The Catalytic Mechanism of Kynureninase from *Pseudomonas fluorescens*: Insights from the Effects of pH and Isotopic Substitution on Steady-State and Pre-Steady-State Kinetics†

Srinagesh V. Koushik,‡,§ Joseph A. Moore III,‖ Bakthavatsalam Sundararaju,‡,‖ and Robert S. Phillips*,‡,‖,⊥

Department of Biochemistry and Molecular Biology, Department of Chemistry, and the Center for Metalloenzyme Studies, University of Georgia, Athens, Georgia 30602-2556

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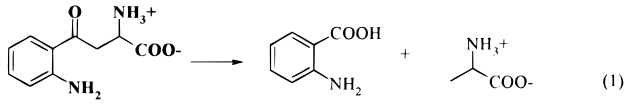
ABSTRACT: The effects of pH and isotopic substitution of substrate and solvent on the reaction of kynureninase from *Pseudomonas fluorescens* have been determined. The pH dependence of k_{cat}/K_m for L-kynurenine is bell-shaped, with apparent pK_a's of 6.25 ± 0.05 on the acidic limb and 8.9 ± 0.1 on the basic limb, and with a pH-dependent value of k_{cat}/K_m of 2×10^5 M^{-1} s^{-1}. The pH dependence of k_{cat}/K_m for 3-hydroxykynurenine is also bell-shaped, with apparent pK_a's of 6.49 ± 0.07 and 8.55 ± 0.09, and with a pH-dependent value of 2.5×10^3 M^{-1} s^{-1}. The k_{cat} for L-kynurenine decreases at acidic pH values, with an apparent pK_a of 6.43 ± 0.06 and a pH-dependent value of 7 s^{-1}. The solvent kinetic isotope effect on k_{cat} for the reaction of kynurenine in [^{2}H]H_2O is 6.56 ± 0.59, whereas there is no normal kinetic isotope effect on k_{cat}/K_m, at pH 8.1. The proton inventory of k_{cat} fits very well to the Gross−Butler equation, with $x = 0.825 \pm 0.08$, suggesting that only a single proton is transferred in the rate-determining step. In contrast, there is no significant kinetic isotope effect on either k_{cat} or k_{cat}/K_m with α-[^{2}H]-L-kynurenine as the substrate. There is a "burst" of anthranilate (0.7 mol/mol of enzyme) formed in the pre steady state of the reaction of kynureninase, with a rate constant of 54 s^{-1} which is not affected by [^{2}H]H_2O. The partition ratio of alanine to pyruvate formation is 2.3×10^4 in H_2O and 6.9×10^3 in [^{2}H]H_2O. Taken together, these data indicate that the rate-limiting step in the reaction of kynureninase occurs subsequent to the first irreversible step, which is anthranilate release, is general base catalyzed, and involves transfer of only a single proton. On the basis of these observations, we propose that the rate-limiting step in the reaction of kynureninase is C-4′ deprotonation of the pyruvate pyridoxamine 5′-phosphate ketimine intermediate.

Kynureninase [EC 3.7.1.1] is a pyridoxal 5′-phosphate (PLP)[1] dependent enzyme which catalyzes the hydrolytic cleavage of L-kynurenine to anthranilic acid and L-alanine (eq 1). This reaction is a key step in the catabolism of



(1)

L-tryptophan by *Pseudomonas fluorescens* and some other bacteria (Hayaishi & Stanier, 1951). In eukaryotic organisms, a similar enzyme reacts preferentially with 3-hydroxykynurenine in the catabolism of L-tryptophan (Soda & Tanizawa, 1979). In eukaryotes, this pathway is also responsible for the biosynthesis of NAD via the intermediacy of quinolinate. Quinolinate is a neurotoxin due to its agonist effects on the NMDA receptor, and excessive levels have been implicated in the etiology of a wide range of diseases such as epilepsy, stroke, and neurological disorders, including AIDS-related dementia (Sei et al., 1995, 1996; Saito et al., 1993a−c; Heyes et al., 1991a,b; Achim et al., 1993).

We have studied the stereochemistry of the aldol and retroaldol reactions catalyzed by kynureninase from *P. fluorescens*, and we found that (4*S*)-dihydro-L-kynurenine is a potent competitive inhibitor, with a K_i value of 0.3 μM (Phillips & Dua, 1991). On the basis of these results, we concluded that there is a *gem*-diolate intermediate in the reaction mechanism. We then designed and synthesized a series of "transition-state analogue" inhibitors of kynureninase, based on this hypothesis, that contain a sulfone group in place of the reactive carbonyl moiety. The most active of these compounds, *S*-(2-aminophenyl)-L-cysteine-*S*,*S*-dioxide, has a K_i value of 0.07 μM at pH 7.8, more than 300-fold lower than the K_m for L-kynurenine (Dua et al.,

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* To whom correspondence should be addressed at the Department of Chemistry, University of Georgia, Athens, GA 30602-2556. Phone: (706) 542-1996. FAX: (706) 542-9454. E-mail: phillips@bscr.uga.edu.

‡ Department of Biochemistry and Molecular Biology.

§ Present address: Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, GA 30912-2650.

‖ Department of Chemistry.

⊥ Center for Metalloenzyme Studies.

[1] Abbreviations: pyridoxal 5′-phosphate (PLP); high-performance liquid chromatography (HPLC); nicotinamide adenine dinucleotide (NAD).

