Inhibition of Tyrosine Phenol-lyase from *Citrobacter freundii* by 2-Azatyrosine and 3-Azatyrosine†

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ABSTRACT: The interactions of 2-azatyrosine and 3-azatyrosine with tyrosine phenol-lyase (TPL) from *Citrobacter freundii* have been examined. 2-Aza-DL-tyrosine and 3-aza-DL-tyrosine were synthesized by standard methods of amino acid synthesis, while the L-isomers were prepared from 3-hydroxypyridine and 2-hydroxypyridine, respectively, with TPL (Watkins, E. B., and Phillips, R. S. (2001) *Bioorg. Med. Chem. Lett. 11*, 2099−2100). 3-Azatyrosine was examined as a potential transition state analogue inhibitor of TPL. Both compounds were found to be competitive inhibitors of TPL, with K_i values of 3.4 mM and 135 μM for 3- and 2-aza-L-tyrosine, respectively. Thus, 3-azatyrosine does not act as a transition state analogue, possibly due to the lack of tetrahedral geometry at C-1. However, 2-aza-L-tyrosine is the most potent competitive inhibitor of TPL found to date. The K_i value of 2-aza-L-tyrosine is half that of 2-aza-DL-tyrosine, indicating that the D-enantiomer is inactive as an inhibitor. Neither azatyrosine isomer was shown to be a substrate for β-elimination, based on coupled assays with lactate dehydrogenase and on HPLC measurements. Both isomers of azatyrosine form equilibrium mixtures of external aldimine and quinonoid intermediates when they bind to TPL. However, 2-azatyrosine reacts about 10-fold faster to form a quinonoid intermediate than does 3-azatyrosine. Since 2-azatyrosine is in the zwitterion or phenolate ion form at all the pH values examined, the strong binding of this compound suggests that L-tyrosine may be bound to the active site of TPL as the phenolate anion.

Tyrosine phenol-lyase (TPL, [EC 4.1.99.2])[1] is a pyridoxal-5′-phosphate (PLP)-dependent enzyme which catalyzes the β-elimination reaction of L-tyrosine to give phenol and ammonium pyruvate (eq 1) (*1*, *2*). TPL also catalyzes β-replacement reactions, as well as the racemization of alanine (*2*−*4*). TPL has been found in a number of different strains of bacteria, most of which belong to *Enterobacteriaceae*, specifically to the genera *Citrobacter* (*Escherichia*), *Proteus*, and *Erwinia* (*5*−*8*), in addition to two symbiotic, thermophilic bacterial species: *Symbiobacterium* sp. SC-1 and *Symbiobacterium thermophilum* (*9*, *10*).

$$V_{max} = V[S]/(K_m(1 + [I]/K_i) + [S])$$

The currently accepted mechanism for the β-elimination of phenol from L-tyrosine catalyzed by TPL is shown in Scheme 1. Tyrosine reacts initially to give an external aldimine (**I**), which is then deprotonated on the α-carbon to give a quinonoid intermediate (**II**). The phenol ring must then be tautomerized to a keto form (**III**) to make it a practical leaving group, and elimination can then proceed to give phenol and an aminoacrylate intermediate (**IV**), which then releases ammonium pyruvate. According to the transition-state analogue theory (*11*, *12*), compounds that resemble the transition state leading to the highest energy intermediate in the mechanism should bind strongly to TPL. The highest energy species in the proposed mechanism of TPL is the ketoquinonoid intermediate (**III**), which has lost the aromatic character in the phenol ring. Thus, in this study, we were interested in synthesizing compounds that were structurally similar to this intermediate. The key structural characteristic of this intermediate is the carbonyl rather than enol oxygen in the *para* position. With this feature in mind, we studied 3-azatyrosine (**1**), which exists predominantly in the keto tautomer, and 2-azatyrosine (**2**) (Scheme 2), which exists as a zwitterion or anion, to determine if they are substrates or inhibitors of tyrosine phenol-lyase. The results of these studies are reported herein.

MATERIAL AND METHODS

General. ^{1}H and ^{13}C NMR were obtained on Bruker AC 250 MHz or AC 300 MHz instruments. Enzyme assays and kinetic experiments were performed with a Varian Cary 1E UV/visible spectrophotometer. High-pressure liquid chromatography (HPLC) was carried out on an instrument with two Rainin Rabbit HP pumps and a LDC Milton Roy Spectro-monitor 3000 variable wavelength detector using Gilson Unipoint software.

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[1] Abbreviations: TPL, tyrosine phenol-lyase [EC 4.1.99.2]; SOPC, *S*-(*o*-nitrophenyl)-L-cysteine; PLP, pyridoxal-5′-phosphate.

