The Catalytic Mechanism of Kynureninase from *Pseudomonas fluorescens*: Evidence for Transient Quinonoid and Ketimine Intermediates from Rapid-Scanning Stopped-Flow Spectrophotometry

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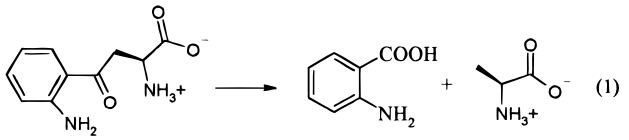
Received January 9, 1998; Revised Manuscript Received March 26, 1998

ABSTRACT: The reaction of *Pseudomonas fluorescens* kynureninase with L-kynurenine and L-alanine has been examined using rapid-scanning stopped-flow spectrophotometry. Mixing kynureninase with 0.5 mM L-kynurenine results in formation of a quinonoid intermediate, with $\lambda_{max} = 494$ nm, within the dead time (ca. 2 ms) of the stopped-flow mixer. This intermediate then decays rapidly, with $k = 743$ s^{-1}, and this rate constant is reduced to 347 s^{-1} in [^{2}H]H_2O, suggesting that protonation of this intermediate by a solvent exchangeable proton takes place. Rapid quench experiments demonstrate that covalent changes in the cofactor occur, as pyridoxal 5′-phosphate is converted to pyridoxamine 5′-phosphate in about 30 mol % within 5 ms after mixing. Under single turnover conditions in the reaction of kynureninase with L-kynurenine, a transient shoulder absorbing at 335 nm is observed that may be a pyruvate ketimine intermediate. In contrast, the reaction of kynureninase with 0.5 mM L-kynurenine in the presence of 10 mM benzaldehyde results in the formation of a quinonoid intermediate ($k = 67.4$ s^{-1}) with a very strong absorbance peak at 496 nm. The reaction of L-alanine with kynureninase exhibits the rapid formation (386 s^{-1} at 0.1 M) of an external aldimine intermediate absorbing at 420 nm, followed by slower formation of a quinonoid intermediate with a peak at 500 nm ($k = 2.5$ s^{-1}). The 420 nm peak then decays slowly with concomitant formation of a peak at 320 nm corresponding to a pyruvate ketimine. These data demonstrate that quinonoid and ketimine intermediates are catalytically competent in the reaction mechanism of kynureninase, and provide additional support for our proposed mechanism for kynureninase from steady-state kinetic studies [Koushik, S. V., Sundararaju, B., and Phillips, R. S. *Biochemistry* 1998, *37*, 1376−1382].

Kynureninase [E.C. 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 L-tryptophan by *Pseudomonas fluorescens* and some other bacteria (*1*). In eukaryotes, a similar enzyme reacts with



(1)

3-hydroxykynurenine in the catabolism of L-tryptophan and in the biosynthesis of NAD (*2*) to produce 3-hydroxyanthranilate. 3-Hydroxyanthranilate dioxygenase then converts 3-hydroxyanthranilate to quinolinate, which is a neurotoxin due to its agonist effects on the *N*-methyl-D-aspartate (NMDA) receptor. Excessive levels of quinolinate have been implicated in the etiology of a wide range of neurological disorders, such as epilepsy, stroke, and AIDS-related dementia (*3*−*10*).

In previous studies, we have designed and synthesized a series of "transition-state analogue" inhibitors of kynureninase that contain a carbinol or 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 (*11, 12*). On the basis of these results, we concluded that there is a *gem*-diolate intermediate in the reaction mechanism. Recently, we have examined the effects of pH, substrate, and solvent isotopic substitution on the steady-state and pre-steady-state kinetics of kynureninase (*13*). We concluded that ketimine intermediates are formed and that the rate determining step in the mechanism is deprotonation at C4′ of a pyruvate ketimine intermediate. In the present work, we have examined the mechanism of kynureninase using rapid-scanning stopped-flow spectrophotometry and rapid chemical quench methods in order to

[†] This work was partially supported by a grant from the National Institutes of Health to R.S.P. (GM42588-08) and by a contract with Pharmacia-Farmitalia.

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[1] Abbreviations: pyridoxal 5′-phosphate (PLP); pyridoxamine 5′-phosphate (PMP); high-performance liquid chromatography (HPLC).

S0006-2960(98)00066-X CCC: $15.00
Published on Web 05/27/1998

observe and identify transient reaction intermediates. These results show that both quinonoid and ketimine intermediates are rapidly formed in the reaction of kynureninase with L-kynurenine, providing additional support for our proposed mechanism.