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1993). These previous results have provided convincing evidence that the mechanism of kynureninase proceeds via a *gem*-diolate intermediate, but gave no insight into the identity of the rate-determining step in the reaction. In the present work we have examined the effects of pH and of substrate and solvent isotopic substitution on the steady state and pre-steady-state kinetics to determine the mechanism of kynureninase.

EXPERIMENTAL PROCEDURES

Materials. Kynureninase was purified from *P. fluorescens* (ATCC 11250) cells grown on a medium with 0.2% L-tryptophan as the carbon and nitrogen source (Hayaishi & Stanier, 1951), using modifications of previously described protocols (Moriguchi et al., 1971; Ishikawa et al., 1989), or from *E. coli* cells containing plasmid pTZ18U with the *kyn* gene of *P. fluorescens* inserted into the linker (Koushik et al., 1997). The enzyme preparations exhibited specific activities of 7−16 μmol/(min·mg) at pH 7.8 and 25 °C. The deuterium oxide ($[^2H]H_2O$) used in the solvent isotope effect studies was obtained from either Isotec or Aldrich and was greater than 99.8% deuterium. L-Kynurenine sulfate and 3-hydroxy-DL-kynurenine were products of United States Biochemical Corp. α-[^{2}H]-L-Kynurenine was synthesized from L-kynurenine by exchange of the α-proton catalyzed by tryptophan indole-lyase in $[^2H]H_2O$, and purified as described earlier (Faleev et al., 1990). ^{1}H NMR of the α-[^{2}H]-L-kynurenine showed that exchange was >98% complete, since no signal corresponding to the α-hydrogen was observed.

Instruments. The steady-state kinetic measurements were performed on a Cary 1E UV/vis spectrophotometer equipped with a 6 × 6 thermoelectric cell changer, controlled by a Gateway 2000 80486 DX2/66 PC using OS/2 (IBM) software provided by Varian Instruments.

Rapid chemical quench experiments were performed on an apparatus from KinTek Instruments (State College, PA). Anthranilic acid in the chemical quench experiments was measured by high-performance liquid chromatography (HPLC) on an instrument consisting of two Gilson model 502 pumps, equipped with a Gilson filter fluorescence detector model 121, using an excitation filter with a band-pass of 305−395 nm and an emission filter with a band-pass of 420−650 nm, and controlled by a Dell 80486 DX2/66 PC running Gilson Unipoint software. The column used was a Whatman Partisphere C18, 4.6 × 250 mm.

Methods. Steady-state kynureninase reactions were performed at 25 °C by following the decrease in absorbance of L-kynurenine at a λ_{max} of 360 nm ($\Delta\epsilon = -4500$ M^{-1} cm^{-1}). The kynurenine was varied at concentrations of about 0.5, 1, 2, 4, and 6 times K_m, in 0.04 M potassium phosphate, pH 7.8, 0.04 mM PLP, in 1-mL reaction volumes, as described earlier (Phillips & Dua, 1991). V_{max} and V_{max}/K_m values were obtained by nonlinear fitting to eq 2 using Enzfitter

$$v = V_{max}[S]/(K_m + [S]) \quad (2)$$

(Elsevier), and divided by the enzyme concentration to obtain k_{cat} and k_{cat}/K_m values. Reactions of 3-hydroxy-DL-kynurenine were performed at a λ_{max} of 369 nm ($\Delta\epsilon = -4500$ M^{-1} cm^{-1}). Because of the high K_m for 3-hydroxy-DL-kynurenine, the values of k_{cat}/K_m were calculated from the slopes of the plots of initial rate against concentration (calculated on the basis of the L-enantiomer) under pseudo-first-order conditions. The protein concentration was determined using the 280-nm absorbance, with $A^{1\%} = 14$ (Moriguchi et al., 1971), assuming a molecular weight of 4.6×10^4 based on the amino acid sequence (Koushik et al., 1997).

The pK_a values in the pH dependencies were determined by fitting the data to eqs 3 and 4 with the compiled

$$\log V = \log C/(1 + [H^+]/K_a) \quad (3)$$

$$\log V = \log (C/(1 + [H^+]/K_a + K_b/[H^+])) \quad (4)$$

$$^Dk_{obs} = (1 - n + n\sqrt[X]{^Dk})^X \quad (5)$$

FORTRAN programs of Cleland, HABELL, and BELL, while the steady-state kinetic isotope effects were determined with ISOVKV (Cleland, 1979). The proton inventory data were fitted to the Gross−Butler equation, eq 5 (Schowen and Schowen, 1982; Kiick, 1991), using Enzfitter (Elsevier). The pK_a values for anthranilic acid, kynurenine, and 3-hydroxykynurenine in aqueous solution were estimated with the SPARC pK_a calculator program (Hilal et al., 1994). For measurements in the pH range 6.0−9.5, potassium phosphate and triethanolammonium phosphate buffers were used, while sodium acetate and trisodium citrate buffers were used in the pH range 5.0−6.5. For reactions in $[^2H]H_2O$, all buffers and solutions were prepared in 99.8% $[^2H]H_2O$ from a freshly opened bottle. pH measurements were corrected for the isotope effect on the glass electrode by addition of 0.4 to the observed reading. Inactivation experiments were performed in assay solutions containing 0.36 mM L-kynurenine excluding PLP, and the absorbance changes at 360 nm were recorded for 120 min at 1-min intervals. The inactivation rate constants were determined from fitting of the time courses to eq 6, where ΔA is the difference between the absorbance values at times t and $t - 1$. The inactivation obeyed first-order kinetics through at least 3 half-lives.