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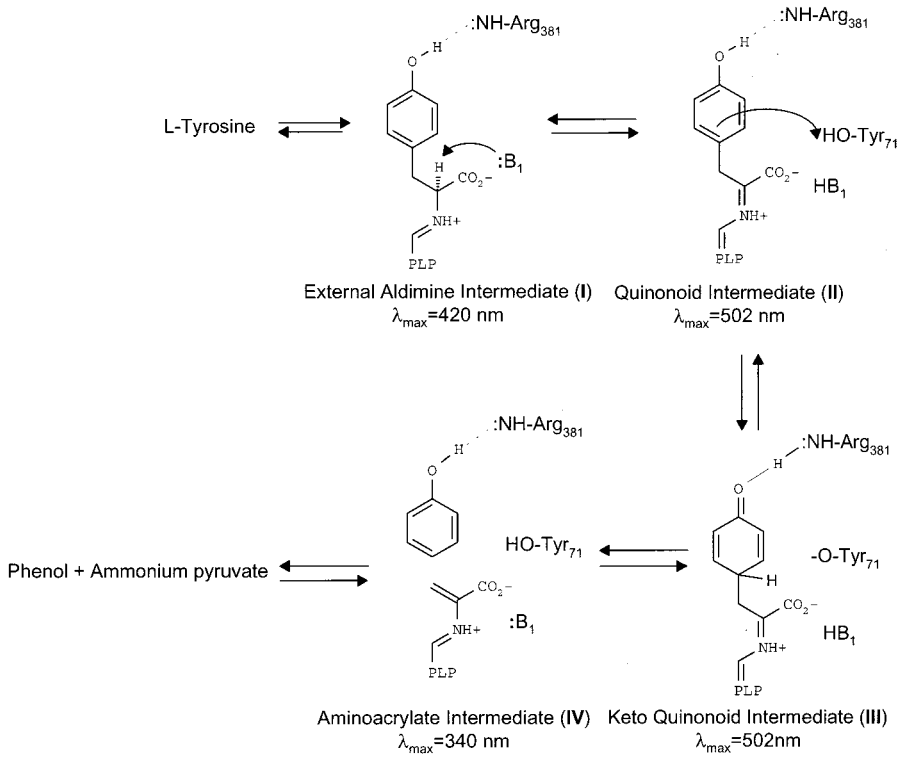
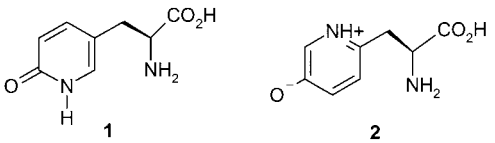
Scheme 1



Scheme 2



Lactate dehydrogenase from rabbit muscle, PLP, and NADH were purchased from United States Biochemical Co. *S*-(*o*-Nitrophenyl)-L-cysteine (SOPC) was prepared as described previously (*13*). Reagents used in the preparation of the tyrosine analogues were purchased from Aldrich Chemical Co. 2-Aza-L-tyrosine and 3-aza-L-tyrosine were prepared from 3-hydroxypyridine and 2-hydroxypyridine using TPL as described elsewhere (*14*).

Preparation of TPL. Single colonies of *Escherichia coli* SVS370/pTZTPL cells, grown on Luria broth/ampicillin agar plates, were removed and rinsed into 1 L of LB medium containing 1% (w/v) casein enzymic hydrolysate, 0.5% yeast extract, 0.5% NaCl, and 0.01% ampicillin. The cells were grown at 37 °C for 20 h with gentle agitation (150 rpm). The cells were then collected by centrifugation for 30 min at 10 000*g* and stored at −78 °C until used for enzyme purification. Enzyme purification was performed as previously described (*15*). The concentration of purified tyrosine phenol-lyase was determined from the absorbance at 278 nm (*A*1% = 8.37) (*16*) assuming a subunit mass of 51 kDa (*17*).

Enzyme Assays. The β-elimination reactions were measured using the coupled assay with lactate dehydrogenase and NADH, measured at 340 nm ($\Delta\epsilon = -6.22 \times 10^3$ M^{-1} cm^{-1}), as previously described by Morino and Snell (*18*) for tryptophan indole-lyase. Reaction mixtures contained, in a total volume of 0.6 mL, 50 mM potassium phosphate, pH 7.8, 5 mM 2-mercaptoethanol, 50 μM PLP, 0.2 mM NADH, 0.02 mg lactate dehydrogenase, and various concentrations of amino acid at 25 °C.

The inhibitory effects of 2-aza-DL-tyrosine and 3-aza-DL-tyrosine on TPL were determined by using varying amounts of SOPC as substrate and measuring the decrease in absorbance at 370 nm ($\Delta\epsilon = -1.86 \times 10^3$ M^{-1} cm^{-1}) at 25 °C in 50 mM potassium phosphate, pH 7.8, 5 mM 2-mercaptoethanol, and 50 μM PLP at various concentrations of azatyrosine. The reactions were initiated by the addition of TPL. A unit of TPL activity is defined as the amount of enzyme that produces 1 μmol of product/min.