EXPERIMENTAL PROCEDURES

Materials. Kynureninase was purified from *P. fluorescens* (ATCC 11250) cells grown on a medium with 0.2% L-tryptophan as carbon and nitrogen source (*1*), using modifications of previously described protocols (*14*, *15*), or from *Escherichia coli* cells containing plasmid pTZ18U with the *kyn* gene of *P. fluorescens* inserted into the linker (*13*). The enzyme preparations exhibited specific activities of 7−16 μmol min^{-1} mg^{-1} at pH 7.8 and 25 °C. The deuterium oxide ([^{2}H]-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.

Instruments. The steady-state kinetic measurements were performed on a Cary 1E UV/visible spectrophotometer equipped with a 6 × 6 thermoelectric cell changer, controlled by a Gateway 2000 80486 DX2/66 PC using software provided by Varian Instruments. Rapid-scanning stopped-flow experiments were performed on an RSM instrument from OLIS, Inc., equipped with a stopped-flow mixer from OLIS with an optical path length of 10 mm. The dead time of the stopped-flow unit is about 2 ms; thus, a reaction with a rate constant of 350 s^{-1} will lose half of its amplitude during the mixing process. Spectra containing 200 data points over a range of 230 nm were collected at a rate of 1 kHz. For some experiments, a fixed 0.6 μm slit was used to collect data at a single wavelength (494 nm) in order to collect 1000 data points in 0.15 s.

Rapid chemical quench experiments were performed on an apparatus from KinTek Instruments (State College, PA). Pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate in the chemical quench experiments were 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. Kynureninase assays were performed at 25 °C by following the decrease in absorbance of L-kynurenine at λ_{max} of 360 nm ($\Delta\epsilon = -4500$ M^{-1} cm^{-1}). For experiments on benzaldehyde inhibition, 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 (*11*). V_{max} and V_{max}/K_m values were obtained by nonlinear fitting to

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

eq 2 using Enzfitter (Elsevier). The protein concentration was determined using the 280 nm absorbance, with $A^{1\%} =$ 14 (*14*), assuming a molecular weight of 4.6 × 10^4 based on the amino acid sequence (*16*). Rate constants in the rapid-scanning stopped-flow experiments were determined by Robust Global Fitting of the spectra, using software provided by OLIS, Inc. (*17*, *18*). Time courses at 494 nm were analyzed by fitting with the SIFIT or LMFIT programs (OLIS, Inc.), which can fit up to three exponentials with amplitudes and an offset, where A_t is the absorbance at time t, a_i is the amplitude of each phase, k_i is the rate constant for each phase, and c is the final absorbance, if nonzero (eq 3). Quality of fit was judged by analysis of the residuals and by the Durbin−Watson value (*19*). The rate constants are reported together with standard errors obtained from the fitting.

$$A_t = \sum_{i}^{1-3} a_i e^{-k_i t} + c \quad (3)$$

For rapid-scanning stopped-flow studies, the kynureninase solutions were preincubated with 0.5 mM PLP for 30 min at 30 °C, then passed through a short gel filtration column (Pharmacia, PD-10) equilibrated with 0.05 M potassium phosphate, pH 7.8, to remove excess PLP. For experiments in [^{2}H]H_2O, the enzyme after gel filtration was concentrated in a 3 mL Amicon cell with a YM-30 membrane to about 0.2 mL, then diluted 10-fold with buffer prepared in [^{2}H]-H_2O. This process of concentration and dilution was repeated twice more before the enzyme was used in the experiments. Solutions of kynurenine at 1 mM were prepared in normal and deuterated buffers. In some experiments, 20 mM benzaldehyde was added to the kynurenine solution. The measurements were performed at 25 °C, with the stopped-flow syringes and observation cell thermostated by an external circulating water bath.

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. 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 PMP determination. The solvent was 0.05 M NaH_2PO_4, pH 3.0/methanol, with a constant composition of 5% methanol for the first 10 min, then a gradient from 20% to 50% methanol was run over the next 20 min. Under these conditions, PMP elutes at 3.7 min after injection, and PLP elutes at 7 min. A standard curve was generated from dilutions of authentic PMP, and the lower limit of detection by fluorescence in this experiment was 1 pmol.

RESULTS

Rapid-Scanning Stopped-Flow Studies of Kynureninase Reaction with L-Kynurenine. When kynureninase is mixed with 0.5 mM L-kynurenine, there is very fast formation (complete within the dead time of the stopped-flow instrument, about 2 ms) of an absorbance peak at 494 nm, which decreases rapidly to a very low steady-state level (Figure 1A). Identical spectra were obtained using either enzyme isolated from L-tryptophan induced *P. fluorescens* cells or recombinant enzyme from *E. coli* DH5α cells containing pTZKYN. The reaction was analyzed by global fitting to all the data in the range from 420 to 560 nm for the first

