Site-Directed Mutagenesis of Tyrosine-71 to Phenylalanine in *Citrobacter freundii* Tyrosine Phenol-Lyase: Evidence for Dual Roles of Tyrosine-71 as a General Acid Catalyst in the Reaction Mechanism and in Cofactor Binding†

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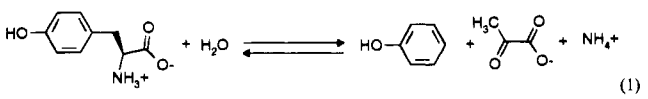
ABSTRACT: Tyr71 is an invariant residue in all known sequences of tyrosine phenol-lyase (TPL). The substitution of Tyr71 in TPL by phenylalanine results in a mutant Y71F TPL with no detectable activity (greater than 3×10^5-fold reduction) for β-elimination of L-tyrosine. Y71F TPL can react with *S*-alkylcysteines, but these substrates exhibit k_{cat} values reduced by 10^3-10^4-fold, while the k_{cat}/K_m values are reduced by 10^2-10^3-fold, compared to wild-type TPL. However, for substrates with good leaving groups (*S*-(*o*-nitrophenyl)-L-cysteine, β-chloro-L-alanine, and *O*-benzoyl-L-serine), Y71F TPL exhibits k_{cat} values 1.85−7% those of wild-type TPL. Y71F TPL forms very stable quinonoid complexes with strong absorbance at 502 nm from L-phenylalanine, tyrosines (L-tyrosine, 3-fluoro-L-tyrosine, and [α-^{2}H]-3-fluoro-L-tyrosine), and *S*-alkylcysteines (*S*-methyl-L-cysteine, *S*-ethyl-L-cysteine, and *S*-benzyl-L-cysteine). The time courses of the formation of quinonoid intermediates in these reactions are biphasic. The slow phase shows a dependence on concentration of PLP and is due to the cofactor binding steps, while the fast phase is due to the amino acid α-deprotonation and reprotonation steps. The rate constants for the fast phase of the reactions of Y71F TPL with L-phenylalanine and *S*-methylcysteine are similar to those for α-deprotonation or reprotonation steps in the reactions of wild-type TPL. The PLP binding constant of Y71F TPL is estimated to be 1 mM by spectrophotometric titration, compared to 0.6 μM for wild-type TPL. Thus, the mutation of Tyr71 to phenylalanine results in a 1700-fold increase in the K_D for PLP. This difference in PLP binding constants corresponds to a $\Delta\Delta G$ value of 4.4 kcal/mol at 25 °C. Tyr70 in aspartate aminotransferase, which exhibits a similar three-dimensional structure, forms a hydrogen bond with the pyridoxal 5′-phosphate (PLP) phosphate oxygen OP2 [Smith, D., Almo, S., Toney, M., & Ringe, D. (1989) *Biochemistry 28,* 8161−8167] and is responsible for cofactor binding, but is not essential for catalysis [Toney, M. D., & Kirsch, J. F. (1987) *J. Biol. Chem. 262,* 12403]. Therefore, in contrast to Tyr70 in aspartate aminotransferase, Tyr71 in *Citrobacter freundii* TPL plays a dual role, both in cofactor binding in the absence of substrate and also as a general acid catalyst in the elimination of leaving groups from quinonoid intermediates.

Tyrosine phenol-lyase (TPL)[1] is a pyridoxal 5′-phosphate (PLP) dependent enzyme which catalyzes the β-elimination of L-tyrosine to produce phenol and ammonium pyruvate (eq 1). This enzyme has been found in various bacteria, most



(1)

of which belong to the *Enterobacteriaceae*, particularly to the genera *Escherichia*, *Citrobacter*, and *Erwinia* (Enei et al., 1972; Kumagai et al., 1970a). In addition to the physiological reaction, TPL also efficiently catalyzes the β-elimination of a number of β-substituted amino acids with good leaving groups, such as *S*-alkylcysteines, β-chloroalanine (Kumagai et al., 1970b), and S-(*o*-nitrophenyl)-L-cysteine (SOPC) (Phillips, 1987). Moreover, TPL has been shown to catalyze the racemization of alanine (Kumagai et al., 1970c; Chen & Phillips, 1993). The three-dimensional structure of the apoenzyme of TPL from *Citrobacter intermedius* has been reported at a resolution of 2.3 Å. The backbone fold of the large, pyridoxal 5′-phosphate binding domain and the location of the active site are similar to that found in aspartate aminotransferase (AspAT) (Antson et al., 1993), even though the amino acid sequence of TPL from *Citrobacter freundii* is quite different from that of *Escherichia coli* AspAT (Smith et al., 1989). However, most of the residues of AspAT which are utilized in binding PLP (Kirsch et al., 1984) are conserved in TPL.

Crystallographic analyses (Jansonius & Vincent, 1987; Smith et al., 1989) have shown that Tyr70 in AspAT is positioned close to the 5′-phosphate group of PLP, and a

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[1] Abbreviations: TPL, tyrosine phenol-lyase (EC 4.1.99.2); PLP, pyridoxal 5′-phosphate; SOPC, *S*-(*o*-nitrophenyl)-L-cysteine; LDH, L-lactate dehydrogenase (EC 1.1.1.27); Trpase, tryptophan indole-lyase (EC 4.1.99.1); AspAT, aspartate aminotransferase (EC 2.6.1.1).

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hydrogen bond is formed between the phenolic hydroxyl group of Tyr70 and the coenzyme phosphate. Toney and Kirsh (1987, 1991a,b) and Inoue et al. (1991) have studied the mutant of AspAT, in which Tyr70 is replaced by phenylalanine by site-directed mutagenesis. Both groups have shown that the main function of Tyr70 in AspAT is to stabilize the holoenzyme complexes and prevent the dissociation of the coenzyme from the enzyme molecule. X-ray crystallographic studies demonstrated that mutation of Tyr70 to Phe did not alter the active-site conformation of the enzyme (Smith et al., 1989; Inoue et al., 1991).