$$\Delta A_t = \Delta A_0 e^{-kt} \quad (6)$$

Rapid quench kinetic measurements were performed at room temperature (ca. 25 °C) by mixing 40 μL portions of enzyme (8.4 μM) and 1 mM L-kynurenine in 0.04 M potassium phosphate, pH 7.8. Corresponding solutions were prepared in $[^2H]H_2O$ for measurement of the solvent isotope effect. Following quench, using 0.5 M $HClO_4$, 50 μL of 1.0 M K_2HPO_4 was added to each sample, and 50 μL aliquots were injected into the HPLC for anthranilic acid determination. The solvent was 0.05 M NaH_2PO_4, pH 3.0/methanol, with a constant composition of 20% methanol for the first 5 min, and then a gradient from 20% to 50% methanol was run over the next 20 min. Under these conditions, anthranilic acid elutes 14 min after injection. A standard curve was generated from dilutions of authentic anthranilic acid, and the lower limit of detection by fluorescence in this experiment was 1 pmol. The pre-steady-state kinetic data were fitted to eq 7 using FITSIM (Zimmerle & Frieden, 1989), where k_{+2} is the rate constant for the formation of anthranilate and k_{+3} is the rate constant for alanine formation. The data did not permit explicit fitting of k_{+1} and k_{-1}, so the initial

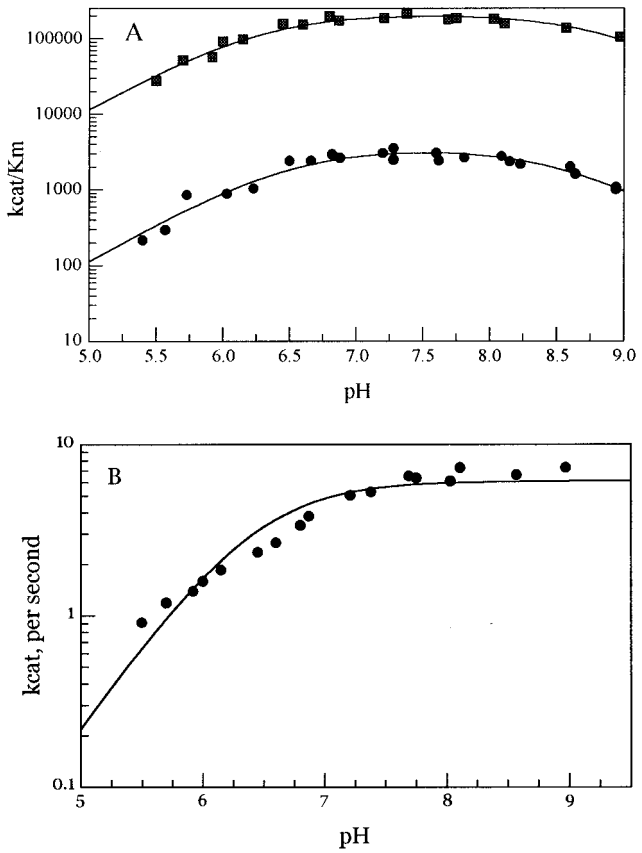


FIGURE 1: pH dependence of kynureninase activity. (A) pH dependence of k_{cat}/K_m. The data points for L-kynurenine are shown as filled squares, and those for 3-hydroxy-DL-kynurenine are shown as filled circles. The lines are the calculated curves obtained from fitting the data to the BELL FORTRAN program of Cleland (1979), with the parameter values given in Results. (B) pH dependence of k_{cat}. The data points are shown as filled circles. The line is the calculated curve obtained from fitting the data to the HABELL FORTRAN program of Cleland (1979), with the parameter values given in Results.

binding step was treated as a rapid equilibrium, with a fixed value of 1×10^{-5} M. Variation of this value had no effect on the fitting of the data to k_{+2} and k_{+3}, since the concentration of L-kynurenine used in these experiments was 5×10^{-4} M.

RESULTS

pH Dependence of Kynureninase. The expressions for k_{cat} and k_{cat}/K_m, based on the minimal mechanism of eq 7, are given in eqs 8 and 9. Due to the low K_m value of

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} EP + Q \xrightarrow{k_{+3}} E + P \qquad (7)$$

$$k_{cat}/K_m = k_{+1}k_{+2}/(k_{-1} + k_{+2}) \qquad (8)$$

$$k_{cat} = k_{+2}k_{+3}/(k_{+2} + k_{+3}) \qquad (9)$$

kynureninase for L-kynurenine (30 μM at pH 7.8) and the high K_i values for L-alanine (>1 mM at pH 7.8) and anthranilic acid (about 0.6 mM at pH 7.8), it is reasonable to assume that the release of both products is irreversible under initial rate conditions. The pH dependences of k_{cat} and k_{cat}/K_m for kynureninase are shown in Figure 1. Identical pH dependence data were obtained either using enzyme isolated from tryptophan-induced *P. fluorescens* cells or with recombinant *P. fluorescens* kynureninase overexpressed in *E. coli* cells containing pTZKYN (Koushik et al., 1997). The k_{cat}/K_m data for L-kynurenine in Figure 1A, obtained with the recombinant enzyme, fit well to a bell-shaped curve for a single basic group, with an apparent pK_a of 6.25 ± 0.05 in the acidic limb, a single acidic group, with an apparent pK_a of 8.9 ± 0.1 on the basic limb, and a pH-dependent value of k_{cat}/K_m of 2×10^5 M^{-1} s^{-1}. The pH profile of k_{cat}/K_m for 3-hydroxykynurenine shows a similar bell-shaped curve, with a single basic group with an apparent pK_a of 6.49 ± 0.07 in the acidic limb, a single acid, with an apparent pK_a of 8.55 ± 0.09 on the basic limb, and a pH-dependent value of k_{cat}/K_m of 2.5×10^3 M^{-1} s^{-1}. In contrast, the k_{cat} pH profile for L-kynurenine in Figure 1B shows a dependence on a single basic group, with an apparent pK_a of 6.43 ± 0.06, in the acidic limb, and with a pH-dependent value of 7 s^{-1}. Due to the high K_m value and low solubility of 3-hydroxykynurenine, the pH dependence of k_{cat} for this compound was not determined.

