Role of Lysine-256 in *Citrobacter freundii* Tyrosine Phenol-lyase in Monovalent Cation Activation†

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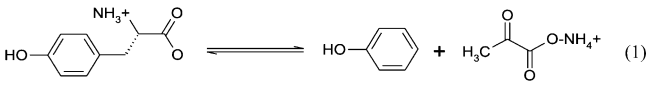
ABSTRACT: Tyrosine phenol-lyase (TPL) from *Citrobacter freundii* is dependent on monovalent cations, K^+ or NH_4^+, for high activity. We have shown previously that Glu-69, which is a ligand to the bound cation, is important in monovalent cation binding and activation [Sundararaju, B., Chen, H., Shillcutt, S., and Phillips, R. S. (2000) *Biochemistry 39*, 8546−8555]. Lys-256 is located in the monovalent cation binding site of TPL, where it forms a hydrogen bond with a structural water bound to the cation. This lysine residue is highly conserved in sequences of TPL and the paralogue, tryptophan indole-lyase. We have now prepared K256A, K256H, K256R, and E69D/K256R mutant TPLs to probe the role of Lys-256 in monovalent cation binding and activation. K256A and K256H TPLs have low activity (k_{cat}/K_m values of 0.01−0.1%), are not activated by monovalent cations, and do not exhibit fluorescence emission at 500 nm from the PLP cofactor. In contrast, K256R TPL has higher activity (k_{cat}/K_m about 10% of wild-type TPL), is activated by K^+, and exhibits fluorescence emission from the PLP cofactor. K256A, K256H, and K256R TPLs bind PLP somewhat weaker than wild-type TPL. E69D/K256R TPL was prepared to determine if the guanidine side chain could substitute for the monovalent cation. This mutant TPL has wild-type activity with *S*-Et-L-Cys or *S*-(*o*-nitrophenyl)-L-Cys but has no detectable activity with L-Tyr. E69D/K256R TPL is not activated by monovalent cations and does not show PLP fluorescence. In contrast to wild-type and other mutant TPLs, PLP binding to E69D/K256R is very slow, requiring several hours of incubation to obtain 1 mol of PLP per subunit. Thus, E69D/K256R TPL appears to have altered dynamics. All of the mutant TPLs react with inhibitors, L-Ala, L-Met, and L-Phe, to form equilibrating mixtures of external aldimine and quinonoid intermediates. Thus, Lys-256 is not the base which removes the α-proton during catalysis. The results show that the function of Lys-256 in TPL is in monovalent cation binding and activation.

Tyrosine phenol-lyase (TPL)[1] catalyzes the reversible *β*-elimination of L-Tyr to form phenol and ammonium pyruvate (eq 1). The enzyme has been isolated and characterized from a number of bacteria, but the enzymes from *Citrobacter freundii* and *Citrobacter intermedius* (*1*) have been studied most extensively. This enzyme requires pyridoxal 5′-phosphate (PLP) (*2*) as a cofactor, as well as a monovalent cation (K^+, NH_4^+, or Rb^+) (*3*, *4*) for maximal activity. The structure of TPL complexed with a competitive inhibitor, 3-(4-hydroxyphenyl)propionic acid (HPPA), has been determined by X-ray crystallography (*5*). The crystals, which were grown in the presence of Cs^+, show that the monovalent cation binding site is located about 9 Å away from the bound substrate and PLP, where it coordinates with one of the carboxylate oxygens of Glu-69. However, Cs^+ is not a strongly activating cation of TPL (*3*, *4*). A structure of TPL from *Erwinia herbicola* has been obtained with K^+ and 5′-phosphopyridoxyl-L-tyrosine bound (Figure 1) (*6*). In this structure, the K^+ is in a similar environment, bound by an O^{ϵ} of Glu-69, and it coordinates to the peptide backbone carbonyl oxygens of Gly-67 and Asn-262, as well as three water molecules (red balls in Figure 1). Mutagenesis of Glu-69 to Gln and Asp has been performed previously, and while E69Q TPL was found to exhibit monovalent cation activation and activity similar to wild-type TPL, E69D TPL has very low activity (0.1%) and is not stimulated by monovalent cations (*4*). Thus, we concluded that Glu-69 is necessary for monovalent cation activation of TPL by transmission of