Tryptophan indole-lyase (Trpase) is another member of the family of β-eliminating lyases. The chemical mechanisms proposed for TPL and Trpase are very similar (Kiick & Phillips, 1988a,b). The Trpases from *E. coli* and *Proteus vulgaris* (Kamath & Yanosfsky, 1992) have substantial sequence homology with *C. freundii* TPL (Antson et al., 1993). Recent crystallographic studies of the holoenzyme of *P. vulgaris* Trpase have revealed that the spatial structure of Trpase is virtually identical with that of *C. intermedius* TPL (Dementieva et al., 1994). Furthermore, the tyrosine residue corresponding to Tyr71 is conserved in all known seqences of TPL and Trpase (Chen & Phillips, 1995). Hence, we were interested to determine if these tyrosine residues in TPL and Trpase would play a similar role to that of Tyr70 in AspAT.

The *tpl* gene has been cloned from *C. freundii* (Antson et al., 1993), *Erwinia herbicola* (Iwamori et al., 1992), *C. intermedius* (Kurusu et al., 1991), and *Symbiobacterium thermophilum* (Hirahara et al., 1993). We have now replaced Tyr71 in TPL by phenylalanine using site-directed mutagenesis (Phillips et al., 1994), and we have studied the catalytic properties of the resultant Y71F TPL. The results suggest that Tyr71 plays a critical role for both PLP binding and in the β-elimination reaction.

MATERIALS AND METHODS

Materials. Lactate dehydrogenase (LDH) from rabbit muscle, PLP, and NADH were purchased from United States Biochemical Co. (USB), as were D-alanine, L-alanine, L-tyrosine, *S*-benzyl-L-cysteine, and *S*-ethyl-L-cysteine. L-Cysteine hydrochloride hydrate and L-phenylalanine were obtained from Sigma. *S*-Methyl-L-cysteine was a product of ICN Biochemicals. *S*-(*o*-nitrophenyl)-L-cysteine (SOPC) (Phillips et al., 1989) and 3-fluoro-L-tyrosine (Phillips et al., 1990) were prepared as previously described. [α-^{2}H]-3-Fluoro-L-tyrosine was prepared by performing the enzymatic synthesis reaction in 99% [^{2}H]H_2O, as previously described for L-tyrosine (Kiick & Phillips, 1988a). The content of the α-[^{2}H] was determined to be >98% by ^{1}H NMR.

Preparation of Y71F TPL. Plasmid pTZTPL, which contains the *tpl* gene from *C. freundii* (Antson et al., 1993), was purified from its host cell, *E. coli* SVS 370. Uracil-containing single-stranded DNA of this plasmid was prepared by introducing pTZTPL into *E. coli* strain CJ236 (*dut*$^-$, *ung*$^-$). Site-directed mutagenesis was performed by the method of Kunkel (1985) using a kit from Bio-Rad. The mutagenic oligonucleotide was GATGAAGCCTTCG-CGGGCA.

Clones obtained after the mutagenesis procedure 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. A primer complementary to nucleotides 847−864 (as defined in Antson et al., 1993) was used for the sequencing. Both clones that were selected for sequencing contained the desired mutation. The resulting plasmid containing the Tyr71 to Phe change was designated pTZTPL Y71F. *E. coli* SVS370 cells were used as the host for the plasmid, since these cells are *tnaA*$^-$, 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 enzyme was purified as previously described (Chen & Phillips, 1995).

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 described by Morino and Snell for Trpase (1970). Reaction mixtures contained, in a total volume of 1 mL, 50 mM potassium phosphate, pH 8.0, 5 mM 2-mercaptoethanol, 50 μM PLP, 0.2 mM NADH, 0.18 mg of lactate dehydrogenase, and various amounts of amino acid substrate at 25 °C. The reaction was initiated by the addition of enzyme solution (10 μL). Enzyme activity during purification was routinely measured with 0.6 mM 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}). A unit of TPL activity is defined as the amount of enzyme which produces 1 μmol of product/min. 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 tyrosine phenol-lyase. Steady-state kinetic data were fit to the Michaelis−Menten equation (eq 2), using ENZFITTER (Elsevier).

$$v = k_{cat}[E_0][S]/(K_m + [S]) \quad (2)$$

Protein Determination. Protein was determinated 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 kDa (Antson et al., 1993). PLP content 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 Å C18 column (4.6 × 250 mm) equilibrated with 0.1% trifluoroacetic acid/20% acetonitrile, and eluted with a linear gradient from 20% to 80% 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.

Spectra of Tyrosine Phenol-Lyase. Prior to performing the measurement of the CD spectra, 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 (Excellose, Pierce) equilibrated with 50 mM phosphate buffer, pH 8.0. Circular dichroism spectra were measured with a Jasco J500C spectropolarimeter. Apo Y71F TPL was prepared by repetitive precipitation with $(NH_4)_2SO_4$, and the spectroscopic titration was performed by addition of aliquots of PLP to the enzyme solution in 0.05 M potassium phosphate, pH 8, with measurement of spectra after 30 min of incubation. A

Table 1: Steady-State Kinetic Constants for Wild-Type and Y71F TPL

substrate	wild-type TPL			Y71F TPL			$(k_{cat})_{Y71F}/(k_{cat})_{WT}$	$(k_{cat}/K_m)_{Y71F}/K_{cat}/K_m)_{WT}$
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})		
L-tyrosine	3.5	0.20	1.8×10^4	$<1.3 \times 10^{-5}$				
S-ethyl-L-Cys	3.9	6.6	5.9×10^2	2.1×10^{-4}	0.30	0.72	5.4×10^{-5}	1.2×10^{-3}
S-methyl-L-Cys	0.9	3.4	2.8×10^2	3.8×10^{-4}	0.52	0.72	4.2×10^{-4}	2.6×10^{-3}
S-benzyl-L-Cys	0.5	0.2	2.7×10^3	1.2×10^{-4}	0.05	2.4	2.4×10^{-4}	8.9×10^{-4}
SOPC	5.1	0.10	4.6×10^4	0.25	0.16	1.6×10^3	0.049	0.035
β-chloro-L-Ala	0.7	2.0	3.5×10^2	0.053	3.2	16.4	0.076	0.047
O-benzoyl-L-Ser	8.3	2.9	2.9×10^3	0.15	0.8	1.9×10^2	0.018	0.066

Cary 1E spectrophotometer was used to measure absorption spectra, time course spectra, and time course measurements at single wavelengths for Y71F TPL, while a single wavelength stopped-flow spectrophotometer and rapid scanning stopped-flow spectrophotometer were used to measure the time courses for wild-type TPL at single wavelengths and time dependent spectra, respectively, as described previously (Chen & Phillips, 1993). The concentration dependence of relaxations was fit to a hyperbolic equation for first-order reactions preceded by a rapid binding equilibrium (eq 3), where k_f is the rate constant for the forward

$$1/\tau_{obs} = k_f[L]/(K_{eq} + [L]) + k_r \quad (3)$$

reaction and k_r is the rate constant for the reverse reaction.

