The Crystal Structure of *Citrobacter freundii* Tyrosine Phenol-lyase Complexed with 3-(4′-Hydroxyphenyl)propionic Acid, Together with Site-Directed Mutagenesis and Kinetic Analysis, Demonstrates That Arginine 381 Is Required for Substrate Specificity[†,‡]

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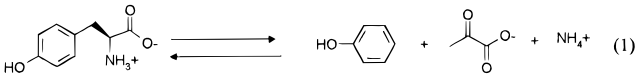
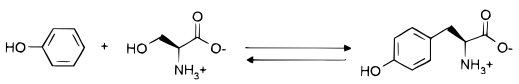
ABSTRACT: The X-ray structure of tyrosine phenol-lyase (TPL) complexed with a substrate analog, 3-(4′-hydroxyphenyl)propionic acid, shows that Arg 381 is located in the substrate binding site, with the side-chain NH_1 4.1 Å from the 4′-OH of the analog. The structure has been deduced at 2.5 Å resolution using crystals that belong to the $P2_12_12$ space group with $a = 135.07$ Å, $b = 143.91$ Å, and $c = 59.80$ Å. To evaluate the role of Arg 381 in TPL catalysis, we prepared mutant proteins replacing arginine with alanine (R381A), with isoleucine (R381I), and with valine (R381V). The β-elimination activity of R381A TPL has been reduced by 10^{-4}-fold compared to wild type, whereas R381I and R381V TPL exhibit no detectable β-elimination activity with L-tyrosine as substrate. However, R381A, R381I, and R381V TPL react with *S*-(*o*-nitrophenyl)-L-cysteine, β-chloro-L-alanine, *O*-benzoyl-L-serine, and *S*-methyl-L-cysteine and exhibit k_{cat} and k_{cat}/K_m values comparable to those of wild-type TPL. Furthermore, the K_i values for competitive inhibition by L-tryptophan and L-phenylalanine are similar for wild-type, R381A, and R381I TPL. Rapid-scanning-stopped flow spectroscopic analyses also show that wild-type and mutant proteins can bind L-tyrosine and form quinonoid complexes with similar rate constants. The binding of 3-(4′-hydroxyphenyl)-propionic acid to wild-type TPL decreases at high pH values with a pK_a of 8.4 and is thus dependent on an acidic group, possibly Arg404, which forms an ion pair with the analog carboxylate, or the pyridoxal 5′-phosphate Schiff base. R381A TPL shows only a small decrease in k_{cat}/K_m for tyrosine at lower pH, in contrast to wild-type TPL, which shows two basic pK_as with an average value of about 7.8. Thus, it is possible that Arg 381 is one of the catalytic bases previously observed in the pH dependence of k_{cat}/K_m of TPL with L-tyrosine [Kiick, D. M., & Phillips. R. S. (1988) *Biochemistry 27*, 7333−7338], and hence Arg 381 is at least partially responsible for the substrate specificity of TPL.

Tyrosine phenol-lyase (TPL, EC 4.1.99.2)[1] is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyzes the β-elimination of L-tyrosine to produce phenol and ammonium pyruvate (eq 1). In addition to L-tyrosine, L- and D-serine,



(1)

S-methyl-L-cysteine (Kumagai *et al.*, 1970), *S*-ethyl-L-cysteine, *S*-(*o*-nitrophenyl)-L-cysteine, *O*-benzoyl-L-serine (Phillips, 1987), and β-chloro-L-alanine act as substrates for β-elimination, with the formation of ammonium pyruvate. TPL also catalyzes the reverse reaction of β-elimination (Yamada *et al.*, 1972; Enei *et al.*, 1972a) and the β-replacement reaction between the substrates for β-elimination and phenol derivatives (eq 2) (Kumagai *et al.*, 1969; Ueno *et al.*, 1970).



(2)

The molecule of TPL consists of four chemically identical subunits (Kazakov *et al.*, 1987), each with molecular mass of 51.4 kDa, and binds 4 mol of PLP per tetramer. This

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[‡] Crystallographic coordinates have been deposited with the Brookhaven Protein Data Bank under file name 2TPL.

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[1] Abbreviations: TPL, tyrosine phenol-lyase [EC 4.1.99.2]; TRPase, tryptophan indole-lyase [EC 4.1.99.1]; AspAT, aspartate aminotransferase; PLP, pyridoxal 5′-phosphate; HPPA, 3-(4′-hydroxyphenyl)-propionic acid; SOPC, *S*-(*o*-nitrophenyl)-L-cysteine.

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enzyme is found primarily in enterobacteria (Enei *et al.*, 1972), although it has been reported to occur in some arthropods (Duffey & Blum, 1977; Duffey *et al.*, 1977). We have previously reported the X-ray crystal structure of apo-TPL (Antson *et al.*, 1993). From comparison of the three-dimensional structures of AspAT and TPL, it appears that two arginine residues of TPL, Arg 381 and Arg 404, are close to the position of the Arg 386 of AspAT, the guanidinium group of which binds the α-carboxyl group of the substrate (Kirsch *et al.*, 1984). One of these, Arg 404, is conserved in the structure of tryptophan indole-lyase (TRPase), a highly homologous enzyme (Antson *et al.*, 1993). Arg 404 resides on one of the β-strands of the small domain β-sheet, similar to the location of Arg 386 in the AspAT structure (Jansonius & Vincent, 1987). Thus, Arg 404 of TPL is likely to be involved in binding the α-carboxyl of amino acid substrates (Antson *et al.*, 1993).

The chemical mechanisms proposed for TPL and TRPase are very similar (Kiick & Phillips, 1988a,b; Faleev *et al.*, 1988). However, these enzymes are specific for their respective physiological substrates. The elimination of a carbon leaving group, in contrast to a heteroatom leaving group, would be expected to require general acid/base catalysis for activation. Thus, the substrate specificity of TPL should be controlled during the β-elimination step, by the interactions of the phenol side chain with a catalytic base within a hydrophobic pocket, rather than at the stage of substrate binding. The chemical mechanism of TPL appears to require two basic groups, one of which (pK_a ca. 7.6−7.8) abstracts the proton from the α-position of the substrate to form a quinonoid intermediate, and the second of which (pK_a ca. 8.0−8.2), acting in concert with proton transfer from the first group to C-1 of the phenolic ring, facilitates cyclohexadienone formation and subsequent elimination of phenol (Kiick & Phillips, 1988a; Faleev *et al.*, 1988).

In the present work, we performed X-ray crystallographic studies of TPL cocrystallized with the substrate analog 3-(4′-hydroxyphenyl)propionic acid (HPPA). These results demonstrate that the Arg 381 side chain is located in front of the phenolic OH of the bound substrate analog, while Arg 404 forms an ion pair with the carboxylate. To determine the role of Arg 381 in TPL catalysis, we mutated it to alanine, isoleucine, and valine by site-directed mutagenesis, and determined the activity of these mutant proteins with tyrosine and other substrates. The results suggest that Arg 381 is at least partly responsible for the substrate specificity of TPL.

