Mechanistic Studies of the Flavoprotein Tryptophan 2-Monooxygenase. 2. pH and Kinetic Isotope Effects†

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ABSTRACT: pH and kinetic isotope effects on steady-state kinetic parameters have been determined for the flavoprotein tryptophan 2-monooxygenase with tryptophan, phenylalanine, 2-hydrazino-3-propanoic acid, and methionine as substrates. The V/K values of the amino acid substrates show that a residue with an apparent pK_a value of 5 must be unprotonated for activity, a residue with a pK_a value equal to that of the amino group of the substrate must be protonated, and deprotonation of a residue with pK_a value of 10 increases the V/K value. A group in the free enzyme with a pK_a value of 6 must be deprotonated for tight binding of amide inhibitors and protonated for tight binding of acids, establishing this as the intrinsic pK_a value. The temperature dependence of this pK_a value is consistent with involvement of a histidinyl residue. Deprotonation of the residue with a pK_a value of 10 decreases binding of amide inhibitors. The $^D(V/K_{trp})$ value is less than 1.7 between pH 5 and 10, consistent with a forward commitment to catalysis of 7−15 with this substrate. The $^D(V/K)_{met}$ value is pH dependent, increasing from a minimal value of 1.8 at pH 8.3 to a limiting value of 5.3 at both high and low pH, with pK_a values of 5.1 and 10. The increase in both the isotope effect and the V/K_{met} value at high pH is consistent with a conformational change to a more open active site above pH 10. The $^D(V/K)_{ala}$ value is 5.3 at pH 8.3; this is probably the intrinsic isotope effect with this substrate. The β-secondary isotope effect with [β,β,β-$^2H_3$]alanine is 0.965 ± 0.041, consistent with a carbanion mechanism. The proposed role of the residue with a pK_a value of 6 is to remove the substrate α-proton to form the carbanion. The V/K_{O_2} values for phenylalanine and tryptophan are essentially insensitive to pH between pH 5 and pH 10. The V/K_{O_2} value with methionine increases severalfold with a pK_a value of 6.8; this is assigned to the reduced FAD. There is no solvent isotope effect on the V/K_{O_2} value with tryptophan, consistent with rate-limiting electron transfer in the reaction with oxygen. With all three substrates, the V_{max} value decreases 20−50-fold when a single residue is protonated. This pK_a value varies with the identity of the substrate; it is assigned to a conformational change which precedes product release. The solvent isotope effect on the V_{max} value with tryptophan is 2.5. This is consistent with slow proton transfer being coupled to product release.

The flavoprotein tryptophan 2-monooxygenase catalyzes the oxidative decarboxylation of tryptophan to indoleacetamide. This is the first step in a two-step pathway for the formation of the plant growth hormone indoleacetic acid by a number of bacteria. The high local concentrations of indoleacetic acid at the sights of infection result in the formation of galls on the plants (Comai & Kosuge, 1980; Klee et al., 1984). While the enzymes from the various bacteria are homologous (Klee et al., 1984; Thomashow et al., 1986; Clark et al., 1993), the sequence of tryptophan 2-monooxygenase is different from those of known proteins (Comai & Kosuge, 1982; Yamada et al., 1985). Little mechanistic work has been reported for flavoproteins such as tryptophan 2-monooxygenase which oxidatively decarboxylate amino acid substrates.

Tryptophan 2-monooxygenase can be considered a member of the family of flavoprotein oxidases (Massey & Hemmerich, 1980). Although the overall reaction is monooxygenation of the amino acid substrate, the enzyme first oxidizes the amino acid to the imino acid, transferring the electrons to the FAD (Emanuele & Fitzpatrick, 1995). This is identical to the reaction catalyzed by flavoprotein oxidases such as D-amino-acid oxidase (Curti et al., 1991). We have been using kinetic isotope effects to analyze the details of carbon−hydrogen bond cleavage by flavoprotein oxidases. Previous reports have dealt with D-amino-acid oxidase, a heavily studied member of this group (Denu & Fitzpatrick, 1992, 1994a,b). Tryptophan 2-monooxygenase has been chosen for study because of the identity of the cofactor, FAD, and the reactivity of its amino acid substrates to those of D-amino-acid oxidase. Comparison of the details of catalytic mechanism and, eventually, structure should provide general insight into the mechanisms of cleavage of carbon−hydrogen bond cleavage by flavoproteins.

Tryptophan 2-monooxygenase from *Pseudomonas savastanoi* has been overexpressed in *Escherichia coli*, providing sufficient material for mechanistic studies (Emanuele et al., 1995). The kinetic mechanism of the enzyme has been determined (Emanuele & Fitzpatrick, 1995) and is shown in Scheme 1. Here, EFl_{ox} and EFl_{red} are enzyme with

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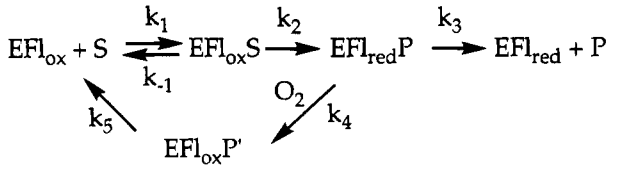
Scheme 1



oxidized and reduced FAD, respectively; P is the imino acid of the amino acid substrate; and P′ is the respective amide. As the next step in the analysis of the catalytic mechanism of this enzyme and of the role of amino acid residues in catalysis, the effects of pH and isotopic substitution on kinetic parameters have been determined. The results of those studies are reported here.