Substrate Kinetic Isotope Effect. α-[^{2}H]-L-Kynurenine was synthesized to study the effect of α-deuteration on the rate of the reaction of kynureninase. Assuming that the isotope-sensitive step is k_{+2} in eq 7, the observed isotope effects on k_{cat}/K_m and k_{cat} are given by eqs 10 and 11.[2] However, we

$$^D(k_{cat}/K_m) = (^Dk_{+2} + k_{+2}/k_{-1})/(1 + k_{+2}/k_{-1}) \qquad (10)$$

$$^D(k_{cat}) = (^Dk_{+2} + k_{+2}/k_{+3})/(1 + k_{+2}/k_{+3}) \qquad (11)$$

did not observe a significant kinetic isotope effect with α-[^{2}H]-L-kynurenine on either k_{cat} or k_{cat}/K_m for the reaction of kynurenine with kynureninase at various pH values from 5.58 to 8.60 in H_2O or [^{2}H]H_2O (Table 1).

Solvent Isotope Effects. We then examined the effect of solvent deuterium substitution on k_{cat} and k_{cat}/K_m for kynureninase. In contrast to the substrate isotope effect, the effect of solvent deuterium could be on any step in the mechanism, or on multiple steps. In [^{2}H]H_2O, we observe a strikingly high isotope effect on k_{cat} that has a value of 6.56 ± 0.59 at pH 8.1 (Table 1). In contrast, there is no significant normal solvent kinetic isotope effect on k_{cat}/K_m, and it may be slightly inverse, as the value of 0.82 ± 0.16 was obtained at pH 8.1 (Table 1). A double-isotope experiment was performed with α-[^{2}H]-L-kynurenine in [^{2}H]H_2O, and a somewhat larger value of 10 for the k_{cat} isotope effect was obtained (Table 1). However, the error is also larger on this single determination, and since there is no corresponding normal isotope effect with α-[^{2}H]-L-kynurenine in H_2O, this difference is probably not significant. The inverse solvent isotope effect on k_{cat}/K_m, although barely larger than experimental error, is quite reproducible, and was also seen in the experiment with α-[^{2}H]-L-kynurenine in H_2O and [^{2}H]H_2O (Table 1). Thus, since there is no significant normal isotope effect on k_{cat}/K_m, the solvent-sensitive step in k_{cat} occurs after the first irreversible step, and the solvent isotope effect is given by

[2] The isotope effects are designated using the leading superscript nomenclature of Northrop (1977), where $^D(k_{cat})$ is the kinetic isotope effect on k_{cat} and $^D(k_{cat}/K_m)$ is the kinetic isotope effect on k_{cat}/K_m.

Table 1: Isotope Effects on the Reaction of Kynureninase

substrate and solvent	pH	$^{D}(k_{cat}/K_m)$	$^{D}k_{cat}$
L-kynurenine and [α-^{2}H]-L-kynurenine, in H_2O	5.58	1.003 ± 0.003	1.003 ± 0.004
L-kynurenine and [α-^{2}H]-L-kynurenine, in H_2O	6.81	0.96 ± 0.03	1.007 ± 0.007
L-kynurenine and [α-^{2}H]-L-kynurenine, in H_2O	7.78	0.95 ± 0.02	1.027 ± 0.006
L-kynurenine and [α-^{2}H]-L-kynurenine, in H_2O	8.60	1.08 ± 0.01	1.020 ± 0.0006
L-kynurenine and [α-^{2}H]-L-kynurenine, in [^{2}H]H_2O	7.80	0.954 ± 0.362	1.239 ± 0.124
L-kynurenine, in H_2O and [^{2}H]H_2O	7.80	0.82 ± 0.16	6.56 ± 0.59
[α-^{2}H]-L-kynurenine, in H_2O and [^{2}H]H_2O	7.80	0.65 ± 0.22	10.0 ± 1.5

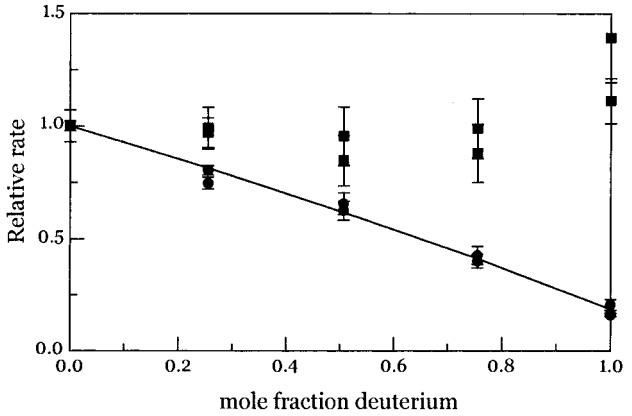


FIGURE 2: Proton inventory for kynureninase at pH 7.8. Kinetic parameters were obtained as described in Experimental Procedures, with buffers prepared by mixing various amounts of [^{2}H]H_2O. The solid line through the k_{cat} values (filled circles) is the calculated line obtained by fitting to eq 10, with $x = 0.825$ protons. The corresponding k_{cat}/K_m values (filled squares) are also shown.

