Reaction of *Pseudomonas fluorescens* Kynureninase with β-Benzoyl-L-alanine: Detection of a New Reaction Intermediate and a Change in Rate-Determining Step†

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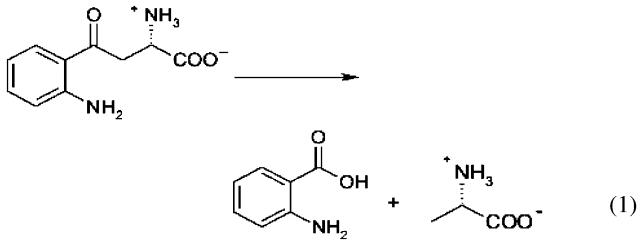
ABSTRACT: β-Benzoyl-DL-alanine was synthesized from α-bromoacetophenone and diethyl acetamidomalonate. The racemic amino acid was resolved by carboxypeptidase A-catalyzed hydrolysis of the *N*-trifluoroacetyl derivative. β-Benzoyl-L-alanine is a good substrate of kynureninase from *Pseudomonas fluorescens*, with k_{cat} and k_{cat}/K_m values of 0.7 s^{-1} and 8.0 × 10^4 M^{-1} s^{-1}, respectively, compared to k_{cat} = 16.0 s^{-1} and k_{cat}/K_m = 6.0 × 10^5 M^{-1} s^{-1} for L-kynurenine. In contrast to the reaction of L-kynurenine, β-benzoyl-L-alanine does not exhibit a significant solvent isotope effect on k_{cat} ($^{H}k/^{D}k$ = 0.96 ± 0.06). The pre-steady-state kinetics of the reaction of β-benzoyl-L-alanine were investigated by rapid scanning stopped-flow spectrophotometry. The spectra show the formation of a quinonoid intermediate, with λ_{max} = 490 nm, in the dead time of the instrument, which then decays, with k = 210 s^{-1}, to form a transient intermediate with λ_{max} at 348 nm. In the presence of benzaldehyde, the 348 nm intermediate decays, with k = 0.7 s^{-1}, to form a quasistable quinonoid species with λ_{max} = 492 nm. Previous studies demonstrated that benzaldehyde can trap an enamine intermediate formed after the $C_\beta-C_\gamma$ bond cleavage [Phillips, R. S., Sundararaju, B., and Koushik, S. V. (1998) *Biochemistry 37*, 8783−8789]. Thus, the 348 nm intermediate is kinetically competent. The position of the absorption maximum and shape of the band is consistent with a PMP−ketimine intermediate. The results from chemical quenching analysis do not show a burst of benzoate and, thus, also support the formation of benzoate as the rate-determining step. These data suggest that, in contrast to L-kynurenine, for which the rate-determining step was shown to be deprotonation of the pyruvate-ketimine intermediate [Koushik, S. V., Moore, J. A., III, Sundararaju, B., and Phillips, R. S. (1998) *Biochemistry 37*, 1376−1382], the rate-determining step in the reaction of β-benzoyl-L-alanine with kynureninase is $C_\beta-C_\gamma$ bond cleavage.

Kynureninase (EC 3.7.1.3) is a pyridoxal 5′-phosphate (PLP)[1] dependent enzyme which catalyzes the hydrolytic cleavage of L-kynurenine to anthranilic acid and L-alanine (*1*) (eq 1). The reaction is a key step in the catabolism of



(1)

L-tryptophan by *Pseudomonas fluorescens* and some other bacteria (*2*), and in these organisms, kynureninase is induced by growth in the presence of L-tryptophan. In animals and some fungi, a similar constitutive enzyme reacts preferentially with 3-hydroxy-L-kynurenine in the catabolism of L-tryptophan (*1*). In these eukaryotes, this pathway is responsible for the biosynthesis of NAD^+, via the intermediacy of quinolinate. However, quinolinate is also a neurotoxin, due to its agonist effects on the *N*-methyl-D-aspartate receptor, and excessive levels of quinolinate have been implicated in the etiology of a wide range of diseases such as epilepsy, stroke, and neurological disorders, including AIDS-related dementia (*3*, *4*). Thus, inhibitors of kynureninase are of interest as possible drugs for the treatment of a range of neurological disorders (*5*). It is important to determine the structural features that determine the reaction specificity of kynureninase to assist in the design of potent and specific inhibitors. Previously, we found that dihydrokynurenines and *S*-(2-aminophenyl)cysteine *S*,*S*-dioxide are potent inhibitors of bacterial kynureninase, with K_i values in the micromolar to nanomolar range (*6*−*8*). The presence of the aromatic amino group at the ortho position was found to be important for inhibitor potency (*7*). Botting and coworkers examined a number of bicyclic analogues of kynurenine which do not contain aromatic amino groups, but these compounds were relatively weak inhibitors of bacterial kynureninase (*9*).

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

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In previous work, we studied the mechanism of *P. fluorescens* kynureninase with L-kynurenine by both steady-state and stopped-flow kinetic methods (*10*, *11*). We demonstrated that the rate-determining step in the reaction of L-kynurenine is the deprotonation of a pyruvate−ketimine intermediate (*10*) and that a quinonoid intermediate is formed in the dead time of the stopped-flow instrument (<2 ms) that decays at 743 s^{-1} to form a reactive intermediate, which was proposed to be a PMP−ketimine of kynurenine (*11*). However, the strong UV−visible absorption of L-kynurenine ($\lambda_{max} = 360$ nm, $\epsilon = 4500$ M^{-1} cm^{-1}) prevented observation in the region of the spectrum below 400 nm where the proposed ketimine intermediate would be expected to absorb. In the current work, we synthesized β-benzoyl-L-alanine, which is the desamino analogue of kynurenine, and we examined it as a substrate for kynureninase with steady-state and pre-steady-state kinetic methods. β-Benzoyl-L-alanine ($\lambda_{max} = 246$ nm) does not absorb beyond 300 nm, allowing observation of the spectrum of intermediates in kynureninase during the pre-steady-state of the reaction. The data show that an intermediate with $\lambda_{max} = 348$ nm is formed and also that the rate-determining step in the reaction of β-benzoyl-L-alanine is changed from deprotonation of the pyruvate−ketimine to the formation of the first product, benzoate.

EXPERIMENTAL PROCEDURES

Materials. α-Bromoacetophenone (**1**), diethyl acetamidomalonate (**2**), and NaH (60% dispersion in mineral oil) were obtained from Acros Organics and used as is. Carboxypeptidase A was obtained from Sigma as a crystalline suspension. Solvents and other reagents were obtained from Fisher Scientific.