MATERIALS AND METHODS

Materials. Lactate dehydrogenase from rabbit muscle, PLP, and NADH were purchased from U.S. Biochemical Corp. (USB), as were L-tyrosine, L-tryptophan, *S*-benzyl-L-cysteine, and *S*-ethyl-L-cysteine. β-Chloro-L-alanine and L-phenylalanine were obtained from Sigma. *S*-Methyl-L-cysteine was a product of ICN Biochemicals. *S*-(*o*-Nitrophenyl)-L-cysteine (SOPC) and *O*-benzoyl-L-serine were prepared as previously described (Phillips, 1987; Phillips *et al.*, 1989). 3-(4′-Hydroxyphenyl)propionic acid (HPPA) was obtained from Lancaster.

Site-Directed Mutagenesis. Plasmid pTZTPL, which contains the *tpl* gene from *Citrobacter freundii* (Antson *et al.*, 1993), was purified from its host cell, *Escherichia coli* SVS370, and used to transform *E. coli* CJ236 (*dut*$^-$ *ung*$^-$). Uracil-containing single-stranded DNA of this plasmid was prepared as previously described by Chen *et al.* (1995a). Site-directed mutagenesis was performed by the method of Kunkel (1985) using a kit from Bio-Rad. The mutagenic oligonucleotides were AGTATGGAG*GC*CGGAATT for the Arg to Ala mutation, AGTATGGAG*AT*CGGAATT for the Arg to Ile mutation, and AGTATGGAG*GT*CGGAATT for the Arg to Val mutation. Mutated clones obtained after the mutagenesis were screened by sequencing the gene in the mutated region using an Applied Biosystems Model 373A DNA sequencer operated in the Molecular Genetics Instrumentation Facility at the University of Georgia. The primer nucleotide prepared from 1681 to 1700 [as defined in Antson *et al.* (1993)] was used for sequencing the region containing the desired mutation. The resulting plasmid containing the Arg 381 to Ala mutation was designated pTZTPL R381A, the plasmid containing the Arg 381 to Ile mutation was designated pTZTPL R381I, and the plasmid containing the Arg 381 to Val mutation was designated pTZTPL R381V. *E. coli* SVS370 cells were used as the host for the plasmid, since these cells are *tna*A$^-$ and thus do not produce tryptophan indole-lyase, which would interfere with the use of SOPC for assay in cell extracts. The cells were grown and the enzymes were purified as previously described (Chen *et al.*, 1995a).

Crystallization of TPL Complex with HPPA. Crystallization of the wild-type TPL holoenzyme−inhibitor complex was performed with monomethyl ether poly(ethylene glycol) 5000 (Fluka) as precipitant using hanging drop vapor diffusion. Typically, 1 μL of the protein solution (20 mg/mL TPL and 50 mM HPPA in 50 mM triethanolamine buffer with 1 mM DTT and 2×10^{-4} M PLP) was half diluted with a reservoir solution. The best crystals were obtained with 30−45 % (w/v) monomethyl ether poly(ethylene glycol) 5000 in the presence of monovalent cations salts (0.15−0.6 M KCl or CsCl).

X-ray Data Collection. X-ray data from crystals of TPL in complex with HPPA were collected at the Daresbury synchrotron radiation source (Daresbury, U.K.) on station 9.5 operating at 0.918 Å wavelength using a Marresearch image plate scanner. The crystals belong to the $P2_12_12$ space group (a = 135.07 Å, b = 143.91 Å, c = 59.80 Å), with two TPL subunits in the asymmetric part. Data collection was carried out at room temperature with 1.2° oscillation per image. Three different crystals were used with about 30° oscillation per crystal in total. X-ray data were processed using the program DENZO (Otwinowski, 1993) and programs SCALA and TRUNCATE from the CCP4 package (Collaborative Computational Project Number 4, 1994). The statistics of the data set are summarized in Table 1.

Structure Solution and Refinement. The starting model for the refinement was the wild-type TPL holoenzyme structure (Antson *et al.*, manuscript in preparation). All computations were performed using the CCP4 program package. Rebuilding of the atomic model was performed using programs XFIT (T. Oldfield, University of York) and QUANTA (Molecular Simulations) and Fourier maps with coefficients $2m|F_o| - D|F_c|$ (Murshudov et al., 1996). Refinement of the model was performed using the maximum likelihood approach as implemented in the program REFMAC (Murshudov *et al.*, 1996). Throughout refinement-main-chain atoms were restrained by noncrystallographic symmetry. In the later stages five water molecules were added at the end of each two refinement cycles using the program ARP (Lamzin & Wilson, 1993).

Table 1: Statistics of the X-ray Data

resolution ranges	$\langle I \rangle$	$\langle I/\sigma(I) \rangle$	redundancy[a]	R_{merge}[b]	completeness (%)
15.0−7.80	2830	6.5	2.9	0.063	98.2
7.80−5.51	1491	7.6	2.9	0.084	98.1
5.51−4.50	2274	7.8	3.0	0.087	98.1
4.50−3.90	2450	6.8	3.0	0.101	98.0
3.90−3.49	1879	5.2	2.9	0.133	96.7
3.49−3.18	1267	3.7	2.8	0.188	94.8
3.18−2.95	887	2.5	2.7	0.274	93.1
2.95−2.76	652	2.1	2.4	0.315	74.4
2.76−2.60	439	2.3	2.0	0.293	57.6
2.60−2.50	383	1.9	1.7	0.361	50.3
total	1528	4.7	2.7	0.139	81.0

[a] Average number of observations of the same reflection. [b] Value of the merging *R*-factor between equivalent measurements of the same reflection, $R_I = \sum|I - \langle I \rangle|/\sum I$.

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 (1970) for TRPase. Reaction mixtures contained, in a total volume of 0.6 mL, 50 mM potassium phosphate, pH 8.0, 5 mM 2-mercaptoethanol, 50 μM PLP, 0.2 mM NADH, 0.02 mg of lactate dehydrogenase, and various amounts of amino acid substrate at 25 °C. The reaction was initiated by the addition of enzyme solution. Determination of the pH dependence of R381A TPL was carried out by measuring the activity at high concentrations of enzyme (1−2 mg/mL) with varying [L-tyrosine] at different pH values from 6.2 to 8.6 using potassium phosphate buffers. The final pH values of the reaction mixtures were measured after the kinetic measurements were completed. Enzyme activity during purification was routinely measured with 0.6 mM *S*-(*o*-nitrophenyl)-L-cysteine (SOPC) in 50 mM potassium phosphate, pH 8.0 at 25 °C (Phillips, 1987), following the decrease in absorbance at 370 nm ($\Delta\epsilon = -1.86 \times 10^3$ M^{-1} cm^{-1}). Determination of the kinetic parameters for SOPC was performed at 25 °C in 50 mM potassium phosphate, pH 8.0, 5 mM 2-mercaptoethanol, and 50 μM PLP, with varying amounts of SOPC and appropriate dilutions of wild-type and mutant TPL. A unit of TPL activity is defined as the amount of enzyme which produces 1 μmol of product/min.

