6-(Difluoromethyl)tryptophan as a Probe for Substrate Activation during the Catalysis of Tryptophanase

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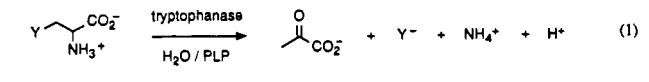
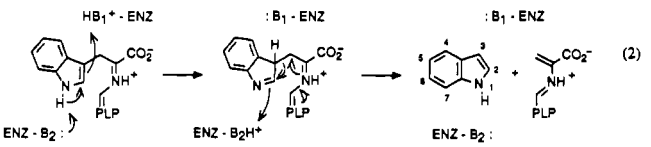
ABSTRACT: A substrate analogue, 6-(difluoromethyl)tryptophan, was developed and characterized for mechanistic investigation of tryptophanase. The utility of this derivative was based on its ability to partition between fluoride elimination and carbon–carbon bond scission during tryptophan metabolism. The nonenzymatic hydrolysis to 6-formyltryptophan occurred slowly under neutral conditions with a first-order rate constant of 0.0039 min^{-1}. This process, however, was accelerated by 10^4-fold upon deprotonation of the indolyl nitrogen (N-1) at high pH. Tryptophanase did not detectably facilitate this hydrolysis reaction, since no protein-dependent conversion of the difluoromethyl group was detected. Instead, the enzyme accepted the fluorinated species as an analogue of tryptophan and catalyzed the corresponding formation of 6-(difluoromethyl)indole, pyruvate, and ammonium ion. Anionic intermediates are therefore not expected to form during the catalytic activation of the indolyl moiety. Instead, aromatic protonation likely promotes the release of indole during enzymatic degradation of tryptophan.

Tryptophanase (tryptophan–indole lyase, E.C. 4.1.99.1) catalyzes a series of β-elimination reactions that are common to many pyridoxal-dependent enzymes (eq 1). Active substrates for this enzyme include a variety of amino acids that possess a good leaving group (Y in eq 1) at the β position as evident in *O*-methyl-L-serine, *O*-acetyl-L-serine, *O*-benzoyl-L-serine, *S*-methyl-L-cysteine, *S*-benzyl-L-cysteine, β-chloro-L-alanine, and *S*-(*o*-nitrophenyl)-L-cysteine (Newton & Snell, 1964; Suelter et al., 1976; Phillips, 1987). Tryptophan, the notable exception to this series, is of course also a substrate for the elimination reaction (Y = indole), but in this case, the cleavage of an unactivated carbon–carbon bond is required. Since the C-3 anion of indole [estimated pK_a of ca. 44 (Phillips et al., 1984)] is an extremely poor leaving group, tryptophanase is thought to provide a method for activating the indolyl moiety prior to its expulsion from the tryptophan skeleton.





A particularly appealing mechanism for stabilizing the nascent indole has been proposed to include a tautomerization of the pyrrole ring of indole via deprotonation at N-1 and reprotonation at C-3 (eq 2) (Davis & Metzler, 1972; Phillips et al., 1984). Elimination of the resulting indolenine species would then allow for rearomatization and final protonation at N-1 [pK_a of ca. 17 (Yagil, 1967)]. Two compounds that mimic the tetrahedral geometry at the C-3 position of the indolenine intermediate, oxindolyl-L-alanine and 2,3-dihydro-L-tryptophan, have already been shown to behave as reaction-intermediate analogues (Phillips et al., 1984, 1985). Both species are competitive inhibitors of tryptophan degradation with K_i values 100-fold less than the K_m for tryptophan. More recently, the active-site acid/base groups thought to interact with the N-1 and C-3 of tryptophan have been described (pK_a 6.0 and 7.6) by multiple kinetic and solvent deuterium isotope effects on the steady-state turnover of tryptophanase (Kiick & Phillips, 1988a). Pre-steady-state kinetics have also been measured and found to be consistent with the release of indole subsequent to a (partially) rate-determining tautomerization at N-1 and C-3 (Phillips, 1989).

This report presents the development and application of a substrate analogue, 6-(difluoromethyl)tryptophan, designed for a more detailed analysis of the proton transfer steps associated with indole activation. Halomethyl substituents have been used previously to identify carbanionic intermediates of catalysis (Dirmaier et al., 1986; Lin et al., 1988; Reynolds et al., 1988) and a similar approach was envisioned for the heterocyclic system within tryptophan. Deprotonation of the heteroatom (N-1) of this analogue greatly facilitates the loss of fluoride ions to yield 6-formyltryptophan. A similar enhancement would be expected if tryptophanase stabilized the indolyl anion on route to tautomerization at N-1/C-3 (eq 2) or in the process of indole expulsion. This work, however, suggests that aromatic protonation initiates the tautomerization and activation of the indolyl group during catalysis.