Stopped-Flow Reactions. Prior to performing the rapid kinetics experiments, the stock enzyme was incubated with 1 mM PLP for 1 h at 30 °C and then separated from excess PLP on a short desalting column (PD-10, Pharmacia) equilibrated with 50 mM potassium phosphate, pH 8.0. Rapid-scanning stopped-flow kinetic data were obtained with an RSM-1000 instrument from OLIS, Inc. For absorbance measurements, the observation cell has a 10 mm path length, and a 150 W Xe lamp was used as the light source, with a 0.2 mm scanning disk and 0.6 mm exit slit on the monochromator. This instrument has a dead time of less than 2 ms and is capable of collecting either absorbance or fluorescence emission spectra in the visible region from 300 to 600 nm at 1 kHz. Typically, spectra were collected for 1−2 s after flow stop. The rate constants for quinonoid intermediate formation were then evaluated by global fitting of the spectra using the program provided by OLIS (*19*). The validity of the fitting was evaluated by inspection of the residuals and standard deviations.

Data Analysis. Steady-state data were analyzed using the FORTRAN programs of Cleland (*20*) adapted to run on IBM-compatible computers. Data for linear competitive inhibition were fit to eq 2 using the COMPO program. In eq 2, [S] is the substrate concentration, [I] is the inhibitor concentration, K_m is the Michaelis constant, and K_i is the inhibition constant. For pre-steady-state kinetic studies, the effects of concentra-

tion on rate constants for formation of quinonoid and aldimine intermediates were evaluated by fitting to eq 3, where k_f is the rate constant for the formation of the quinonoid intermediate, k_r is the rate constant for reprotonation of the quinonoid intermediate, K_d is the binding constant for the amino acid, and [L] is the concentration of the amino acid.

$$\log V = V[S]/(K + [S]) \quad (2)$$

$$k_{obs} = k_f[L]/(K_d + [L]) + k_r \quad (3)$$

*Synthesis of Aza-DL-tyrosines. Ethyl 2-Acetamido-2-(2′-fluoro-5′-pyridylmethyl)malonate (**3**).* To a solution of diethyl acetamidomalonate (13.6 g, 62.6 mmol) in ethyl alcohol (200 mL) containing sodium (1.4 g, 62.6 mmol) was added 2-fluoro-5-bromomethylpyridine (*21*) (11.9 g, 62.5 mmol). The mixture was refluxed overnight until the pH of an aliquot dissolved in distilled water had decreased to 5−6. The salt was removed by filtration, and the liquid was taken to dryness in vacuo. The product was taken up in ether. The ether was removed by vacuum, and the product was recrystallized from hot water (14.6 g, 45 mmol, 72%), mp 125−127 °C. 1H NMR ($CDCl_3$): δ 7.87 (d, J = 2.0 Hz, 1H), 7.44 (dt, J = 2.7, 8.2 Hz, 1H), 6.86 (dd, J = 2.9, 8.2 Hz, 1H), 6.59 (bs, 1H), 4.35 (m, 4H), 3.66 (s, 2H), 2.04 (s, 3H), 1.28 (t, J = 7.0 Hz, 6H).

*3-Aza-DL-tyrosine (**1**).* Ethyl 2-acetamido-2-(2′-fluoro-5′-pyridylmethyl)malonate (1.3 g, 4 mmol) and aqueous HCl (6 N, 20 mL) were added to a round-bottom flask. The solution was refluxed for 12 h, the solvent was removed by rotary evaporation, and the residue was dissolved in a small amount of water. Sodium bicarbonate solution (saturated) was added to neutralize the acid, and acetone was added to precipitate the amino acid (*22*) (0.51 g, 71%), mp 220−221 °C (dec). 1H NMR (D_2O/NaOD): δ 7.65 (dd, J = 2.4, 9.0 Hz, 1H), 7.42 (d, J = 2.4 Hz, 1H), 6.63 (d, J = 9.0 Hz, 1H), 3.89 (t, J = 6.5 Hz, 1H), 3.01 (t, J = 6.5 Hz, 2H). MS (ESI) m/z: 183 (MH^+).