The inhibitory effects of phenylalanine and tryptophan on wild-type and mutant TPL were determined using SOPC as substrate as described above. The inhibitory effect of 3-(4′-hydroxyphenyl)propionic acid on wild-type TPL was studied with L-tyrosine as substrate. The reaction mixtures contained 50 mM triethanolamine hydrochloride, pH 6.8−8.8, or 50 mM Bis-tris propane hydrochloride, pH 8.3−9.4, 2.5 mM dithiothreitol, 50 μM PLP, 0.2 mM NADH, 2 units of LDH, and varying amounts of L-tyrosine, and the reaction was initiated by the addition of TPL.

Protein Determination. Protein was determined by the method of Bradford (1976), with purified TPL as a standard. The concentration of purified TPL was determined from the absorbance at 278 nm ($E^{1\%} = 8.37$) (Muro *et al.*, 1978) assuming a subunit molecular mass of 51.4 kDa (Antson *et al.*, 1993). The PLP content of mutant enzymes was determined by measuring the spectrum of the enzyme in 0.1 N NaOH, assuming $\epsilon^{390} = 6600$ M^{-1} cm^{-1} (Peterson & Sober, 1954). Samples for mass spectrometry were applied to a Vydac 300 A C18 column (4.6 × 250 mm) equilibrated with 0.1% trifluoroacetic acid/20% acetonitrile. The protein was detected by UV absorbance at 278 nm, and the large peak eluting at 17.3 min was collected. Mass spectra were then obtained on these samples using a PE SCIEX electrospray ionization mass spectrometer.

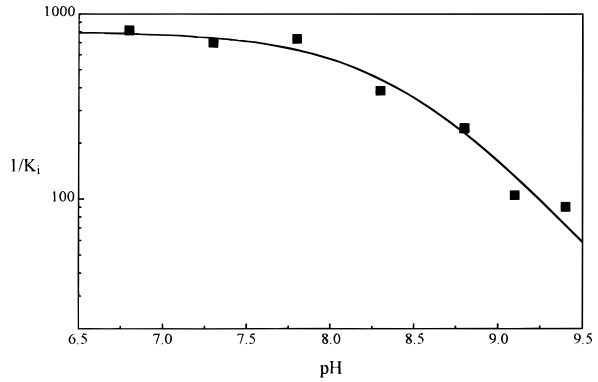


FIGURE 1: pH dependence of inhibition of TPL by HPPA. The filled squares are the $1/K_i$ values for the inhibitor at the given pH, and the line is calculated from eq 4 using a value of 8.4 for the pK_a and a pH-independent value of 1.25 mM for K_i.

Spectra of Tyrosine Phenol-lyase. Absorbance spectra were obtained on a Cary 1E UV/vis spectrophotometer, and CD spectra were obtained with a Jasco J-700 spectropolarimeter. Enzyme samples were dialyzed overnight against 0.05 M potassium phosphate buffer, pH 8.0, containing 0.05 mM PLP and were passed through PD-10 desalting columns equilibrated with 0.05 M potassium phosphate, pH 8.0, prior to measurement of the spectra.

Stopped-Flow Measurements. Prior to the rapid kinetic 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 instrument from OLIS, Inc. This instrument has a dead time of ca. 2 ms, and is capable of collecting spectra in the visible region from 300 to 600 nm at 1 kHz. The enzyme solutions in 50 mM potassium phosphate, pH 8.0, were mixed with 2 mM L-tyrosine, and the absorbance changes at 500 nm were followed for either 1 s or 1 min, in different experiments, in order to observe both fast and slow processes. The rate constants were then evaluated by exponential fitting using the LMFT or SIFIT programs provided by OLIS. The validity of the fitting was evaluated by the standard deviation or the Durbin−Watson parameters. Typically, rate constants were estimated to a standard deviation of less than 5%.

Data Analysis. Steady-state kinetic values of k_{cat} and K_m were obtained by fitting the data (initial velocity vs substrate concentration) to the Michaelis−Menten equation

$$V = K_{cat}[E][S]/(K_m + [S]) \quad (3)$$

using a hyperbolic regression program (Hyper). In eq 3, S is the substrate, [E] is the total enzyme concentration, and K_m is the Michaelis constant. The enzyme concentrations were calculated using a subunit molecular mass of 51 440 Da (Antson *et al.*, 1993). The pH dependence of inhibition by HPPA was fit to eq 4 using the HBBELL Fortran program of Cleland (1979):

$$1/(K_i)_{app} = (1/K_i)[H^+]/([H^+] + K_a) \quad (4)$$

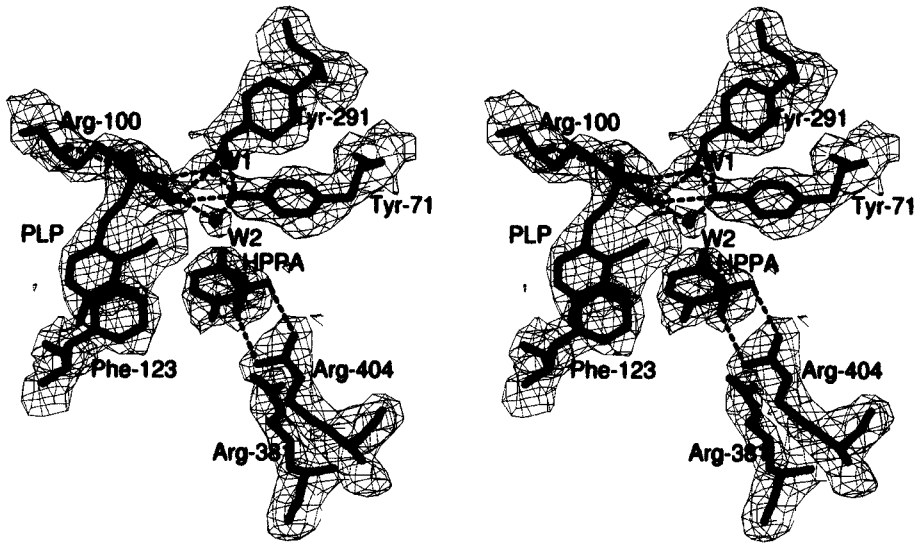


FIGURE 2: Electron density maps around the HPPA calculated with coefficients $2m|F_o| - D|F_c|$ (Murshudov *et al.*, 1996). The final refined model is shown with two water molecules near the substrate analog labeled as W1 and W2. For the PLP-bound Lys-257 only the ϵ-amino group is shown.