RESULTS

Characterization of Y71F Tyrosine Phenol-Lyase. The desired mutation was confirmed by automated DNA sequencing in the region of the mutation. In addition, the mass spectra of wild-type and Y71F 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). Y71F TPL exhibited a molecular mass of 51 421.8 ± 4.9 Da. The difference (WT − Y71F) of 15.2 Da is in good agreement with the expected loss of 16 Da for the Tyr to Phe mutation.

Catalytic Activity of Tyrosine Phenol-Lyases. The steady-state kinetic parameters of wild-type and Y71F mutant TPL for six substrates are shown in Table 1. The mutation of Tyr71 to Phe results in an enzyme with no measurable activity for L-tyrosine; thus, we can estimate that the lower limit for the reduction of activity is 3×10^5-fold. We have also examined the reaction of Y71F TPL with 3-fluoro-L-tyrosine, which is a good substrate for wild-type TPL (Faleev et al., 1988; Chen et al., 1995), but no measureable activity was observed for this substrate. However, Y71F TPL can use *S*-alkylcysteines as substrates (Table 1), but these reactions exhibit k_{cat} values reduced by 10^3-10^4-fold and k_{cat}/K_m values reduced by 10^2-10^3-fold compared to the reactions catalyzed by wild-type TPL. The K_m values for these substrates are also decreased by 10-fold, compared with those of wild-type TPL (Table 1). Y71F TPL can also use β-chloro-L-alanine, *O*-benzoyl-L-serine, and SOPC as substrates, exhibiting about 7%, 1.85%, and 4.9% of the k_{cat} of the wild-type enzyme, respectively (Table 1). The k_{cat}/K_m values for these latter substrates are reduced only 16−30-fold compared to wild-type TPL (Table 1). The time courses of the reactions of Y71F TPL with substrates are nonlinear, with a rather long time lag (2−5 min) which is dependent on [PLP], before the linear steady-state region. Therefore, we determined the reaction velocities from the slopes in the linear region. We found that the activities for SOPC and *S*-methylcysteine are PLP dependent, but there is no significant change in steady-state activity when [PLP] is higher than 30 μM. The PLP concentration used for all the assays was 50 μM. Toney and Kirsch (1987, 1991b) found that the Y70F mutant of AspAT also shows nonlinear kinetics, and linearity can be restored by adding an excess of PLP.

Spectra of Tyrosine Phenol-Lyases. The absorption band near 420 nm in wild-type TPL is generally assigned to the internal aldimine of PLP formed with lysine 257 (Antson et al., 1993). Y71F TPL as isolated in buffers containing 0.1 mM PLP contains a substoichimetric amount (one preparation contained 0.27 mol of PLP/mol of TPL) of PLP, most of which is apparently unbound, as it can be readily removed by dialysis or $(NH_4)_2SO_4$ precipitation. However, Y71F TPL exhibits a visible absorption peak at 420 nm in buffers containing 0.5 mM PLP. Under these conditions, Y71F TPL exhibits a positive CD band for the internal aldimine with λ_{max} at 421 nm, similar to wild-type TPL. We have attempted to perform titration of Y71F TPL using the visible absorption at 420 nm. Because of the weak binding and high absorbance background at millimolar [PLP], it was not possible to saturate the enzyme, but we are able to estimate that Y71F TPL binds PLP with a K_D of about 1 mM, much weaker than wild-type TPL, which has a K_D of 0.6 μM (Kazakov et al., 1988). However, the similarity in the shape and position of the absorption and CD spectra of Y71F TPL suggests that the structure of the PLP binding site is not significantly changed by the mutation of Tyr71 to phenylalanine.

Reactions of Tyrosine Phenol-Lyases with Phenylalanine and Tyrosine. It has been found that L-phenylalanine, L-alanine, and D-alanine can interact with wild-type TPL and form stable quinonoid complexes which exhibit visible absorbance peaks at about 502 nm (Kumagai et al., 1970b; Faleev et al., 1988; Chen & Phillips, 1993), although they are not substrates of TPL for the β-elimination reaction. When an excess of L-phenylalanine (10 mM), L-alanine (0.25 M), or D-alanine (0.25 M) is incubated with Y71F TPL, absorption peaks at 502 nm are also observed (Figure 1A), that form much more slowly than those of wild-type TPL. The final absorbance of theY71F TPL complex at 502 nm with L-phenylalanine at pH 8 (0.77 for 1 mg/mL enzyme; $\epsilon_{502} = 3.96 \times 10^4$ M^{-1} cm^{-1}) is much higher than that of the wild-type TPL complex with L-phenylalanine (0.4 for 1 mg/mL enzyme; Faleev et al., 1988; $\epsilon_{502} = 2.1 \times 10^4$ M^{-1} cm^{-1}) under the same reaction conditions. L-Phenylalanine is a competitive inhibitor of wild-type TPL, with a K_i of about 2 mM (Kiick & Phillips, 1988a). L-Phenylalanine is also a competitive inhibitor of Y71F TPL; however, the K_i