Experimental Procedures

General. UV/vis studies were conducted on a Perkin Elmer λ-5 spectrophotometer. Mass spectrometry was performed on a VG Analytical ZAB-HF system for fast atom bombardment. High-performance liquid chromatography (HPLC)[1] was performed on a Spherex 5-μm C-18 column (Phenomenex) by using a Varian 5000 instrument equipped with a Varian 2050 variable-wavelength detector and Hewlett-Packard integrator (3390A).

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[1] Abbreviations: DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; LDH, lactate dehydrogenase; NAD and NADH, β-nicotinamide adenine dinucleotide, oxidized and reduced, respectively; PLP, pyridoxal 5′-phosphate.

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Indole and 6-methylindole (purified via flash silica chromatography) were purchased from Aldrich Chemical Co. 6-(Difluoromethyl)indole and 6-formylindole were prepared by the method of Woolridge and Rokita (1989), and 6-trifluoromethyl)indole was prepared by the method of Kalir and Pelah (1966). L-Lactic dehydrogenase (LDH), β-nicotinamide adenine dinucleotide, oxidized and reduced forms (NAD and NADH), pyridoxal 5'-phosphate (PLP), and L-tryptophan were purchased from Sigma Chemical Company. Tryptophanase (*Escherichia coli*) and tryptophan synthase (*Salmonella typhimurium*) were generously provided by Dr. Robert S. Phillips (University of Georgia) and Dr. Edith Wilson Miles (National Institutes of Health), respectively. All other reagents were of the highest commerical quality and were used without further purification.

Preparation of 6-(Difluoromethyl)tryptophan. 6-(Difluoromethyl)indole (0.2 mM) was incubated at room temperature with 50 mM potassium phosphate buffer, pH 8.0, 30 mM L-serine, 180 mM NaCl, 25 μM PLP, and 0.41 mg/mL tryptophan synthase in a total volume of 1.5 mL. After 30 min, 1 M triethylammonium acetate, pH 6 (167 μL), was added to the reaction mixture in order to lower the pH to 6, and 1.6 mL of this mixture was separated by reverse-phase HPLC under the same conditions as those described below. Product was collected and immediately frozen on dry ice. These procedures were repeated four times and product fractions from each incubation were combined. The solvent was removed via lyophilization to yield 6-(difluoromethyl)-tryptophan as a white solid: λ_{max} 280 nm (H_2O), ϵ = 11.2 mM^{-1} cm^{-1}; FAB/MS (glycerol matrix, positive ion) m/z 255.0939 $(M + H)^+$, calcd for $C_{12}H_{13}N_2O_2F_2$ m/z 255.0945. According to UV spectroscopy, this compound was stable for at least several weeks when stored as a solution (4.9 mM) in dimethyl sulfoxide (DMSO) at −20 °C.

Preparation of 6-Formyltryptophan. A solution of 6-(difluoromethyl)indole (3 mg, 17.9 μmol) in 375 μL of ethanol was added to a 10-mL solution containing 50 mM potassium phosphate buffer, pH 8.0, 180 mM NaCl, 30 mM L-serine, 25 μM PLP, and 0.18 mg/mL tryptophan synthase. The reaction was maintained overnight at room temperature to ensure complete hydrolysis of the difluoromethyl group attached to the indole and tryptophan analogues. Triethylammonium acetate (final concentration, 0.1 M) was then added, and the product was isolated by reverse-phase HPLC. Column fractions were dried under reduced pressure to yield 6-formyltryptophan as a pale yellow solid: λ_{max} 304 nm (H_2O), ϵ = 16.9 mM^{-1} cm^{-1}.

pH–Rate Profile for the Nonenzymatic Hydrolysis of 6-(Difluoromethyl)tryptophan. A DMSO solution of 6-(difluoromethyl)tryptophan was diluted into the following buffers (0.1 M) at 25 °C: potassium acetate, pH 3.99; potassium phosphate, pH 5.61, 7.81, 11.98, 12.30; potassium carbonate, pH 9.51, 10.51; potassium hydroxide, 13.06. All buffers were adjusted to an ionic strength (μ) of 1.0 M with potassium chloride (Skibo & Bruice, 1983). The hydrolysis was followed at 304 nm, the λ_{max} of 6-formyltryptophan. The pH-dependent rate constants (k_{obs}) were determined from a semilogarithmic plot of $(A_\infty - A_t)/(A_\infty - A_0)$ vs time by using a least-squares analysis (Bednar & Hadcock, 1988). This method was repeated at each pH with no less than three different concentrations of the (difluoromethyl)tryptophan; the concentrations typically employed were 20, 39, and 79 μM.