RESULTS

Inhibition of Tyrosine Phenol-lyase by 3-(4′-Hydroxyphenyl)propionic Acid. 3-(4′-Hydroxyphenyl)propionic acid (HPPA) acts as a competitive inhibitor of L-tyrosine β-elimination. The pH dependence of the K_i value shows that HPPA binding decreases above pH 8 (Figure 1), and the data fit well to a single pK_a of 8.4 $\pm$ 0.08, with a pH-independent value of K_i of 1.25 mM. This suggests that an acidic group in the enzyme active site with a pK_a of 8.4 is critical for the binding of HPPA. Addition of HPPA to solutions of wild-type TPL results in a red shift of the visible absorbance peak from 420 to 425 nm, and there is a small decrease in the dissymetry factor of the CD spectra, from 6.4×10^{-6} to 5.6×10^{-6} (data not shown).

Structure of Complex of Tyrosine Phenol-lyase with 3-(4′-Hydroxyphenyl)propionic Acid. The X-ray structure of this complex was refined at 2.5 Å resolution to a crystallographic *R*-factor of 18.3% (R_{free} = 26.3%). The final electron density in the region of the active-site cleft is shown in Figure 2. The final model contains 7252 protein atoms and 253 water molecules. All residues have allowed main-chain conformational angles (Figure 3). Summary statistics of the refined model are given in Table 2.

HPPA was present in the crystallization medium at a concentration of 25 mM, i.e., about 12 times higher than the inhibition constant. However, electron density maps indicate it is present in only one of the two independent subunits in the crystallographic asymmetric unit. In kinetic studies, TPL has not been found to exhibit any significant cooperativity between subunits (Kiick & Phillips, 1988a). The differences in substrate binding must be due to the different crystallographic environment around the two subunits, perhaps coupled with an increased dissociation constant for the HPPA in the crystallization medium.

In one of the two subunits, the substrate analog binds with its α-carboxyl, making a salt bridge with the Arg 404 guanidinium group (Figure 4). The HPPA Cα atom is 4.3 Å from the C4′ of the PLP, and in a geometry which would allow the amino group of the real substrate to interact with the C4′ and form the external aldimine intermediate. It is not evident which protein group would act to abstract proton from the substrate Cα in the first stage of the reaction. The

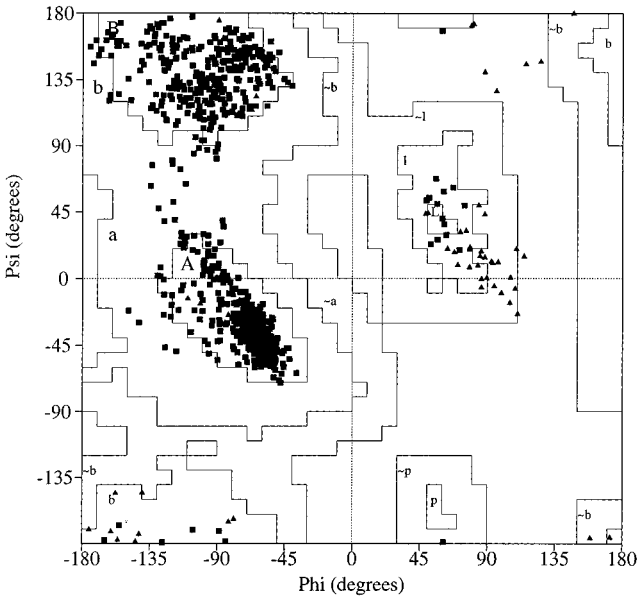


FIGURE 3: Ramachandran (ϕ, ψ) plot for the two TPL subunits present in the asymmetric unit prepared using PROCHECK (Laskowski *et al.*, 1993).

HPPA Cα atom is located 4.6 Å from the Lys 257 Nϵ and 4.7 Å from a water molecule, Figure 5. One possibility for the Cα proton abstraction is Lys 257, where NH_2 would be released on the formation of the external aldimine. Another possibility is the water molecule coordinated by the Tyr 71 and Tyr 291 phenol hydroxyls, by NH_2 of Gln 98 and NH_2 of Arg 100, and also by the Lys 256 Nϵ, which itself is 4.2 Å from the monovalent cation. Our modeling studies indicate that the lysine side chain could be flexible with the Nϵ moving between the cation and the water molecule (W1 in Figure 5). The monovalent cation may thus lower the pK_a for the lysine side-chain amino group, which may abstract a proton from the water molecule and thus activate it. The nascent hydroxide could act to abstract the proton from the substrate Cα. Another water molecule is 3.0 Å from the substrate Cγ and is H-bonded to Arg 100 and Tyr 71. The Tyr 71 phenol hydroxyl, H-bonded to this water molecule, is 3.7 Å from the substrate analog Cα and has been confirmed by mutation to phenylalanine to be involved

Table 2: Refinement and Model Correlation

resolution (Å)		2.5−15.0
no. of protein atoms		7252
no. of water molecules		253
no. of reflections used in refinement		32 151
R-factor[a] (%)		18.3
no. of reflections used for R_{free}		957
R_{free}[a] (%)		26.3
average atomic *B*-factor (Å^2)	main chain	29.2
	side chain	31.0
	waters	29.4
Deviations from Ideal Geometry[b]		
bond distance (Å)		0.014 (0.020)
angle distance (Å)		0.046 (0.040)
planes (Å)	aromatic	0.012 (0.020)
	peptide	0.043 (0.050)
torsion angles (deg)	planar	7.0 (7.0)
	staggered	20.7 (15.0)
	orthonormal	29.5 (25.00)
B-factors correlation (Å^2)	main-chain bond	2.5 (3.0)
	main-chain angle	3.4 (4.0)
	side-chain bond	9.6 (8.0)
	side-chain angle	11.2 (10.0)
noncrystallographic symmetry[c]	main-chain bonds (Å)	0.125 (0.100)
	main-chain *B*-factors (Å^2)	0.94 (1.0)

[a] Crystallographic *R*-factor, $R_{(free)} = \Sigma||F_o| - |F_c||/\Sigma|F_o|$. For R_{free} calculation the sum was taken over 957 randomly chosen reflections (3.0% of the total), which were excluded from refinement. All other reflections in the resolution range 15.0−2.5 Å were used in the refinement. [b] Rms deviation from the standard values are given with target values in parentheses. [c] Noncrystallographic symmetry restraints were applied to the main-chain atoms only. Rms deviations from the average position and from the average *B*-factor are given.