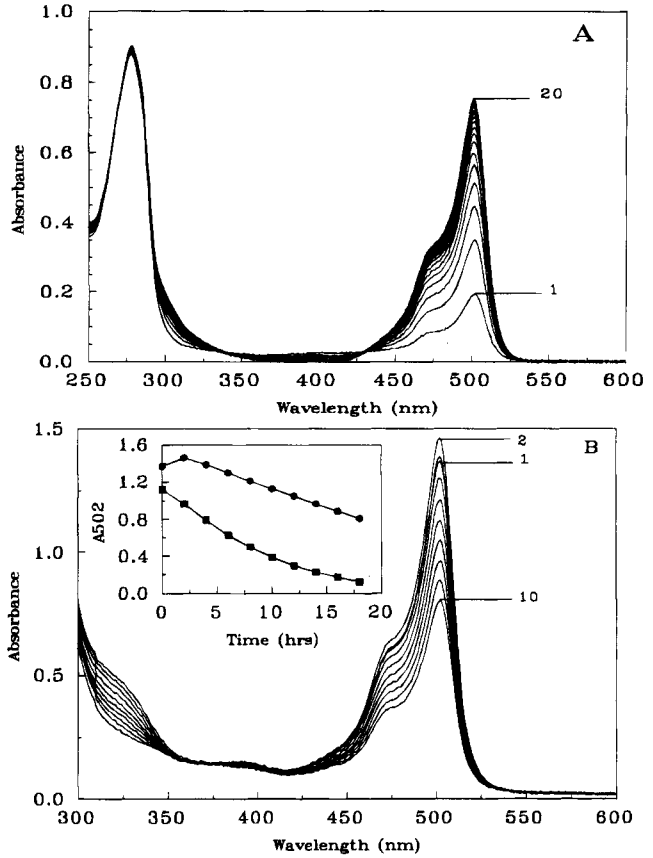


FIGURE 1: Absorbance changes during reaction of Y71F TPL with L-phenylalanine and L-tyrosine. (A) The solution contained Y71F TPL (19.4 μM) in 0.05 M potassium phosphate, pH 8.0, 0.1 mM PLP, and 1 mM L-phenylalanine. Scans were taken at 0.5 min intervals. (B) The solution contained Y71F TPL (38.8 μM) in 0.05 M potassium phosphate, pH 8.0, 38.8 μM PLP, and 1 mM L-tyrosine at 25 °C. Scans were taken at 2 h intervals. Inset: The absorbance at 502 nm of this solution (filled circles) as a function of time. The filled squares are from a similar reaction mixture containing 20 mM L-phenylalanine.

value is reduced 15-fold to 130 μM. In constrast, the absorbances of the Y71F TPL complexes at 502 nm with L-alanine (0.03 for 1 mg/mL enzyme) and with D-alanine (0.02 for 1 mg/mL enzyme) are much less than those of the wild-type TPL complexes under the same reaction conditions (0.35 for 1 mg/mL enzyme for L-alanine and 0.27 for 1 mg/mL enzyme for D-alanine (Chen and Phillips, 1993)). The CD spectra of the wild-type TPL complex with L-phenylalanine and the Y71F TPL complex with L-phenylalanine are virtually identical, exhibiting a broad positive CD band between 470 and 490 nm (Figure 2). Therefore, based on the absorption and CD spectra, Y71F TPL can form quinonoid intermediates with amino acids that are very similar or identical to those formed by wild-type TPL.

Pre-steady-state kinetic studies of the reaction of L-phenylalanine with Y71F mutant TPL demonstrated that the formation of the quinonoid intermediate was biphasic; the fast phase, with a very small amplitude, was detected by using a single-wavelength stopped-flow spectrophotometer. The observed rate constant for the fast phase was found to increase in a hyperbolic manner with increasing [L-phenylalanine]. The rate constants, k_f and k_r, and the binding constant K_{eq} were determined to be 10 s^{-1}, 1.0 s^{-1} and 20.5 mM, respectively (Table 2), by fitting the data to eq 3. These constants are not significantly different from those for

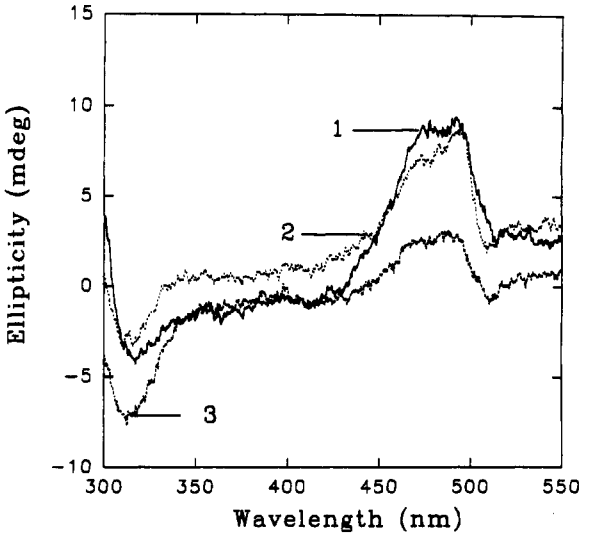


FIGURE 2: Circular dichroism spectra of TPL complexes with L-phenylalanine and L-tyrosine in 50 mM potassium phosphate buffer, pH 8.0, at 25 °C. Curve 1, wild-type TPL (43 μM) with L-phenylalanine (50 mM); curve 2, Y71F mutant TPL (37 μM) with L-phenylalanine (50 mM); curve 3, Y71F mutant TPL (74 μM) with L-tyrosine (1 mM).

quinonoid intermediate formation in the reaction of wild-type TPL with L-phenylalanine (Table 2). The slow phase, with a very large amplitude (70-fold greater than that of the fast phase), can be measured by using a conventional spectrophotometer (Figure 1A). The relaxation rate of the slow phase was also dependent on [L-phenylalanine] in a hyperbolic manner. The rate constants, k_f and k_r, and the binding constant, K_{eq}, were found to be 4.0 × 10^{-3} s^{-1}, 1.7 × 10^{-3} s^{-1}, and 3.0 mM, respectively (Table 2).