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

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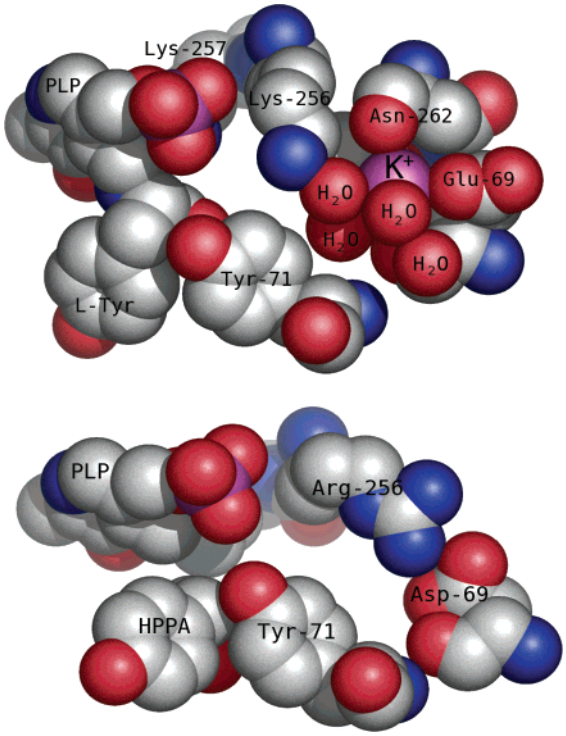

FIGURE 1: Top: Structure of the monovalent cation binding site of *E. herbicola* TPL. Bottom: Model of the E69D/K256R double mutant of *C. freundii* TPL.

structural changes to the active site. Lys-256, which precedes the PLP-binding Lys-257 in the sequence of *C. freundii* TPL, is highly conserved in sequences of TPL. Furthermore, a strictly conserved lysine is located in the same location in the structure of a paralogous enzyme, tryptophan indole-lyase (Trpase), which is also activated by monovalent cations (*7*). The ϵ-amino group of Lys-256 in TPL is located in the structure about 3.5 Å from the monovalent cation, forming a hydrogen bond to a water coordinated to the K^+ ion (Figure 1, top). Thus, on this basis it seemed likely that Lys-256 is also required for monovalent cation binding and activation of TPL. In the present work, we have mutated Lys-256 of wild-type TPL to Ala, His, and Arg to probe the role of this residue in monovalent cation binding and activation. In addition, we have prepared the double mutant E69D/K256R TPL, based on the hypothesis that the Arg mutation could place the guanidinium side chain in the cation site and complement the low activity of E69D TPL (Figure 1, bottom). The results of these studies are reported herein.

MATERIALS AND METHODS

Materials. Lyophilized lactate dehydrogenase (LDH) from rabbit muscle, pyridoxal 5′-phosphate (PLP), and NADH were purchased from USB, as were L-alanine, L-tyrosine, and *S*-ethyl-L-cysteine. L-Cysteine hydrochloride hydrate and L-phenylalanine were products of Sigma. *S*-(*o*-Nitrophenyl)-L-cysteine (SOPC) was prepared as previously described (*8*).

Preparation of Mutant TPL. K256A and K256H TPLs were prepared by the method of Kunkel (*9*) using a MutaGene kit from Bio-Rad. Uracil-containing single-stranded DNA of pTZTPL, which contains the *tpl* gene from *C. freundii* (*10*), was prepared by introducing pTZTPL into *Escherichia coli* strain CJ236 (*dut*$^-$, *ung*$^-$). The mutagenesis was performed according to the directions using the appropriate oligonucleotides with the Lys → Ala and Lys → His mutations.

K256R TPL and the double mutant, E69D/K256R TPL, were prepared using the QuickChange mutagenesis procedure (Stratagene). The wild-type and E69D pTZTPL plasmids (*4*, *10*) were used as templates for the mutagenesis procedure with an oligonucleotide with the desired Lys → Arg mutation. 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. *E. coli* SVS370 cells were used as the host for the plasmids, 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 mutant enzymes were purified as previously described (*11*).

Enzyme Assays. The β-elimination reactions were measured on a Cary 1 UV/vis spectrophotometer with a Peltier-controlled 6 × 6 cell compartment or a SpectraMAX 340PC microplate reader from Molecular Devices, Inc. Reactions of L-tyrosine and *S*-alkyl-L-cysteines were performed 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 (*12*). Reaction mixtures contained, in a total volume of 0.3 mL (microplate reader) or 0.6 mL (Cary 1), 50 mM potassium phosphate, pH 8.0, 5 mM 2-mercaptoethanol, 200 μM PLP, 0.2 mM NADH, 0.01 mg of lactate dehydrogenase, and various amounts of amino acid substrate at 25 °C. The reaction was initiated by the addition of a small amount 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 (*13*), 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 200 μM PLP, with varying amounts of SOPC and appropriate dilutions of TPL. Steady-state kinetic data were fit to the Michaelis−Menten equation (eq 2), using HYPERO (*14*).