EXPERIMENTAL PROCEDURES

Materials. L-Tryptophan and L-methionine were from United States Biochemical Co., Cleveland, OH. L-Alanine, D-tryptophan, D-methionine, D-phenylalanine, L-phenylalanine, L-tyrosine, phenylpyruvate, indole-3-acetamide, hydrocinnamic acid, and phenyllactic acid were from Sigma Chemical Co., St. Louis, MO. Phenyl-3-acetamide was from TCI America, Portland, OR. [α-^{2}H]-D,L-Phenylalanine and [α-^{2}H]-D,L-alanine were from MSD Isotopes, Montreal, Canada. [β,β,β-$^2H_3$]-D,L-Alanine was from Cambridge Isotope Laboratories Inc., Andover, MA. 3-Phenyl-2-hydrazinopropanoic acid was synthesized by a modification of the method of Glamkowski et al. (1967). [α-^{2}H]-L-Tryptophan was synthesized by the method of Kiick and Phillips (1988). [β,β,β,α-$^2H_4$]-L-Methionine was synthesized by resolution of [β,β,β,α-$^2H_4$]-D,L-methionine (LeMaster & Richards, 1977), using D-amino-acid oxidase to destroy the D-enantiomer; when the enantiomeric excess was greater than 95%, the L-enantiomer was purified by cation exchange chromatography. NMR analysis indicated >95% incorporation of deuterium at the 2-carbon. *P. savastanoi* tryptophan 2-monooxygenase was purified from *E. coli* as described by Emanuele et al. (1995). D-Amino-acid oxidase was purified from hog kidneys by the method of Fitzpatrick and Massey (1982), and benzoate was removed by the method of Brumby and Massey (1968). Catalase was from Boehringer Mannheim, Indianapolis, IN. Tryptophan indole-lyase was a generous gift from Dr. Robert S. Phillips of the University of Georgia.

Assays. Activity assays were performed by following the rate of oxygen consumption using a Yellow Springs Instrument Co. Model 5300 biological oxygen monitor. All assays were conducted at 25 °C in either 2- or 3-mL volumes. Assays not run in air-saturated buffer were equilibrated with the appropriate mixture of O_2/N_2 by bubbling the gas through the assay mixture for 10 min. V_{max} values were determined by varying both substrates or by varying one substrate while the other was at a concentration of 8−10 times its K_m. Above pH 10, all parameters were determined by varying both substrates. From pH 5 to pH 10, the buffer was 0.052 M Tris[1] -HCl, 0.052 M ethanolamine hydrochloride, and 0.1 M ACES; from pH 4.5 to pH 5.5, it was 0.05 M MES, 0.1 M Tris-HCl, and 0.05 M acetic acid; and from pH 10 to pH 11, 100 mM EDTA was used as a buffer. Assays were performed at overlapping points whenever a transition was made from one buffer to another. The concentrations of tryptophan 2-monooxygenase and substrates were determined as described in Emanuele and Fitzpatrick (1995). The concentrations of [α-^{2}H]-D,L-alanine, [β,β,β-$^2H_3$]-D,L-alanine, and D,L-alanine were determined by enzymatic end point assays with D-amino-acid oxidase. $^DV/K$ values were typically determined in air-saturated buffer, since the V/K values for the amino acids are independent of the oxygen concentration (Emanuele & Fitzpatrick, 1995). For solvent isotope effects, 0.052 M Tris-HCl, 0.052 M ethanolamine hydrochloride, and 0.1 M ACES, pH 8.3, was made up in H_2O. It was then lyophilized and dissolved in D_2O. This was repeated before adding solid tryptophan to a final concentration of 3 mM and dissolving the mixture in D_2O. To determine isotope effects on V_{max} and V/K_{O_2} values, the concentration of oxygen was varied from 64 μM to 1.3 mM at 3 mM tryptophan (K_{trp} = 40 μM).

Data Analysis. Kinetic data were fit to eqs 1−8 using the programs of Cleland (1979) in the KinetAsyst software (IntelliKinetics, State College, PA) and to equations 9-13 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%. Equation 1 was used when only one substrate concentration was varied. Equations 2 and 3 were used when the concentrations of both substrates were varied. Equation 4 is for a kinetic isotope effect on V_{max} only; eq 5 is for a kinetic isotope effect on V/K only; and eq 6 is for separate isotope effects on V_{max} and V/K. Equation 7 was used to fit data from pH rate profiles which showed a decrease in log V, log V/K, or −log K_i values of unit slope at low pH. Equation 8 was used to fit data which decreased with unit slope at both high and low pH. Equation 9 was used to fit data which exhibited two plateaus, Y_L and Y_I, connected by a transition region with unit slope. Equation 10 was used to fit data which exhibited two plateaus, Y_L and Y_H, connected by a transition region, and decreased above Y_H with unit slope. Equation 11 was used to fit data which exhibited two plateaus connected by a transition region and which decreased with unit slope at both low and high pH. The effects of pH on the $^D(V/K_{met})$ value were fit to eq 12; here, Y_L is the limiting isotope effect at low pH, Y_I is the minimum isotope effect, and Y_H is the limiting isotope effect at high pH.