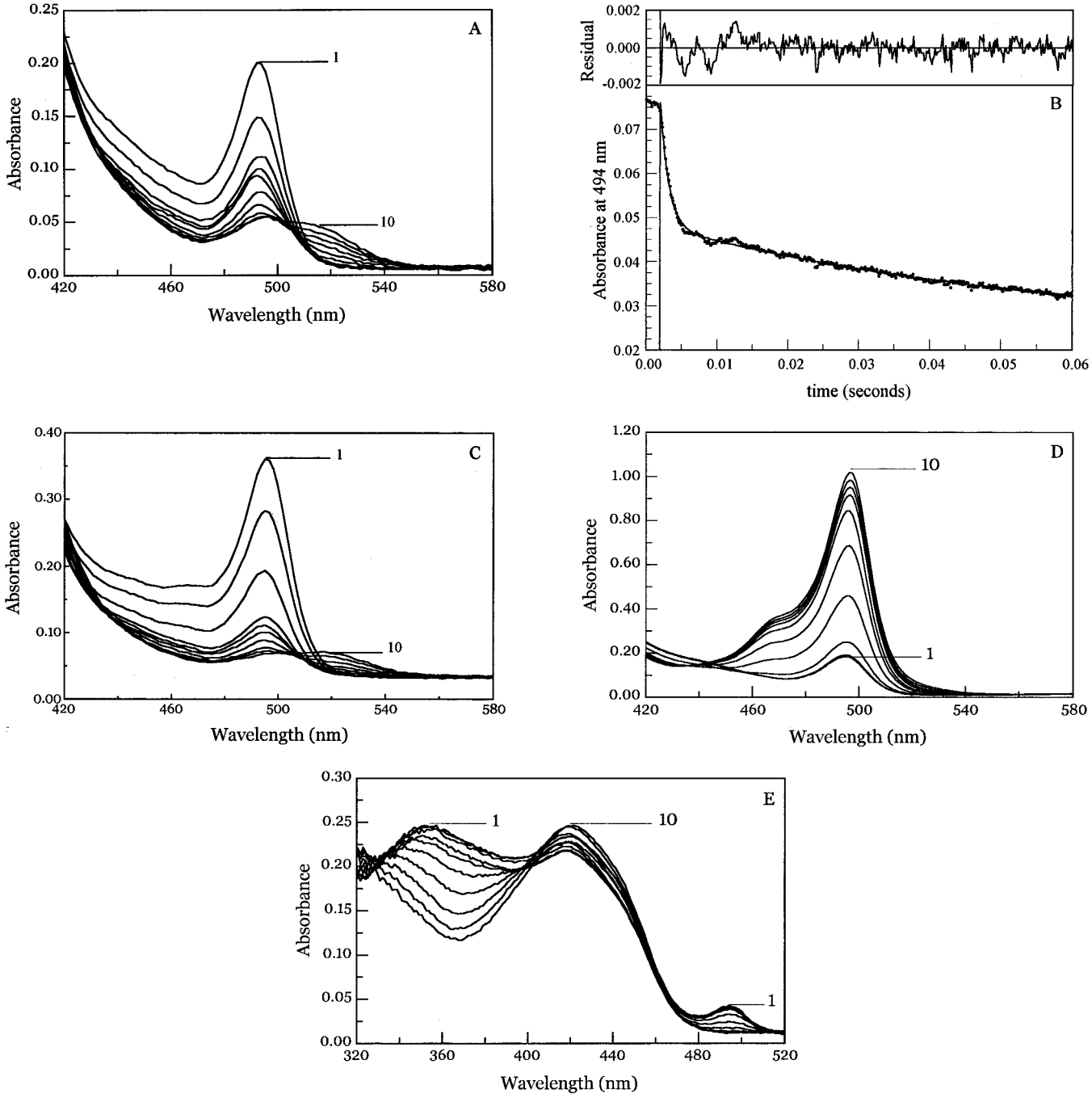


FIGURE 1: (A) Rapid-scanning stopped-flow spectra of the reaction of 0.033 mM kynureninase with 0.5 mM L-kynurenine. Scanning times are (1) 0.001 s, (2) 0.003 s, (3) 0.005 s, (4) 0.010 s, (5) 0.020 s, (6) 0.040 s, (7) 0.080 s, (8) 0.160 s, (9) 0.320 s, and (10) 0.640 s. (B) Time course at 494 nm for the reaction of 0.022 mM kynureninase with 0.5 mM L-kynurenine. One thousand data points were collected in a total of 150 ms. The line is the calculated curve from fitting of the data to a two exponential equation with the parameters $1/\tau_1 = 773 \pm 17$ s^{-1} and $1/\tau_2 = 15.5 \pm 0.8$ s^{-1}. The residual shows the difference between the experimental data and the calculated curve. (C) Rapid-scanning stopped-flow spectra of the reaction of 0.033 mM kynureninase with 0.5 mM L-kynurenine in $[^2H]H_2O$. Scanning times are the same as in Figure 1A. (D) Rapid-scanning stopped-flow spectra of the reaction of 0.033 mM kynureninase with 0.5 mM L-kynurenine in the presence of 10 mM benzaldehyde. Scanning times are the same as in Figure 1A. (E) Rapid-scanning stopped-flow spectra of the reaction of 0.0375 mM kynureninase with 0.0375 mM L-kynurenine. Scanning times are the same as in Figure 1A.