Instruments. Synthesis products were analyzed by ^{1}H and ^{13}C NMR spectroscopy (Varian, 400 MHz) and mass spectrometry. Elemental analysis was performed by Atlantic Microlab, Inc., Norcross, GA. The steady-state kinetic measurements were performed on a Cary 1E UV/vis spectrophotometer equipped with a 6 × 6 thermo electric cell changer, controlled by a PC using Windows 2000 software provided by Varian Instruments. Stopped-flow kinetic measurements were performed on an OLIS (Bogart, GA) RSM-1000 rapid scanning spectrometer with a stopped-flow attachment, as previously described (*11*). The dead time of the stopped-flow mixer is <2 ms, and the observation path length is 10 mm. The monochromator has an exit slit of 0.6 mm, corresponding to a bandwidth of 4 nm. Typically, spectra consisting of 256 points were collected over the spectral range from 200 to 800 nm, at a rate of 1000 scans s^{-1}. Rapid chemical quench experiments were performed on a preparative quench apparatus from Kintek Instruments (Austin, TX).

Purification of Enzyme. Kynureninase was purified from *Escherichia coli* XL-1 Blue cells that were transformed with the pTZKYN plasmid containing the *kyn* gene cloned from *P. fluorescens* (*12*). A single colony of the XL-1 Blue cells containing the pTZKYN plasmid was inoculated into 1 L of liquid Luria broth medium containing 100 mg/L ampicillin and grown overnight at 37 °C with shaking at 225−250 rpm. The cells were collected by centrifugation at 5000*g* for 30 min. The enzyme was isolated by chromatography on phenyl-Sepharose as previously described (*12*). The concentration of the purified protein was determined using the value of $A_{280} = 1.4$ mg^{-1} mL^{-1} (*13*). The preparation of kynureninase used in these studies exhibited a specific activity of 19 units/mg and was >95% pure by PAGE. The enzyme contained 1.0 mol of PLP/mol of subunit, based on the absorbance of PLP at 390 nm in 0.1 M NaOH (*14*).

Synthesis of β-Benzoylalanine. (A) Ethyl 4-Phenyl-4-oxo-2-acetamido-2-ethoxycarbonylbutyrate (***3***). Sodium hydride (1.39 g, 34.9 mmol, 2.5 equiv) (60% in mineral oil) was suspended in dry DMF (14 mL). A solution of diethyl acetamidomalonate (**2**, 4.56 g, 21 mmol, 1.5 equiv) in dry DMF was added. The solution was stirred at 0 °C under a nitrogen atmosphere for 3 h until the anion had formed. A solution of α-bromoacetophenone (**1**, 13.9 mmol, 2.78 g) in dry DMF (10 mL) was added and the solution warmed to room temperature and stirred overnight under nitrogen. The mixture was poured into distilled water (100 mL), acidified to pH 3 with 1 M hydrochloric acid in an ice bath, and extracted into diethyl ether (4 × 70 mL). The ether extracts were dried over $MgSO_4$, and the solvent was removed under reduced pressure to give white crystals of ethyl 4-phenyl-4-oxo-2-acetamido-2-ethoxycarbonylbutyrate (**3**): yield 1.53 g (68%); ^{1}H NMR ($CDCl_3$) δ (ppm) 1.23−1.27 (t, 3H), 2.042 (s, 3H), 4.27 (s, 2H), 7.135 (s, 1H), 7.45−7.49 (t, 2H), 7.58−7.61 (m, 1H), 7.94−7.98 (m, 2H).

*(B) β-Benzoyl-DL-alanine (2-Amino-4-oxo-4-phenylbutyric Acid) Hydrochloride (****4****).* Ethyl 4-phenyl-4-oxo-2-acetamido-2-ethoxycarbonylbutyrate (**3**, 1.6 g, 4.7 mmol) was dissolved in 1,4-dioxane (50 mL) and 6 M hydrochloric acid (70 mL) added. The reaction was heated under reflux for 8 h until no starting material was visible by TLC (silica, petroleum ether: ethyl acetate; 1:1). The solution was then cooled and washed with ethyl acetate (50 mL). The aqueous phase was concentrated under reduced pressure to give a brown syrup, which was triturated with acetone to produce β-benzoyl-DL-alanine hydrochloride (**4**) as an off-white crystalline solid: yield 0.68 g (74%); mp 198−200 °C; ^{1}H NMR (D_2O + DCl) δ (ppm) 3.732−3.746 (d, 2H, $J = 5.715$ Hz), 4.34−4.365 (t, 1H), 7.396−7.431 (t, 2H), 7.55−7.587 (t, 1H), 7.839−7.855 (d, 2H, $J = 8.329$ Hz); MS (ESI) (M + H) 194. Anal. Calcd for $C_{10}H_{11}NO_3$·HCl: C, 52.29;, H, 5.266; N, 6.1. Found: C, 52.34; H, 5.22; N, 6.14.

(C) 4-Oxo-4-phenyl-2-(2,2,2-trifluoroacetylamino)butyric Acid (***5***). β-Benzoyl-DL-alanine (**4**, 280 mg, 1.5 mmol) was suspended in 2.0 mL of dry DMF, and 1.5 equiv of dry tetramethylguanidine and ethyl trifluoroacetate was added. After being allowed to stand overnight, the reaction mixture was diluted with 20 mL of water, and the pH was adjusted to 1.0 with careful addition of concentrated HCl. A white precipitate of 4-oxo-4-phenyl-2-(2,2,2-trifluoroacetylamino)-butyric acid (**5**) formed, which was extracted into ethyl acetate. Evaporation of the organic extract after drying over Na_2SO_4 gave 380 mg of 4-oxo-4-phenyl-2-(2,2,2-trifluoroacetylamino)butyric acid (90%): ^{1}H NMR ($CDCl_3$) δ (ppm) 3.72−3.76 (d, 2H, $J = 5.73$ Hz), 5.07−5.084 (t, 1H), 7.53−7.57 (t, 2H), 7.65−7.68 (t, 1H), 8.0309−8.052 (d, 2H, $J = 8.43$ Hz), 8.6 (s, 1H).