*2-(2′,2′-Biscarboethoxy-2′-acetamidoethyl)-5-benzenesulfonyloxypyridine (**4**).* A 100 mL three-neck flask was flame-dried under nitrogen with an attached condenser and a calcium chloride drying tube. Dry ethanol (5 mL) was added to the flask, and sodium (67 mg, 2.9 mmol) was added to make sodium ethoxide. Diethyl acetamidomalonate (585 mg, 2.7 mmol) was added with stirring to the solution of sodium ethoxide. Once all of the material had dissolved, 2-iodomethyl-5-benzenesulfonyloxy pyridine (*23*) (1.1 g, 2.9 mmol) was added, and the solution was allowed to stir at room temperature. The reaction was monitored by thin-layer chromatography (TLC) (silica gel, 4:1 CH_2Cl_2:hexanes) every 15 min until no starting material remained (1.5 h). Afterward, the solution was diluted with water and neutralized (pH 6.5−7.5) with glacial acetic acid. The ethyl alcohol was removed, and the remaining aqueous solution was extracted with ethyl acetate (30 mL × 2). The ethyl acetate layer was dried with anhydrous sodium sulfate and concentrated in vacuo. The residue was purified on silica gel (10:1 CH_2Cl_2:ethyl acetate) to give the desired product (730 mg, 1.6 mmol, 54%). 1H NMR ($CDCl_3$): δ 8.04 (d, J = 2.7 Hz, 1H), 7.82 (d, J = 7.4 Hz, 2H), 7.71 (t, J = 7.8 Hz, 1H), 7.56 (t, 7.8 Hz, 2H), 7.27 (dd, J = 3.2, 8.7 Hz, 1H), 7.04 (d, J = 8.5 Hz, 1H), 6.68 (s, 1H), 4.22 (m, 4H), 3.82 (s, 2H), 1.96 (s, 3H), 1.23 (m, 6H).

*N-Acetyl-DL-2-azatyrosine (**5**).* To a 50 mL flask fitted with a reflux condenser was added 2-(2′,2′-biscarboethoxy-2′-acetamidoethyl)-5-benzenesulfonyloxypyridine (2.8 g, 6 mmol) followed by 10 mL of water and 12 mL of dioxane. Sodium hydroxide (780 mg, 19.5 mmol) was added, and the solution was refluxed until no starting material was seen on TLC (reverse phase, C18, 70:30 water:methanol). The solvent was removed by rotary evaporation, and the residue was dissolved in a small amount of water. Acetic acid was added to neutralize the solution, and it was loaded onto a Dowex 50W-X8 ion exchange column (H^+ form). The column was washed with water, and the product was eluted with 0.75 M aqueous ammonia. The solvent was removed by rotary evaporation to yield a tan oil. Water was added and removed by rotary evaporation twice more. Absolute ethanol was added to the oil and removed by vacuum to yield the product as a tan solid (1.2 g, 5 mmol, 84%). 1H NMR (D_2O): δ 7.95 (s, 1H), 7.52 (d, J = 8.6 Hz, 1H), 7.40 (d, J = 8.7 Hz, 1H), 4.47 (dd, J = 5.0, 14.4 Hz, 1H), 3.30 (dd, J = 5.0, 14.4 Hz, 1H), 3.03 (dd, J = 5.0, 14.4 Hz, 1H), 1.87 (s, 3H).

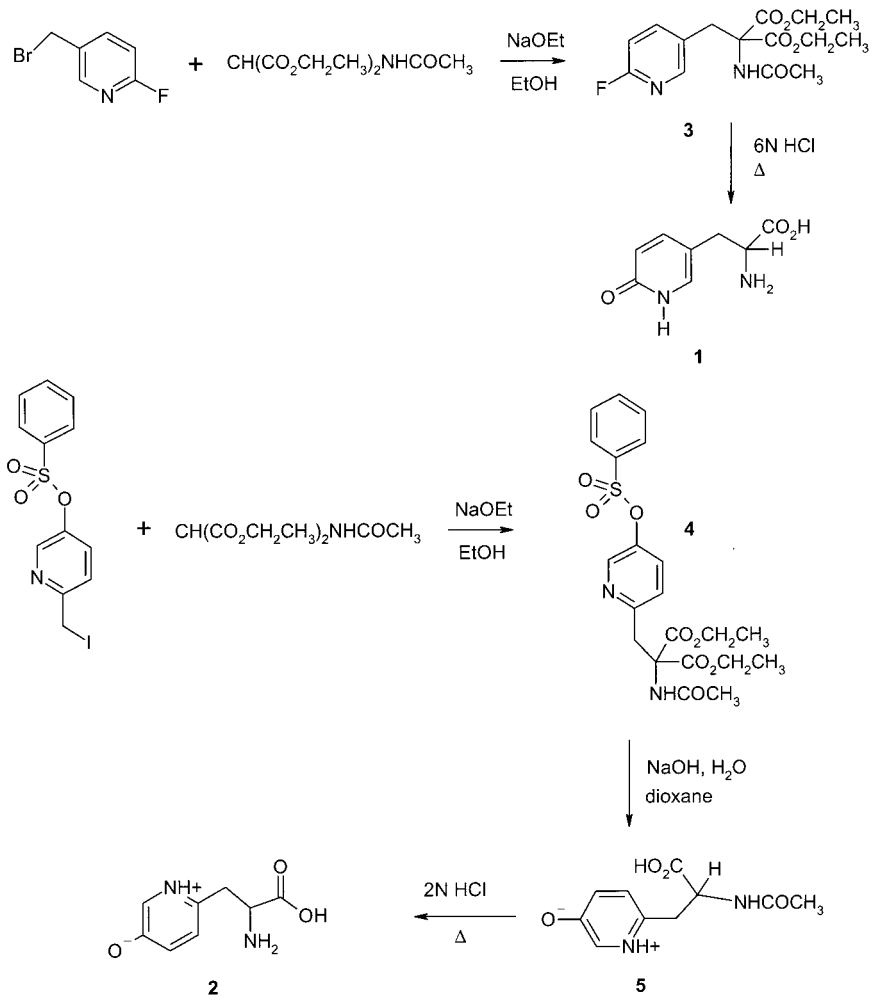
*2-Aza-DL-tyrosine (**2**).* *N*-acetyl-2-aza-DL-tyrosine (350 mg, 1.6 mmol) was added to 2 N HCl (20 mL), and the solution was refluxed overnight. The solution was then evaporated, and the residue was dissolved in a small amount of water. The pH was adjusted to 5.5 with aqueous ammonia, and the amino acid crystallized. The amino acid (**2**) (220 mg, 1.2 mmol, 78%) was filtered and washed with ethanol and ether, mp 247−253 °C (dec). 1H NMR (D_2O): δ 7.69 (bs, 1H), 6.99 (d, J = 8.8 Hz, 1H), 6.89 (dd, J = 2.0, 8.8 Hz, 1H), 3.42 (dd, J = 3.4, 8.6 Hz, 1H), 2.89 (dd, J = 5.4, 13.3 Hz, 1H), 2.63 (dd, J = 8.6, 13.3 Hz, 1H). MS (ESI) m/z: 183 (MH^+).