When either L-tyrosine or 3-fluoro-L-tyrosine are incubated with Y71F TPL, a strong absorbance peak at 502 nm forms, with an isosbestic point between the 400 and 502 nm peaks, indicating that these two species directly interconvert. This reaction is very slow, requiring several minutes to reach a maximum absorbance, compared to the reactions of L-tyrosine or 3-fluoro-L-tyrosine with wild-type enzyme, which are complete within 100 ms (Muro et al., 1978; Chen & Phillips, 1995); however, this behavior is very similar to the reaction of Y71F TPL with L-phenylalanine (Figure 1A). It is interesting that L-tyrosine forms a complex that exhibits a prominent 502 nm peak with Y71F TPL (Figure 1B), with absorbance comparable to that of the L-phenylalanine complex, while L-tyrosine forms a complex with wild-type TPL with weak 502 nm absorbance (Muro et al., 1978). The spectra of the L-tyrosine and L-phenylalanine complexes were found to be stable for at least 2 days at 25 °C in the presence of excess PLP. However, if only a stoichiometric concentration of PLP is present, the absorbance at 502 nm slowly decreases, concomitant with an increase at about 325 nm (Figure 1B), consistent with a slow abortive transamination process, as has been reported for wild-type TPL (Demidkina et al., 1987). The decay of these quinonoid species (inset, Figure 1B) is first-order, with a rate constant of 1.0 × 10^{-5} s^{-1} for L-tyrosine and 3.3 × 10^{-5} s^{-1} for L-phenylalanine. The rate constant for the decay of the Y71F TPL L-phenylalanine quinonoid complex is 24-fold slower than that reported for the abortive transamination of L-phenylalanine (8 × 10^{-4} s^{-1}) by wild-type TPL (Demidkina et al., 1987). Although it is not a substrate, L-tyrosine is a competitive

Table 2: Pre-Steady-State Kinetic Constants for Wild-Type and Y71F TPL

amino acid	wild-type			Y71F		
	K_D (mM)	k_f (s^{-1})	k_r (s^{-1})	K_D (mM)	k_f (s^{-1})	k_r (s^{-1})
3-fluoro-L-Tyr	0.8	140	6.9	1.9	3.0×10^{-3}	8.8×10^{-4}
[α-^{2}H]-3-fluoro-L-Tyr	0.3	23	9.5	1.9	2.9×10^{-3}	9.1×10^{-4}
S-ethyl-L-Cys	4.9	59	3.9	10.3	3.1×10^{-3}	1.5×10^{-3}
S-methyl-L-Cys	21.6	28	1.4	29.0	12.6	0.7
S-methyl-L-Cys (slow phase)				6.9	1.3×10^{-3}	9.6×10^{-4}
S-benzyl-L-Cys	1.8	41	4.2	0.5	1.5×10^{-3}	6.7×10^{-4}
[α-^{2}H]-*S*-benzyl-L-Cys	1.5	7	3.2	0.4	7.5×10^{-4}	3.0×10^{-4}
L-phenylalanine	17.4	13	5.1	20.5	10	1.0
L-Phe (slow phase)				3.0	4.0×10^{-3}	1.7×10^{-3}

inhibitor of Y71F TPL using SOPC as substrate, with a K_i of 110 μM, similar to that for L-phenylalanine. The Y71F TPL complex with L-tyrosine exhibits a CD spectrum similar to that of the wild-type TPL complex with L-phenylalanine (Figure 2). This suggests that both L-tyrosine and L-phenylalanine form very similar quinonoid intermediates with Y71F TPL. It is not possible to observe the CD spectrum of the quinonoid intermediate formed from wild-type TPL and L-tyrosine, due to the very low absorbance at 502 nm during the steady-state of the reaction.

Reactions of S-Alkyl-L-cysteines. We performed similar experiments with *S*-alkyl-L-cysteines, and we also observed the slow formation of intermediates absorbing at 502 nm (Figure 3A). Similar spectra are observed in the reaction of *S*-alkyl-L-cysteines with wild-type TPL (Figure 3B); however, the absorbance changes in Figure 3B are complete in 100 ms. It is noteworthy that there is an isosbestic point at 340 nm in the spectra of Figure 3A. Thus, we are able to simultaneously measure the time courses of quinonoid intermediate formation at 502 nm and pyruvate formation in the LDH coupled assay system at 340. We found that the formation of the quinonoid intermediate corresponds exactly to the time lag in the time course for pyruvate formation (Figure 4). When the absorbance at 502 nm reaches its maximum level, the reaction system is in the steady state; hence, the quinonoid intermediate is catalytically competent. The quinonoid complexes of Y71F TPL with *S*-alkyl-L-cysteines are also very stable, likely due to the very low k_{cat} values of these substrates (Table 1).

The progress curves for quinonoid intermediate formation at 502 nm in the reaction of Y71F TPL with *S*-methyl-L-cysteine are biphasic. Both the fast phase (with a very small amplitude) and the slow phase were found to be dependent on [*S*-methyl-L-cysteine] in a hyperbolic manner. The rate constants and binding constant for the fast phase were found to be not significantly different from those for the reaction of wild-type TPL with *S*-methyl-L-cysteine (Table 2). Furthermore, the rate constants and binding constant for the slow phase were found to be similar to those of the reaction of the Y71F mutant with L-phenylalanine (Table 2).

We have examined the effects of [PLP] on both phases. As shown in Figure 5A, we found that the rate constants of the fast phase are independent of [PLP], while the amplitudes of the fast phase increase with increasing [PLP] (data not shown). In contrast, the rate constants of the slow phase are [PLP]-dependent (Figure 5B), while the amplitudes of the slow phase decrease with increasing [PLP] (data not shown). On the basis of these observations, we propose that the fast phase refers to the α-deprotonation and reprotonation in the formation of the quinonoid intermediate, while the