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

Protein Determination. The concentration of purified wild-type and mutant TPLs was determined from the absorbance at 278 nm ($E^{1\%} = 8.37$) (*15*) assuming a subunit molecular mass of 51 kDa (10). The PLP content was determined by measuring the spectrum of the enzyme in 0.1 N NaOH, assuming $\epsilon^{390} = 6600$ M^{-1} cm^{-1} (*16*).

Spectra of Tyrosine Phenol-lyase. Prior to measurement of the absorbance spectra, the 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 buffer, pH 8.0. A Cary 1 spectrophotometer was used to measure absorption spectra of mutant TPLs, and an RSM-1000 from OLIS, Inc., equipped with a stopped-flow cell compartment, as previously described (*4*) was used to measure the reaction of mutant TPLs with amino acids. Rate constants were

Table 1: Activity of Wild-Type and Lys-256 Mutant TPLs

	wild type[a]		K256A		K256H		K256R		E69D/K256R	
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})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
L-Tyr	3.5	1.8×10^4	1.0×10^{-3}	14	1.3×10^{-4}	1.3	0.12	87	$<10^{-5}$	$<10^{-2}$
SOPC	5.1	4.6×10^4	9.1×10^{-3}	27	2.7×10^{-2}	55	0.17	185	7.4	2.8×10^4
S-Et-L-Cys	3.9	5.9×10^2	2.5×10^{-3}	3.3	8.8×10^{-3}	55	0.02	18	0.37	2.8×10^3

[a] Data taken from Chen et al. (*11*).

obtained by global fitting of the rapid-scanning stopped-flow spectra with the Global Works program provided by OLIS (*17*). The fluorescence spectra were also measured on the RSM-1000, using a standard cell compartment and a 450 W Xe lamp for excitation, with 8 nm slits on the excitation and emission monochromators.

Modeling of Mutant TPL. In silico mutagenesis of TPL was performed using the SPDBV program (*18*), available by download from http://www.expasy.org/spdbv/. The coordinates of the TPL complex with 3-(4-hydroxyphenyl)-propionic acid (HPPA), PDB accession number 2TPL,were loaded. The cesium and associated waters were deleted, and mutagenesis of Glu-69 to Asp and Lys-256 to Arg was performed. The double mutant structure was then minimized using SPDBV, and the side chain conformations of Arg-256 and Asp-69 were searched manually for conformations which would allow close contact between the carboxylate and guanidinium.

RESULTS

Activity of Lys-256 Mutant TPL. Lysine-256 in wild-type TPL was mutated to Ala, His, and Arg by site-directed mutagenesis to give, respectively, K256A, K256H, and K256R TPLs. In addition, the double mutant, E69D/K256R TPL, was prepared by mutagenesis of E69D TPL. K256A and K256H TPLs exhibit very low, but detectable, activity for β-elimination with L-Tyr, SOPC, and *S*-Et-L-Cys (Table 1), with k_{cat}/K_m values reduced about 100−10000-fold compared to wild-type TPL. In contrast, K256R TPL has k_{cat} reduced only about 10-fold, while k_{cat}/K_m values are reduced about 100-fold. However, the double mutant, E69D/K256R, has k_{cat}/K_m values with SOPC and *S*-Et-L-Cys similar to or greater than wild-type TPL (Table 1) but exhibits no detectable catalytic activity with L-Tyr.

Monovalent Cation Activation. Wild-type TPL is activated about 30-fold by K^+, NH_4^+, and Rb^+ (*3*, *4*). The extremely low activity of K256A and K256H TPLs with L-Tyr is not increased by addition of monovalent cations, K^+, Na^+, Li^+, Rb^+, or NH_4^+ (data not shown). Furthermore, the activity of K256A TPL is also not significantly increased by addition of propylamine up to 50 mM, so there is no apparent "chemical rescue", as has been found with a similar mutant of aspartate aminotransferase (*19*). In contrast, the activity of K269R TPL shows a strong increase (50-fold) in activity with K^+ (Figure 2, circles), similar to the 30-fold activation seen with wild-type TPL (*4*). Fitting of the data in Figure 2 to eq 3, where K_d is the apparent activation constant for the cation and V_0 is the observed rate in the absence of the cation, results in a K_d of 16.7 mM for K^+, about 5-fold higher than the binding of K^+ to wild-type TPL (*4*). Since E69D/K256R TPL does not show detectable activity with L-Tyr, the activation of E69D/K256R by K^+ was examined with *S*-Et-L-Cys as the substrate, and the reaction rate is not significantly affected by addition of K^+ (data not shown).