eq 12 (Northrop, 1981), where R_f is the catalytic ratio, k_{+3}/

$$^{D_2O}k_{cat} = (^{D_2O}k_{+3} + R_f/E_f + C_r(^{D}K_{eq}))/ (1 + R_f/E_f + C_r(^{D}K_{eq})) \quad (12)$$

k_{+2}, E_f is the equilibrium preceding catalysis, C_r is the reverse commitment, and $^{D}K_{eq}$ is the isotope effect on the overall equilibrium constant. In eq 12, the effects of solvent deuterium on the reaction equilibrium constant and of the reverse commitment can be neglected, since the kynureninase reaction is functionally irreversible. Furthermore, the catalytic ratio, k_{+3}/k_{+2}, is also small, since anthranilate formation is much faster than alanine formation and the pre-equilibrium term, E_f, is apparently large, based on the pre-steady-state data (see below). Thus, the observed solvent isotope effect on k_{cat} is apparently intrinsic, and hence it is valid to perform a proton inventory.

The proton inventory of [^{2}H]H_2O on the solvent isotope effects in the reaction of kynureninase was measured at pH 7.8. Fitting of the k_{cat} data to the simplified form of the Gross−Butler equation[3] (Schowen and Schowen, 1982; Kiick, 1991) given in eq 5 gives an excellent fit, with $x = 0.825 \pm 0.088$ protons, suggesting the involvement of one proton in the rate-determining step (Figure 2). In contrast, the proton inventory of k_{cat}/K_m shows no significant normal isotope effect, as anticipated from the data in Table 1; however, a small inverse effect is again observable in this experiment at 100 mol % deuterium (Figure 2).

[3] The simplified Gross−Butler equation requires that fractionation factors be approximately 1. This is reasonable for kynureninase, as there is no evidence that the catalytic base is a thiolate.

Table 2: Product Inhibition Constants for Anthranilic Acid

pH	K_{is}, M	K_{ii}, M
5.90	1.65×10^{-5}	1.82×10^{-5}
7.10	1.55×10^{-4}	9.32×10^{-5}
7.80	6.10×10^{-4}	4.63×10^{-4}

It was reported that kynureninase undergoes reversible inactivation due to abortive transamination, with formation of pyruvate and pyridoxamine 5′-phosphate, when PLP is excluded from the assay mixture (Moriguchi & Soda, 1973). From analysis of the progress curves for inactivation, we determined that the rate constant of pyruvate formation from kynurenine in normal water buffer is 3.0×10^{-4} s^{-1} at pH 7.8 and 25 °C. Since the rate constant for alanine formation in normal water is about 7 s^{-1} (Figure 1B), about 1 mol of pyruvate is formed for every 2.3×10^4 mol of L-alanine formed. In deuterated buffer solution, the rate constant for pyruvate formation was found to be 1.6×10^{-4} s^{-1}, and the rate constant for L-alanine formation is 1.1 s^{-1} due to the large solvent isotope effect. The partition ratio has thus been increased in [^{2}H]H_2O to 1 mol of pyruvate for every 6.9×10^3 mol of L-alanine, and there is thus a solvent isotope effect of 3.3 on the partition ratio. This finding provides evidence that the solvent-sensitive step in k_{cat} is on an intermediate which partitions to either pyruvate or L-alanine.

Product inhibition. Anthranilate was reported by Tanizawa and Soda (1979) to be a competitive inhibitor of kynureninase, with a K_i of 1.8 mM. This would be the expected result if anthranilate were the last product released, and it bound to only the free enzyme. In contrast, however, we found that anthranilate is a mixed inhibitor of kynureninase, implying that anthranilate may bind to both the free enzyme and an enzyme−substrate or enzyme−product complex. The K_i values for anthranilate decrease dramatically at lower pH values (Table 2). This is consistent with the preferential binding of the neutral and/or zwitterionic tautomeric forms of anthranilate to the enzyme.

Pre-Steady-State Kinetic Experiments. Rapid chemical quench experiments were performed to examine the pre steady state of the kynureninase reaction. The results of these experiments are presented in Figure 3. There is a pronounced "burst" of anthranilate (Figure 3, filled circles) formed in the pre steady state, with a rate constant of 54 s^{-1} and an amplitude of 0.7 mol of anthranilic acid/mol of enzyme subunit, followed by a slow steady-state increase at 5.8 s^{-1}. The solid line in Figure 3 is the calculated line from fitting the data to eq 7 with FITSIM. In [^{2}H]H_2O, there is also a prominent "burst" of 0.5 mol of anthranilic acid/mol of enzyme subunit (Figure 3, open circles), formed with an identical rate constant of 54 s^{-1}, but with a much slower steady-state rate, as expected from the large solvent isotope effect on k_{cat}. The dashed line in Figure 3 is the calculated