600 ms. The best fit is to three exponential processes, and the rate constants for the three phases are 743 ± 7 s^{-1}, 23.2 ± 1.8 s^{-1}, and 5.2 ± 0.1 s^{-1}. These rate constants are not changed when the L-kynurenine concentration is raised to 1 mM. The first two exponentials correspond to a decrease in the absorbance at 494 nm. Single wavelength measurements were performed at 494 nm over a period of 150 ms in order to obtain more data points for the fast processes; these data were fitted to an equation with two exponentials and gave similar rate constants for the first two phases. Representative data from this experiment are shown in Figure 1B. The points are the data obtained in the stopped-flow experiment, and the line is the fitted curve. The residual calculated from the experimental data and the fitted curve is shown above in Figure 1B. The average of 10 separate single wavelength determinations of the fast phase is 755 ± 23 s^{-1}, in excellent agreement with the rapid-scanning results. During the slowest phase, a shoulder at 520 nm increases

slowly (Figure 1A). There is no isosbestic point between the absorbance peak at 494 nm and the peak at 520 nm, indicating that these complexes are not interconverting (Figure 1A).

When the reaction of kynureninase with L-kynurenine is performed in [^{2}H]H_2O, the initial absorbance of the 494 nm peak is increased by about 2-fold (Figure 1C). Global fitting of these data demonstrate that the rate constant for the fast phase of the absorbance decrease is reduced from 743 s^{-1} in H_2O to 347.0 ± 1.4 s^{-1} in [^{2}H]H_2O. Thus, there is a solvent isotope effect of $^Dk = 2.14 \pm 0.02$ on the fast process of decay of the 494 nm intermediate. The larger initial absorbance of the 494 nm peak is also consistent with this isotope effect, since as the rate constant for its decay is decreased, less of the reaction is lost in the instrument dead time. The rate constant of the second phase is decreased from 23.2 to 15.0 ± 0.5 s^{-1} in [^{2}H]H_2O, so the solvent isotope effect is about 1.5 on this step. The rate constant for formation of the 520 nm intermediate is also reduced from 5.2 to 3.0 ± 0.1 s^{-1} in [^{2}H]H_2O, giving a solvent isotope effect of about 1.7.

Benzaldehyde is a potent uncompetitive inhibitor of kynureninase due to its participation in an aldol reaction with an enzyme intermediate to give γ-phenyl-L-homoserine (*11*, *20*, *21*). We confirmed the inhibitory effects of benzaldehye on the steady-state kinetics of kynureninase. At concentrations of benzaldehyde above 1 mM, the reaction of kynureninase under normal assay conditions becomes virtually undetectable. When the rapid-scanning stopped-flow reaction of kynureninase was done in the presence of 0.5 mM L-kynurenine and 10 mM benzaldehyde, the initial peak at 494 nm forms within the dead time of the stopped-flow instrument, decreases rapidly, as observed without benzaldehyde present, with a rate constant of 636 ± 1 s^{-1}, then the absorbance increases (k = 67.4 ± 0.1 s^{-1}) to form a very intense peak at 496 nm (Figure 1D). The final spectrum of this intermediate resembles that of kynureninase complexed with the potent transition state analogue competitive inhibitor, *S*-(2-aminophenyl)-L-cysteine *S*,*S*-dioxide (*12*).

Because of the strong absorbance peak of L-kynurenine at 360 nm (ϵ = 4500 M^{-1} cm^{-1}), it is not possible to observe reaction intermediates at wavelengths below 420 nm under saturating conditions. To observe intermediates in the region from 320 to 420 nm, single turnover experiments with L-kynurenine were performed (Figure 1E) with 37.5 μM L-kynurenine and 37.5 μM kynureninase (based on subunits). In these reactions, a much smaller peak at 494 nm is formed upon mixing, the L-kynurenine peak at 360 nm decays rapidly, and a transient shoulder in the spectra at about 335 nm is seen (Figure 1E, curve 7). It should be noted that the product, anthranilate, absorbs strongly at 310 nm. Within 640 ms, the spectrum returns to that of free kynureninase (Figure 1E, curve 10).

