Isotope Effects and Alternative Substrate Reactivities for Tryptophan 2,3-Dioxygenase*

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Tryptophan 2,3-dioxygenase (EC 1.13.1.12) is a hemoprotein which catalyzes the first step in the oxidative degradation of tryptophan. The reaction believed to proceed by addition of O₂ across the 2,3-bond of the indole ring, followed by decomposition of the resultant dioxetane to give N-formylkynurenine. A primary D₂O isotope effect of 4.4 on Vₘₐₓ/Kₘ was observed at the pH optimum, pH 7.0. This implies that abstraction of the indole proton is at least partially rate-determining. An inverse secondary isotope effect of 0.96 was observed for L-[2-³H]tryptophan at this pH. The secondary isotope effect signals the formation of the C-0 bond at C-2. As the rate of proton abstraction increased with increasing pH, the D₂O isotope effect decreased to 1.2 at pH 8.5 and the secondary isotope effect increased to 0.92. The rate-determining steps therefore change with increasing pH, and bond formation at C-2 becomes more rate-limiting. The secondary isotope effect did not change significantly with varying O₂ concentration so that substrate binding is primarily ordered with O₂ binding first. The specificity of the enzyme towards substituted tryptophans shows that substitution of the phenyl ring of the indole is sterically unfavorable. Steric hindrance is highest at the 4- and 7-positions, while the 5- and 6-positions are less sensitive. 6-Fluoro-L-tryptophan was more reactive than tryptophan, and the increased reactivity can be explained by an electronic effect that enhances the rate of C-0 bond formation at C-2.

EXPERIMENTAL PROCEDURES

Materials—[L-²³C]Tryptophan was purchased from Research Products Int. L-[³¹C]Tryptophan was purchased from New England Nuclear Research Products. [²³H]Tryptophan was synthesized by catalytic tritiation of the N-1 trifluoroacetyl methyl ester derivative of 2-bromotryptophan (Du Pont-New England Nuclear). The labeled protected tryptophan was resolved and deprotected with chymotrypsin and carboxypeptidase A. Synthesis of the protected 2-bromotryptophan and deprotection were according to Phillips and Cohen (1986). Fluorotryptophan analogues were resolved by the same derivatization and enzymatic protection scheme. The resulting l-isomers were purified by HPLC on a C18 column in 5 mM potassium phosphate, pH 4, using a gradient from 0 to 50% acetonitrile. Enantiomeric purity was confirmed by derivatization with Marfey’s reagent (Marfey, 1984). Specificity of tritium labeling was demonstrated by preparing [²³H]tryptophan in parallel and characterizing the product by deuteron NMR. Additionally, tritium was shown to be released quantitatively as formate after incubation of labeled substrate with tryptophan dioxygenase and hydrolysis of the N-formylkynurenine product (see below). 7-Fluoro-L-tryptophan was a generous gift from Dr. Robert Phillips, Athens, GA. Other substituted tryptophans and L-tryptophan were purchased from Sigma. N-(3-fluorenyl)-methoxycarbonyl-alanine (Fmoc-Ala) was from Applied Biosystems, San Jose, CA. Sprague-Dawley rats were purchased from Charles River.

Purification of Tryptophan 2,3-Dioxygenase—The procedure of Schutz and Feigelson (1972) was adapted for the induction and purification of tryptophan dioxygenase from rat liver with minor modifications. Prednisolone sodium phosphate was used to induce the enzyme at a single dose of 50 mg/kg body weight, which was given intraperitoneally 5.5 h before excising livers. DEAE-cellulose was substituted for DEAE-Sephadex and the final gel electrophoresis step was omitted. Instead, DEAE-cellulose fractions were concentrated using Centricon-10 protein concentrators (Amicon) and desalted on a G-25 column. The DEAE-cellulose-purified tryptophan dioxygenase was loaded onto a Mono Q column (Pharmacia LKB Biotechnology).