*(D) β-Benzoyl-L-alanine (****6****).* 4-Oxo-4-phenyl-2-(2,2,2-trifluoroacetylamino)butyric acid (**5**, 350 mg) was suspended in 10 mL of water, and the pH was adjusted to 7.5 by addition of triethylamine. A 100 μL portion of the carboxypeptidaseA suspension (53 units) was added. The reaction mixture was allowed to stand for a few hours. The reaction

progress was monitored by HPLC using a chiral hydroxyproline column (Serva) eluted with 0.2 mM $CuSO_4$. When the analysis showed that hydrolysis was 50% complete, it was then stopped by addition of a few drops of glacial acetic acid to bring the pH to 5.0. After filtration to remove precipitated protein, the filtrate was acidified to pH 1 and extracted with ethyl acetate to remove the unreacted *N*-trifluoroactetyl D-isomer (**7**). The aqueous layer was concentrated to dryness and redissolved in a minimum volume of water, and the pH was then adjusted to 4.8−5.0. The resultant β-benzoyl-L-alanine (**6**) was obtained as white crystals (129 mg, 89.7%) [$[\alpha]^{25}_D = +41.2$ [lit. +42.9 (*15*, *16*)], $c = 1.00$, 6 M HCl]. The isolated L-isomer was found by HPLC on the chiral column to be >98% homochiral. The organic extract was dried, filtered, and concentrated. The unreacted *N*-trifluoroacetyl D-isomer obtained from the extraction was suspended in 10 mL of water. Sodium hydroxide was then added (1.2 equiv, 8.4 mmol, 0.336 g), and the reaction was stirred for 18 h. The reaction was stopped by adjusting the pH to 4.6−5.0 with 4 N sulfuric acid. The β-benzoyl-D-alanine (**8**) which crystallized from the solution was filtered and dried to yield 94 mg (65.4%).

Steady-State Kinetic Measurements. Kynureninase activity was measured by following the decrease in absorbance at 360 nm due to consumption of L-kynurenine ($\Delta\epsilon = -4500$ M^{-1} cm^{-1}) (*17*) in 0.04 M potassium phosphate, pH 7.8, containing 40 μM PLP. Competitive inhibition was measured by variation of [L-kynurenine] at several fixed concentrations of inhibitor. K_m, V_{max}, and K_i values were determined by fitting of initial rate data to eqs 2 and 3 using the compiled

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

$$v = V_{max}[S]/\{K_m(1 + [I]/K_i) + [S]\} \quad (3)$$

FORTRAN programs HYPER and COMP of Cleland (*18*). The activity of β-benzoyl-L-alanine was measured at its absorption maximum, 246 nm ($\Delta\epsilon = -4500$ M^{-1} s^{-1}).

Pre-Steady-State Kinetics. The reactions were performed in 0.04 M potassium phosphate, pH 8, at 25 °C. The final concentration of kynureninase was 31 μM, and that of β-benzoyl-L-alanine was 0.5 mM. The data were fit to appropriate models (one, two, three, or four exponentials) using the Globalworks software provided by OLIS, Inc. (*19*). The goodness of fit was determined by examination of the residuals, the variance, and the validity of the calculated SVD spectra.

The chemical quench kinetic measurements were performed at room temperature (ca. 25 °C) by mixing 40 μL portions of enzyme (88.0 μM) and 40 μL portions of 1.0 mM β-benzoyl-L-alanine in 0.04 M potassium phosphate, pH 7.8. The reactions were quenched by mixing with an equal volume of 0.5 M $HClO_4$ in the quench syringe. Reaction times ranged from a minimum of 5 ms to 1 s. Benzoic acid in the solutions was determined by HPLC on a C18 reverse-phase column (Luna, Phenomenex, Inc.) using a gradient pump and diode array detector from Thermo Separations. The solvent was water/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, benzoic acid elutes 17.0 min after injection.

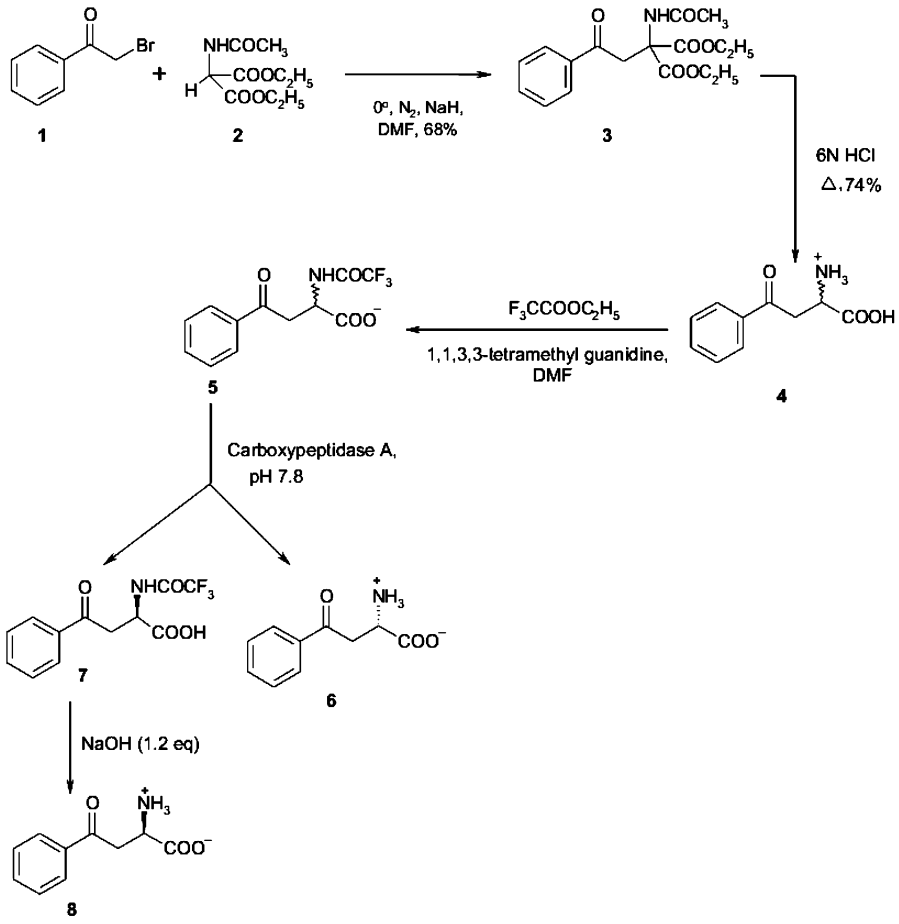
RESULTS

Synthesis of β-Benzoyl-L-alanine. The synthesis of this compound was reported previously from α-bromoacetophenone (**1**) and a malonic ester by Alerto et al. (*20*) using sodium ethoxide as the base in ethanol, but when we attempted to follow this procedure, we got an unacceptably low yield of alkylated product. We then employed the synthetic method Fitzgerald et al. (*9*) had used for the synthesis of bicyclic analogues of kynurenine, which uses NaH as the base in DMF solvent, and we obtained much better yield (68%) of ethyl 4-phenyl-4-oxo-2-acetamido-2-ethoxycarbonylbutyrate (**3**) (Scheme 1), as described in Experimental Procedures. Acid hydrolysis of **3** gave β-benzoyl-DL-alanine (**4**) in 74% isolated yield. A practical method for preparative enzymatic resolution of **4** was then developed using carboxypeptidase A digestion of the *N*-trifluoroacetyl derivative (**5**) (Scheme 1), providing β-benzoyl-L-alanine (**6**) and β-benzoyl-D-alanine (**8**) in good isolated yield and high enantiomeric purity, based on optical rotation and chiral HPLC. β-Benzoyl-L-alanine (**6**) has also been prepared via chiral alkylation of a diketopiperazine (*21*). After this work was completed, a procedure for preparation of **6** from L-aspartic acid anhydride and benzene by a Friedel−Crafts reaction appeared in a patent (*22*).