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

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

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

$$v = \frac{VA}{K_m + A(1 + F_i(E_v))} \quad (4)$$

$$v = \frac{VA}{K_m(1 + F_i(E_{vk})) + A} \quad (5)$$

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; ACES, *N*-(2-acetamido)-2-aminoethanesulfonic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

$$v = \frac{VA}{K_m(1 + F_i(E_v)) + A(1 + F_i(E_{vk}))} \quad (6)$$

$$\log Y = \log \frac{C}{1 + \frac{H}{K_1}} \quad (7)$$

$$\log Y = \log \frac{C}{1 + \frac{K_2}{H} + \frac{H}{K_1}} \quad (8)$$

$$\log Y = \log \frac{Y_L + Y_H\frac{K_1}{H}}{1 + \frac{K_1}{H}} \quad (9)$$

$$\log Y = \log \frac{Y_L + \frac{Y_H K_1}{H}}{1 + \frac{K_1}{H} + \frac{K_1K_2}{H^2}} \quad (10)$$

$$\log Y = \log \left(\frac{\left(Y_L\frac{K_1}{H} + \frac{Y_1K_1K_2}{H^2}\right)\left(1 + \frac{H}{K_3}\right)}{1 + \frac{K_1}{H} + \frac{K_1K_2}{H^2}} \right) \quad (11)$$

$$\log Y = \log \frac{Y_L + \frac{Y_1K_1}{H} + \frac{Y_HK_1K_2}{H^2}}{1 + \frac{K_1}{H} + \frac{K_1K_2}{H^2}} \quad (12)$$

RESULTS

Effect of pH on V/K$_a$ Values. The effects of pH on the *V/K* values for several amino acid substrates were determined. The data obtained with tryptophan and phenylalanine fit well to eq 8 (Figure 1A). With both substrates, two groups appear to be titrating over the pH range studied; one group with an apparent pK_a value of 5.0 must be deprotonated for activity, and a second group with a pK_a value of about 10.0 must be protonated for activity (Table 1). In contrast, with methionine as substrate (Figure 1B), the data fit significantly better to eq 11 than to eq 8 (σ value of 0.072 versus 0.166). Equation 11 describes a pH dependence in which one group must be deprotonated and a second group must be protonated for activity, and deprotonation of a third group generates an enzyme with increased activity. These three pK_a values with methionine were 5.0, 9.4, and 9.8 (Table 1). The pK_a value of the methionine amino group is 9.4, suggesting that it must be protonated for activity. The *V/K* versus pH profiles obtained with tryptophan and phenylalanine also fit well to eq 11 when the pK_a value of the group which must be protonated was set equal to the pK_a value of the substrate's amino group. However, the σ values did not change significantly with these two substrates.

To determine directly if one of these pK_a values was indeed that of the substrate amino group, the effect of pH on the *V/K* value of the alternate substrate 3-phenyl-2-hydrazinopropanoic acid was determined (Figure 1B). The data fit to

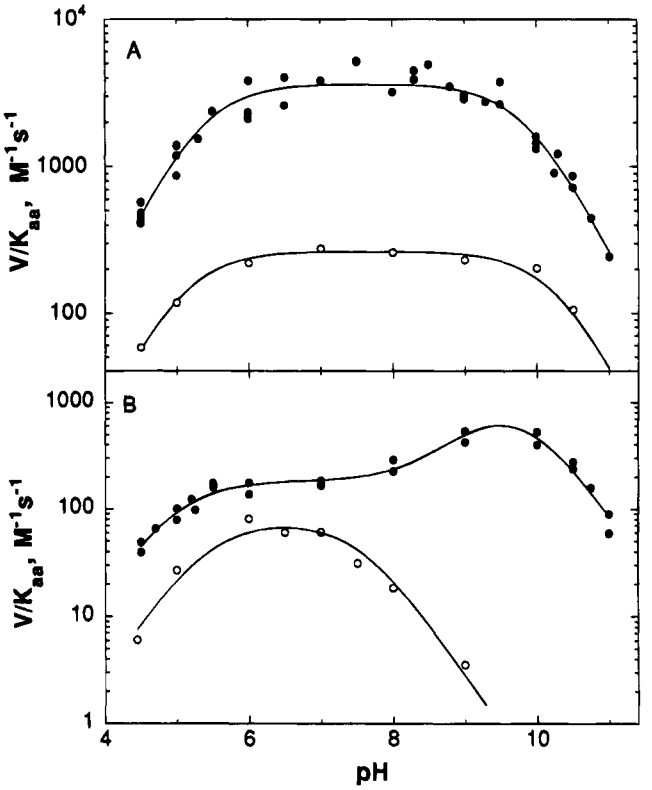


FIGURE 1: Effect of pH on the *V/K* values for amino acid substrates for tryptophan 2-monooxygenase. (A) *V/K* values for tryptophan (●) and phenylalanine (○). The lines are from fits of the data to eq 8. (B) *V/K* values for methionine (●) and 3-phenyl-2-hydrazinopropanoic acid (○). The lines are from fits of the data to eq 11 (methionine) or eq 8 (3-phenyl-2-hydrazinopropanoic acid).

eq 8 with pK_a values of 5.3 and 7.3 (Table 1). The higher pK_a value is equal to the pK_a value of the substrate's hydrazino group (Purdy, 1982), consistent with a requirement that the substrate amino group be protonated for activity. The data with all four substrates suggest that the substrate amino group must be protonated for catalysis. In addition, one group on the enzyme with an apparent pK_a value of about 5.0 must be unprotonated for activity with all substrates. Deprotonation of a second group with an apparent pK_a value of 10 gives rise to a form of the enzyme which shows increased activity with methionine as substrate.