Rapid-Scanning Stopped-Flow Studies of Kynureninase Reaction with L-Alanine. When kynureninase is mixed with 0.1 M L-alanine, the 420 nm peak of the resting enzyme initially decreases rapidly, with an apparent rate constant of 386 ± 23 s^{-1}. Thus, the formation of the external aldimine from L-alanine is relatively slow. This intermediate is then converted to a quinonoid structure with a peak at 500 nm (Figure 2). The rate constant for the formation of the 500 nm intermediate from L-alanine is 2.5 s^{-1} at 0.1 M L-alanine,

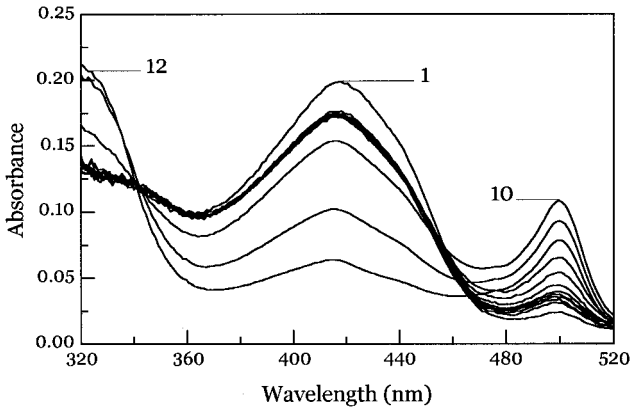


FIGURE 2: Rapid-scanning stopped-flow spectra of the reaction of L-kynurenine with 0.1 M L-alanine. Scanning times are (1) 0.001 s, (2) 0.005 s, (3) 0.010 s, (4) 0.020 s, (5) 0.040 s, (6) 0.080 s, (7) 0.160 s, (8) 0.320 s, (9) 0.640 s, (10) 24 s, (11) 150 s, and (12) 300 s.

about 3-fold slower than k_{cat} of L-kynurenine (*13*). Within a few minutes, the 420 and 500 nm peaks begin to decline, and a new species absorbing at 320 nm is seen, as the pyruvate ketimine forms by the abortive transamination reaction (Figure 2).

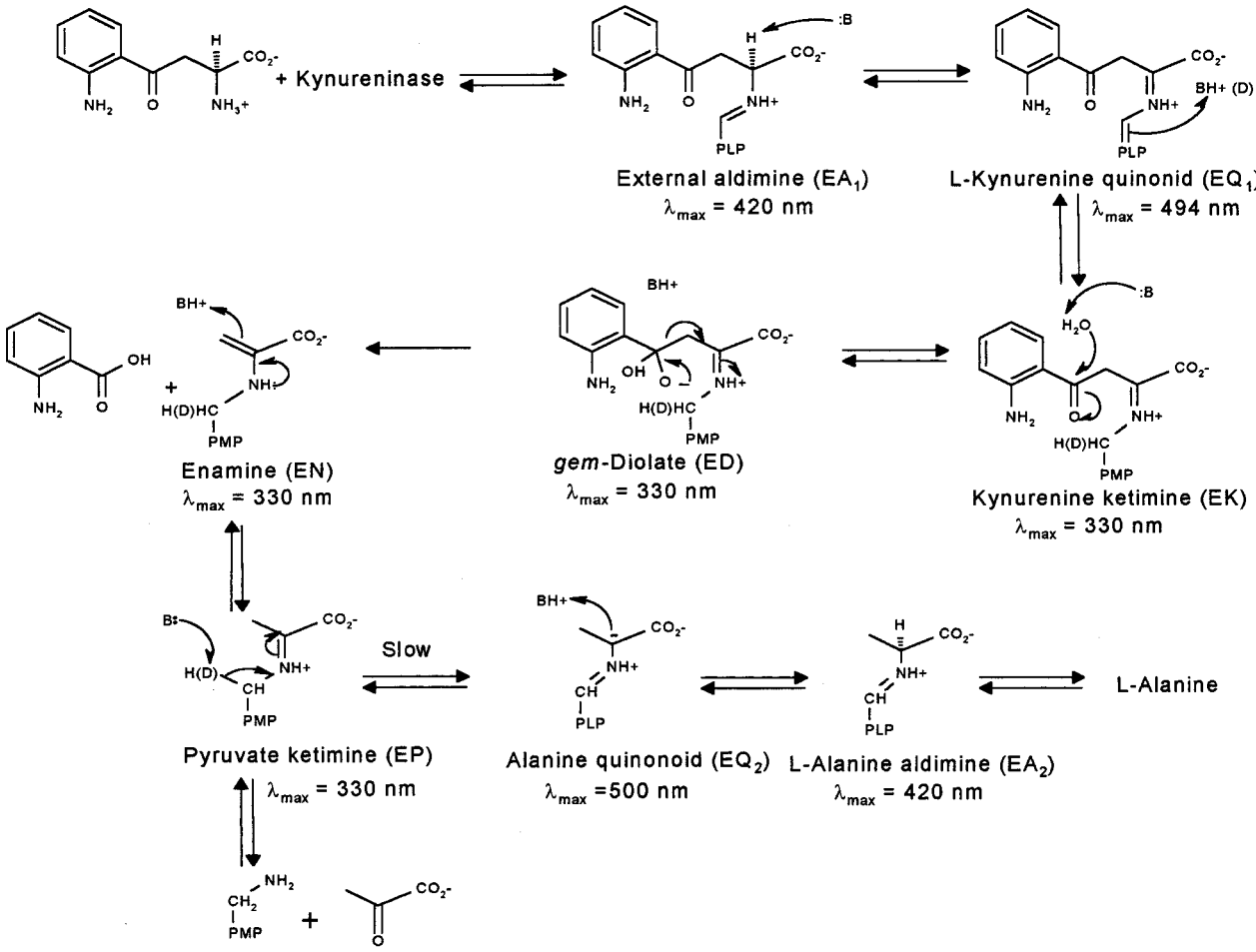
Rapid-Quench Studies of Kynureninase. Previous studies had shown the formation of PMP during the reaction of kynureninase by a relatively slow abortive transamination, which takes place over a period of minutes (*22*). We performed rapid chemical quench experiments to determine if PMP also forms at a rate fast enough to be a competent reaction intermediate. There is no significant PMP observed by HPLC if kynureninase is premixed with 0.5 M $HClO_4$ before addition of L-kynurenine. However, a very large fluorescence peak of PMP, corresponding to more than 30 mol % of the subunit concentration of enzyme, is found in reaction mixtures quenched at 0.005, 0.010 and 0.020 s (data not shown). These results suggest that PMP is formed as part of a catalytically competent intermediate in the reaction of kynureninase.