Steady-State Kinetics. β-Benzoyl-L-alanine was found to be a substrate of *P. fluorescens* kynureninase. The K_m for β-benzoyl-L-alanine was determined to be 8 μM, whereas the K_m for L-kynurenine is about 30 μM (*6*). The k_{cat} and k_{cat}/K_m values for β-benzoyl-L-alanine are 0.7 s^{-1} and 8.0×10^4 M^{-1} s^{-1}, respectively, at pH 7.8. These values can be compared to $k_{cat} = 16$ s^{-1} and $k_{cat}/K_m = 6.0 \times 10^5$ M^{-1} s^{-1} for L-kynurenine under the same conditions. β-Benzoyl-L-alanine, as expected for a substrate, is also a competitive inhibitor of the reaction of L-kynurenine with kynureninase, with a K_i value of 10 μM, comparable to K_m. We investigated the solvent isotope effects on the reaction of β-benzoyl-L-alanine in order to see if the rate-determining step was affected. In contrast to our previous results with L-kynurenine, which exhibits a large normal solvent isotope effect of 6.56 ± 0.59 on k_{cat} (*10*), we did not observe any significant solvent isotope effect on k_{cat} for β-benzoyl-L-alanine ($^Hk/^Dk = 0.96 \pm 0.06$). This result suggests that the rate-determining step is changed in the reaction of β-benzoyl-L-alanine. There is also a large inverse solvent isotope effect on k_{cat}/K_m ($^Hk/^Dk = 0.102 \pm 0.029$) for β-benzoyl-L-alanine, whereas L-kynurenine was found previously to exhibit a small inverse solvent isotope effect on k_{cat}/K_m ($^Hk/^Dk = 0.82 \pm 0.16$) (*10*).

Stopped-Flow Kinetic Experiments. The reaction of β-benzoyl-L-alanine with kynureninase was investigated by rapid scanning stopped-flow spectrophotometery (Figure 1A). Kynureninase exhibits a 423 nm absorption maximum in the absence of substrate, due to the bound PLP in an external aldimine (Figure 1A, dashed line). The stopped-flow spectra show the initial formation of a quinonoid intermediate, with an absorption maxima at about 490 nm, which is formed within the dead time (<2 ms) of the stopped-flow instrument. This is similar to our previous results obtained with L-kynurenine (*11*). The 490 nm intermediate then decays with $1/\tau_1 = 210 \pm 20$ s^{-1} to form an intermediate with λ_{max} at 348 nm (Figure 1B), with a good isosbestic point at 392 nm. This rate of decay is somewhat slower than the reaction

Scheme 1



of the quinonoid intermediate of L-kynurenine, which decays with a rate constant of 743 s^{-1} (*11*). There is a second slower phase in the reaction of β-benzoyl-L-alanine, which proceeds with an increase in absorbance at 348 nm and a decrease in absorbance at 420 nm, with $1/\tau_2 = 9 \pm 1$ s^{-1}. This second phase does not exhibit any significant absorbance change at 490 nm (Figure 1A). The amplitudes of both phases of formation of the 348 nm species are comparable, and this may reflect a half-site reactivity for kynureninase. The spectra in Figure 1A show that the 348 nm species is the major intermediate populated in the steady state, and there is no significant absorption at 490 or 420 nm, from quinonoid or external aldimine complexes, respectively. In constrast to previous studies with L-kynurenine (*11*), a quinonoid species which forms slowly and absorbs with low intensity at 520 nm is not formed. The time courses for the reaction of β-benzoyl-L-alanine at 490 and 348 nm are shown in Figure 1B. When the reaction shown in Figure 1 is performed with stoichiometric amounts of kynureninase and β-benzoyl-L-alanine, the 348 nm intermediate forms rapidly and decays slowly to return to the resting enzyme spectrum (data not shown).

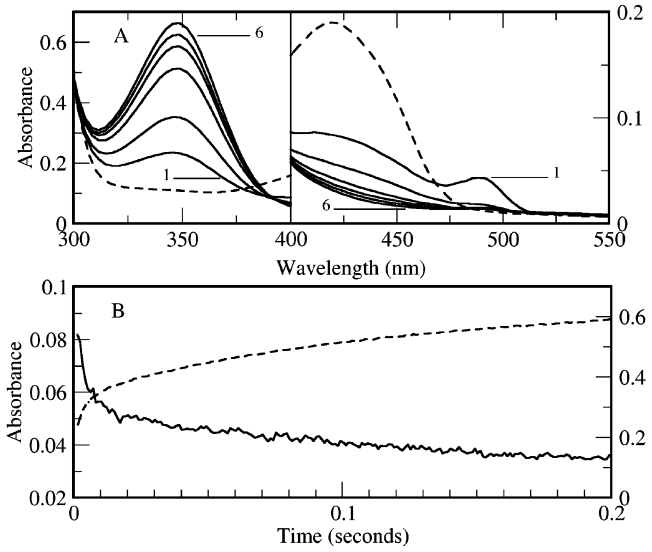


FIGURE 1: Rapid scanning stopped-flow spectra of the reaction of kynureninase (31 μM) with 0.5 mM β-benzoyl-L-alanine. (A) Selected spectra for the reaction of β-benzoyl-L-alanine collected during the first second of the reaction. The dashed line is the free enzyme spectrum. Curves 1−6 were collected at 0.002, 0.010, 0.090, 0.180, 0.270, and 0.650 s. (B) Time courses for the reaction at 348 nm (dashed line, right *y*-axis) and 490 nm (solid line, left *y*-axis).