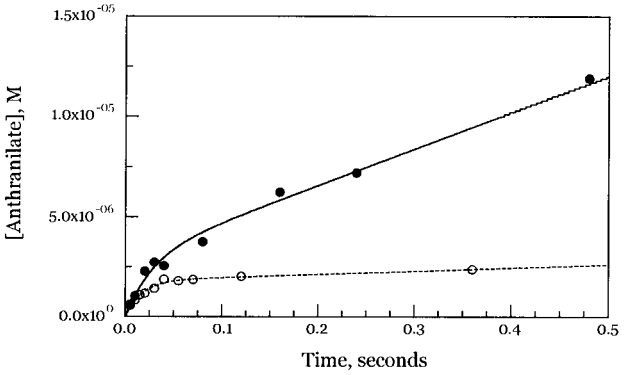


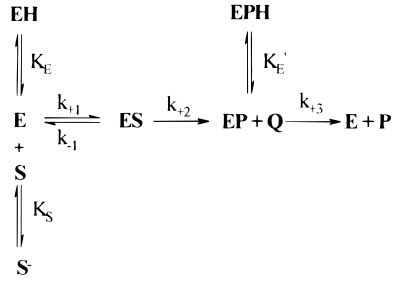
FIGURE 3: Pre-steady-state kinetics of the kynureninase reaction. Portions of kynureninase (40 μL, 8.4 μM) in 0.04 M potassium phosphate, pH 7.8, were mixed with 40 μL of 1 mM L-kynurenine then quenched with 0.5 M $HClO_4$ (80−120 μL, depending on reaction loop) at varying time intervals. The anthranilic acid product was measured by HPLC with fluorescence detection, as described in Experimental Procedures. The filled circles represent the concentration of product formed when the reaction was performed in H_2O, and the solid line is the calculated curve from fitting to eq 7 with FITSIM. The open circles represent the concentration of product formed when the reaction was performed in $[^2H]H_2O$, and the dashed line is the calculated curve from fitting of these data to eq 4 with FITSIM.

line from fitting the data obtained in $[^2H]H_2O$ to eq 7 with FITSIM.

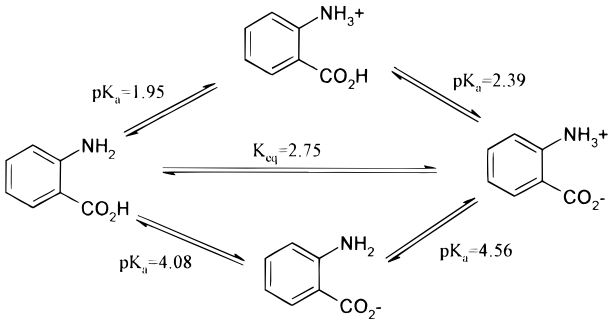
DISCUSSION

The mechanism of kynureninase has been the subject of much speculation since the discovery of the enzyme by Braunstein, who proposed that hydroxide attacks the carbonyl, followed by Cβ−Cγ cleavage (Braunstein et al., 1949). Longenecker and Snell (1955) suggested a mechanism with β-elimination of an acyl anion, followed by redox electron transfer to the α-aminoacrylate intermediate. Akhtar et al. (1984) and Walsh (1979) have speculated that kynureninase utilizes a nucleophilic mechanism, generating an acyl−enzyme intermediate which subsequently undergoes hydrolysis. Tanizawa and Soda (1979) proposed a mechanism similar to that of Braunstein with a *gem*-diol intermediate, followed by formation of a pyruvate ketimine. We concluded, as a result of the potent inhibition of kynureninase by "transition-state analogs" (Dua et al., 1993; Phillips & Dua, 1991), that kynureninase catalyzes the hydrolysis of L-kynurenine by a general base mechanism with a *gem*-diolate intermediate. On the basis of the results of Kishore (1984) with mechanism-based inactivators, we had proposed that there were two catalytic bases in the mechanism of the kynureninase reaction (Phillips & Dua, 1991). However, the present work shows that the pH dependence of k_{cat}/K_m fits to only a single pK_a in the acidic limb (Figure 1A), demonstrating that there can be only a single base involved in the reaction mechanism. This conclusion is in agreement with the results of the stereochemical studies of Palcic et al. (1985). The decrease in k_{cat}/K_m values at high pH is likely due to the preferential binding of the zwitterionic form of kynurenine to the enzyme, since the pK_a for ionization of the zwitterion to the anion of kynurenine calculated by the SPARC pK_a calculator program is 8.87. The observation that the substrate isotope effect on k_{cat}/K_m for α-[^{2}H]-L-kynurenine is insignificant and does not increase at pH values