Effect of pH on V/K$_{O_2}$ Values. Values of *V/K*$_{O_2}$ were determined over the pH range 4.5−11.0 with tryptophan, phenylalanine, and methionine as substrates. The effect of pH on the *V/K*$_{O_2}$ values with tryptophan could be fit to eq 8, but the pK_a values obtained from these fits were outside of the pH range over which assays were done (Figure 2). The data with phenylalanine also fit to eq 8, consistent with two groups titrating over this range (Figure 2). One group with a pK_a value of 4.5 has to be protonated while a second group with a pK_a value of 10.7 has to be unprotonated. In contrast, the data with methionine fit best to eq 9 (Figure 2); one group with an apparent pK_a value of 6.8 has to be unprotonated to obtain maximum *V/K*$_{O_2}$ values. These results are summarized in Table 1.

Effect of pH on V$_{max}$ Values. The effects of pH on V_{max} values were determined with tryptophan, phenylalanine, and methionine as substrates; the data fit best to eq 9. The results with methionine are shown in Figure 3; the results with all three substrates are summarized in Table 1. One group appeared to titrate over the pH range 4.5−11, with an

Table 1: pK_a Values for Tryptophan 2-Monooxygenase

parameter	eq	pK_1	pK_2	pK_3
V/K_{trp}	8	5.0 (5.0−5.2)[a]	10.1 (9.9−10.3)	
V/K_{phe}	8	5.3 (5.2−5.4)	9.8 (9.4−10.8)	
V/K_{met}	11	5.0 (4.9−5.1)	9.8 (9.6−9.9)	9.4 (9.2−9.6)
$V/K_{phenylhydrazinopropanoate}$	8	5.3 (5.1−5.6)	7.3 (7.0−7.5)	
$^DV/K_{met}$	12	5.1 (4.7−5.5)	10.0 (9.7−10.4)	
$V/K_{O_2(trp)}$	8	4.0 (3.8−4.3)	11.3	
$V/K_{O_2(phe)}$	8	4.5 (4.4−4.6)	10.7 (10.6−10.8)	
$V/K_{O_2(met)}$	9	6.8 (6.7−6.9)		
V_{trp}	9	6.3 (6.2−6.4)		
V_{phe}	9	7.7 (7.5−7.9)		
V_{met}	9	8.5 (8.3−8.7)		
$K_{indoleacetamide}$	10	6.0 (5.7−6.2)	10.4 (10.3−10.7)	
$K_{phenylacetamide}$	10	6.0 (5.7−6.2)	10.6 (10.3−11.1)	
$K_{hydrocinnamic\ acid}$	9	6.3 (6.2−6.4)		
$K_{phenylpyruvic\ acid}$	9	6.3 (6.2−6.4)		

[a] Range of values at a confidence interval of 67%.

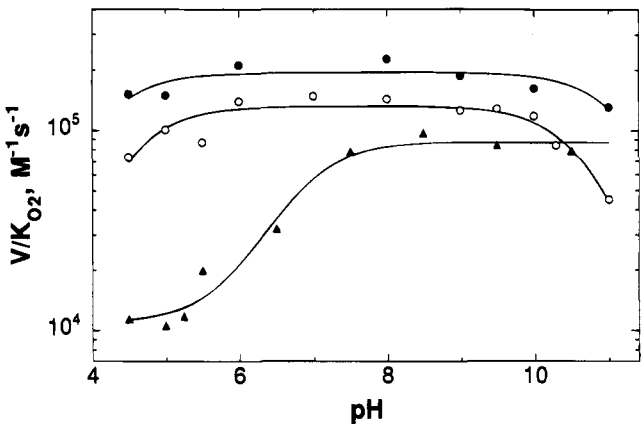


FIGURE 2: Effect of pH on the V/K values for oxygen as a substrate for tryptophan 2-monooxygenase with tryptophan (●), phenylalanine (○), and methionine (▲) as the amino acid substrate. The lines are from fits of the data to eq 8 (tryptophan and phenylalanine) or eq 9 (methionine).

increase in the V_{max} value when the group was deprotonated. The apparent pK_a value varied with the identity of the amino acid substrate, establishing that this is a pK_a value for an enzyme species with a form of the substrate still bound.

Effect of pH on pK_i Values for Competitive Inhibitors. The effects of pH on the pK_i values were determined for several inhibitors that are competitive versus the amino acid substrate. With both indole-3-acetamide (Figure 4) and phenyl-3-acetamide, the data fit best to eq 10. Binding affinity decreased above a pK_a value of about 10.5 and decreased to a finite value below a pK_a of 6.0. The effect of temperature on the lower pK_a value for inhibition by indoleacetamide was determined over the temperature range

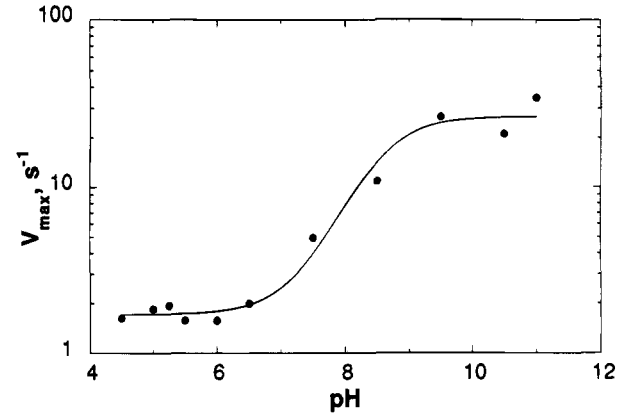


FIGURE 3: Effect of pH on the V_{max} value for tryptophan 2-monooxygenase with methionine as substrate. The line is from a fit of the data to eq 9.