We demonstrated in our previous work with L-kynurenine that benzaldehyde undergoes an aldol reaction with an enzyme-bound enamine intermediate to form a relatively stable quinonoid complex, with $\lambda_{max} = 496$ nm (*11*). The formation of the quinonoid species in the reaction of L-kynurenine with benzaldehyde occurs with a rate constant of 67 s^{-1}, which was shown to be comparable to the rate constant of anthranilate formation, 54 s^{-1}, found in the burst phase in chemical quench experiments (*11*). Thus, the reaction with benzaldehyde can be used as a convenient kinetic probe for the $C_\beta-C_\gamma$ carbon−carbon bond cleavage

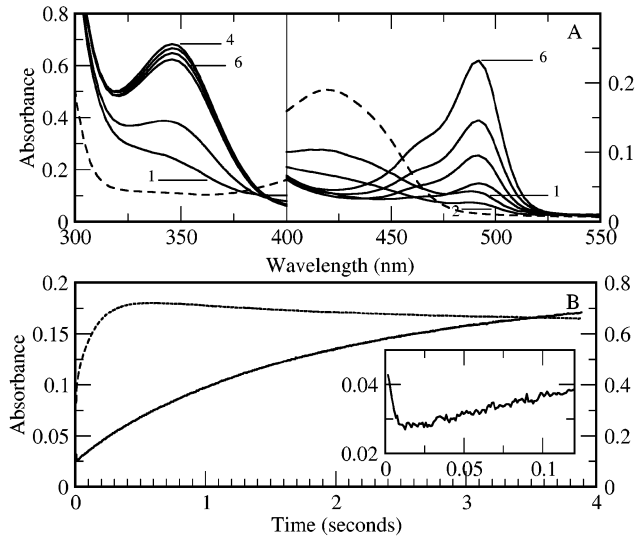


FIGURE 2: Rapid scanning stopped-flow spectra of the reaction of kynureninase (31 μM) with 0.5 mM β-benzoyl-L-alanine in the presence of 10 mM benzaldehyde. (A) Selected spectra for the reaction of β-benzoyl-L-alanine in the presence of 10 mM benzaldehyde. The dashed line is the free enzyme spectrum. Curves 1−6 were collected at 0.002, 0.013, 0.290, 1.00, 2.60, and 11.80 s. (B) Time courses for the reaction at 348 nm (dashed line, right *y*-axis) and 490 nm (solid line, left *y*-axis). Inset: Expansion of the first 0.1 s of the reaction at 490 nm.

step in the reaction. Similarly, when the reaction of β-benzoyl-L-alanine is performed in the presence of benzaldehyde, the initial 490 nm intermediate forms from β-benzoyl-L-alanine in the dead time of the stopped-flow instrument and decays rapidly to the 348 nm intermediate, and then it in turn decays slowly, with $1/\tau = 0.7$ s^{-1}, to form a second quinonoid complex with $\lambda_{max} = 492$ nm (Figure 2A). Time courses at 490 and 348 nm for the reaction of β-benzoyl-L-alanine in the presence of benzaldehyde are shown in Figure 2B. The inset in Figure 2B shows the rapid decay of the initially formed quinonoid intermediate. There is a very slow gradual buildup of the final quinonoid species over a period of more than 30 s, probably due to accumulation of the γ-phenylhomoserine aldol product (*23*), which is an inhibitor (*6*).

Chemical Quench Experiments. Rapid chemical quench experiments were performed to examine the pre-steady-state of the kynureninase reaction with β-benzoyl-L-alanine. The results of these experiments are presented in Figure 3. In contrast to the reaction of L-kynurenine, which shows a prominent "burst" of 0.7 mol of anthranilate/mol of kynureninase in the pre-steady-state (*10*), we do not observe any burst of benzoate formed in the pre-steady-state with β-benzoyl-L-alanine. The best fit of the data in Figure 3 is to a simple linear equation, and we were not able to obtain a satisfactory fit of the data to an exponential equation with either a burst or a lag. The slope of the line in Figure 3 is 36.1 μM s^{-1}, and the enzyme concentration after mixing was 44 μM, so the value of k_{cat} taken from this experiment is 0.82 s^{-1}, in good agreement with k_{cat} of 0.7 s^{-1} obtained in steady-state kinetic measurements.

DISCUSSION

Tanizawa and Soda studied a number of kynurenine analogues as substrates or inhibitors of kynureninase from

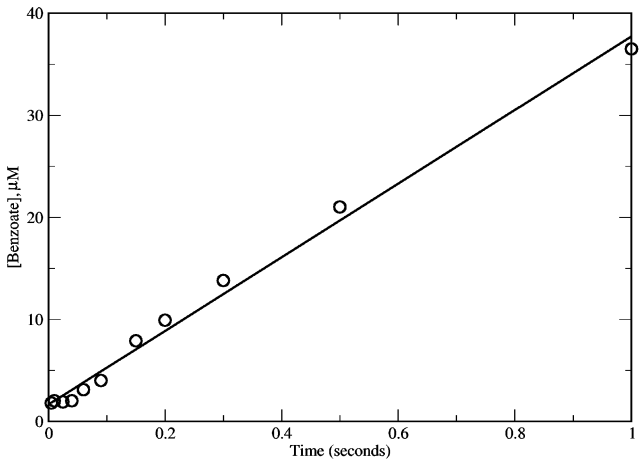
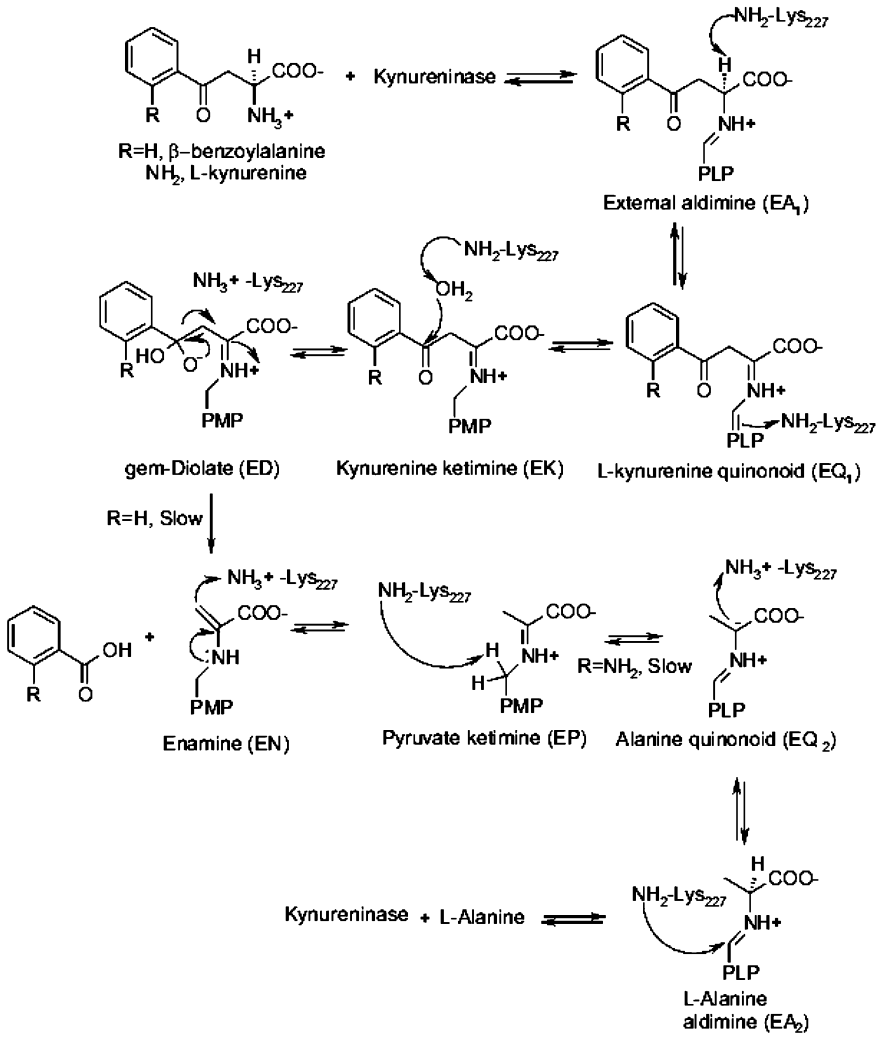