Dissociation Constants for the Binding of Indole Derivatives to Tryptophanase. Each indole derivative was added over the concentration ranges described below to a solution (990 μL) containing 0.25 mg of tryptophanase, 260 mM L-alanine, and 0.1 M potassium phosphate buffer, pH 8.0. After indole addition, the spectrum from 460 to 550 nm was observed and the absorbance at 501 nm recorded (Kazarinoff & Snell, 1980). Plots of ΔA_{501} vs ΔA_{501}/[indole] were prepared and the calculated slopes provided the dissociation constants (K_s values) from eq 3 (Kazarinoff & Snell, 1980; Fersht, 1985).

$$\Delta A_{501} = (\Delta A_{501})_{max} - K_S(\Delta A_{501}/[\text{indole}]) \quad (3)$$

The concentration ranges examined were as follows: 6-methylindole, 0.075–1.1 mM; 6-(trifluoromethyl)indole, 0.051–0.25 mM (solubility limit ≈ 0.3 mM); 6-formylindole, 0.051–0.78 mM; indole, 0.011–0.54 mM; 6-(difluoromethyl)indole, 0.024–0.71 mM. In all cases, aliquots of the indoles were added from ethanol stock solutions; the final concentration of ethanol did not exceed 4% in any case. Absorbance values were adjusted for the volume change (maximally 4%) that occurred during indole addition. The studies involving 6-(difluoromethyl)indole were completed within 15 min to avoid interference from the hydrolysis products formed upon fluoride elimination.

Steady-State Analysis of Tryptophanase Turnover. The catabolic degradation of tryptophan and its difluoromethyl derivative was monitored by the concomitant formation of pyruvate. This product was quantified by the oxidation of NADH in the presence of excess LDH (Morino & Snell, 1970). A stock dilution of enzyme (0.13 mg/mL, 2.3 μM active site) was prepared daily and incubated at 37 °C with 100 μM PLP and 10 mM β-mercaptoethanol in 0.1 M potassium phosphate buffer, pH 8.0, for 1 h prior to use to ensure maximum formation of the holoenzyme (Phillips, 1987). 6-(Difluoromethyl)tryptophan was prepared as a stock solution (4.9 mM) in DMSO. Assays were initiated by the addition of this substrate (17–150 μM) to a 1-mL mixture (25 °C) containing 0.1 M potassium phosphate buffer, pH 8.0, 100 μM NADH, 50 μM PLP, 19 units/mg LDH, and 0.038 mg/mL tryptophanase (0.69 μM active site, 3.5 units/mg); this latter enzyme was added only just before inclusion of 6-(difluoromethyl)tryptophan. The initial velocity was observed over the first 3 min by the absorbance increase at 340 nm. The final concentration of DMSO (3% v/v) used in these assays was found to have no effect on the enzymatic reaction of tryptophanase.

Product Analysis of Substrate Turnover. 6-(Difluoromethyl)tryptophan (98 μM) was added to a solution (1 mL) of 0.1 M potassium phosphate buffer, pH 8.0, 50 μM PLP, and 0.13 mg/mL tryptophanase (2.3 μM active site, 2.2 units/mg) under ambient conditions. Three minutes after the addition of the substrate, 111 μL of 1 M triethylammonium acetate buffer, pH 6.0 was added to yield a final concentration of 0.1 M. At 4 min, 1050 μL of this mixture was applied to the reverse-phase HPLC column and separated over the next 40 min by using 0.1 M triethylammonium acetate buffer (pH 6) with an initial isocratic elution of 5% acetonitrile for 5 min and then a gradient of 5–25% acetonitrile over the next 20 min, 25–35% acetonitrile for the following 10 min, and finally 35–45% in the last 5 min. The elution profile was monitored at 304 nm. For quantitative analysis, the detector response was standardized by a parallel analysis using known quantities of each component.