RESULTS

Chemistry. The racemic azatyrosines were synthesized by coupling the necessary halomethylpyridine with the anion of diethyl acetamidomalonate followed by hydrolysis (Scheme 3). S_N2 displacement of the bromide from 2-fluoro-5-bromomethylpyridine using the anion generated from diethyl acetamidomalonate (*21*) resulted in the desired substituted malonate (**3**) (72%). Treatment of product **3** with refluxing 6 N HCl led to simultaneous saponification, decarboxylation, deprotection, and hydrolysis. Neutralization of the reaction mixture and workup resulted in the target compound, 3-aza-DL-tyrosine (**1**), in 71% yield for two steps (*21*).

2-Aza-DL-tyrosine was synthesized in a similar manner. 5-(Benzenesulfonyloxy)-2-iodomethyl pyridine (*23*), when treated with diethylacetamidomalonate in sodium ethoxide under reflux, afforded a complex mixture of products. We found that the desired malonate (**4**) was produced in moderate yield (54%) by treating 5-(benzenesulfonyloxy)-2-iodomethyl pyridine with diethyl acetamidomalonate in sodium ethoxide at room temperature. The *N*-acetyl-DL-2-azatyrosine (**5**) was obtained in 84% yield by refluxing the malonate ester with NaOH in dioxane/H_2O. Finally, the target molecule, 2-aza-DL-tyrosine (**2**), was formed in 78% yield by refluxing the acetyl amino acid in 2 N HCl overnight. We were unable to resolve the acetyl aza-DL-tyrosines using either pig kidney or fungal acylase (*24*). We subsequently found that TPL

Scheme 3



could be used to prepare both aza-L-tyrosines, albeit in low yield, from the corresponding hydroxypyridines and ammonium pyruvate (*14*).

Steady-State Kinetics. 3-Aza-DL-tyrosine was found to act as a competitive inhibitor of TPL, although it is rather weak, since the K_i value was determined to be 3.4 mM.[2] Surprisingly, 3-aza-DL-tyrosine was not detectable as a substrate under the conditions employed in these studies. Using the coupled assay to detect pyruvate formation with lactate dehydrogenase and NADH, no change in the rate of absorbance decrease at 340 nm above the blank rate was detected, even at high concentrations of TPL (ca. 1 mg/mL). To further verify this, 3-aza-DL-tyrosine was incubated with TPL for 24 h. An aliquot was analyzed by HPLC (reverse phase, C18, 5% MeOH/95% aqueous TFA (0.1%)), but no additional peak with the retention time of 2-hydroxypyridine was detected.

2-Aza-DL-tyrosine was found to be a more potent competitive inhibitor of TPL than was 3-aza-DL-tyrosine, with a K_i of 135 μM. 2-Aza-DL-tyrosine also was not found to be a substrate under the conditions employed in this study. Using the coupled assay for pyruvate formation with lactate dehydrogenase and NADH, no decrease in absorbance at 340 nm above the blank rate was seen, even at high concentrations of TPL (ca. 1 mg/mL). To further verify this, 2-aza-DL-tyrosine was incubated with TPL for 24 h. An aliquot was analyzed by HPLC (reverse phase, C18, 5% MeOH/95% aqueous TFA (0.1%)), but no additional peak with the retention time of 3-hydroxypyridine was detected.