FIGURE 3: Chemical quench experiments with β-benzoyl-L-alanine (**6**). Reactions were performed for the time shown on the *x*-axis, quenched with 0.5 M $HClO_4$, and benzoate was measured by HPLC analysis. The line shown is a fit to a linear equation, $y = mx + b$, with $m = 36.1$ μM/s and $b = 1.64$ μM.

Pseudomonas marginalis (*24*). They reported that racemic β-benzoylalanine was a slow substrate for kynureninase. Our data also demonstrate that β-benzoyl-L-alanine is a substrate for kynureninase from *P. fluorescens*. In fact, the value of k_{cat}/K_m for β-benzoyl-L-alanine is only about 7-fold less than that for L-kynurenine. Thus, the presence of the aromatic amino group in the substrate is not necessary, either for binding or substrate activity, with kynureninase. This result is in contrast to our previous studies with *S*-phenyl-L-cysteine *S*,*S*-dioxide inhibitors, which display about a 100-fold increase in K_i when the aromatic amino group is lacking or is located at the 4-position instead of the 2-position (*7*). However, in contrast to L-kynurenine, for which the rate-determining step is deprotonation of the PMP−pyruvate ketimine complex prior to release of alanine (*10*), the present data show that the rate-determining step in the reaction of β-benzoyl-L-alanine with kynureninase is $C_\beta-C_\gamma$ bond cleavage to form benzoate. The results from quenching analysis (Figure 3), which show linear production, not a burst, of benzoate, support the proposition that the formation of benzoate is the rate-determining step. Furthermore, the steady-state k_{cat} value is 0.7 s^{-1}, which is identical with the rate constant for the reaction of benzaldehyde to form a quinonoid species in the stopped-flow experiments (Figure 2). Benzaldehyde was shown previously to undergo an aldol reaction with an enamine intermediate that is formed when the acyl carbon−carbon bond is cleaved (*6*, *23*). Hence, the reaction of substrates with kynureninase in the presence of benzaldehyde serves as a convenient kinetic probe for the rate of the $C_\beta-C_\gamma$ bond cleavage step. Although the presence of the amino group of L-kynurenine is not required for steady-state activity, there is apparently a 100-fold decrease in the microscopic rate constant of benzoate formation (0.7 s^{-1}) compared to anthranilate (67 s^{-1}) (*11*). Thus, the aromatic amino group of substrate makes an important contribution to the mechanism primarily in this step. This suggests that the aromatic amino group must interact with active site residues, probably by hydrogen bonding, to facilitate the hydrolysis. This interaction probably orients the aromatic ring and the carbonyl for optimal reaction in the hydration and retro-Claisen steps of the mechanism. Although the structure of *P. fluorescens* kynureninase has been recently determined

Scheme 2



(*25*), the residues which contact the substrate side chain are not yet known, since a structure of a complex with a ligand has not yet been obtained.

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} EP + Q \xrightarrow{k_{+3}} E + P \qquad (4)$$

$$k_{cat}/K_m = k_{+1}k_{+2}/(k_{-1} + k_{+2}) \qquad (5)$$

$$k_{cat} = k_{+2}k_{+3}/(k_{+2} + k_{+3}) \qquad (6)$$

The lack of a significant solvent isotope effect on k_{cat} with β-benzoyl-L-alanine is also consistent with a change in rate-determining step, since we showed in previous studies that the unusually large solvent isotope effect of about 6.6 observed with L-kynurenine arises from rate-determining deprotonation of C-4′ of the PMP−ketimine, which is exchanged when the reaction is performed with $[^2H]H_2O$ (*10*). For the minimal mechanism for kynureninase shown in eq 4, k_{cat} and k_{cat}/K_m are given by eqs 5 and 6, respectively. If k_{+2} is smaller than k_{+3}, then $k_{cat} = k_{+2}$, which is the apparent rate constant for formation of benzoate or anthranilate. For L-kynurenine, k_{+2}, the rate constant for anthranilate formation, is 67 s^{-1} (*11*). The apparent rate constant, k_{+3}, for release of the second product, L-alanine, should be the same for both L-kynurenine and β-benzoyl-L-alanine and should be approximately equal to k_{cat} for the reaction of kynurenine, 16 s^{-1}. Clearly, this is much faster than the value of k_{cat} for β-benzoyl-L-alanine, 0.7 s^{-1}, and this also supports our conclusion that benzoate formation is rate-determining. In contrast to L-kynurenine, there is a quite large inverse isotope effect on k_{cat}/K_m ($^Hk/^Dk = 0.1$) for the reaction of β-benzoyl-L-alanine. This may reflect larger contributions from desolvation in the binding of β-benzoyl-L-alanine.