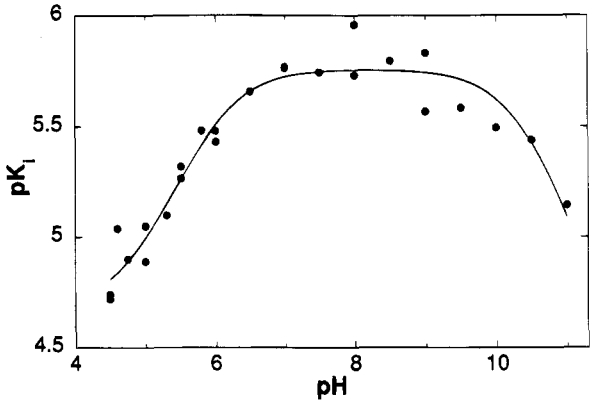


FIGURE 4: Effect of pH on the pK_i value for indoleacetamide as a competitive inhibitor of tryptophan 2-monooxygenase. The line is from a fit of the data to eq 10.

15−45 °C. These data gave an enthalpy of ionization of 6.3 ± 2.0 kcal mol^{-1}. This value is consistent with the residue responsible for this pK_a value being a histidine (Cleland, 1977).

Hydrocinnamic acid and phenylpyruvic acid are also competitive inhibitors versus the amino acid substrate. In both cases, the binding affinity decreased when a single residue on the enzyme was deprotonated. Because binding did not go to zero at high pH, the data were fit to eq 9. In both cases, tight binding of these inhibitors required protonation of a group with a pK_a value of about 6.3 (Table 1).

Primary Isotope Effects. Primary deuterium isotope effects were measured using methionine, phenylalanine, and tryptophan as substrates for tryptophan 2-monooxygenase. $^DV/K$ and $^DV_{max}$ values with [α-^{2}H]-L-tryptophan were small from pH 5 to pH 10. The $^DV_{trp}$ value was 1.59 ± 0.12 at pH 5.0, 1.05 ± 0.11 at pH 8.3, and 1.1 ± 0.10 at pH 10.0. The $^D(V/K_{trp})$ was also small over the pH range that tryptophan 2-monooxygenase is stable; the values were 1.15 ± 0.17 at pH 5.0, 1.09 ± 0.2 at pH 8.3, and 1.00 ± 0.10 at pH 10. Similarly, the $^D(V/K_{phe})$ value, measured with [α-^{2}H]-D,L-phenylalanine, was 1.12 ± 0.05 at pH 5.0 and 1.18 ± 0.034 at pH 10.0. $^DV_{phe}$ values were not determined since preliminary experiments with the racemic substrate at ambient oxygen concentration gave very small effects. In contrast, there was a large pH-dependent kinetic isotope effect on the $^D(V/K_{met})$ value (Figure 5), which increased from 1.8 ± 0.18 at pH 8.5 to approximately 5 at both low and high pH. The data were fit to eq 12, which assumes that

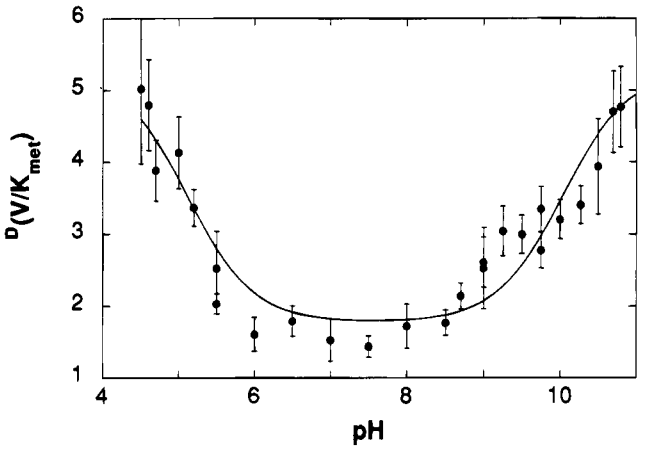


FIGURE 5: Effect of pH on the $^{D}(V/K)_{met}$ value. Initial rates were determined with tryptophan and [α-^{2}H]tryptophan as substrates for tryptophan 2-monooxygenase at 25 °C. Isotope effects at each pH were calculated using eq 3. The line is a fit of the data to eq 12.

the isotope effect increases to a maximum at both high and low pH. This gave limiting values for $^{D}(V/K_{met})$ of 5.3 ± 0.8 at low pH and 5.1 ± 0.8 at high pH. If the assumption were made that the limiting $^{D}(V/K_{met})$ value at high and low pH is the same, a value of 5.3 ± 0.8 was obtained. Protonation of a group with a pK_a value of 5.1 ± 0.4 or deprotonation of a group with a pK_a value of 10 ± 0.4 resulted in the increase in the observed $^{D}(V/K_{met})$ values. These pK_a values are identical to those found in V/K_{met} versus pH profiles.

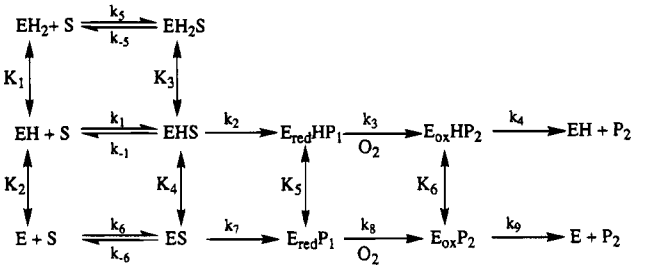
Secondary Isotope Effects. Secondary kinetic isotope effects provide information about bond rehybridization in the transition state. It was not possible to determine an α-secondary effect for tryptophan 2-monooxygenase since glycine is not a substrate. However, a β-secondary effect could be determined. This was done with alanine, a very slow substrate (Emanuele et al., 1995). The $^{D}V/K$ value with [α-^{2}H]-D,L-alanine at pH 8.3 was 5.3 ± 0.5. This value is similar to the limiting value of $^{D}(V/K_{met})$ found at the pH extremes and to the intrinsic $^{D}(V/K_{D\text{-}ala})$ value of 5.7 measured with D-amino-acid oxidase (Denu & Fitzpatrick, 1994a). The β-secondary kinetic isotope effect with [β,β,β-^{2}H$_3$]-D,L-alanine at pH 8.3 was 0.965 ± 0.041.