Scheme 1



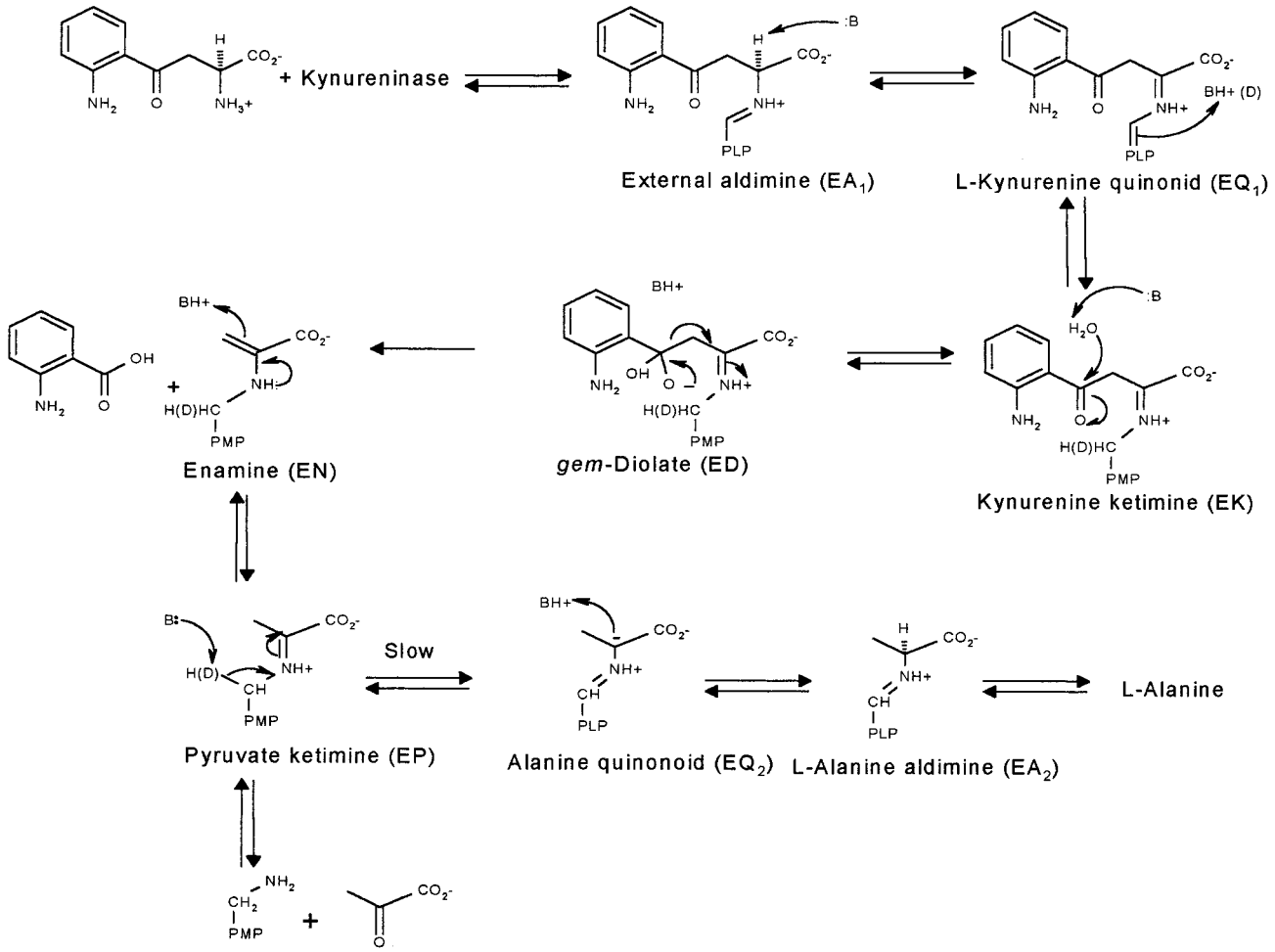
Scheme 2



below the pK_a (Table 1) suggests that kynurenine will only bind to the correct protonation state of enzyme (Cook & Cleland, 1981). The mechanism shown in Scheme 1 is an expansion of the simple mechanism shown earlier in eq 7 to include ionization of enzyme, substrate, and enzyme−product complexes. For this mechanism, if k_{+2} is equal to or greater than k_{-1}, the pK_a observed in the pH profile of k_{cat}/K_m is an apparent pK_a of the enzyme base, $(pK_a)_{obs} = pK_a - \log(1 + k_{+2}/k_{-1})$. Since k_{cat}/K_m for 3-hydroxykynurenine is reduced 80-fold, it is likely that k_{+2} is reduced and k_{-1} is increased. Hence, the reaction of 3-hydroxykynurenine should show a much smaller commitment, and the pK_a of 6.49 observed in its pH dependence should be the true pK_a. Thus, since the apparent pK_a for the reaction of kynurenine is 6.25, it is pushed out by 0.24 pH units, and the forward commitment for kynurenine, k_{+2}/k_{-1}, is 0.7.

The large solvent isotope effect on k_{cat}, together with neither a normal solvent isotope effect nor a substrate kinetic isotope effect on k_{cat}/K_m, shows that the rate-determining step in the mechanism of kynureninase occurs subsequent to the first irreversible step. The small inverse isotope effect on k_{cat}/K_m may be due to the viscosity difference between H_2O and $[^2H]H_2O$ (Karsten et al., 1995). The magnitude of the inverse isotope effect on k_{cat}/K_m may also be reduced as a result of a commitment (Kiick, 1991). The fitting of the proton inventory for k_{cat} (Figure 2) suggests that only a single proton is transferred in the rate-determining step. The magnitude of the solvent isotope effect on k_{cat} is much larger than normal for a single solvent proton and is in the range expected for an isotope effect on the breakage of a C−H bond (Williams, 1984), suggesting that the observed isotope effect determined in $[^2H]H_2O$ is a primary isotope effect on the abstraction of a carbon-bound proton which originated from solvent on the enzyme−product complex. A nonspecific solvent isotope effect can be ruled out on the following bases: (1) there is a negligible normal solvent isotope effect on k_{cat}/K_m; and (2) there is no solvent isotope effect on the pre-steady-state kinetics.