The position of the absorption maximum of the newly detected intermediate at 348 nm (Figure 1) is significantly red shifted from the position expected for a PMP−ketimine, which is generally observed at about 330 nm in aminotransferases (*26*). This intermediate could not be directly observed in the previous pre-steady-state experiments with L-kynurenine as substrate (*11*), because of the strong absorption peaks of the substrate, L-kynurenine, at 360 nm ($\epsilon = 4500$ M^{-1} cm^{-1}) and the product, anthranilate, at 310 nm ($\epsilon = 2820$ M^{-1} cm^{-1}), which completely obscure the spectrum below 400 nm. However, a single turnover rapid scanning stopped-flow experiment with L-kynurenine did show a transient species absorbing at about 350 nm, as a weak shoulder (*11*). Furthermore, chemical quench experiments demonstrated that more than 30% of the PLP of kynureninase is converted to PMP within 5 ms after mixing with L-kynurenine (*11*), suggesting that a PMP complex is a significant component

of the steady state. Since β-benzoyl-L-alanine and the product, benzoate, do not exhibit significant absorption above 300 nm, we hoped that we could detect the previously proposed ketimine intermediate. With β-benzoyl-L-alanine as substrate, the 348 nm species is the major enzyme form present in the steady state, and there is no significant external aldimine or quinonoid intermediate present, since the steady-state spectra show no significant absorption peaks at 420 or 490 nm (Figure 1A). The only common PLP-derived species which is known to exhibit λ_{max} ~350 nm is the α-aminoacrylate Schiff base, as seen in tryptophan synthase (*27*) and tryptophan indole-lyase (*28*−*30*). However, an α-aminoacrylate Schiff base intermediate is not compatible with the chemistry of kynureninase, which catalyzes a reaction with electrophilic, rather than nucleophilic, substitution at the β-carbon. The intensity of the absorption of the 348 nm intermediate is surprising, since it exhibits an apparent extinction coefficient of about 2×10^4 M^{-1} cm^{-1}. PMP has a molar extinction coefficient of about 8×10^3 M^{-1} cm^{-1} at 325 nm (*14*), and PMP−ketimines seen in transaminases typically exhibit extinction coefficients which are similar in magnitude (*26*). Kynureninase exhibits a peak at 423 nm and apparent $\epsilon = 5800$ M^{-1} cm^{-1} (dashed line, Figure 1A) for the internal aldimine. There is a very weak band at about 340 nm in the absorption (dashed line, Figure 1A) or CD spectra (S. Lima and R. S. Phillips, unpublished results), indicating the presence of a minor enolimine tautomer. Furthermore, there are no absorbance changes in the region below 300 nm in the reaction with β-benzoyl-L-alanine that are not attributable to the substrate (data not shown). Normally, there is an aspartate side chain accepting a hydrogen bond from the pyridinium hydrogen of the PLP in enzymes of the aminotransferase family (*31*). In the structure of kynureninase, there is an additional conserved aspartate, Asp-132, as well as the expected Asp-201, which both can form hydrogen bonds to each other and to the PLP pyridinium hydrogen in kynureninase (*25*). The δ-oxygens of these two aspartates are only 2.5 Å apart (*25*), suggesting a possible low-barrier hydrogen bond (*32*). The tautomeric equilibrium between ketoenamine and enolimine forms of PLP−Schiff bases is known to be affected by the polarity of the environment (*26*). A polar environment favors the ketoenamine form, with λ_{max} at 423 nm, as is seen in the spectrum of wild-type kynureninase (dashed line, Figure 1A). The presence of the two aspartates may create a highly polar local environment for the PLP, which may be at least partially responsible for the unusual spectroscopic properties of this putative ketimine species. In support of this proposition, the mutation of Asp-132 to Ala in kynureninase results in an increase in the 340 nm band and a decrease in the 423 nm band in absorption and CD spectra (S. Lima and R. S. Phillips, unpublished results), showing a significant decrease in the polarity of the PLP environment.

The proposed mechanism of kynureninase reacting with L-kynurenine and β-benzoyl-L-alanine is shown in Scheme 2. The formation of external aldimine (EA_1) and quinonoid (EQ_1) intermediates is very fast for either L-kynurenine or β-benzoyl-L-alanine, as a quinonoid species is completely formed within the dead time (<2 ms) of the stopped-flow instrument for both compounds. The decay of the initial quinonoid complex to the ketimine form (EK), by proton transfer to C-4′, is somewhat slower for β-benzoyl-L-alanine (210 s^{-1}) compared to L-kynurenine (743 s^{-1}). General base-catalyzed hydration of the carbonyl of the ketimine gives the *gem*-diolate (ED), which undergoes a retro-Claisen reaction to give the first product, anthranilate or benzoate, and an alanine enamine (EN). The 348 nm intermediate observed in these studies can include contributions from EK, ED, and EN, all of which are PMP derivatives in the steady state. Finally, release of alanine takes place by β-protonation of the enamine, EN, to give the pyruvate ketimine (EP), which then undergoes deprotonation of C-4′ of the pyruvate ketimine, followed by protonation of the alanine quinonoid complex (EQ_2) at C_α to give the external aldimine of L-alanine (EA_2), which releases the product. At present, we cannot distinguish if *gem*-diolate (ED) formation or the retro-Claisen step to give the enamine (EN) is rate-determining in benzoate formation from β-benzoyl-L-alanine. The lack of an observed solvent kinetic isotope effect on k_{cat} favors rate-determining $C_\beta-C_\gamma$ bond cleavage, since slow formation of the *gem*-diolate would be expected to show a normal solvent kinetic isotope effect on ketone hydration. The analysis of substitutent effects on benzoate formation in a series of substituted β-benzoyl-L-alanines may provide some insight into this question. These experiments are now in progress.