Results

Halomethyl Derivatives of Tryptophan. The appropriate halomethyl analogue of tryptophan was chosen for this investigation through model studies assessing the relative stability of (monohalomethyl)-, (dihalomethyl)-, and (trihalo-

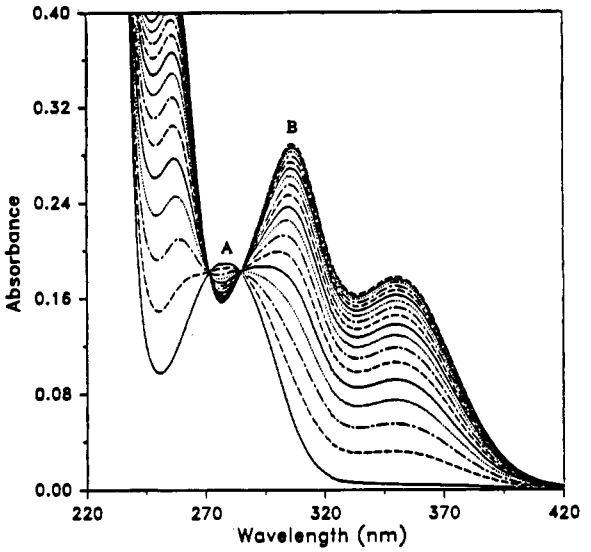


FIGURE 1: Hydrolysis of 6-(difluoromethyl)tryptophan under aqueous conditions to form 6-formyltryptophan. 6-(Difluoromethyl)tryptophan (17 μM) was incubated at 25 °C in 0.1 M potassium phosphate buffer, pH 11.9. Spectra were recorded every 10 min starting with A (0.0 min) and ceasing with B (170 min).

methyl)indoles [for example, see Woolridge and Rokita (1989)]. Unlike the (monohalomethyl)phenyl compounds developed by the laboratories of Kozarich and Kenyon (Dirmaier et al., 1986; Lin et al., 1988; Reynolds et al., 1988), the related derivatives of indole were extremely unstable to ambient conditions. Full substitution of the methyl group to form 6-(trifluoromethyl)indole resulted in a species that, in contrast, resisted hydrolysis even when treated at pH 13 (2 h, 25 °C). However, the disubstituted 6-(difluoromethyl)indole exhibited the most appropriate balance of stability under neutral conditions and lability under basic conditions. This latter indole was elaborated into its amino acid derivative by the action of tryptophan synthase, an enzyme that had previously been used to prepare a photochemically labile derivative of indole (Miles & Phillips, 1985). 6-(Difluoromethyl)tryptophan exhibits a reactivity similar to that of its parent indole derivative. The amino acid is stable in protic and aprotic organic solvents (e.g., ethanol and DMSO) but hydrolyzes under aqueous conditions to form 6-formyltryptophan. The kinetics of this process are easily followed by monitoring the absorption at 304 nm (λ_{max} of the aldehyde) and no intermediates (for example, quinone methide; see below) were detected during the steady-state reaction (Figure 1).

pH Dependence of 6-(Difluoromethyl)tryptophan Hydrolysis. The first-order rate for conversion of the difluoromethyl amino acid to its formyl derivative was examind at various pH values ranging from ca. 4 to 13 under constant ionic strength. The pH profile distinguishes three modes of reaction and all data satisfy the experimental rate equation (eq 4) (see Figure 2). The observed rate constant (k_{obs}) is dependent on the rate constant (k_w) for the reaction of the zwitterion, the equilibrium constant for ionization (K_a) of the α-amine, the rate constant (k_m) for the reaction of the α amino free base (the tryptophan monoanion), the equilibrium constant (K_a') for the ionization of the heterocyclic nitrogen, and the rate constant (k_d) for the free base species that has been deprotonated at N-1 (the tryptophan dianion). The pK_a' value was estimated from a Hammett plot used previously to correlate substituent effects and ionization constants of various (23) indoles (Yagil, 1967); a value of 16.3 was suggested by a ρ value of 1.91 and a σ_p value of 0.32 for the difluoromethyl group (Smart, 1983). The following parameters were obtained

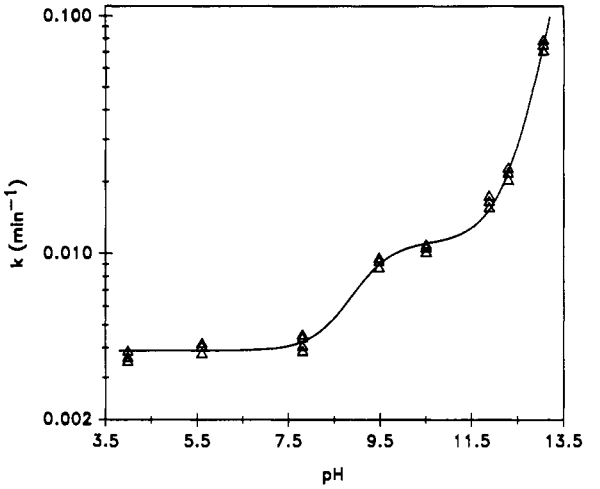


FIGURE 2: pH–rate profile for the first-order hydrolysis of 6-(difluoromethyl)tryptophan to 6-formyltryptophan in 0.1 M buffer (μ = 1.0 M, KCl). Experimental procedures are described in the text. The solid line represents a nonlinear regression analysis of k_{obs} fit to the logarithmic transformation of eq 4.