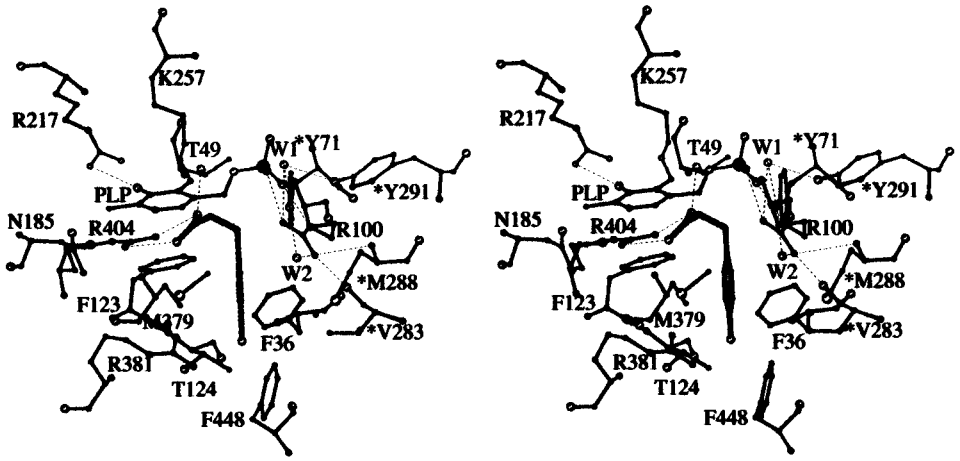


FIGURE 4: Stereo plot of the TPL substrate binding site, prepared using MOLSCRIPT (Kraulis, 1991). The bound HPPA is shown with bold lines. Residues from an adjacent subunit are marked by stars. The two water molecules near the HPPA are labeled as in Figure 2.

in donation of a proton to the substrate Cγ (Chen *et al.*, 1995).

The phenolic hydroxyl of the substrate analog is 4.1 Å from the Arg 381 side-chain NH_1 atom. It is at an angle of 85° to the NH_1 and 103° to the NH_2 atoms of Arg 381. Although these angles are not energetically favorable for potential H-bonding, the real substrate may well cause conformational changes in the active site upon formation of the Schiff base with PLP and upon further Cα proton abstraction. Other protein groups in contact with substrate phenol are the side chains of residues Thr 124, Met 288, and Phe 448. Of these, only Thr 124 has polar character, with the side-chain hydroxyl situated 4.9 Å from the substrate hydroxyl. The Arg 381 side chain appears to be the closest group with potential catalytic activity to the hydroxyl group of the substrate analog.

Characterization of R381A, R381I, and R381V Tyrosine Phenol-lyase. In order to determine the role of Arg 381 in the reaction of TPL, this arginine was mutated to alanine, isoleucine, and valine by site-directed mutagenesis. The presence of the desired mutation and the absence of other mutations was confirmed by automated DNA sequencing in the region of the expected mutation. In addition, the mass spectra of wild-type, R381A, R381I, and R381V TPL were obtained using electrospray ionization. Wild-type TPL exhibited a molecular mass of 51 437.0 ± 4.3 Da, in good agreement with the calculated molecular mass of 51 440.9 Da from the amino acid sequence (Antson *et al*., 1993) using the average residue mass values of Biemann (1990). R381A, R381I, and R381V TPL exhibited molecular masses of 51 351.9 ± 7.1, 51 398.8 ± 9.9, and 51 381.4 ± 5.0, respectively, which are in excellent agreement with the expected masses of 51 355.7 Da for the Arg to Ala mutation, 51 397.9 Da for the Arg to Ile mutation, and 51 383.9 for the Arg to Val mutation. The PLP content of the mutant proteins was found to be 1 mol/subunit, as was shown previously for wild-type TPL (Kazakov *et al.*, 1987). Thus, the mutations do not significantly affect the binding of PLP to the protein. The visible spectrum of R381A TPL, with peaks at 340 and 500 nm in addition to the expected internal

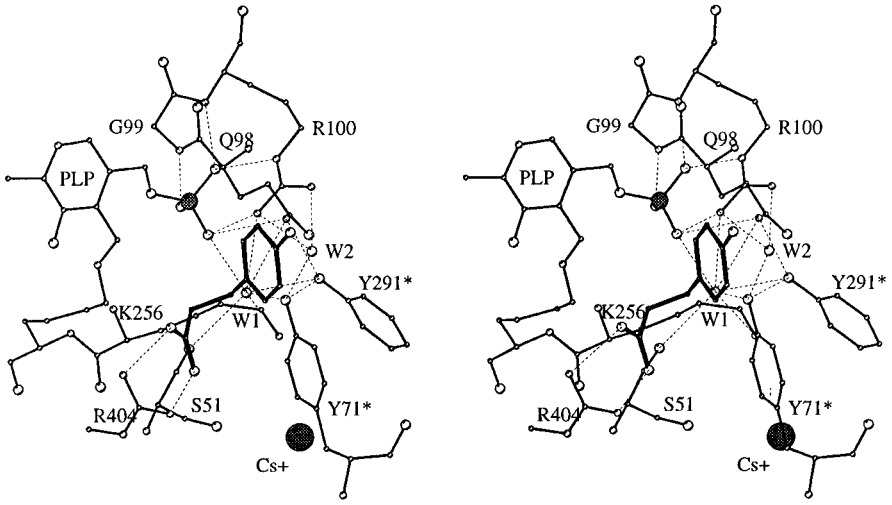


FIGURE 5: Stereo plot of the environment around the two water molecules that point toward the HPPA, prepared using MOLSCRIPT (Kraulis, 1991). The monovalent Cs^+ cation is represented by a large sphere. HPPA is drawn with bold lines.

Table 3: Substrate Specificity of TPL and Mutant Forms

	wild-type TPL		R381A TPL		R381I TPL		R381V TPL	
substrate	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
SOPC	9.7	3.6×10^4	2.1	3.1×10^4	0.46	2×10^4	9.7	1.0×10^5
L-ty	2.2	8.0×10^3	1.8×10^{-4}	0.48	$<1.4 \times 10^{-5}$	$<1.4 \times 10^{-3}$	$<1.4 \times 10^{-5}$	$<1.4 \times 10^{-3}$
S-methyl-L-Cys	0.9[a]	2.8×10^2	0.033	2.5×10^3	0.20	1.2×10^2	0.67	1.3×10^4
S-ethyl-L-Cys	1.7	7.4×10^2	0.017	2.4×10^3	0.19	24	0.61	6.1×10^3
O-benzyl-L-Ser	3.9	3.3×10^3	0.012	3.2×10^3	0.68	2.7×10^3	2.5	7.8×10^3
O-benzoyl-L-Ser	8.3[a]	2.9×10^3	0.37	2.9×10^2	0.95	3.0×10^2	2.9	1.2×10^3
β-chloro-L-Ala	3.0	1.8×10^3	0.025	8.6×10^4	0.76	4.5×10^3	0.84	2.7×10^3

[a] Data from Chen *et al*. (1995).

aldimine band at 415 nm (Figure 6A), is distinct from that of wild-type TPL, which exhibits an internal aldimine absorbance peak at 420 nm. The visible spectra of R381I (Figure 6A) and R381V TPL (data not shown) also exhibit peaks at 340 and 415 nm but do not show the 500 nm peak. The CD spectra of R381A and R381I TPL, shown in Figure 6B, show positive bands for the 415 nm peaks, as was previously observed for the 420 nm band of wild-type TPL (Demidkina and Myagkikh, 1989; Chen *et al*., 1995b). In addition, the 340 nm peak is seen as a positive CD band for both mutant proteins, but the 500 nm absorbance peak of R381A TPL is absent from the CD spectrum.