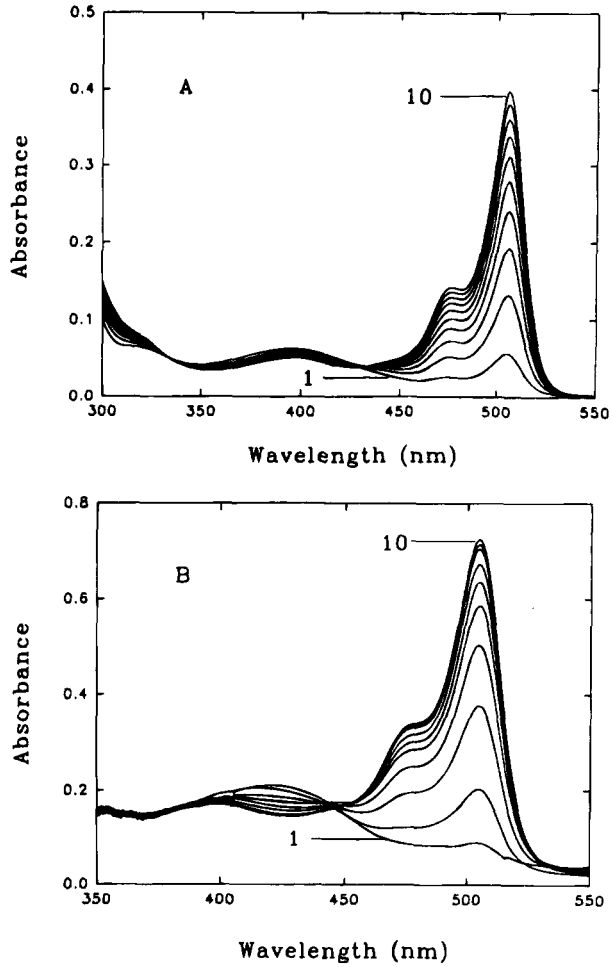


FIGURE 3: Absorption spectra of TPL complexes with *S*-ethyl-L-cysteine in 50 mM potassium phosphate buffer, pH 8.0, at 25 °C. (A) Spectra of Y71F TPL complex (26 μM) with *S*-ethyl-L-cysteine (5 mM) recorded at 0.5-min intervals, beginning at 0.5 min (curve 1). (B) Rapid-scanning spectra of wild-type enzyme complex (35 μM) with *S*-ethyl-L-cysteine (5 mM). Scans were taken at 0.01-s intervals, beginning at 0.01 s (curve 1).

slow phase involves the PLP binding equilibria. A kinetic model shown in Scheme 1 was employed to explicate the mechanism of this reaction. This mechanism is characterized by four relaxation times, since it includes five states. We assume that two of the relaxations are associated with the two substrate binding steps, and they are too fast to be measured by our stopped-flow methods. The fast phase refers to the relaxation between the PLP-bound ES and EQ. The rate equation for this relaxation time is the same as eq 3, in which k_f is the rate of deprotonation, k_r is the rate of reprotonation, and K_{eq} is the binding constant of substrate

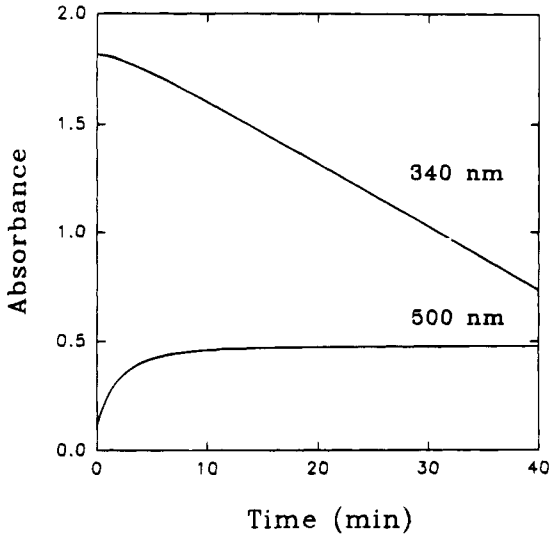


FIGURE 4: Time courses of quinonoid intermediate (500 nm) and product (pyruvate) (340 nm) formation in the reactions of Y71F mutant TPL (218 μM) with *S*-methyl-L-cysteine (10 mM). Pyruvate produced by Y71F mutant TPL from *S*-methyl-L-cysteine was reduced by LDH with NADH (see Materials and Methods).

to the holoenzyme. The rate constants and binding constants of the fast phase for both L-phenylalanine and *S*-methyl-L-cysteine demonstrate that the replacement of Tyr71 by Phe has no significant effects on binding of the substrates to holoenzyme, and on the rates of α-deprotonation and reprotonation. Because the rate equation does not contain a [PLP] term, the relaxation shows [PLP]-independent kinetic behavior. However, the presence of PLP converts the apoenzyme to holoenzyme; thus, the amplitude of the fast phase increases with increasing [PLP].

The slow phase is due to the relaxations between apoenzyme and holoenzyme, and substrate-bound apoenzyme and substrate-bound holoenzyme. By using the methods described by Bernasconi (1976), the rate equation is given by:

$$1/\tau = k_4[\text{PLP}]K_2/(S + K_2) + k_{-4}K_1/((1 + k_3/k_{-3})S + K_1) + k_5[\text{PLP}]S/(S + K_2) + k_{-5}S/((1 + k_3/k_3)S + K_1) \quad (4)$$

The intercept at [substrate] = 0 is equal to the rate of interconversion of the apoenzyme and the holoenzyme (k_4[PLP] + k_{-4}). The replots of the intercepts in Figure 5B against [PLP] are linear (Figure 5C, filled circles) and give $k_4 = 0.6 \times 10^{-3}$ min^{-1} μM^{-1}, and $k_{-4} = 0.07$ min^{-1}. Thus, in the absence of *S*-methyl-L-cysteine, the binding constant of PLP to Y71F TPL can be calculated as 117 μM, about 9-fold stronger than the results of spectroscopic titration. Equation 4 predicts that the maximum value of $1/\tau$ at infinite [substrate] is for the interconversion of the substrate-bound apoenzyme and the substrate-bound holoenzyme. The replot of the values in Figure 5B against [PLP] is also linear (Figure 5C, open circles) and gives $k_5 = 1.4 \times 10^{-3}$ min^{-1} μM^{-1} and $k_{-5} = 0.06$ min^{-1}. Hence, in the presence of *S*-methyl-L-cysteine, the apparent binding constant of PLP to Y71F TPL can be calculated as 43 μM.