by a nonlinear least-squares fit of a log k_{obs} vs pH transformation of equation 4: k_w, 0.0039 ± 0.0001 min^{-1}; k_m, 0.0111

$$k_{obs} = k_w\left[\frac{[H^+]}{([H^+] + K_a)}\right] + k_m\left[\frac{K_a}{([H^+] + K_a)}\right] + k_d\left[\frac{K_a'}{([H^+] + K_a')}\right] \quad (4)$$

± 0.0004 min^{-1}; k_d, 110 ± 5 min^{-1}; pK_a, 9.1 ± 0.1. This pK_a value is quite similar to the value (9.4) generally quoted in the literature for the α-amino group of tryptophan (Dawson et al., 1986).

Characterization of the Indole-Binding Site Formed by Tryptophanase. Little structural information is currently available to describe the active-site region of tryptophanase that surrounds the indole moiety of substrate. However, this region becomes critical to the application of such mechanistic probes as 6-(difluoromethyl)tryptophan and its products. The immediate environment of the aromatic binding pocket was therefore examined by interaction with a series of indole analogues. This was used to identify the steric and dipolar factors that influence the binding and reactivity of substrates, products, and their derivatives. The corresponding amino acids were not studied because the additional functional groups of these species would likely obscure the specific behavior of the indole moiety on the enzyme surface.

The dissociation constants for the indole derivatives were measured by their characteristic destabilization of the alanyl–quinonoid complex that forms spontaneously in the presence of enzyme and alanine (Kazarinoff & Snell, 1980). A single methyl substitution attached to C-6 weakened the binding of indole by 2-fold as indicated by an increase in the K_s value from 71 μM (indole) to 150 μM (6-methylindole). A more polar analogue of the methyl group, however, restored the binding affinity. 6-(Difluoromethyl)indole exhibited a K_s value (75 μM) approximately equivalent to that of indole. Even the hydrolysis product, 6-formylindole, was accommodated in the active site; the K_s value of this compound (130 μM) was lower than that of the methyl analogue. Only the 6-(trifluoromethyl) species produced a dissociation constant (170 μM) greater than that of 6-methylindole. Although polar substituents are generally well tolerated in the active site, the great steric bulk of the trifluoromethyl group may begin to

interfere with efficient binding. The Taft steric parameters suggest that a trifluoromethyl group (−2.40) is significantly larger than a methyl group (−1.24) and may be more accurately compared to an *iso*-propyl (−1.71) or even *tert*-butyl (−2.78) group (Hansch & Leo, 1979; Smart, 1983).

Tryptophanase-Induced Scission of the Carbon Skeleton of a Substrate Analogue. The enzymatic processing of 6-(difluoromethyl)tryptophan was first evaluated by the typical β-elimination assay in which the production of pyruvate was coupled to the oxidation of NADH. Detection of catalytic activity was therefore based on an absorbance (340 nm) that was unaffected by the concurrent hydrolysis of 6-(difluoromethyl)tryptophan to 6-formyltryptophan. Under standard conditions (pH 8), tryptophanase catalyzed the release of pyruvate from the difluoromethyl amino acid in a simple Michaelis–Menten process. Double-reciprocal analysis of substrate concentration vs initial velocity yielded a K_m value of 140 μM, similar to the corresponding value determined for tryptophan (210 μM). In contrast, the k_{cat} value for this difluoromethyl analogue (3.6 min^{-1}) was only 0.6% of that for tryptophan (620 min^{-1}). This analogue exhibited a k_{cat} value similar to that determined previously for 7-azatryptophan (1.8 min^{-1}) (Phillips, 1989).

Tryptophanase-Induced Elimination of Fluoride from the Difluoromethyl Derivatives. The spontaneous and catalytic conversion of 6-(difluoromethyl)indole to 6-formylindole was observed at 304 nm in analogy to that for the reaction illustrated in Figure 1. No activation of this process was detected (±1.5%) at pH 8 in the presence of 72 μM substrate and as much as 0.063 mg/mL tryptophanase (1.1 μM active site). The alternative addition of alanine (26 mM), serine (10 mM), and glycine (100 mM) had no effect on the enzymatic activity even though these amino acids would bind to the PLP and form a turnoverlike complex (Kazarinoff & Snell, 1980).