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* The abbreviations used are: HPLC, high performance liquid chromatography; Fmoc, N-(9-fluorenyl) methoxycarbonyl.
equilibrated with 20 mM HEPES, pH 7.0, containing 10 mM L-tryptophan. Activity was eluted with a linear gradient from 0 to 1.0 M KCl in the equilibrating buffer. Purified tryptophan dioxygenase was stored at -80 °C in the presence of 10 mM L-tryptophan. This preparation had no contaminating kynurenine formamidase activity. Assays for Tryptophan Dioxygenase Activity—All determinations were initiated by addition of enzyme. After 30 min at 37 °C, 100 μl of pH 7, 100 mM potassium phosphate buffer with 4.9 mM L-tryptophan, 2.0 mM sodium ascorbate (prepared fresh daily), and 0.92 μCi hematin (prepared fresh daily) were added to each tube. Routine radiometric assays were based on the fact that N-formylkynurenine is readily hydrolyzed to kynurenine and formic acid in the presence of 3% perchloric acid (Ozaki et al., 1986). The assay solution contained: 4.9 mM L-tryptophan, 20 mM sodium ascorbate, 0.46 μM hematin, and L-[2-14C]tryptophan (typically 400 dpm/nmol) in 0.1 M KH2PO4, pH 7.0. The reaction was initiated by addition of enzyme. After 30 min at 37 °C, 100 μl of 6% perchloric acid was added and the samples were incubated an additional 30 min. 1.0 ml of 10% charcoal (w/v) suspended in 3% perchloric acid containing 1.0 mM formic acid was added, and the samples were vortexed and then centrifuged for 5 min at 15,000 rpm to remove unreacted tryptophan. A 0.6-ml aliquot from each tube was counted to determine the extent of labeled formate formation.

HPLC analysis of reaction mixtures was performed on a C18 column using gradient elution with acetonitrile/water buffered with either 5 mM KH2PO4, pH 4.0 (Yim et al., 1987), or 10 mM ammonium acetate. Molecules were separated on four, 250 × 4.6-mm Brownlee ODS columns (5-μm particles, Perkin-Elmer) were used in series at a flow rate of 1.5 ml/min. The columns were eluted for 5 min with 100 mM potassium phosphate and 100 mM Tris in either H2O or D2O at pH 7.0, 8.0, and 8.4. pH meter readings in D2O were corrected for the known shift of 0.4 units from the true pD (Schowen and Schowen, 1982). 3 mM α-Methyl-DL-tryptophan was included in all the reaction mixtures in order to saturate allosteric interactions with the enzyme (Schutz et al., 1972). The enzyme was concentrated and exchanged into buffers of the appropriate pH containing 90% H2O and 10% D2O by desalting on a P-10 column. Either 5 or 10 μl were added to reaction mixtures to give a final volume of 100 μl so that the isomeric purity of the water was >95% in the reaction mixtures. The rate of oxidation of L-tryptophan was determined using the charcoal precipitation assay described above. The rates of 5-fluoro-L-tryptophan and 6-fluoro-L-tryptophan oxidation relative to L-tryptophan were determined by HPLC analysis as described above.

RESULTS

Substrate Specificity—Reactivities of 4-, 5-, 6-, and 7-substituted tryptophans relative to tryptophan are given in Table I. A number of other tryptophan analogues were tested but turned over at <1% of the rate for tryptophan: 7-aza-DL-tryptophan, 5-hydroxy-α-methyl-DL-tryptophan, 1-thio-DL-tryptophan, 5-hydroxy-DL-tryptophan, and N-methyl-DL-tryptophan. Relative V/K values for tryptophan analogues were measured directly by comparing the ratio of turnover of each analogue relative to tryptophan when the two substrates were incubated together in the same reaction mixture (Abelès et al., 1960). Note that rates for L-isomers were determined in the presence of α-methyltryptophan, an allosteric effector. In all cases, substrates with methyl or bromo substituents were poorer substrates than tryptophan. This agrees with previous studies (Civen and Knox, 1960) and most probably reflects steric hindrance, since these substituents have only a small electronic effect on the pKs of tryptophan (Yagil, 1967). This effect is greatest at positions 4 and 5 and smallest at positions 5 and 6.

The 5-, 6-, and 7-fluorotryptophans also showed a wide range of reactivities. In parallel with the methyl-substituted analogues, 7-fluorotryptophan was least reactive, and the 6-fluoro analog was most reactive. The general trend (7 < 5 < 6) follows that predicted by steric effects, even for a substituent as small as fluorine. 6-Fluoro-L-tryptophan was actually a better substrate than tryptophan, however. This may well represent an electronic effect of fluorine that is large enough to dominate steric effects at this position.