The time courses of the reactions of Y71F TPL with other *S*-alkylcysteines (*S*-benzylcysteine, and *S*-ethylcysteine) also exhibit two exponential phases. For L-tyrosine and 3-fluoro-L-tyrosine, the fast phase is not detectable in the stopped-flow spectrophotometer, probably due to the combination

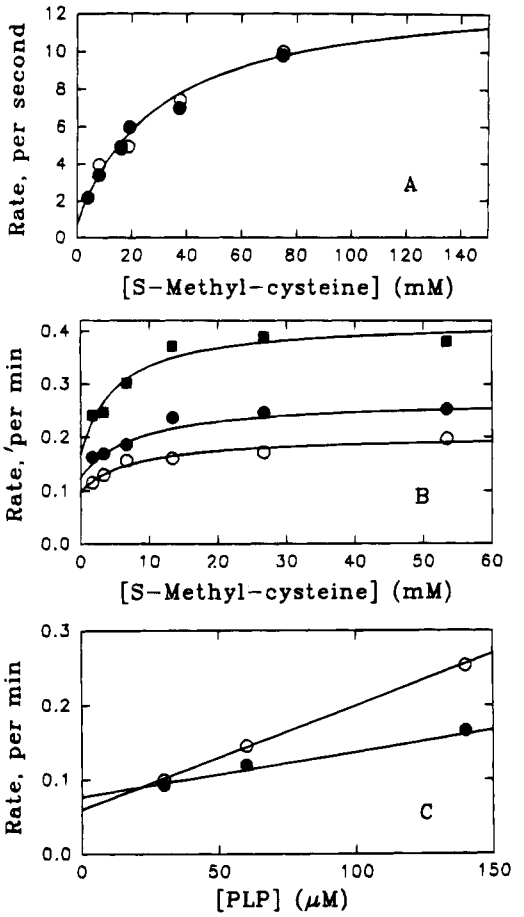
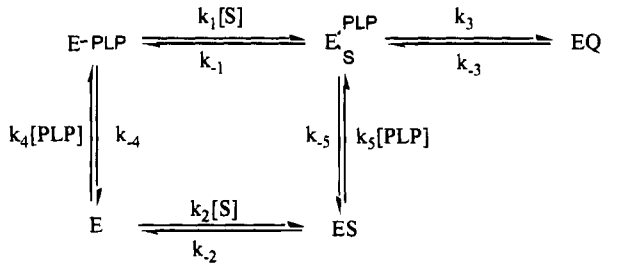


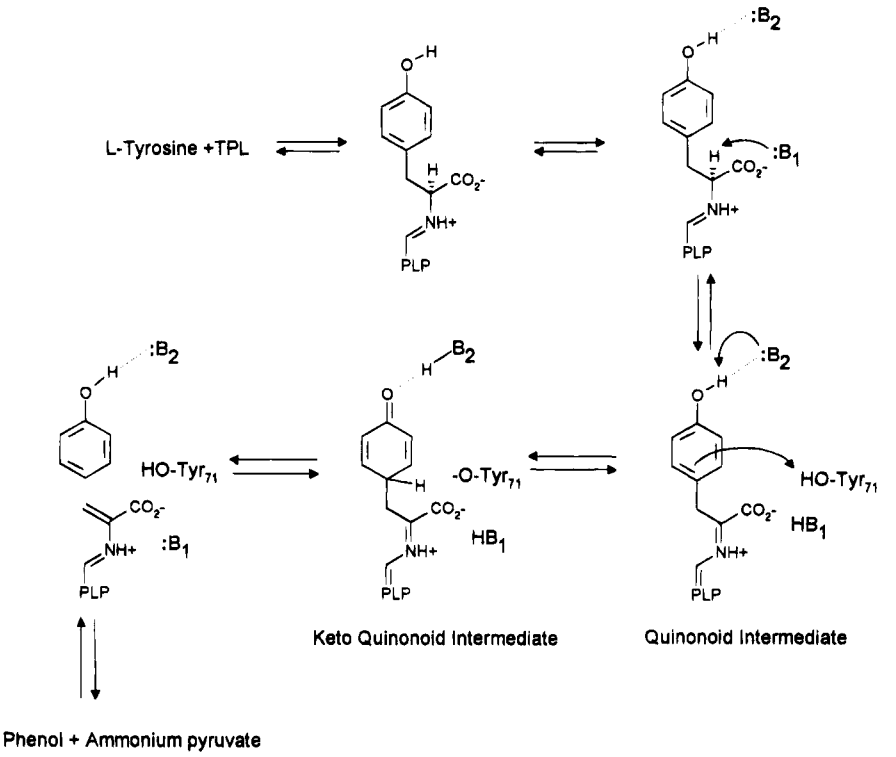
FIGURE 5: Effects of PLP on the pre-steady-state kinetic measurements for the reactions of the reactions of Y71F TPL with *S*-methyl-L-cysteine. (A) Dependence of the fast phase relaxation on the concentration of *S*-methyl-L-cysteine in the presence of 30 μM PLP (open circles) and 60 μM PLP (filled circles). (B) Dependence of the slow phase relaxation on the concentration of *S*-methyl-L-cysteine in the presence of 30 μM PLP (open circles), 60 μM PLP (filled circles), and 140 μM PLP (filled squares). (C) Plots of the values at [*S*-methyl-L-cysteine] = 0 (filled circles) or at infinite [*S*-methyl-L-cysteine] (open circles) *versus* [PLP].

Scheme 1



of low amplitude and high rates. The rate constants for the slow phase of the reactions of all amino acids are similar, as shown in Table 2. These results suggest that the slow phase may refer to the same reaction step. Comparing the rate constants, k_f, for 3-fluoro-L-tyrosine to that for [α-^{2}H]-3-fluoro-L-tyrosine, we find the ratio is almost equal to 1 (Table 2). This demonstrates that the rate-determining step in the slow phase of quinonoid intermediate formation is not due to α-deprotonation. We have found that the rate constants for the slow phase of these reactions are also [PLP]-dependent (data not shown). Thus, the slow phase of these reactions must involve PLP binding.