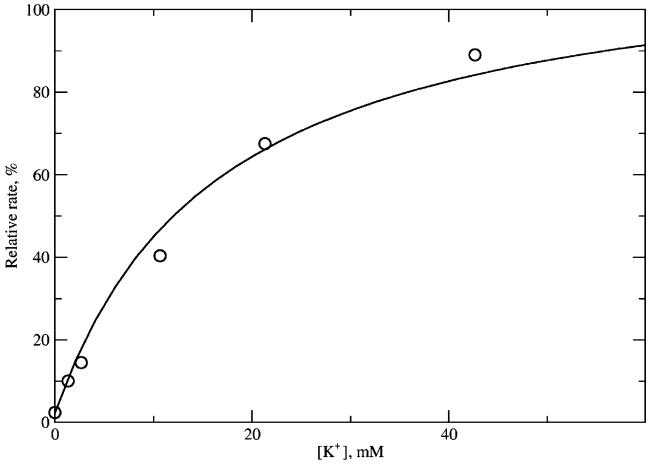

FIGURE 2: Activation of K256R TPL by K^+. Activity was measured with 2 mM L-Tyr, 0.1 mM NADH, and 10 μg/mL lactate dehydrogenase in 0.05 M triethanolamine hydrochloride, pH 8.0, 100 μM PLP, and variable [K^+]. The reaction was followed by the decrease in absorbance at 340 nm. The line is the best fit by nonlinear least squares regression to eq 3.

$$V_{obs} = V_{max}[M^+]/(K_d + [M^+]) + V_0 \qquad (3)$$

Effect of Lys-256 Mutations on PLP Binding. All Lys-256 mutant TPLs have low stoichiometries of PLP/subunit, less than 0.5 mol/subunit, as isolated, while wild-type TPL contains 1 PLP/subunit (*2*, *5*). Addition of PLP into activity assays strongly activates K256A, K256H, and K256R, with apparent K_d values of 34, 66, and 107 μM, respectively (Figure 3), while inclusion of PLP in assays does not increase the activity of E69D/K256R TPL (data not shown). In comparison, wild-type TPL exhibits a K_d for PLP of 19 μM by activity measurement (Figure 3, circles). Thus, the mutations of Lys-256 have a modest effect of weakening PLP binding. It should be noted that the measurement of PLP binding by activity assays typically gives a value higher than the true equilibrium constant, since equilibration is slow, with a half-life on the order of minutes at catalytic concentrations (*20*, *21*). In the case of *E. coli* Trpase, the apparent binding of PLP based on activity is 2 μM (*22*), while the true equilibrium constant for PLP binding is 0.32 μM (*20*). Nevertheless, the comparison of activation of wild-type and mutant TPLs by PLP in Figure 3 shows that the Lys-256 mutations influence PLP binding. Incubation of K256A, K256H, and K256R mutant TPLs at high concentration (~20 mg/mL, ~0.4 mM) with 1 mM PLP for 5 min at room temperature, followed by rapid gel filtration, gave protein with ca. 1 mol of PLP/mol of subunit (Figure 4). However, under the same conditions, E69D/K256R TPL did

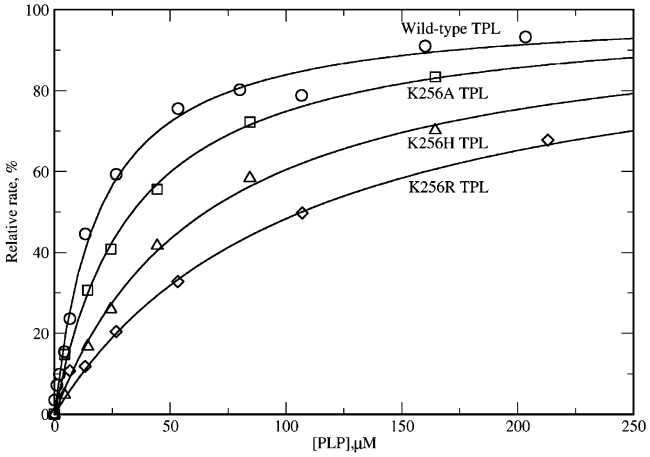

FIGURE 3: Activation of wild-type, K256A, K256H, and K256R TPLs by PLP. The conditions were identical to those used in Figure 1, except that [PLP] was varied and [K^+] was constant at 100 mM. Key: wild-type TPL, circles; K256A TPL, squares; K256H TPL, triangles; K256R TPL, diamonds. The lines are calculated curves using eq 2 and the parameters from fitting the data with HYPERO (*14*).