Solvent Isotope Effects. Solvent isotope effects were determined on the oxidative half-reaction with tryptophan as substrate at pH 8.3, where both the V_{max} and V/K_{O_2} values are independent of pH. There was no significant effect on the V/K_{O_2} value. The solvent isotope effect on the V_{max} value was 2.48 ± 0.18.

DISCUSSION

Previous studies of the mechanism of tryptophan 2-monooxygenase utilizing steady-state and rapid reaction kinetics are consistent with the kinetic mechanism of Scheme 1 (Emanuele & Fitzpatrick, 1995). The effects of pH on the activity of tryptophan 2-monooxygenase provide evidence for the expanded mechanism shown in Scheme 2. The amino acid substrate binds with its amino group protonated. This is clearly established by the *V/K* pH profile obtained with 3-phenyl-2-hydrazinopropanoic acid. With this substrate, a group with a pK_a value of 7.3 must be protonated for activity. This pK_a value matches the pK_a of the substrate hydrazino group; it is not seen in the pH profiles with the amino acids

Scheme 2



tryptophan, methionine, and phenylalanine where the pK_a values of the amino groups are about 9.2.

Below pH 8, the *V/K* pH profiles with all four substrates are consistent and show that a group on the protein with an apparent pK_a value of about 5 must be unprotonated for activity. This pK_a value is probably due to the same protein residue that must be unprotonated for tight binding of the amide inhibitors. pK_a values determined from *V/K* values can be perturbed if the substrate has a significant commitment to catalysis. This is not the case with pK_a values determined with competitive inhibitors (Cleland, 1982). Thus, the intrinsic value of the pK_a for the amino acid residue that must be unprotonated for tight binding of substrates and amide inhibitors is 6.0. A comparison of this value with the pK_a values obtained from *V/K* profiles suggests that all four substrates partition forward to react faster than they dissociate from the enzyme's surface, i.e., in Scheme 2 k_2 is larger than k_{-1}. These difference in pK_a values can be used to estimate the ratio of k_2, the rate constant for catalysis, to k_{-1}, the rate constant for substrate dissociation, using eq 13

$$\Delta pK_a = \log\left(1 + \frac{k_2}{k_{-1}}\right) \qquad (13)$$

(Cleland, 1977). Estimates of this value are 7 for tryptophan and methionine and 4 for phenylalanine and 3-phenyl-2-hydrazinopropanoic acid.

The kinetic isotope effects serve as independent measurements of the values of the forward commitments for tryptophan and methionine. The $^{D}V/K_{trp}$ value of 1.09 ± 0.2 at pH 8.3 reported here is smaller than the kinetic isotope effect on the bond breaking step (2.4) obtained from rapid reaction data (Emanuele & Fitzpatrick, 1995). If the intrinsic isotope effect is 2.4, a forward commitment of 7 would reduce $^{D}(V/K)_{trp}$ to 1.2, while a commitment of 15 would reduce it to 1.1. The larger value for the commitment is more consistent with the observed $^{D}(V/K_{trp})$ value at pH 5 of 1.15 ± 0.17. Thus, both pH and isotope effects are consistent with a large forward commitment for tryptophan as substrate for tryptophan 2-monooxygenase. In contrast, methionine is a less sticky substrate, so that measurements over the accessible pH range can be used to test the mechanism in Scheme 2. Protonation of a group with an apparent pK_a value of 5.1 both decreases the observed V/K_{met} value and increases the observed $^{D}V/K$ value. Such agreement of pK_a values is expected for the upper branch of Scheme 2, where the amino acid substrate can bind to the incorrectly protonated form of the enzyme. The value of k_2/k_{-1} can be calculated from the difference between the limiting $^{D}(V/K_{met})$ value of 5.3 at the pH extremes and the value of 1.8 at the pH optimum. The estimated value is 3.5

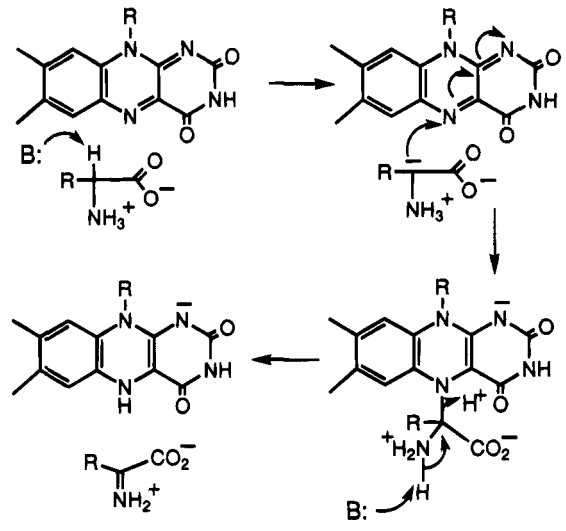
± 0.5; this is in reasonable agreement with the estimate from pH data alone for this substrate.