A direct spectroscopic assay was also used to determine the spontaneous hydrolysis of 6-(difluoromethyl)tryptophan (Figure 1), but this technique was not available for monitoring enzymatic activity. The absorbance increase (304 nm) associated with the elimination of fluoride was obscured by the absorbance decrease [290 nm (Higgins et al., 1979)] associated with the competing release of 6-(difluoromethyl)indole (eq 1). The enzyme-generated partition between carbon–carbon and carbon–fluorine bond scission was instead examined by product analysis. 6-(Difluoromethyl)tryptophan was incubated at $^1/_2V_{max}$ conditions in the presence and absence of tryptophanase and sampled after 24% of the substrate was consumed. Reverse-phase HPLC was used to identify and quantify the enzymatic and nonenzymatic products, 6-formyltryptophan, 6-(difluoromethyl)indole, and 6-formylindole. The compound resulting from standard enzyme turnover, 6-(difluoromethyl)indole, was the only enzyme-dependent species detected (Table I). During the initial 3–4 min of reaction, no spontaneous or catalytic hydrolysis of the difluoromethyl group was evident. The maximum values listed for the alternative products derive from the individual thresholds of detecting each compound by HPLC. These values in turn set the limits for the partition ratios of products: 6-(difluoromethyl)indole:6-formyltryptophan, ≥2500:1; 6-(difluoromethyl)indole:6-formylindole, ≥125:1.

Table I: Spontaneous and Tryptophanase-Catalyzed Degradation of 6-(Difluoromethyl)tryptophan Analyzed by Reverse-Phase Chromatography

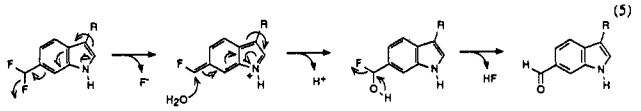
substrate/product	retention time[a] (min)	material detected after treatment[b] (nmol) (−) enz	(+) enz
6-(difluoromethyl)tryptophan	18	93	70
6-formyltryptophan	14	<0.01	<0.01
6-(difluoromethyl)indole	38	<0.4	25
6-formylindole	29	<0.2	<0.2

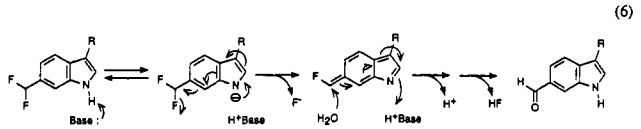
[a] An analytical (150 × 4.6 mm) Spherex column (C-18) was eluted at 1 mL/min with a gradient described under Experimental Procedures. [b] 6-(Difluoromethyl)tryptophan was incubated for 3 min in the absence and presence of tryptophanase (2.3 μM active site) and HPLC analysis begun at the fourth minute. Error in transfer and measurement would suggest an uncertainty of ca. 10%.

Discussion

Few PLP-dependent enzymes catalyze the cleavage of unactivated carbon–carbon bonds and the intrinsic reactivity of PLP could not be expected alone to promote the expulsion of an unstable carbanion. A protein mediated activation of such a process can therefore be expected. The molecular basis for this is now actively under investigation for tyrosine–phenol lyase (Kiick & Phillips, 1988b), tryptophanase (Kiick & Phillips, 1988a; Phillips, 1989), and a related system, tryptophan synthase (Ahmed et al., 1986; Hyde et al., 1988; Woolridge & Rokita, 1991). In each case, the catalytic mechanism may include an enzyme-dependent tautomerization of the aromatic substrate to yield a high-energy intermediate poised for carbon–carbon bond cleavage (and formation). The substrate analogue created for the present study was designed to focus attention specifically on the individual proton transfer steps of the potential tautomerization effected by tryptophanase.

