the value of k_2 (139 s^{-1}). This slow step can reasonably be assigned as product release. The value of k_5, the rate constant for indole-3-acetamide release, can be calculated from the V_{max} value, which is made up of the values of all the first-order rate constants in the reaction. The only kinetically significant first-order rate constants are k_2 and k_5. Since the value of k_2 has been measured, the value of k_5 can be calculated to be 14 s^{-1}. The simulation of the rapid quench results shown

in Figure 4 gave the best fit to the data if the rate of product release was set at 9 s^{-1}. These two values are in reasonable agreement.

The complete kinetic mechanism of tryptophan 2-monooxygenase has been determined using both steady-state and rapid reaction kinetic methods. Determining the kinetic mechanism of an enzyme is the first step in a complete analysis of the catalytic mechanism. These results provide the necessary basis for utilizing pH and kinetic isotope effects to probe details of catalysis. Results of such studies with tryptophan 2-monooxygenase are described in the accompanying manuscript (Emanuele & Fitzpatrick, 1995).

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REFERENCES

Alcock, N. W., Benton, D. J., & Moore, P. (1970) *J. Chem. Soc., Faraday Trans. 1 266*, 2210−2213.
Barshop, B. A., Wrenn, R. F., & Frieden, C. (1983) *Anal. Biochem. 130*, 134−145.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103−138.
Comai, L., & Kosuge, T. (1980) *J. Bacteriol. 143*, 950−957.
Comai, L., & Kosuge, T. (1982) *J. Bacteriol. 149*, 40−46.
Curti, B., Ronchi, S., & Simonetta, M. P. (1991) in *Chemistry and Biochemistry of Flavoenzymes*, Vol. III (Müller, F., Ed.) pp 69−94, CRC Press, Boca Raton.
Dawson, R. M. C., Elliott, D. C., Elliott, W. H., & Jones, K. M. (1986) *Data for Biochemical Research*, Clarendon Press, Oxford.
Denu, J. M., & Fitzpatrick, P. F. (1992) *Biochemistry 31*, 8207−8215.
Denu, J. M., & Fitzpatrick, P. F. (1994) *Biochemistry 33*, 4001−4007.
Diêp Lê, K. H., & Lederer, F. (1991) *J. Biol. Chem. 266*, 20877−20881.
Eberlein, G., & Bruice, T. C. (1983) *J. Am. Chem. Soc. 105*, 6685−6697.
Emanuele, J. J., Jr., & Fitzpatrick, P. F. (1995) *Biochemistry 34*, 3716−3723.
Emanuele, J. J., Jr., Heasley, C. J., & Fitzpatrick, P. F. (1995) *Arch. Biochem. Biophys. 316*, 241−248.
Flashner, M. I. S., & Massey, V. (1974a) *J. Biol. Chem. 249*, 2579−2586.
Flashner, M. I. S., & Massey, V. (1974b) *J. Biol. Chem. 249*, 2587−2592.
Ghisla, S., & Massey, V. (1991) in *Chemistry and Biochemistry of Flavoenzymes*, Vol. II (Muller, F., Ed.), pp 243−289, CRC Press, Boca Raton.
Giegel, D. A., Williams, C. H., Jr., & Massey, V. (1990) *J. Biol. Chem. 265*, 6626−6632.
Greenstein, J. P., & Winitz, M. (1961) *Chemistry of the Amino Acids*, Vol. 3, p 2125, John Wiley and Sons, Inc., New York.
Hutcheson, S. W., & Kosuge, T. (1985) *J. Biol. Chem. 260*, 6281−6287.
Kiick, D. M., & Phillips, R. S. (1988) *Biochemistry 27*, 7339−7344.
Klee, H., Montoya, A., Horodyski, F., Lichtenstein, C., Garfinkel, D., Fuller, S., Flores, C., Peschon, J., Nester, E., & Gordon, M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1728−1732.
Koyama, H. (1982) *J. Biochem. 92*, 1235−1240.
Lockridge, O., Massey, V., & Sullivan, P. A. (1972) *J. Biol. Chem. 247*, 8097−8106.
Magie, A. R., Wilson, E. E., & Kosuge, T. (1963) *Science 141*, 1281−1282.
Massey, V., & Ghisla, S. (1974) *Ann. N. Y. Acad. Sci. 227*, 446−465.
Takeda, H., Yamamoto, S., Kojima, Y., & Hayaishi, O. (1969) *J. Biol. Chem. 244*, 2935−2941.

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