Binding of the inhibitors hydrocinnamic acid and phenylpyruvic acid also shows evidence for an active site residue with a pK_a value of about 6, but in this case the residue must be protonated. The simplest interpretation of these data is that a single residue with a pK_a value of 6 must be unprotonated for binding and catalysis and for binding of neutral inhibitors such as amides and protonated for binding of negatively charged inhibitors such as acids. Protonation of this residue would allow an electrostatic interaction between the positively charged residue and a negatively charged inhibitor, so that binding of acids would increase at lower pH.

The effect of temperature on the pK_a value of 6 for inhibition by indoleacetamide is consistent with a histidinyl residue. A histidinyl residue is also consistent with a pK_a value of 6. Consistent with such a conclusion, diethylpyrocarbonate will slowly inactivate tryptophan 2-monooxygenase (Emanuele et al., 1995). Kinetic isotope effect studies with tryptophan 2-monooxygenase are consistent with the formation of a carbanion intermediate (*vide infra*). Thus, a reasonable role for a histidine in the active site of tryptophan 2-monooxygenase is to act as the active site base responsible for removing the substrate α-hydrogen to form the carbanion. Histidines have been implicated as the active site bases in the FMN-containing enzymes lactate monooxygenase and glycolate oxidase, which are also thought to utilize carbanion mechanisms (Müh et al., 1994; Lindqvist & Brändèn, 1989).

The pH behavior above pH 8 is more complex. With 3-phenyl-2-hydrazinopropanoic acid as substrate, the complete *V/K* pH profile from pH 4.5 to pH 9 can be explained by a requirement that the residue on the protein with a pK_a value of 6 be unprotonated and a requirement that the amino (hydrazino) group of the substrate be protonated for productive binding. This is also the simplest explanation for the *V/K* pH profiles obtained with tryptophan and phenylalanine. However, such a model does not explain the V/K_{met}, $^D(V/K_{met})$, and amide pK_i pH profiles, all of which show the pK_a value of 10 that is not due to the amino group of a substrate. Deprotonation of this group increases the V/K_{met} value about 30-fold, while abolishing inhibition by indoleacetamide and phenylacetamide. This behavior is consistent with deprotonation of this residue being coupled to a conformational change which results in a more open form of the enzyme in which both association and dissociation of the substrate are more rapid, so that the forward commitment becomes zero. The effects of pH on the $^D(V/K_{met})$ value provide strong support for such a model. If the increase in the $^D(V/K_{met})$ values at high pH were due only to binding of the substrate to an incorrectly protonated and therefore inactive form of the enzyme, the V/K_{met} value should *decrease* above a pK_a value of about 10 (Cook & Cleland, 1981). Since it increases, such a mechanism is not appropriate. However, the data are consistent with a mechanism in which there is a change to a more open conformation of the enzyme above pH 10, so that both k_6 and k_{-6} are significantly greater than k_1 and k_{-1}, while k_2 and k_7 are not significantly different. In this case, there will be an increase in the $^D(V/K_{met})$ value, because the forward commitment is equal to k_7/k_{-6} at high pH and $k_{-6} > k_7$. Further indirect evidence for a conformational change in tryptophan 2-monooxygenase comes from the effect of pH on the stability of the enzyme. Tryptophan 2-monooxygenase is 10-fold less stable at pH 10.6 than at pH 8.3, even though the high pH form of the enzyme is more active (Emanuele et al., 1995).

Scheme 3



The change in conformation appears to have much less effect on binding and catalysis with phenylalanine and tryptophan as substrates. If the data from the *V/K* pH profiles with these substrates are fit to the same model as was used for methionine, no significant change occurs in the pK_a values or the quality of the fits. The increase in the *V/K* value above the pK_a of 10 is only 4-fold with phenylalanine and 7-fold with tryptophan. This suggests that any improvement in catalysis is offset to a greater degree by a loss in binding affinity with these two substrates. The open conformation must also bind to amide inhibitors much more weakly, suggesting that there is a larger effect on k_{-1} than on k_1 if the inhibitor or substrate contains an aromatic side chain.

Scheme 3 shows a chemical mechanism for the reductive half-reaction of D-amino-acid oxidase and related flavoprotein oxidases that is supported by a number of experimental approaches (Ghisla, 1982). Secondary kinetic isotope effects can serve as probes of the mechanism of carbon−hydrogen bond cleavage to test such a mechanism. Intrinsic α- and β-secondary deuterium kinetic isotope effects have been measured for D-amino-acid oxidase. In neither case is the value significantly different from 1 (Denu & Fitzpatrick, 1994a), consistent with cleavage of the substrate carbon−hydrogen bond by removal of the α-proton to form a carbanion, as shown in Scheme 3. Since glycine is not a substrate for tryptophan 2-monooxygenase, it was not possible to measure an α-secondary isotope effect. However, it was possible to determine a β-secondary isotope effect. The $^DV/K$ value with [α-^{2}H]-D,L-alanine, an extremely slow substrate, is 5.3 ± 0.5 at pH 8.3. This value is identical to the limiting $^D(V/K)_{met}$ value of 5.3 measured at the pH extremes with methionine and comparable to the intrinsic $^DV/K$ value of 5.7 reported for D-amino-acid oxidase with D-alanine as substrate (Denu & Fitzpatrick, 1994a). These data suggest that 5.3 is the intrinsic isotope effect with alanine as a substrate for tryptophan 2-monooxygenase. The β-secondary deuterium kinetic isotope effect determined with alanine as a substrate for tryptophan 2-monooxygenase is not significantly different from one. β-Secondary isotope effects are the result of changes in hyperconjugation which

occur in the transition state (Cleland, 1987). A value of 1.2 has been reported for lactate dehydrogenase, which uses a hydride transfer mechanism (Cook et al., 1981). The absence of a significant β-secondary isotope effect under conditions where carbon−hydrogen bond cleavage is fully rate limiting is inconsistent with a hydride transfer mechanism. These results suggest that the mechanisms of carbon−hydrogen bond cleavage by D-amino-acid oxidase and tryptophan 2-monooxygenase are the same.