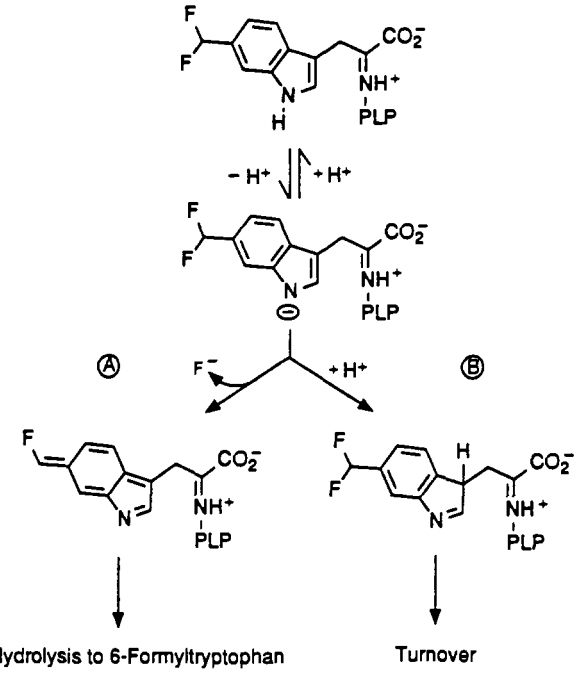
Development of an Indicator for Heteroatomic Ionization. The central compound, 6-(difluoromethyl)tryptophan, culminated our studies on a series of derivatives that were designed to include a halomethyl group as a probe for anionic intermediates (Dirmaier et al., 1986; Lee & Skibo, 1987). The electron-rich nature of indole (Sundberg, 1970) precluded the traditional application of a monohalogenated derivative (Woolridge & Rokita, 1989), but the difluoro species was found to be stable enough for manipulation under neutral conditions. Fluoride elimination from this difluoromethyl species is relatively slow and independent of hydrogen ion concentration between pH 4 and 8 (Figure 2). Hydrolysis likely proceeds under these conditions through the intermediate formation of an iminium quinone methide (eq 5). No buffer dependence (0.02–1.0 M) was detected for the related conversion of 6-(difluoromethyl)indole to 6-formylindole at these pH values or at any others tested (data not shown).





Deprotonation of the α-ammonium group appears to promote the hydrolysis of the difluoromethyl group of the amino acid analogue by approximately 3-fold as illustrated by the increase in rate from pH 8 to 10. More accurately, however, an inhibitory effect of the ammonium cation is likely neutralized by the rise in pH, since the enhanced rate of hydrolysis (pH 8–10) is equivalent to that exhibited by 6-(difluoromethyl)indole (Woolridge & Rokita, 1991). Regardless, this effect is quite minor when compared to the significant acceleration in rate (10^4-fold) due to the deprotonation at N-1

Scheme I: Fluoride Elimination versus Indole Tautomerization during Aromatic Activation



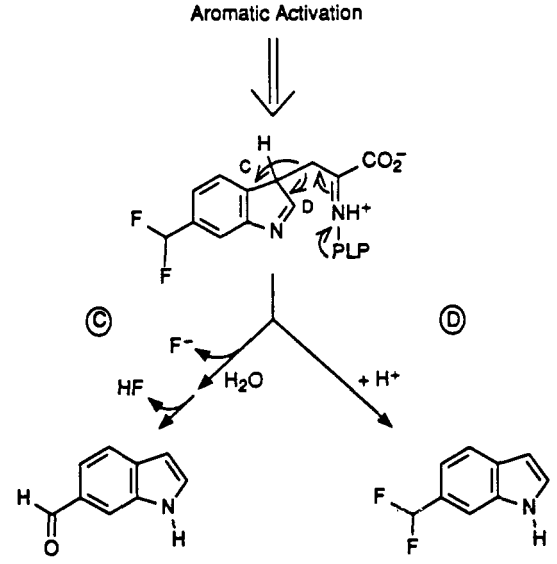
(eq 6). The consequence of this latter acceleration is even detectable at pH 11, for which less than 1 in 10^5 molecules of indole is deprotonated [assuming a pK_a of 16.3 (this report; Yagil, 1967)]. The dramatic effect of ionization now serves as the basis for this investigation on the aromatic proton transfer catalyzed by tryptophanase. Diagnostic decomposition of the difluoromethyl group is used as a benchmark to examine the possibility of forming an indolyl anion during catalytic turnover.

Application of 6-(Difluoromethyl)tryptophan. The steric and electronic effects of the difluoromethyl group did not prohibit the corresponding indole and tryptophan derivatives from interacting with tryptophanase. The binding constants for indole and its difluoromethyl analogue are almost equivalent. Polar substituents at C-6 actually appear to stabilize binding relative to the effect of a nonpolar group such as methyl. The active site environment surrounding this portion of the indole is thus not likely to be very lipophilic or sterically restrictive. This in turn would support the assumption implied below that fluoride release is equivalent for the indolyl derivatives free in solution or bound to the enzyme.