When comparing the reactivity of substrate analogues in a two-substrate reaction, the relative Vmax/Km values for one substrate versus its analogues can change when the concentr...
tration of the second substrate is varied. Variations in relative $V_{\text{max}}/K_m$ values depend on the order of substrate binding ("Appendix"). Accordingly, the $K_m$ for $O_2$ was calculated by fitting oxygen electrode data for $O_2$ consumption to the integrated Michaelis-Menten equation in order to establish a meaningful range for varying $O_2$ concentrations. The $K_m$ was 7.2 $\mu$M at pH 7.0 and 6.9 mM tryptophan. The relative $V_{\text{max}}/K_m$ values for 6-fluorotryptophan compared to tryptophan were measured at $O_2$ concentrations above and below the $K_m$ and were 2.14 ± 0.06 at 5 $\mu$M $O_2$, compared to 2.13 ± 0.06 at 250 $\mu$M $O_2$. These results are consistent with ordered binding with $O_2$ binding first.

**D_{2}O Isootope Effects**—The initial velocities for tryptophan oxidation by tryptophan dioxygenase are presented in Fig. 1 as a function of pH in H$_2$O and D$_2$O. Velocities were measured below the $K_m$ for tryptophan and reflect $V_{\text{max}}/K_m$ values. These reactions were all performed in the presence of 3 mM $\alpha$-methyl-DL-tryptophan to minimize ambiguities due to allosteric effects (Schutz et al., 1972). Rates of tryptophan oxidation in H$_2$O were optimum at pH 7.0. The pH rate profile in D$_2$O differed substantially, however. The reaction in D$_2$O was 5.25-fold slower than the reaction in H$_2$O at low pH, pH 6.5. When the pH was increased, the D$_2$O isotope effect decreased to only 1.19 at pH 8.4. The pH optimum in D$_2$O was pH 8.4 or higher. The relative rates of turnover of 6-fluoro-L-tryptophan versus [2-14C]tryptophan were also determined over a range of pH values in both H$_2$O and D$_2$O (Fig. 2). The 6-fluoro substituent effect did not change with pH in D$_2$O and was small, approximately 1.5. In H$_2$O, the substituent effect was similarly small at low pH but increased to 2.8 at pH 8. Changes in the substituent effect will reflect changes in rate-limiting steps with pH or solvent, and steps sensitive to 6-fluoro substitution apparently predominate at high pH in H$_2$O. The data for 6-fluorotryptophan are also plotted as absolute rates in the inset for Fig. 2. The substrate analogue shows a solvent isotope that is comparable in magnitude to that for tryptophan and is similarly sensitive to pH, although the maximum isotope effect persists up to pH 7.5 for this substrate.

**$^3$H Isotope Effects**—The $^3$H isotope effect on $V_{\text{max}}/K_m$ for [2-$^3$H]tryptophan was determined in competition with L-[2-$^14$C]tryptophan (Table II). In the initial experiment at pH 7.0, the observed isotope effect was 0.96 ± 0.01. At pH 8.4, the isotope effect was significantly larger, 0.92 ± 0.01. The variation of isotope effects with substrate concentration can provide information about order of substrate binding if isotope effects below and above the substrate $K_m$ are compared (Klinman et al., 1980). The $^3$H isotope effect for tryptophan dioxygenase did not change significantly when measured at 5 $\mu$M $O_2$ compared to a normal $O_2$ concentration of 250 $\mu$M, again indicating ordered addition with $O_2$ binding first.

**DISCUSSION**

Based on solvent and substrate isotope effects, alternative substrate reactivities, and pH rate profiles, the mechanism of Fig. 3 is proposed for tryptophan 2,3-dioxygenase. It is assumed that oxidative cleavage of the indole ring proceeds via formation and decomposition of a dioxetane intermediate. Our results provide information regarding the steps leading to dioxetane formation as outlined below.

Large D$_2$O isotope effects were observed for tryptophan at low pH: 5.2 at pH 6.5 and 4.4 at pH 7.0 (Fig. 1). A similarly large isotope effect of 6.1 was observed for 6-fluorotryptophan at pH 7.5 (Fig. 2, inset). There are numerous possible sources of solvent isotope effects (Klinman, 1978; Schowen and