Catalytic Activity of TPL and Mutant Proteins. The steady-state kinetic parameters of wild-type, R381A, R381I, and R381V mutant TPL for six substrates are shown in Table 3. Although R381I and R381V TPL have no detectable activity with tyrosine, R381A TPL has a very low, but readily measurable, activity with tyrosine. The k_{cat} for L-tyrosine has been decreased 10^4-fold, and the k_{cat}/K_m has been reduced by 5×10^3-fold for R381A TPL, compared to wild-type TPL. Wild-type, R381A, R381I, and R381V TPL can all utilize SOPC as a substrate, with only modest effects on k_{cat} and k_{cat}/K_m, indicating that Arg 381 is not an essential residue for the reaction of SOPC. All of these enzymes can also utilize *S*-alkylcysteines as substrates (Table 3). For R381A TPL, these substrates exhibit k_{cat} values reduced by about 100-fold and k_{cat}/K_m values increased by about 10-fold compared to the reactions catalyzed by wild-type TPL (Table 3). This is due to the very low K_m values of R381A for these substrates. In contrast, R381I and R381V TPL exhibit k_{cat} values larger than that of R381A TPL, but somewhat smaller than that of wild-type TPL, with *S*-alkyl-L-cysteines. The k_{cat}/K_m values of R381I TPL are smaller than those of either wild-type, R381A, or R381V TPL with *S*-alkyl-L-cysteines. Other known substrates of TPL, *O*-benzyl-L-serine, *O*-benzoyl-L-serine, and β-chloro-L-alanine, are relatively good substrates for all of these mutant enzymes (Table 3).

We then examined the effect of pH on the reaction of R381A TPL with L-tyrosine. R381A TPL shows very similar k_{cat} values at pH 6.60, 7.70, and 8.36, with only a 3-fold decrease at the lowest pH (Table 4). Similarly, there is only about a 3-fold decrease in k_{cat}/K_m between pH 8.36 and 6.60 (Table 4). In contrast, we have previously shown that wild-type TPL exhibits two basic pK_as of about 7.8 in the pH profile of k_{cat}/K_m (Kiick & Phillips, 1988a; Chen *et al*., 1995a) and thus would be expected to show about a 200-fold decrease in k_{cat}/K_m between pH 8.36 and 6.60. These results suggest that Arg 381 may be one of the catalytic bases in wild-type TPL that contributes to the pH dependence.

Effect of Inhibitors. The inhibition of wild-type, R381A, and R381I TPL by L-tryptophan and L-phenylalanine was studied, and the inhibitory constants for L-tryptophan and L-phenylalanine are 2.9 mM and 2.5 mM, respectively, for wild-type TPL, 1.8 mM and 8.5 mM, respectively, for R381A TPL, and 1.1 mM and 0.3 mM, respectively, for R381I TPL (Table 5). Thus, the mutation of Arg 381 to either alanine or isoleucine does not significantly reduce the binding of L-phenylalanine and L-tryptophan to TPL. Indeed, L-phenylalanine binds about 10-fold more strongly to R381I TPL than to wild type (Table 5).

Rapid-Scanning Stopped-Flow Reactions of TPL with L-Tyrosine. L-Tyrosine forms a steady-state quinonoid complex with wild-type TPL that exhibits a rather weak

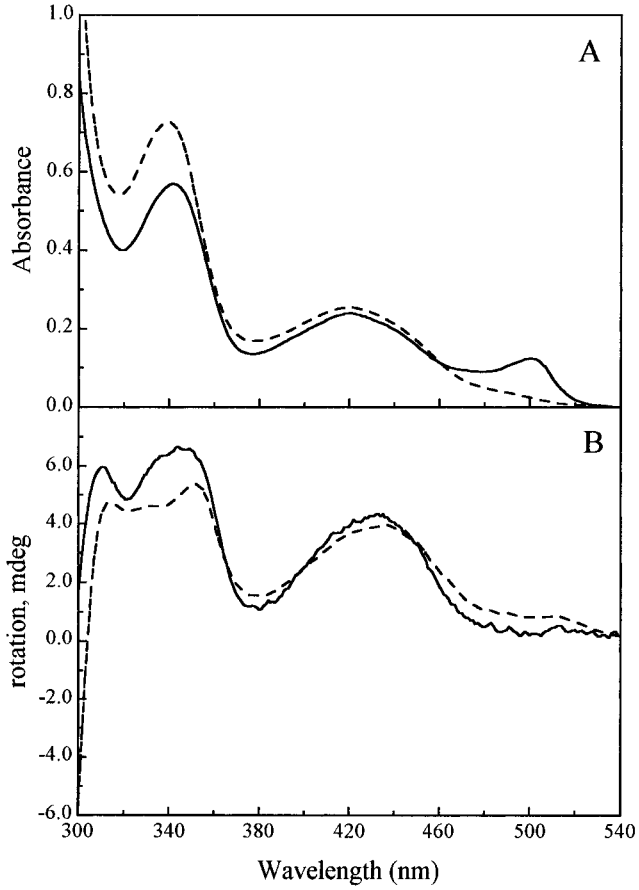


FIGURE 6: (A) Absorbance spectra of Arg 381 TPL mutant proteins. Solid line, R381A TPL, 7 mg/mL. Dashed line, R381I TPL, 12 mg/mL. (B) CD spectra of Arg 381 TPL mutant proteins. Solid line, R381A TPL, 7 mg/mL. Dashed line, R381I TPL, 12 mg/mL.

Table 4: Effect of pH on TPL Activity

	R381A TPL		wild-type TPL[a]	
pH	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
6.60	2.3×10^{-4}	0.46	3.5	6.53×10^{1}
7.70	2.0×10^{-4}	0.46	3.5	4.56×10^{3}
8.36	6.0×10^{-4}	1.80	3.5	1.30×10^{4}

[a] Data calculated from Chen *et al.* (1995).