The pH profiles for the V/K_{O_2} values are consistent with phenylalanine and tryptophan fitting more tightly into the active site than does methionine. Thus, the V/K_{O_2} value with tryptophan is essentially independent of pH, while that for phenylalanine is only affected at the pH extremes. In contrast, with methionine there is clear evidence for the importance of a group with a pK_a value of 6.8. This is likely to be the FAD itself, which has a pK_a value of 6.2 when free in solution (Ehrenberg & Hemmerich, 1968). Deprotonation of the flavin N(1) increases the rate of the reaction with oxygen by about an order of magnitude (Eberlein & Bruice, 1983); this is very similar to the behavior of the V/K_{O_2} value with methionine as substrate. With the other two substrates the FAD N(1) proton is not accessible to solvent, so that the $FADH_2$ would be in the anionic form above pH 5.

The lack of a solvent isotope effect on the V/K_{O_2} value is consistent with the mechanism proposed by Eberlein and Bruice (1983) for the reaction of reduced flavins and O_2. The initial and rate-determining step is single electron transfer from the reduced flavin to O_2 The resultant superoxide and flavin radicals then rapidly couple to form the flavin 4a-peroxide. Rate-limiting electron transfer will not generate a solvent isotope effect. There is also no solvent isotope effect on the V/K_{O_2} value with D-amino-acid oxidase (Denu & Fitzpatrick, 1994b). That enzyme does not catalyze the oxidative decarboxylation of its substrate. Conversion of the imino acid to the amide by tryptophan 2-monooxygenase occurs at a rate indistinguishable from the rate of reaction of the reduced enzyme−imino acid complex with oxygen. The pH dependence of this step and the lack of a solvent isotope effect are common to both tryptophan 2-monooxygenase and D-amino-acid oxidase. This suggests that the decarboxylation step occurs very rapidly once hydrogen peroxide is formed at the active site upon flavin oxidation, as suggested by Lockridge et al. (1972). The different fates of the imino acids with the two enzymes must be due to differences in the active site structure which are not reflected in the other steps in the reaction.

The pH behavior of the V_{max} values reported here is similar with all three substrates examined, in that protonation of a group on the enzyme results in an increase in rate. With tryptophan as substrate, product release is the rate-limiting step in turnover (Emanuele & Fitzpatrick, 1995). Consequently, the pK_a values measured from V_{max} profiles would be for enzyme−product complexes and will vary with the substrate used. There is also a significant solvent isotope effect on the V_{max} value with tryptophan as substrate at pH 8.3; this is consistent with slow transfer of an exchangeable proton being coupled to product release. An identical situation has been observed with D-amino-acid oxidase; in that case, a proton inventory has shown that a single exchangeable proton is being transferred in a step coupled to product release (Denu & Fitzpatrick, 1994b). The pH and solvent isotope effects on the V_{max} values for tryptophan 2-monooxygenase can similarly be attributed to rate-limiting product release involving a pH-dependent conformational change which is coupled to transfer of a solvent-exchangeable proton.

Values of $^DV_{trp}$ are small across the accessible pH range. This is consistent with at least one other step in the mechanism being slower than carbon−hydrogen bond cleavage. Rapid reaction studies at pH 8.3 (Emanuele & Fitzpatrick, 1995) give an estimated rate for the isotope sensitive step with tryptophan of 139 s^{-1} (k_2 in Scheme 1). The calculated value of k_4 is 14.9 s^{-1}; this gives a value for k_2/k_4 of 9.3. A commitment of this magnitude would reduce an isotope effect of 2.4 to about 1.15; this value is in good agreement with the observed $^DV_{max}$ value of 1.1 at this pH.

The results presented here in combination with those in the previous manuscript present a detailed picture of the kinetic and chemical mechanisms of tryptophan 2-monooxygenase. This enzyme is a member of a poorly characterized group of flavoproteins that catalyze the oxidative decarboxylation of imino acids. In addition, the sequence of tryptophan 2-monooxygenase is distinct from that of known proteins. Despite these differences in reaction and structure, many of the details of the reaction are similar to those of D-amino-acid oxidase, which has been studied in great detail. Thus, carbon−hydrogen bond cleavage likely involves a carbanion in both enzymes (Curti et al., 1991; Denu & Fitzpatrick, 1994a). However, the pH dependencies of this step are very different in the two enzymes. With D-amino-acid oxidase, the pH dependence of V/K values for amino acid substrates is consistent with a residue with a pK_a value of 8.8 acting as the active site base (Denu & Fitzpatrick, 1992), while with tryptophan 2-monooxygenase a histidine with a pK_a value of 6 is the likely candidate. In both enzymes, the initial reaction of reduced enzyme with oxygen appears to be similar, but tryptophan 2-monooxygenase then catalyzes the rapid decarboxylation of the imino acid. These differences suggest that the two enzymes evolved separately and that their active sites are distinct. Three-dimensional structural information will be required to definitively address this point.

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