The inhibitory effect of 2-aza-L-tyrosine, prepared from ammonium pyruvate and 3-hydroxypyridine using TPL (*14*), was determined to compare with the results for 2-aza-DL-tyrosine. The K_i for 2-aza-L-tyrosine at pH 7.69 was determined to be 0.53 ± 0.16 mM, while 2-aza-DL-tyrosine gave a corrected K_i value of 0.48 ± 0.14 mM. These results confirm our expectation that the D-enantiomers of azatyrosines do not significantly inhibit TPL.

Stopped-Flow Kinetic Analysis. We did not examine the reaction of the racemic azatyrosines by stopped-flow spectrophotometry, due in part to their limited solubility. However, 2-aza-L-tyrosine and 3-aza-L-tyrosine are considerably more soluble, and hence their reactions were readily examined by rapid-scanning stopped-flow spectrophotometry. The fitted absorbance spectra for the reaction of 5 mM 2-aza-L-tyrosine with tyrosine phenol-lyase are presented in Figure 1A. The reaction displays an absorbance peak at 410 nm in the initial spectrum (solid line) that decreases in the first phase (dashed line), followed by a subsequent second phase with an absorbance increase at 504 nm (dashed and dotted line). The 504 nm absorbance peak (corresponding to the quinonoid intermediate) reaches a maximum within 400 ms

[2] All K_i values for racemates were corrected by dividing by 2, assuming that the D-amino acids are inactive as inhibitors.

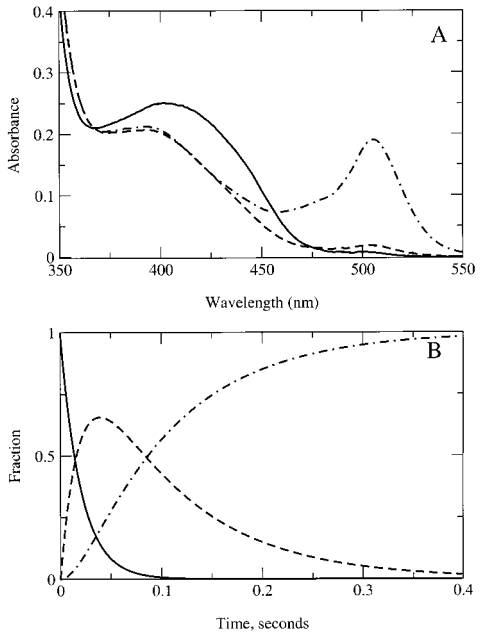


FIGURE 1: (A) Rapid-scanning stopped-flow spectra for the reaction of TPL with 2 mM 2-aza-L-tyrosine in 50 mM potassium phosphate, pH 8.0, at 25 °C. Fitted spectra, obtained by global analysis of the data, are shown. Solid line, initial spectrum; dashed line, spectrum after first exponential phase; dashed and dotted line, spectrum after second exponential phase. (B) Time courses for reaction of 2-aza-L-tyrosine with TPL.

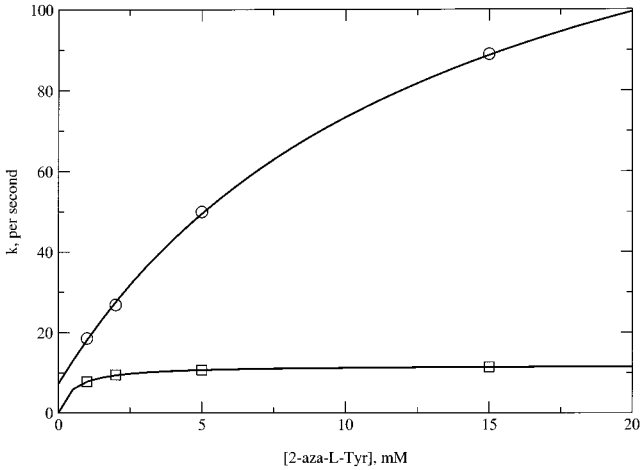


FIGURE 2: Fitting of the observed rate constants for the reaction of 2-aza-L-tyrosine to eq 4. Circles, fast phase; squares, slow phase. The lines are the calculated curves for eq 4 using the parameters given in Table 1.

and then is stable. The calculated time courses for the different spectroscopic species are shown in Figure 1B. The apparent rate constants at each concentration of 2-aza-L-tyrosine were determined by global fitting of the spectra. The rate constants, k_f and k_r, and the binding constant (K_d), for both phases, were then determined by fitting the data at varying concentrations of 2-aza-L-tyrosine to eq 3 (Figure 2), and the results are given in Table 1. Based on the parameters given in Table 1 for the slow phase of quinonoid intermediate formation, the estimated K_i for 2-aza-L-tyrosine is 107 μM, in good agreement with the measured value of 135 μM from steady-state kinetics.