DISCUSSION

The reaction mechanism of kynureninase has been the subject of experiment and speculation for nearly half a century (*2*, *23*−*26*). In our previous paper, we studied the effects of pH and isotopic substitution on the steady-state kinetics of the reaction of *P. fluorescens* kynureninase (*13*). We found no primary isotope effect on either k_{cat} or k_{cat}/K_m with α-[^{2}H]L-kynurenine, but a large kinetic isotope effect (^Dk = 6.56) on k_{cat} in [^{2}H]H_2O. Furthermore, this large solvent isotope effect was not observed on the "burst" of anthranilate seen in pre-steady-state rapid chemical quench measurements. Thus, the solvent isotope effect is on a step in the reaction mechanism after anthranilate release, and we proposed that the rate-determining step in the reaction of kynureninase is proton abstraction from C4′ of a pyridoxamine−pyruvate ketimine intermediate.

When kynureninase is mixed with L-kynurenine in the rapid-scanning stopped-flow spectrophotometer, a quinonoid intermediate (EQ_1 in Scheme 1), with λ_{max} = 494 nm, is formed within the dead time of the mixer (about 2 ms) (Figure 1A). This transient intermediate then decays with a rate constant of 743 s^{-1}, and with an apparent isotope effect

Scheme 1

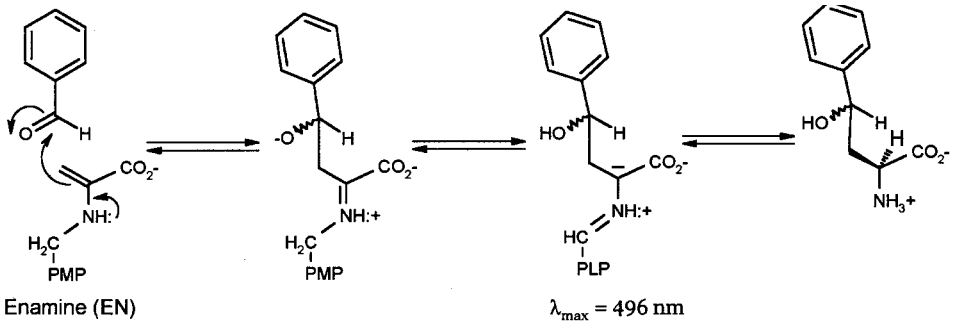


of 2.1 in [^{2}H]H_2O (Figure 1B). Thus, it is apparent that this transient quinonoid species decays by proton transfer from a solvent exchangeable acid. The only basic sites where a quinonoid intermediate is likely to be protonated are at Cα or C4′. Reprotonation of the carbanionic species at Cα would be mechanistically futile, since it would lead back to the external aldimine of L-kynurenine (EA_1). Thus, it seems more likely that the decay of the 494 nm intermediate is due to protonation at C4′ to give a kynurenine ketimine intermediate (EK in Scheme 1). The ketimine intermediate would be expected to exhibit an absorbance peak at 325−340 nm, but in these experiments this region was obscured by the high background absorbance of the L-kynurenine. To test for the presence of this intermediate, we performed rapid chemical quench experiments with kynureninase, and, as predicted, we found that indeed a large amount of PMP (about 30 mol %) is formed within 5 ms after mixing with L-kynurenine, in a time frame comparable to the fast phase of decay of the 494 nm absorbance peak. Thus, the results of these experiments demonstrate that kynurenine quinonoid (EQ_1) and ketimine (EK) intermediates form rapidly and are kinetically competent in the reaction mechanism of kynureninase. The high rate of formation of these intermediates is consistent with our previous conclusion that all species up to EK must be in fast equilibrium, based on the lack of a k_{cat}/K_m isotope effect with α-[^{2}H]L-kynurenine (*13*).