Table 5: Effect of Mutation of Arg 381 on Binding of TPL Inhibitors

	K_i (mM)		
inhibitor	wild-type TPL	R381A	R381I
L-tryptophan	2.9	1.8	1.1
L-phenylalanine	2.5	8.5	0.3

visible absorbance peak at about 500 nm (Muro *et al.*, 1978). L-Phenylalanine, L-tryptophan, L-alanine, and D-alanine form complexes with a similar, but more intense, peak at about 500 nm (Kumagai *et al.*, 1970b; Faleev *et al.*, 1988; Chen & Phillips, 1993), although they are not substrates of TPL for the β-elimination reaction. We examined the reactions of wild-type, R381A, and R381I TPL with L-tyrosine using rapid-scanning stopped-flow spectrophotometry. The reaction of wild-type TPL with 2 mM L-tyrosine exhibits rapid formation of a quinonoid complex with a peak at 501 nm (Figure 7A), as previously reported by Muro *et al.* (1978). The reaction of R381A TPL with L-tyrosine is more complex, with a small rapid increase in the absorbance peak at 500 nm, followed by a slow decrease at 500 nm with a

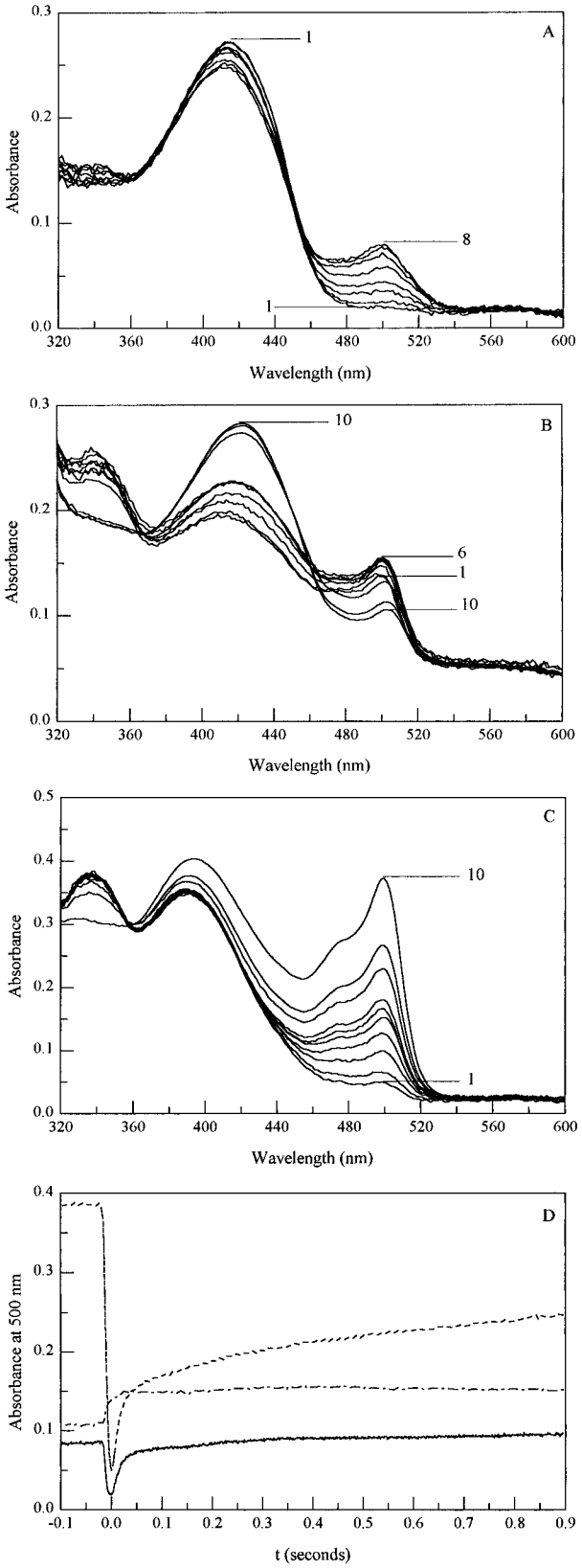
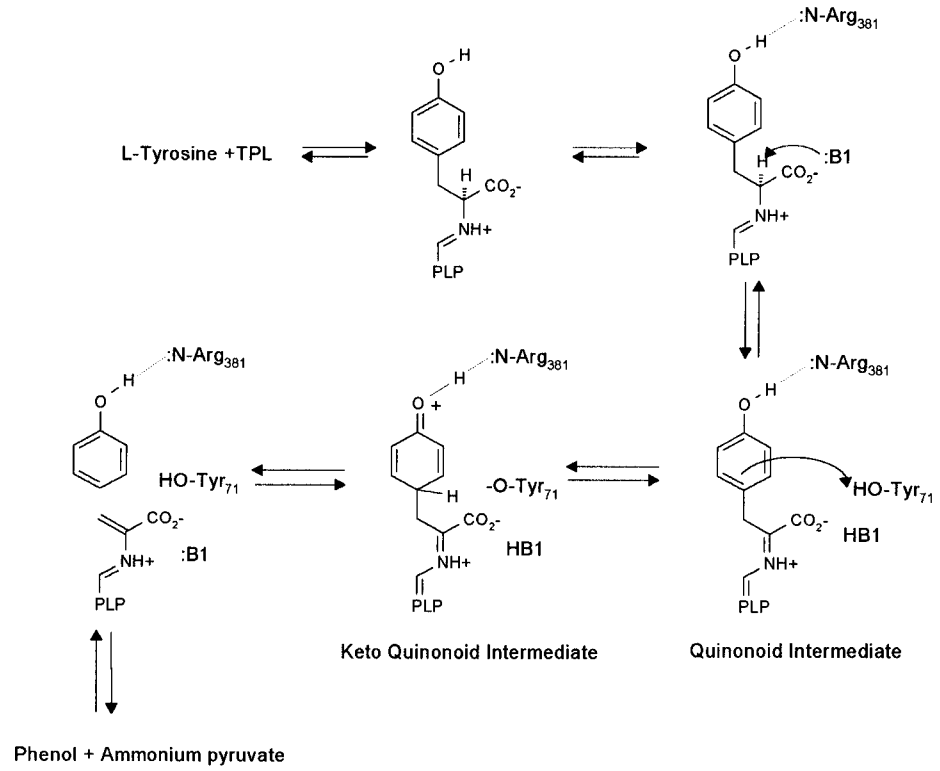


FIGURE 7: (A) Rapid-scanning stopped-flow spectra of wild-type TPL (1 mg/mL; 19.4 μM) reaction with 2 mM L-tyrosine. Spectra are displayed at 0.001, 0.005, 0.010, 0.020, 0.040, 0.080, 0.160, and 0.320 s after the start of the reaction. (B) Rapid-scanning stopped-flow spectra of R381A TPL (1 mg/mL; 19.4 μM) reaction with 2 mM L-tyrosine. Spectra are displayed at 0.001, 0.005, 0.020, 0.160, 0.320, 0.640, 1.1, 13.5, 38.5, and 57.7 s after start of the reaction. (C) Rapid-scanning stopped-flow spectra of R381I TPL (1 mg/mL; 19.4 μM) reaction with 2 mM L-tyrosine. Spectra are displayed at 0.001, 0.005, 0.020, 0.040, 0.160, 0.320, 0.640, 1.39, 5.39, and 9.07 s after start of the reaction. (D) Time courses for reaction of TPL and mutant proteins with 2 mM L-tyrosine at 500 nm.