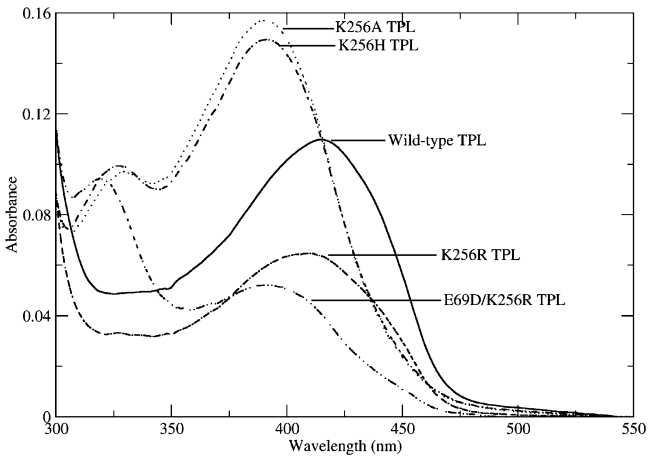

FIGURE 4: Visible absorption spectra of wild-type and Lys-256 mutant TPLs. The solutions contained 1 mg/mL TPL in 0.05 M potassium phosphate, pH 8.0. Key: wild-type TPL (—); K256A TPL (···); K256H TPL (— · —); K256R TPL (- - -); E69D/K256R TPL (— ·· —).

not increase in PLP content. The stoichiometry for E69D/K256R TPL can be increased to ca. 1 mol of PLP/mol of subunit by 2 h of incubation with excess PLP at 35 °C, followed by gel filtration. Thus, the low binding stoichiometry of PLP in the isolated protein and the lack of activation of E69D/K256R TPL by PLP under assay conditions appears to be due to very slow PLP binding kinetics. The spectra of K256A, K256H, and E69D/K256R TPLs exhibit peaks at about 390 and 325 nm, K256R TPL exhibits a peak at 409 nm, while wild-type TPL exhibits a maximum at 420 nm for the internal aldimine (Figure 4).

We showed previously that activation of TPL by monovalent cations results in a significant increase in fluorescence emission at 500 nm arising from the PLP cofactor (*4*). The fluorescence spectra of wild-type, K256A, K256H, K256R, and E69D/K256R mutant TPLs were measured with both 280 and 420 nm excitation. All of the enzymes show an emission band at about 335 nm, with 280 nm excitation, due to the tryptophan residue (Figure 5). However, only wild-

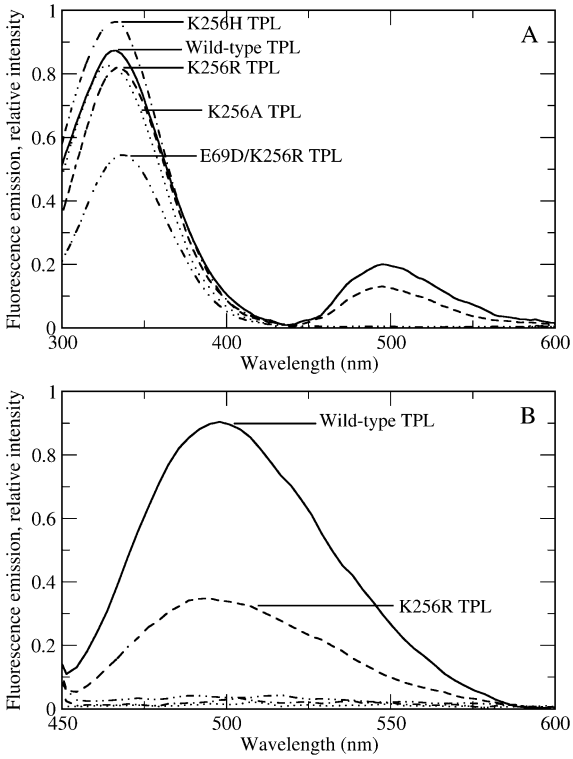

FIGURE 5: Fluorescence emission spectra of wild-type and Lys-256 mutant TPLs. The spectra were obtained with 0.5 mg/mL TPL in 0.05 M potassium phosphate, pH 8.0. Key: wild-type TPL (—); K256A TPL (- - -); K256H TPL (— · —); K256R TPL (···); E69D/K256R TPL (— ·· —). (A) Excitation at 280 nm; (B) excitation at 420 nm.

type and K256R TPLs show an additional emission band at 500 nm, presumably resulting from Förster resonance energy transfer from the tryptophan to the PLP (Figure 5A). Similarly, when the internal aldimines are directly excited at 420 nm, only wild-type and K256R TPLs show the 500 nm emission (Figure 5B).