The role of the second phase of the absorbance decrease at 494 nm ($k = 23.2$ s^{-1}) is not obvious. It cannot be due to anthranilate formation, since it is considerably slower than the rate of anthranilate formation ($k = 54$ s^{-1}) measured previously in chemical quench experiments. It is possible that there is some conformational hetereogeneity of the kynurenine quinonoid intermediate (EQ_1). Modeling of the data in Figure 1B using KINSIM (*27*) shows that the time course at 494 nm can be simulated by assuming two species of EQ_1 in a 3:1 ratio that react at 750 and 20 s^{-1}, respectively (data not shown). This is consistent with the results of the rapid quench experiments, which show 0.7 mol anthranilate formed in the burst phase (*13*). Finally, the complex absorbing at about 520 nm (Figure 1, A and C) does not appear to be in the direct catalytic pathway, since it forms slowly, with a rate constant less than k_{cat}, and shows a much smaller isotope effect in [^{2}H]H_2O than k_{cat}.

The reaction of kynureninase with L-kynurenine in the presence of benzaldehyde results in an aldol reaction, giving γ-phenyl-L-homoserine rather than L-alanine as product (*11*, *21*). This reaction is diastereoselective, giving a 3:1 mixture of γ-R to γ-S diastereomers (*11*). Addition of benzaldehyde, a potent uncompetitive inhibitor of kynureninase (*20*), to the rapid-scanning stopped-flow experiments results in the formation of an intense absorbance peak at 496 nm that can be attributed to a different quinonoid structure (Figure 1D). This new complex is formed after the rapid phase of the quinonoid intermediate decay, and the rate constant for formation of this species is 67.4 s^{-1}, which is approximately the same as the rate constant for anthranilate formation in

Scheme 2



the "burst" measured by rapid quench studies, 54 s^{-1} (*13*). This suggests that benzaldehyde reacts very rapidly with the enzyme enamine intermediate (EN) (Scheme 2) formed after release of anthranilate. The 496 nm absorbance peak seen in the presence of benzaldehyde is quite stable and persists for at least 30 min. Thus, the deprotonation of the ketimine of the benzaldehyde aldol product at C4′ to form the quinonoid intermediate must be fast (Scheme 2). The equilibrium between the γ-phenylhomoserine external aldimine and quinonoid intermediates must lie strongly in favor of the quinonoid complex absorbing at 496 nm. We then performed a rapid-scanning stopped-flow study of kynureninase under single turnover conditions, with only a stoichiometric concentration of L-kynurenine present. These data show the transient formation of a shoulder at about 335 nm, which may be due to the pyruvate ketimine intermediate (EP in Scheme 1). The expected λ_{max} for a ketimine intermediate is 320−340 nm. The lack of an isosbestic point in the 320−340 nm region in Figure 1E indicates that there are more than two intermediates contributing to the spectra.

When kynureninase is mixed with L-alanine, there is rapid formation of an external aldimine intermediate in the rapid-scanning stopped-flow spectra, corresponding to a decrease in the absorbance peak at 420 nm (Figure 2). The rate constant for subsequent formation of the quinonoid intermediate, absorbing at 500 nm, is 2.5 s^{-1}, and is independent of [L-alanine] in the range from 0.05 to 0.5 M. Within 1−2 min, the absorbance peak of the L-alanine external aldimine at 425 nm decreases, and there is an increased absorbance at 320 nm, corresponding to formation of a pyruvate ketimine intermediate. The rate constant for this reaction is 0.007 s^{-1}, which is about one order of magnitude faster than the overall rate of inactivation by abortive transamination (*13*). Thus, this peak must be that of the pyruvate ketimine intermediate, and the hydrolysis to release pyruvate and PMP must be slower. It is interesting to note that the absorbance peak at 500 nm does not decrease to the same extent as the aldimine peak (Figure 2), suggesting that the equilibrium between the L-alanine aldimine (EA_2), ketimine (EP), and quinonoid (EQ_2) intermediates lies in favor of the ketimine and quinonoid species. These data demonstrate that L-alanine forms both quinonoid and ketimine intermediates with kynureninase that should be comparable to those formed by nascent alanine formed from kynurenine in the forward reaction. The rates of quinonoid and ketimine intermediate formation from L-alanine are slower than k_{cat} for the overall reaction of L-kynurenine to produce L-alanine. However, the apparent rate constant for formation of the quinonoid intermediate from L-alanine is concentration independent, whereas if it were a simple binding equilibrium followed by deprotonation, it should show a hyperbolic dependence (*28*). This result suggests that there may be a slow conformational change limiting quinonoid intermediate formation in the direction from L-alanine.