Scheme 1: Mechanism of Tyrosine Phenol-lyase



concomitant increase at 420 nm and decrease at 340 nm (Figure 7B). The reaction of R381I TPL with L-tyrosine shows a fast phase of absorbance increase at 501 nm, followed by a very slow further increase over 10 s, with concomitant decrease in the 340 nm peak (Figure 7C). The time courses for the reactions of wild-type (solid line), R381A (dots and dashes), and R381I TPL (dashes) at 500 nm are shown in Figure 7D. All three enzymes show a rapid phase with a similar rate constant of about 70 s^{-1}, indicating that the mutations have not significantly affected the ability of the enzymes to form quinonoid intermediates from L-tyrosine.

DISCUSSION

The k_{cat}/K_m value for the reaction of L-tyrosine or 3-fluoro-L-tyrosine with TPL decreases at acidic pH, showing a dependence on two basic groups with an average pK_a value of 7.8, while the value of k_{cat} is pH-independent (Kiick & Phillips, 1988a; Chen *et al.*, 1995a). The pH dependence of k_{cat}/K_m reflects ionizations occurring on the free enzyme and free substrate, while those of k_{cat} reflect ionizations on the enzyme−substrate complex. Since L-tyrosine does not have a basic group with a pK_a of 8, these basic groups seen in the pH dependence of k_{cat}/K_m must be catalytic residues of TPL. In contrast to L-tyrosine, the reaction of S-methyl-L-cysteine with TPL exhibits only a single pK_a of 7.6 in its pH dependence of k_{cat}/K_m, suggesting that the reaction mechanism for β-elimination of this substrate requires only a single proton abstraction to form the quinonoid intermediate (Kiick & Phillips, 1988a). Hence, we concluded that the second basic group, with a pK_a of about 8.0, is required to catalyze the elimination of the phenol moiety of tyrosine. A mechanism was proposed that suggested that this second base interacts with the phenolic OH of the tyrosine substrate and, by abstraction of the proton, would facilitate the tautomerization of the phenol to the cyclohexadienone intermediate (Kiick & Phillips, 1988a; Faleev *et al.*, 1988). In the present work, the structure of the complex of TPL with 3-(4′-hydroxyphenyl)propionic acid demonstrates that Arg 381 is the only group near the phenolic OH of the substrate analog in a position to function as this second catalytic base (Figure 3). In order to confirm this hypothesis, we have performed site-directed mutagenesis of Arg 381 of TPL to alanine, isoleucine, and valine.

R381A TPL has very low activity, while R381I and R381V TPL have no detectable activity, for elimination of phenol from L-tyrosine (Table 3); however, the k_{cat}/K_m values for SOPC are hardly affected by the mutations. It should be noted that the leaving group of SOPC, *o*-nitrobenzenethiol, has a pK_a of 5.2 (Suelter *et al.*, 1976) and thus requires no additional assistance from the enzyme beyond formation of the quinonoid intermediate. Furthermore, the rapid-scanning stopped-flow data (Figure 7) and the steady-state inhibitor data (Table 5) indicate that the mutations of Arg 381 do not reduce the ability of TPL to bind amino acids and to form quinonoid intermediates. In contrast to wild-type TPL, k_{cat}/K_m of L-tyrosine for R381A TPL exhibits very little pH dependence, as expected if Arg 381 is one of the basic groups contributing to the pH profile. The crystallographic results, taken together with the mutagenic and kinetic studies, provide strong evidence for the role of Arg 381 as a catalytic residue in TPL. The pK_a of about 8 is rather low for the guanidine functionality of an arginine, since the expected value in solution is about 12.5. However, Arg 381 is located in a hydrophobic pocket with few ionic or polar groups nearby, which should destabilize a positive charge, thus decreasing the pK_a. Although not operative in this case, electrostatic effects have been shown to decrease the pK_a of lysine residues in enzyme active sites by as much as 5 pK_a units (Westheimer, 1995).

In contrast to the reaction of L-tyrosine, the binding of HPPA is dependent on an *acidic* group rather than a basic

group, as the K_i for HPPA increases at higher pH with a pK_a of 8.4 (Figure 1). This is consistent with the structure, as the main contact of HPPA with the protein is the ion pairing with Arg 404 (Figure 4), which would be weakened if Arg 404 is deprotonated. However, the pK_a of 8.4 could also be due to ionization of the PLP Schiff base at high pH, since HPPA binding induces a red shift in the PLP spectrum. In aromatic amino acid aminotransferase, the binding of phenylpropionic acid was shown to increase the pK_a of the PLP Schiff base (Iwasaki *et al.*, 1994).

The reaction mechanism for TPL as currently understood is presented in Scheme 1. The PLP is bound to the resting enzyme as a Schiff base. Binding of L-tyrosine to the enzyme results in a transaldimination and forms the external aldimine, with the release of Lys 257. An as yet unidentified base (probably Lys 257, but also possibly the water molecule that could be activated by Lys 256, from the present crystal structure) subsequently removes the α-proton to form the quinonoid intermediate. It is then necessary to form the cyclohexadienone tautomer of the phenol, which requires both general acid and general base catalysis, for elimination to proceed. General acid catalysis is provided by Tyr 71, which transfers a proton to the C-1 position of the aromatic ring, possibly mediated via an intervening water molecule (Figure 4) (Chen *et al.*, 1995b). The results of the present study suggest that this protonation at C-1 is assisted by the Arg 381 interacting with the OH at C-4 across the ring. The small residual activity of R381A TPL suggests that removal of the phenolic proton is not absolutely necessary for catalysis, and the main function of Arg 381 may thus be to serve as a hydrogen-bond acceptor rather than as a true base. The 16 700-fold reduction in k_{cat}/K_m for R381A TPL corresponds to a 5.75 kcal/mol increase in activation free energy at 298 K, within the expected range of hydrogen-bond energies. Why then do we see a pK_a in the pH profile, if Arg 381 is not really acting as a base? If the guanidine moiety of Arg 381 does indeed function catalytically as a hydrogen-bond *acceptor*, it could not perform this role efficiently if it were in the protonated form. Proton transfer to the substrate at C-1 would generate a resonance stabilized cation, with significant positive charge on the oxygen (Scheme 1). This would be destabilized by electrostatic repulsion if Arg 381 were in the cationic guanidinium form, and thus at acidic pH values, the k_{cat}/K_m of TPL for phenol elimination from L-tyrosine decreases. Thus, the data reported herein demonstrate that Arg 381 is required for the substrate specificity of tyrosine phenol-lyase.

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