Scheme 3. Mechanism of Kynureninase



PLP-dependent enzymes that catalyze substitutions and eliminations at the β- or γ-carbon of amino acids typically bind the amino acid as the first substrate and release the amino acid (or ketoacid) as the last product, since it remains covalently attached to the PLP cofactor throughout the catalytic cycle. Confirmation of the order of product release in kynureninase by measuring product inhibition by anthranilic acid is complicated by the various tautomers and ionization states (neutral, zwitterion, and anion) of anthranilate in the reaction mixture (Scheme 2). Anthranilate was found to exhibit mixed inhibition, as shown in Table 2. The anthranilate zwitterion, which structurally resembles an amino acid substrate, likely binds at the amino acid binding site. The neutral tautomer of anthranilic acid is the predicted product of kynureninase (Scheme 3) and is the species expected to exhibit product inhibition. The tautomeric equilibrium favors the zwittwerion over the neutral form by a factor of 2.75 (Scheme 2). The values of both K_{is} and K_{ii} decrease dramatically with pH (Table 2), suggesting that the anthranilate anion binds weakly, if at all. The results of the rapid quench experiments (Figure 3) convincingly demonstrate that anthranilate is the first product released and that the solvent-sensitive step is a step subsequent to anthranilate formation.

The pH dependence of k_{cat} (Figure 1B) shows a decrease at acidic pH values, and the data fit best to a single base with pK_a = 6.43. Thus, k_{cat} reflects a step which requires general base catalysis. However, since k_{cat} is limited by a step in the mechanism after the release of the first product, the pK_a observed in the pH dependence of k_{cat} is not that of an ES complex, but rather an EP complex (Scheme 1). These data provide evidence that the rate-determining step in the reaction of kynureninase involves a general base catalyzed proton abstraction which occurs after release of anthranilate.

Scheme 3 shows a mechanism for kynureninase that incorporates all the data presented here as well as the results of previous investigations by ourselves and others. Since the pH dependence data are consistent with a single base mechanism, we propose that the same basic group that abstracts the α-proton from the external aldimine of L-kynurenine (EA_1) to form a quinonoid intermediate (EQ_1) must also be available to catalyze the subsequent carbonyl hydration step. In order to regenerate the free base, the quinonoid intermediate must be protonated at C-4′ of the PLP to give a ketimine intermediate (EK), as is well-documented in the mechanism of transaminases (Kirsch et al., 1984). All steps up to EK must be in fast equilibrium, since there is no observed k_{cat}/K_m isotope effect with α-[^{2}H]-L-kynurenine. Furthermore, the fitting of the rapid chemical quench data with FITSIM required assumption of rapid equilibrium for all steps up to anthranilate formation. Hydration of the ketone carbonyl by general base catalysis can then occur to give the Braunstein *gem*-diolate intermediate (ED) (Braunstein, 1949). Elimination of anthranilate from the *gem*-diolate intermediate is relatively fast (54 s^{-1}) and apparently irreversible to give an enamine intermediate

(EN). The irreversibility may be due to rapid ionization of anthranilate to the anion at the pH values examined, since the enamine can be trapped by other electrophiles, such as benzaldehyde, to give aldol products (Phillips and Dua, 1991; Bild and Morris, 1984). After protonation of the enamine at the β-carbon, the pyruvate ketimine (EP) is formed (Tanizawa and Soda, 1979). In order for L-alanine to be released, it is necessary to abstract the proton from C-4′ to regenerate a quinonoid intermediate (EQ_2), and transfer it to C-α to give the alanine aldimine (EA_2). This is the only step subsequent to anthranilate release in the proposed mechanism which requires general base catalysis in the removal of a single proton; thus, all the data are consistent with this step being rate-limiting in the steady state. In this regard, kynureninase is similar to cytosolic aspartate aminotransferase, where it has been shown that ketimine−aldimine interconversion is rate-limiting in the steady state (Julin & Kirsch, 1989). However, the proton inventory of kynureninase suggests that only a single proton is in flight in the transition state, so the proton shift from C-4′ to C-α cannot be concerted, as is the case for cytosolic aspartate aminotransferase (Julin & Kirsch, 1989). Hence, the quinonoid intermediate of L-alanine (EQ_2) must exist on the reaction coordinate (Scheme 3). In agreement with this prediction, preliminary rapid-scanning stopped-flow kinetic studies of the reaction of kynureninase with L-alanine have demonstrated the formation of a quinonoid intermediate with a peak at 500 nm (R. S. Phillips, unpublished). It should be noted that kynureninase is known to undergo a slow abortive transamination under turnover conditions in the absence of added PLP, resulting in pyruvate and the inactive pyridoxamine-5′-phosphate enzyme (Moriguchi & Soda, 1973). A solvent isotope effect of 3.3 on the alanine/pyruvate partition ratio requires the isotope effect to be associated with an intermediate at a branch point in the mechanism leading to both products. This is in agreement with our proposed mechanism, since both L-alanine and pyruvate arise by partitioning of the pyruvate ketimine intermediate, EP, between deprotonation and hydrolysis (Scheme 3). EP must be at least partially exposed to solvent, since both solvent protonation of the base and hydrolysis of the ketimine to give pyruvate can occur. The proposed mechanism also explains the observed small (1.2%) internal transfer of label from C-α of α-[^{3}H]-kynurenine to the alanine product (Palcic et al., 1985), since the original α-proton would be partially transferred to C-4′, then returned to C-α. However, the α-proton must undergo substantial exchange, as the large isotope effect on k_{cat} in [^{2}H]H_2O indicates that most of the protons on C-4′ of the pyruvate ketimine intermediate arise from solvent.

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