The absorption spectra for the reaction of 3-aza-L-tyrosine with TPL are shown in Figure 3A. The global analysis of

Table 1: Pre-Steady-State Kinetic Data for the Reaction of Azatyrosines with TPL

substrate	K_d (mM)	k_f (s^{-1})	k_r (s^{-1})
2-aza-L-tyrosine (slow phase)	0.42	10.5	1.68
2-aza-L-tyrosine (fast phase)	13.9	147.5	7.45

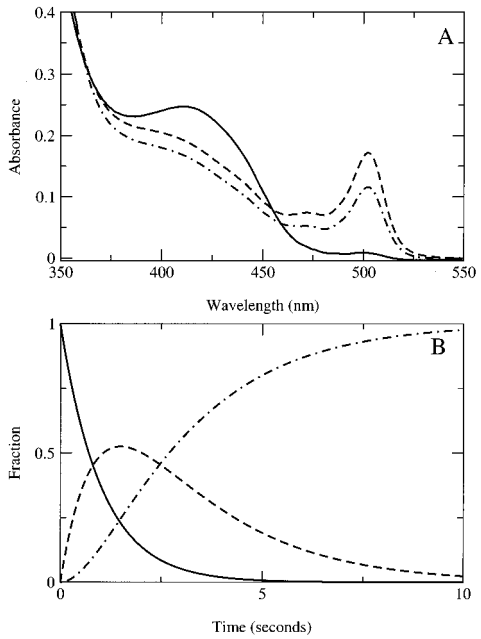


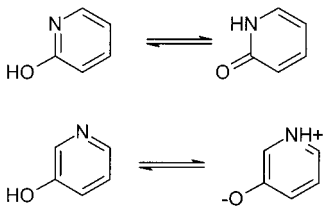
FIGURE 3: (A) Rapid-scanning stopped-flow spectra for the reaction of TPL with 20 mM 3-aza-L-tyrosine in 50 mM potassium phosphate, pH 8.0, at 25 °C. Fitted spectra, obtained by global analysis of the data, are shown. Solid line, initial spectrum; dashed line, spectrum after first exponential phase; dashed and dotted line, spectrum after second exponential phase. (B) Time courses for reaction of 3-aza-L-tyrosine with TPL.

the spectra for the reaction of 3-azatyrosine with TPL demonstrates that the reaction has slow and fast phases. The initial spectrum (solid line) shows an absorbance peak at 410 nm that decreases slowly with time and forms an absorbance peak at 504 nm (dashed line). In a subsequent step, the absorbance at 504 nm decreases slightly (dashed and dotted line). The time courses for the changes in spectra are shown in Figure 3B. There is relatively little change, less than 2-fold, in the rate constants for these phases with concentration changes of 50-fold. At 20 mM 3-aza-L-tyrosine, the observed rate constants are $1/t_1 = 1.08\ s^{-1}$ and $1/t_2 = 0.41\ s^{-1}$.

DISCUSSION

The purpose of this study was to develop novel inhibitors of tyrosine phenol-lyase that could be used to gain additional insights into the binding of substrates and reaction intermediates to the active site. We have synthesized both 3-aza-DL-tyrosine (**1**) and 2-DL-azatyrosine (**2**) to achieve this goal. These compounds are analogues of the keto and phenolate forms, respectively, of L-tyrosine and thus would be expected to provide information on the structural state of the bound tyrosine in the reaction mechanism. 3-Aza-DL-tyrosine (**1**), which contains a 2-hydroxypyridine ring, was found to inhibit TPL rather weakly, with a K_i of 3.4 mM. In aqueous solution, 2-hydroxypyridine has been shown to exist primarily in the keto tautomer (Scheme 4). The keto tautomer also predominates in nonpolar solvents (*25*). Therefore, 3-azatyrosine

Scheme 4



would be expected to bind to the active site of TPL in the keto form as well. The weak binding of 3-azatyrosine to TPL was surprising, since the reaction mechanism predicts that a ketoquinonoid intermediate should form (structure **III** in Scheme 1). Thus, we expected that 3-azatyrosine would be a transition state analogue of TPL and would be a strong competitive inhibitor. However, the binding constant of 3-azatyrosine is similar to that of L-Phe, which is 2 mM (*26*), suggesting that the carbonyl group in the *para* position does not participate in any enhanced binding interactions with the active site, which would be expected for a transition state analogue. Possibly, the planar geometry of 3-azatyrosine precludes the proper orientation for strong interaction of the carbonyl with the active site. The postulated ketoquinonoid intermediate would have tetrahedral geometry at C_1 of the cyclohexadienone ring (Scheme 1). In previous work with tryptophan indole-lyase, we found that oxindolyl-L-alanine and 2,3-dihydro-L-tryptophan, which have tetrahedral geometry at C-3 of the heterocyclic ring, are potent transition state analogue inhibitors (*27*, *28*).