Scheme 2



DISCUSSION

Toney and Kirsch (1987, 1991b) reported that the replacement of Tyr70 in AspAT by phenylalanine results in the diminished binding of PLP or PMP. This was ascribed to the loss of the hydrogen bond between the hydroxyl group of Tyr70 and coenzyme phosphate oxygen OP2 (Smith et al., 1989; Toney & Kirsch, 1991b). We have also found that the dissociation constant of Y71F TPL for PLP is higher ($K_D \cong 1$ mM) than for wild type TPL ($K_D = 0.6$ μM, Demidkina and Myagkikh (1989)). The Tyr71 residue in the apoenzyme of TPL is in a similar location in the active site to that of Tyr70 in holoAspAT (Antson et al., 1993). Hence, we believe that the weaker binding of PLP to Y71F TPL is also due to the loss of a hydrogen bond between the hydroxyl group of Tyr71 and a PLP phosphate oxygen. The approximately 1700-fold tighter binding of PLP to wild-type TPL represents a 4.4 kcal/mol difference in free energy at 25 °C contributed by the hydrogen bond to the stability of bound PLP in the internal aldimine. Toney and Kirsch (1991b) have reported that the replacement of Tyr70 in AspAT by Phe results in a 2.7 kcal/mol difference in free energy for bound PLP.

We have found that Y71F TPL forms quinonoid intermediates which have absorbance and CD spectra similar to those formed by wild-type TPL. These results imply that the hydroxyl group of Tyr71 is not an essential catalyst for the formation of quinonoid intermediates. Furthermore, the rate constants and binding constants for the formation of quinonoid intermediates in the reaction of Y71F TPL with L-phenylalanine are very similar to those in the reaction of wild-type TPL with L-phenylalanine (Table 2). The rate constant for the deprotonation step in the reaction of the Y71F TPL with *S*-methyl-L-cysteine is only 2-fold smaller than that in the reaction of the wild-type enzyme with *S*-methylcysteine (Table 2). Therefore, the contribution of Tyr71 to the α-deprotonation of external aldimines to form quinonoid intermediates is negligible.

Since the rate constants of the PLP binding steps and α-deprotonation steps in the reactions of Y71F TPL with *S*-alkylcysteines are much larger than the k_{cat} values for these reactions (Tables 1 and 2), PLP binding and quinonoid intermediate formation cannot be rate-determining. These results, along with the accumulation of quinonoid intermediates (Figures 1 and 3), imply that the rate-determining step for these reactions is the leaving group elimination. The replacement of Tyr71 by phenylalanine reduces the k_{cat} value for tyrosine by $>10^5$-fold, and for *S*-alkylcysteines by 10^3–10^4 fold (Table 1). Thus, it is likely that the hydroxyl group of Tyr71 plays an essential role in the β-elimination process. In contrast, it has been shown that Tyr70 in AspAT is not an essential component of the catalytic apparatus (Toney & Kirsch, 1991a). The results reported herein demonstrate that conserved residues in structurally homologous enzymes may not always have identical catalytic functions.

In the reaction of TPL with L-tyrosine, it has been proposed that there is an additional step involved in activation of the carbon–carbon bond between C-1 and C-β of tyrosine before the C–C bond cleavage to the aminoacrylate intermediate and phenol release from the enzyme (Scheme 2) (Faleev et al., 1988; Kiick & Phillips, 1988). Thus, after formation of the quinonoid intermediate, the substrate hydroxyl proton is removed, increasing electron density at C-1 of the substrate. A proton is concomitantly donated to C-1 of the substrate to permit formation of the cyclohexadienone tautomer, shown in Scheme 2 as the keto quinonoid intermediate. On the basis of our mutagenesis results, the hydroxyl group of Tyr71 may be the general acid catalyst required for the formation of this intermediate. Y71F TPL is perfectly competent to form the first quinonoid intermediate from L-tyrosine (Figure 1B), but is unable to proceed to the keto quinonoid

intermediate, and the reaction comes to a halt at this point. Previously, it was found that the proton abstracted from C-α of the substrate is partially transferred to C-4 of the phenol product (Faleev et al., 1983; Palcic et al., 1987), suggesting the involvement of a single acid/base group. However, this would also be observed if there is proton transfer from the conjugate acid of the base, B1, which removes the α-proton, to the Tyr71 anion (Scheme 2). In the next catalytic cycle, there would be partial transfer of label to C-4 of the phenol. It is also possible that the Y71F mutation causes a subtle conformational change that affects the ability of the enzyme to protonate weak leaving groups, wtihout direct participation of Tyr71 in proton transfer. However, recent X-ray crystallographic measurements of the holoTPL complex with a competitive inhibitor, 3-(4′-hydroxyphenyl)propionic acid, show that the phenolic oxygen of Tyr71 is within van der Waals contact distance (3.1 Å) of the C-1′ of the substrate analog (T. V. Demidkina and A. A. Antson, unpublished), as expected if proton transfer from the Tyr71 OH to the substrate carbon occurs.

There are relatively few examples of tyrosine functioning as a general acid in enzyme reaction mechanisms. However, tyrosine residues have been shown to function as general acid catalysts in the mechanism of ketosteroid isomerase (Kuliopulos et al., 1989; Brooks & Benisek, 1994) and aldose reductase (Bohren et al., 1994). Because the reaction of Y71F TPL with *S*-alkyl-L-cysteines also shows very low activity, the hydroxyl group of Tyr71 may donate a proton to the thiolate leaving group (with a pK_a of 10.5 for ethanethiol). In contrast, we have found that SOPC, *O*-benzoyl-L-serine, and β-chloro-L-alanine show relatively high activity with Y71F TPL. This may be due to the low pK_a values of the conjugate acids of the leaving groups of these substrates (*o*-nitrothiophenol, with a pK_a of 5.2, benzoic acid, with a pK_a of 4.2, and HCl, with a pK_a of −7, respectively). Thus, since these leaving groups form stable anions at pH 7, they do not require protonation for elimination to proceed. However, even these substrates have significantly lower k_{cat} (1.8−7.6%) and k_{cat}/K_m values (3.5−6.6%) than for wild-type TPL (Table 1). Thus, the OH of Tyr71 must also contribute to transition state stabilization in these elimination reactions, possibly by hydrogen bond donation to the incipient leaving group in the transition state. Surprisingly, we find that L-phenylalanine is bound more strongly (K_i = 130 μM) to Y71F TPL than to wild-type TPL (K_i = 2 mM). This suggests that the proximity of the polar OH of Tyr71 to the substrate aromatic ring results in ground-state destabilization, which is used by the enzyme to facilitate the formation of the high-energy cyclohexadienone ring in the keto quinonoid intermediate.

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