![Fig. 1. The initial velocity of tryptophan 2,3-dioxygenase at five different pH values in H$_2$O and D$_2$O. Assays were performed in triplicate, rates were determined at levels of tryptophan below its $K_m$ ($V_{\text{max}}/K_m$ values), and error bars represent standard errors.](image-url)
It is common, for example, that pk values will shift to approximately 0.5 units higher in D2O, and this may be observed as a shift by as much as 0.5 units to higher pH in enzyme pH rate profiles. The solvent isotope effect for tryptophan dioxygenase, however, does not appear to be related to a simple shift of pH rate profiles. Thus, a shift of the curve for tryptophan turnover in D2O by 0.5 units to lower pH would have no effect on the observed isotope effect at pH 7.0. In addition, the pH rate profile for 6-fluorotryptophan in D2O is very similar to that for tryptophan, while the pH optimum in H2O is pH 7.0 for tryptophan and pH 8.0 for the fluoro derivative. The D2O pH rate profiles for the two substrates, therefore, do not appear to represent simple and equal shifts of the profiles in H2O. In the absence of a major contribution by a simple shift in pk, the size of the observed isotope effects is large enough to be considered a primary kinetic isotope effect for transfer of an exchangeable proton. Considering the nature of the tryptophan dioxygenase reaction, the most straightforward interpretation of the effect would be that it represents deprotonation of the indole nitrogen.

A secondary isotope effect was observed for L-[2-3H]-tryptophan. This isotope effect increased from 0.96 at pH 7.0 to 0.92 at pH 8.4. The secondary isotope effect is inverse and is indicative of a change in hybridization from sp2 to sp3 at position 2, i.e. carbon-oxygen bond formation at C-2. The magnitude of the secondary isotope effect at pH 8.4 is sufficiently large to indicate that bond formation is essentially complete in the rate-limiting step at high pH (Klinman, 1978). Since the 3H isotope effect increased with pH as the solvent isotope effect decreased, proton transfer and bond formation at C-2 must represent different steps in the reaction mechanism. Furthermore, the proton transfer step must precede C-O bond formation. This is because secondary isotope effects will generally be largest if they precede the rate-determining step and will be masked only if they follow the slow step.

The fact that the secondary isotope effect decreased by only a factor of two between pH 8.4 and 7 indicates that C-O bond formation at C-2 is still partially rate-limiting at the pH optimum. This in turn implies that proton abstraction from the indole nitrogen is only partially rate-limiting at pH 7, and the intrinsic solvent isotope effect may therefore be larger than that observed by as much as a factor of two.

Varying the O2 concentration from 5 to 250 μM did not significantly affect the secondary isotope effect, and this can be taken as indication of a predominantly ordered binding pattern with O2 binding first (Klinman et al., 1980). We also found that the rate of turnover of 6-fluorotryptophan relative to tryptophan did not change with varying O2 concentration. The interpretation of the effect of O2 concentration on the relative rates of alternative substrate turnover is similar but not identical to that for competitive isotope effects. An analysis is provided in the "Appendix." The fact that O2 binds first indicates that O2 binds to heme and implies that the function of the heme is to localize and activate O2 rather than tryptophan. This order of binding is contrary to that proposed for the closely related enzymes indoleamine dioxygenase (Ishimura et al., 1970) and tryptophan dioxygenase of P. acidovorans (Koike and Feigelson, 1971) based on equilibrium binding studies. The reason for the discrepancy may be either that the related enzymes have different kinetic mechanisms or that the equilibrium results are misleading in their prediction of kinetics.

Free energy changes for selected steps of the tryptophan dioxygenase reaction are provided in Table III and allow us to both strengthen and extend the above conclusions. Tryptophan is a weak acid (Equation 1, Table III) and we must consider whether it is thermodynamically feasible that the tryptophanyl anion is an intermediate in the reaction. The accessibility of high energy intermediates in enzyme reactions has been discussed by Jencks (1980) and recently by Gerlt and Gassman (1992). The barrier to proton abstraction from tryptophan by a base on the enzyme with pk = 7 would be 13.6 kcal/mol. To this thermodynamic barrier must be added a kinetic barrier which should be no more than the 3 kcal/mol barrier for diffusion-controlled transfer of a proton from a nitrogen base in solution. The total barrier of 16.6 kcal/mol is very close to the predicted allowable limit of 16.7 kcal/mol (Gerlt and Gassman, 1992) for the tryptophan dioxygenase reaction with kcat = 10 s⁻¹ (Schutz and Feigelson, 1972). We
proceeds via the 3-peroxy intermediate (ionic intermediates. We therefore suggest that addition of 1- \(\text{Fe}^{3+} \cdot \text{O}_{2}^{-}\) would catalyze bond formation at C-2 as shown in step 4 of Fig. 3. The protonated intermediate thus formed is enclosed by concerted addition of \(\text{O}_2^+\) and \(\text{Fe}^{2+}\) \(\text{O}_2^-\) to \(\text{Fe}^{3+}\). Reprotonation on the imino nitrogen would permit activation of tryptophan to oxidation by proton \(\text{H}^+\) of \(\text{Kd}\). The anilinium anion formed upon concerted addition of \(\text{H}^+\) would be a strong base with a \(\text{pK}_a\) of approximately 24.5. We conclude that the tryptophanyl anion is a viable intermediate and note that it is probably not fortuitous that the observed rate of reaction is equal to that predicted from the thermodynamics.