Inhibition by 2-azatyrosine (**2**) was found to be more potent than 3-azatyrosine (**1**), with a K_i of 135 μM. This K_i value is lower than any other TPL inhibitor previously investigated, and is similar to the K_m of L-Tyr for TPL, which is about 200 μM (*1*). The reaction of L-tyrosine was shown previously to require two basic groups with an average pK_a of 7.8 (*1*). One of the pK_a values, 7.6, is likely to be that of the base which abstracts the α-proton, while the other, with a pK_a of 8.2, is likely to be from a group which interacts with the substrate phenol (*1*). Previous crystallographic and mutagenesis results suggested that Arg-381 may be the base with pK_a of 8.2 (*29*). 3-Hydroxypyridine is known to exist primarily in the zwitterionic form at neutral pH (Scheme 4) (*30*). Therefore, 2-azatyrosine would be expected to bind to TPL in this form. This would likely interact with the protonated form of Arg-381 to form an ion pair, similar to the interaction of tyrosine with Arg-381 shown in Scheme 1. This result suggests that in the reaction of L-tyrosine, proton transfer may take place from the phenolic OH of substrate to Arg-381 prior to or concomitant with the formation of the quinonoid intermediate. The tyrosine phenolate would be more easily protonated at C-1 to allow the formation of the ketoquinonoid intermediate and carbon−carbon bond cleavage to proceed (Scheme 1). However, the low, but measurable, activity of the R381A mutant TPL that we observed previously suggested that formation of the phenolate anion is not an absolute requirement for the mechanism, although the reaction efficiency is reduced by about 4 orders of magnitude (*28*).

Rapid-scanning stopped-flow analysis of the reactions of TPL with 2-aza-L-tyrosine and 3-aza-L-tyrosine demonstrates that both compounds bind to form equilibrating mixtures of external aldimine intermediates (broad peaks at 400−410 nm) and quinonoid intermediates (peaks at 504 nm in Figures 1A and 3A). The reaction of 2-azatyrosine was found to be biphasic, with a slow phase and a fast phase, as was previously observed with L-tyrosine (*31*). The two phases of quinonoid intermediate formation observed with L-tyrosine are believed to be due to the reaction of two different conformations of TPL which differ in monovalent cation induced activation state (*31*). In contrast to the reaction of L-tyrosine, the observable fast phase of the reaction of 2-aza-L-tyrosine is the formation of the external aldimine (Figure 1A). This step is too fast to be observed in the reaction of L-tyrosine, since it appears to be complete within the dead time of the stopped-flow instrument. Thus, the rate constant for external aldimine formation is at least 6−8-fold slower for 2-azatyrosine, perhaps because of the charged pyridine ring of the substrate analogue. The rate constant for quinonoid intermediate formation for 2-aza-L-tyrosine is about 10 times faster ($k = 10.5\ s^{-1}$) than 3-aza-L-tyrosine ($k = 1\ s^{-1}$), but still much slower than L-tyrosine (80 s^{-1}) (*31*). This is consistent with the greater inhibition seen with 2-azatyrosine. The rate constant for quinonoid intermediate formation seen with 2-azatyrosine is much faster than the slow phase previously seen with L-tyrosine (1−2 s^{-1}), so it is likely to be the deprotonation reaction of the fully activated enzyme. 3-Aza-L-tyrosine reacts also in a biphasic manner, but much slower than 2-aza-L-tyrosine. The first phase shows an increasing absorbance at 504 nm (Figure 2A). Thus, external aldimine formation appears to be faster for 3-aza-L-tyrosine than for 2-aza-L-tyrosine. The second phase of the reaction with 3-aza-L-tyrosine shows a small absorbance decrease at 504 nm. This may be due to a slow conformational change. In contrast to 2-aza-L-tyrosine, there is little change in the observed rate constants for either phase with concentration.

We have found that under the conditions employed in this study, both 2-azatyrosine and 3-azatyrosine fail to act as detectable substrates of tyrosine phenol-lyase for β-elimination. This may be due to the electron-deficient character of the hydroxypyridines, making them less likely to become protonated on an aromatic carbon, which is necessary for β-elimination to take place. Since we found that both 2-aza and 3-azatyrosine can be synthesized by TPL from 3-hydroxypyridine and 2-hydroxypyridine, respectively (*14*), this result is surprising. Thus, it is likely that the β-elimination of azatyrosines takes place, but at a rate too slow to detect under the conditions of our assays. The LDH-coupled assay would detect activity to 10^{-4} that of tyrosine, while the HPLC assay should detect a rate of about 10^{-5} that of tyrosine.

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