REFERENCES

1. Soda, K., and Tanizawa, K. (1979) Kynureninases: Enzymological properties and regulation mechanism, *Adv. Enzymol. Relat. Areas Mol. Biol. 49*, 1−40.
2. Hayaishi, O., and Stanier, R. Y. (1951) The Kynureninase of *Pseduomonas fluorescens*, *J. Bacteriol. 62*, 691−709.
3. Achim, C. L., Heyes, M. P., and Wiley, C. A. (1993) Quantitation of human immunodeficiency virus, immune activation factors, and quinolinic acid in AIDS brains, *J. Clin. Invest. 91*, 2769−2775.
4. Sei, S., Saito, K., Stewart, S. K., Crowely, J. S., Brouwers, P., Kleiner, D. E., Katz, Katz, D. A., Pizzo, P. A., and Heyes, M. P. (1995) Increased human immunodeficiency virus (HIV) type 1 DNA content and quinolinic acid concentration in brain tissues from patients with HIV encephalopathy, *J. Infect. Dis. 172*, 638−647.
5. Stone, T. W. (2000) Inhibitors of the kynurenine pathway, *Eur. J. Med. Chem. 35*, 179−186.
6. Phillips, R. S., and Dua, R. K. (1991) Stereochemistry and mechanism of aldol reactions catalyzed by kynureninase, *J. Am. Chem. Soc. 113*, 7385−7388.
7. Dua, R. K., Taylor, E. W., and Phillips, R. S. (1993) S-Aryl-L-cysteine S,S-dioxides: design, synthesis, and evaluation of a new class of inhibitors of kynureninase, *J. Am. Chem. Soc. 115*, 1264−1270.
8. Heiss, C., Anderson, J., and Phillips, R. S. (2003) Differential effects of bromination on substrates and inhibitors of kynureninase from *Pseudomonas fluorescens*, *Org. Biomol. Chem. 1*, 288−295.
9. Fitzgerald, D. H., Muirhead, K. M., and Botting, N. P. (2001) A comparative study on the inhibition of human and bacterial kynureninase by novel bicyclic kynurenine analogues, *Bioorg. Med. Chem. 9*, 983−989.
10. Koushik, S. V., Moore, J. A., III, Sundararaju, B., and Phillips, R. S. (1998) 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, *Biochemistry 37*, 1376−1382.
11. Phillips, R. S., Sundararaju, B., and Koushik, S. V. (1998) The catalytic mechanism of kynureninase from *Pseudomonas fluorescens*: Evidence for transient quinonoid and ketimine intermediates from rapid-scanning stopped-flow spectrophotometry, *Biochemistry 37*, 8783−8789.
12. Koushik, S. V., Sundararaju, B., McGraw, R. A., and Phillips, R. S. (1997) Cloning, sequence, and expression of kynureninase from

Pseudomonas fluorescens, *Arch. Biochem. Biophys. 344*, 301−308.
13. Moriguchi, M., Yamamoto, T., and Soda, K. (1971) Crystalline kynureninase from *Pseudomonas marginalis*, *Biochem. Biophys. Res. Commun. 44*, 752−757.
14. Peterson, E. A., and Sober, H. A (1954) Preparation of crystalline phosphorylated derivatives of vitamin B_6, *J. Am. Chem. Soc. 76*, 169−175.
15. Bretschneider, T., Miltz, W., Munster, P., and Steglich, W. (1988) New syntheses of α-amino acids based on *n*-acylimino acetates, *Tetrahedron 44*, 5403−5414.
16. Kober, R., Papadopoulos, K., Miltz, W., Enders, D., and Steglich, W. (1985) Synthesis of diastereo- and enantiomerically pure α-amino-γ-oxo acid esters by reaction of acyliminoacetates with enamines derived from six-membered ketones, *Tetrahedron 41*, 1693−1701.
17. Kishore, G. M. (1984) Mechanism-based inactivation of bacterial kynureninase by β-substituted amino acids, *J. Biol. Chem. 259*, 10669−10674.
18. Cleland, W. W. (1979) Statistical analysis of enzyme kinetic data, *Methods Enzymol. 63*, 103−138.
19. Matheson, I. B. C. (1990) A critical comparison of least absolute deviation fitting (robust) and least-squares fitting: The importance of error distributions, *Comput. Chem. 14*, 49−57.
20. Varasi, M., Giordani, A., Speciale, C., Cini, M., and Bianchetti, A. (1995) Preparation of 2-amino-4-phenyl-4-oxobutyric acid derivatives with kynureninase and/or kynurenine-3-hydroxylase inhibiting acitvity, PCT Int. Appl. Pharmacia, S.p.A., Italy, 79 pp.
21. Papadopoulos, A., Lewall, B., Steckhan, E., Ginzel, K. D., Knoch, F., and Martin, N. (1991) Anodic oxidation of *N*-acyl and *N*-alkoxycarbonyl dipeptide esters as a key step for the formation of chiral heterocyclic synthetic building blocks, *Tetrahedron 47*, 563−572.
22. Kim, N. D., Moon, Y. H., Lee, K. I., Kim, K. E., Kim, C. K., Lee, G. S., and Chang, Y. K. (2003) Improved method of preparing 2(S)-amino-4-phenylbutyric acid, PCT Int. Appl., 14 pp.
23. Bild, G. S., and Morris, J. C. (1984) Detection of beta-carbanion formation during kynurenine hydrolysis catalyzed by *Pseudomonas marginalis* kynureninase, *Arch. Biochem. Biophys. 235*, 41−47.
24. Tanizawa, K., and Soda, K. (1979) The mechanism of kynurenine hydrolysis catalyzed by kynureninase, *J. Biochem. (Tokyo) 86*, 1199−209.
25. Momany, C., Levdikov, V., Blagova, L., and Phillips, R. S. (2004) The three-dimensional structure of kynureninase from *Pseudomonas fluorescens*, *Biochemistry* (in press).
26. Kallen, R. G., Korpela, T., Martell, A. E., Matsushima, Y., Metzler, C. M., Metzler, D. E., Morozov, Yu. V., Ralston, I. M., Savin, F. A., Torchinsky, Yu. M., and Ueno, H. (1985) Chemical and spectroscopic properties of pyridoxal and pyridoxamine phosphates, in *Transaminases* (Christen, P., and Metzler, D. E., Eds.) pp 37−108, Wiley-Interscience, New York.
27. Hur, O., Niks, D., Casino, P., and Dunn, M. F. (2002) Proton transfers in the β-reaction catalyzed by tryptophan synthase, *Biochemistry 41*, 9991−10001.
28. Phillips, R. S. (1991) Reaction of indole and analogues with amino acid complexes of *Escherichia coli* tryptophan indole-lyase: detection of a new reaction intermediate by rapid-scanning stopped-flow spectrophotometry, *Biochemistry 30*, 5927−5934.
29. Sloan, M. S., and Phillips, R. S. (1996) Effects of α-deuteration and of aza and thia analogues of L-tryptophan on formation of intermediates in the reaction of *Escherichia coli* tryptophan indole-lyase, *Biochemistry 35*, 16165−16173.
30. Phillips, R. S., Sundararju, B., and Faleev, N. G. (2000) Proton transfer and carbon−carbon bond cleavage in the elimination of indole catalyzed by *Escherichia coli* tryptophan indole-lyase, *J. Am. Chem. Soc. 122*, 1008−1014.
31. Mehta, P. K., Hale, T. I., and Christen, P. (1993) Aminotransferases: Demonstration of homology and division into evolutionary subgroups, *Eur. J. Biochem. 214*, 549−561.
32. Cleland, W. W., and Kreevoy, M. M. (1994) Low-barrier hydrogen bonds and enzymic catalysis, *Science 264*, 1887−1890.

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