Iron-catalyzed formation of dioxygenases has been proposed to be a concerted singlet biradical reaction (Sheu et al., 1990), and we can address the question whether formation of the dioxygen in the tryptophan dioxygenase reaction proceeds by concerted addition of \(\text{O}_2\) across the 2,3-bond. Formation of the C-O bond at C-2 occurs in a step subsequent to and discrete from proton transfer. Furthermore, if deprotonation of nitrogen is catalyzing addition of \(\text{O}_2\), then reprotonation must occur in a step subsequent to addition of \(\text{O}_2\) (Fig. 3). An analysis of the effect of fluorine is greatest at high pH where ring closure to form the dioxetane becomes more rate-limiting, and we propose that the effect of fluorine is to activate the intermediate imine to nucleophilic attack (step 5, Fig. 3).

Finally, we show C-O bond formation at C-3 proceeding in steps of proton abstraction, 1-electron transfer, and bond formation. It is equally likely that C-O bond formation is concerted with proton abstraction, and the proposed intermediates are enclosed in brackets to indicate that protonation of the imine may be a discrete step or may be concerted with C-O bond formation. This mechanism parallels closely the chemistry of electrochemical and photosensitized oxidations of tryptophan (Nguyen et al., 1986; Nakagawa et al., 1977a, 1977b) in terms of formation of a 3-peroxy intermediate and subsequent acyclic-nucleophilic addition at C-2. In the model reactions, however, the nucleophil that adds to C-2 is solvent or the amino group of tryptophan, and the thermodynamically less favorable dioxetane formation is not observed. The reactivity of 6-fluorotryptophan is also consistent with the proposed mechanism. The 6-fluorine analogue is a better substrate than tryptophan, and this is taken to represent an electronic effect. The effect of fluorine does not represent a simple effect on the \(\text{pK}_a\) of tryptophan since the effect is suppressed in \(\text{D}_2\text{O}\) where proton transfer is more rate-limiting. The effect of fluorine is greatest at high pH where ring closure to form the dioxetane becomes more rate-limiting, and we propose that the effect of fluorine is to activate the intermediate imine to nucleophilic attack (step 5, Fig. 3).

APPENDIX

**Kinetic expressions for ordered binding mechanisms** are provided in Table IV. Rate constant ratios defining \(k_{\text{cat}}/K_m\),

![Fig. 4. Minimal kinetic scheme for 2-substrate reactions.](image-url)
for tryptophan are given for the limiting cases of high and low O₂ concentrations. This approach has been used to predict the behavior of competing isotopically labeled substrates (Klinman et al., 1980). The isotope effects are a simplified case in which the assumption can be made that any difference in rates of turnover must arise from steps associated with a chemical reaction (k₁ in this case). The treatment can be generalized to any two competing substrates, in this case 6-fluorotryptophan versus tryptophan, with one important limitation.

If O₂ binds first, then it can be said without qualification that the relative rates of turnover of two competing substrates will not change as the O₂ concentration is varied. This can be seen in the ratio of rate constants at low versus high O₂ concentrations. This ratio depends only on the binding constant for binding of O₂ to free enzyme and will therefore be independent of the identity of the substrate.

If tryptophan binds first, then any differences in rate between alternative substrates that are reflected in rate constants k₁, k₂, k₃, or k₄ will be observed only at low O₂ concentrations. This is parallel to the situation for competing isotopically labeled substrates, for which the differences in rate would be associated only with k₃. However, in the more general case of any two competing substrates, observed differences in rate can also be associated with k₁, if that rate constant is a factor in the rate expression at all O₂ concentrations. In the general case, therefore, it is only possible to say that relative rates may change and that the direction of the change may be to increase or decrease with changing O₂ concentrations, depending on the contribution of the rate of binding, k₁, to the differences in rate.

For 6-fluorotryptophan versus tryptophan we would argue that the difference in k₅/k₄ is not significantly determined by k₁ since the relative rates vary both with pH and D₂O versus H₂O as solvent. We argue that, in the present case, the increased reactivity of 6-fluorotryptophan is an effect on a chemical step of the reaction rather than simply on the rate of binding. The data for 6-fluorotryptophan at high and low O₂, therefore, support a kinetic mechanism in which O₂ binds first.