The presence of the difluoromethyl group also did not prevent the tryptophan derivative from participating in the standard catalytic cycle of tryptophanase, as indicated by a coupled assay based on pyruvate formation. The catalytic specificity (k_{cat}/K_m) for this substrate is less than 1% of that for tryptophan, yet this does not diminish the utility of the partition between diagnostic hydrolysis (A) and turnover (B) established by 6-(difluoromethyl)tryptophan. The ratio of products formed in the presence of enzyme is directly related to the rate for proton exchange at N-1 and C-3 and the competitive rate for hydrolysis of the difluoromethyl group (for example, Schemes I and II). The limits of this approach are set by the intrinsic rates of fluoride elimination and by the sensitivity of product detection.

Only the result of standard catalysis was observed by HPLC analysis after incubating 6-(difluoromethyl)tryptophan with tryptophanase. Therefore, fluoride release and the concomitant hydrolysis to 6-formyltryptophan (Scheme I) must have occurred at a frequency less than 1 in 2500 turnovers as indicated

Scheme II: Fluoride Elimination during Indole Release

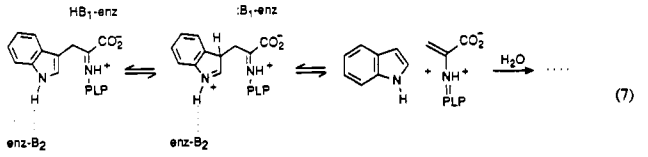


by the minimum partition ratio, 25 nmol/<0.01 nmol (Table I). If deprotonation of N-1 precedes protonation of C-3, then the rate of C-3 protonation would have to be at least (2500 × 110 min^{-1}) 2.75 × 10^5 min^{-1} in order to avoid a measurable elimination of fluoride (k_d, 110 min^{-1}). Similarly, the minimum ratio between 6-(difluoromethyl)indole and 6-formylindole (25 nmol/<0.2 nmol) suggests the minimum partition during indole release of (C) fluoride expulsion and (D) N-1 protonation and rearomatization (Scheme II).

Pre-steady-state kinetic analysis of tryptophanase has suggested that the initial N-1/C-3 tautomerization (eq 2) is at least one of the limiting steps of catalysis (Phillips, 1989). This process has been estimated to proceed at a rate, 440 min^{-1}, quite similar to that of the overall turnover measured in those experiments (360 min^{-1}). Further dissection of this key proton transfer may initially yield three likely alternatives that could control the activation of the aromatic group: (1) a rapid preequilibrium for ionization at N-1 followed by a slow protonation at C-3, (2) a slow ionization at N-1 followed by a rapid protonation at C-3, or (3) a slow protonation at C-3 followed by a rapid deprotonation of the resulting N-1 iminium ion.

Mechanisms 1 and 2 are both described by Scheme I, in which the ionization of nitrogen occurs before carbon protonation. Under this constraint, only mechanism 2 would be compatible with the partition and pre-steady-state data. Carbon protonation (B) would have to be fast (>2.75 × 10^5 min^{-1}) to prevent hydrolysis of the difluoromethyl group (A), and consequently, nitrogen ionization would have to be slow to satisfy the overall rate of tautomerization [440 min^{-1} (Phillips, 1989)]. However, mechanism 2 is not consistent with the low k_{cat} values noted for substrates containing electron-withdrawing substituents on the aromatic system, 7-azatryptophan (Phillips, 1989) and 6-(difluoromethyl)tryptophan. The dipolar effect of these analogues would serve to lower the pK_a of N-1 and thus facilitate its ionization. For mechanism 2, this effect would then be expected to enhance, not diminish, the value of k_{cat}. In addition, the difluoromethyl substituent appears not to affect the aromatic binding, as indicated by the K_s values above.

All of the mechanistic information available on the tryptophanase-catalyzed release of indole could be described by the final mechanism 3. Activation of the aromatic group by initial protonation at C-3 would preclude anion formation and



(7)

prevent fluoride elimination from 6-(difluoromethyl)tryptophan. This pathway would also require an electron-rich aromatic system (for example, indole) and thus turnover would likely be limited for substrates affected by the electron-withdrawing nature of the 7-aza and 6-difluoromethyl groups. The suggested proton transfer at N-1, however, would contribute little to the overall turnover process. Deprotonation of the indolenine cation might stabilize an intermediate, but then reprotonation would likely be necessary before the indolyl group is released. Consequently, a minimal scheme for the elmination of indole is illustrated in eq 7 and includes only a hydrogen bond between an enzyme base and the N-1 proton of the substrate. Construction of a difluoromethyl-substituted tryptophan has therefore provided an intramolecular probe for identifying potential intermediates of catalysis and for differentiating between mechanistic pathways. Application of this substrate analogue now directs attention to the protonation of the indolyl C-3 as the key step in activating the aromatic substitution and degradation of tryptophan. Related derivatives are now under investigation with other aromatic activating systems.

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