The data presented here provide further support for our previously proposed mechanism for kynureninase (*13*). In addition, the data here demonstrate for the first time that catalytically competent quinonoid and ketimine intermediates are formed in the reaction of kynureninase with L-kynurenine.

REFERENCES

1. Hayaishi, O., and Stanier, R. Y. (1951) *J. Bacteriol. 62*, 691−709.
2. Soda, K., and Tanizawa, K. (1979) *Advances Enzymol. Relat. Areas Mol. Biol. 49*, 1−40.
3. Sei, S., Saito, K., Stewart, S. K., Crowley, J. S., Brouwers, P., Kleiner, D. E., Katz, D. A., Pizzo, P. A., and Heyes, M. P. (1995) *J. Infect. Dis. 172*, 638−647.
4. Sei, Y., Paul, I. A., Saito, K., Layar, R., Hartley, J. W., Morse, H. C. R., Skolnick, P., and Heyes, M. P. (1996) *J. Neurochem. 66*, 296−302.
5. Saito, K., Crowley, J. S., Markey, S. P., and Heyes, M. P. (1993) *J. Biol. Chem. 268*, 15496−15503.
6. Saito, K., Nowak, T. S., Jr., Markey, S. P., and Heyes, M. P. (1993) *J. Neurochem. 60*, 180−192.
7. Saito, K., Nowak, T. S., Jr., Suyama, K., Quearry, B. J., Saito, M., Crowley, J. S., Markey, S. P., and Heyes, M. P. (1993) *J. Neurochem. 61*, 2061−2070.
8. Heyes, M. P., Brew, B., Martin, A., Markey, S. P., Price, R. W., Bhalla, R. B., and Salazar, A. (1991) *Adv. Exp. Med. Biol. 294*, 687−690.
9. Heyes, M. P., Papagapiou, M., Leonard, C., Markey, S. P., and Auer, R. N. (1991) *Adv. Exp. Med. Biol. 294*, 679−682.
10. Achim, C. L., Heyes, M. P., and Wiley, C. A. (1993) *J. Clin. Invest. 91*, 2769−2775.
11. Phillips, R. S., and Dua, R. K. (1991) *J. Am. Chem. Soc. 113*, 7385−7388.
12. Dua, R. K., Taylor, E. W., and Phillips, R. S. (1993) *J. Am. Chem. Soc. 115*, 1264−1270.
13. Koushik, S. V., Moore, J. A., III, Sundararaju, B., and Phillips, R. S. (1998) *Biochem. 37*, 1376−1382.
14. Moriguchi, M., Yamamoto, T., and Soda, K. (1971) *Biochem. Biophys. Res. Commun. 44*, 752−757.
15. Ishikawa, T., Okuno, E., Kawai, J., and Kido, R. (1989) *Comp. Biochem. Physiol. 93*, 107−111.
16. Koushik, S. V., Sundararaju, B., McGraw, R. A., and Phillips, R. S. (1997) *Arch. Biochem Biophys. 344*, 301−308.
17. Matheson, I. B. C. (1990) *Comput. Chem. 14*, 49−57.
18. Maeder, M., and Zuberbuhler, A. D. (1990) *Anal. Chem. 62*, 2220−2224.
19. Durbin, J., and Watson, G. S. (1970) *Biometrika 37*, 409−414.

20. Tanizawa, K., and Soda, K. (1979) *J. Biochem. (Tokyo) 86*, 1199−1209.
21. Bild, G. S., and Morris, J. C. (1984) *Arch. Biochem. Biophys. 235*, 41−47.
22. Moriguchi, M., and Soda, K. (1973) *Biochemistry 12*, 2974−2980.
23. Braunstein, A. E., Goryachenkova, E. V., and Paskhina, T. S. (1949) *Biokhimiya 14*, 163.
24. Longenecker, J. B., and Snell, E. E. (1955) *J. Biol. Chem. 213*, 205−235.
25. Walsh, C. (1979), *Enzymatic Reaction Mechanisms* W. H. Freeeman and Co., San Francisco, p 821.
26. Akthar, M., Emery, V. C., and Robinson, J. A. (1984) in *The Chemistry of Enzyme Action* (Page, M. I., Ed.) Elsevier, Amsterdam, p 342.
27. Zimmerle, C. T., and Frieden, C. (1989) *Biochem. J. 258*, 381−387.
28. Strickland, S., Palmer, G., and Massey, V. (1975) *J. Biol. Chem. 250*, 4048−4052.

BI980066V