Rapid-Scanning Stopped-Flow Spectroscopy of Mutant TPLs. The reaction of competitive inhibitors, L-Ala, L-Phe, and L-Met, with solutions of K256A, K256H, K256R, and E69D/K256R TPLs was followed by rapid-scanning stopped-flow spectrophotometry. All of these compounds react to form absorption peaks at about 410 nm, assigned to the external aldimines of the amino acids. They also produce absorption peaks at about 500 nm of variable intensity, due to formation of quinonoid complexes. It should be noted that all of these compounds readily form quinonoid complexes with wild-type TPL, which exhibit strong absorption bands at about 500 nm (*23*, *24*). The rapid-scanning stopped-flow data obtained with K256A TPL are shown in Figure 6. The reactions require at least two exponentials to fit the data (Table 2). There is a relatively slow reaction to form external aldimines, with λ_{max} at 415 nm, and low intensity bands at 500 nm, from quinonoid species, most prominently with L-Phe (Figure 6E). The absorbance changes at 415 and 500 nm occur with a single rate constant; thus, the external aldimine formation must be rate determining. The second very slow phase is most likely due to PLP binding to the apoenzyme. The data obtained with E69D/K256R TPL are shown in Figure 7. In this case, the reactions are much faster than for K256A TPL (Table 2), and the quinonoid bands show more intense absorption. The addition of K^+ has only

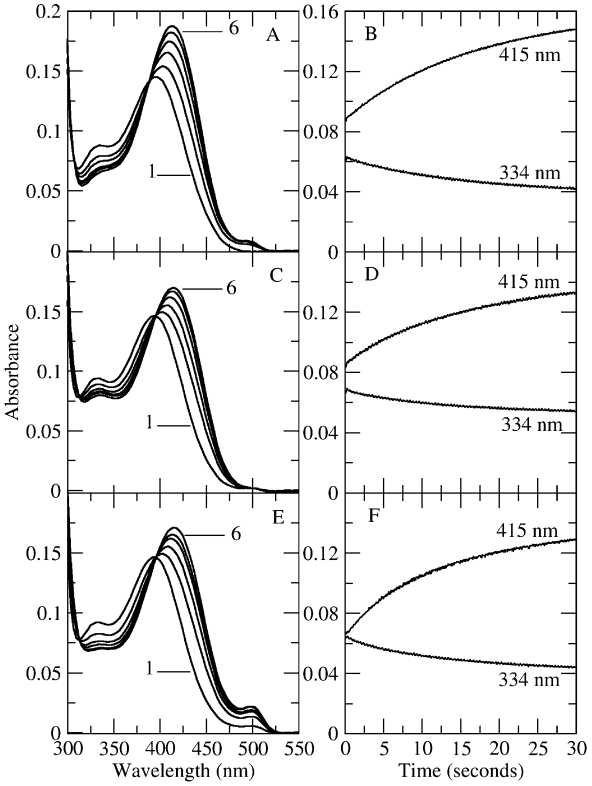

FIGURE 6: Rapid-scanning stopped-flow spectra of the reaction of K256A TPL with L-Ala, L-Met, and L-Phe. (A) Reaction of 250 mM L-Ala. Scans are shown at 0.09, 1.2, 1.8, 2.4, 3.0, and 6.0 s. (B) Time courses of the reaction of 250 mM L-Ala at 334 and 415 nm. (C) Reaction of 50 mM L-Met. Scans are shown at 0.09, 1.2, 1.8, 2.4, 3.0, and 6.0 s. (D) Time courses of the reaction of 50 mM L-Met at 334 and 415 nm. (E) Reaction of 50 mM L-Phe. Scans are shown at 0.09, 1.2, 1.8, 2.4, 3.0, and 6.0 s. (F) Time courses of the reaction of 50 mM L-Phe at 334 and 415 nm.

minor effects on the amplitudes and rates of formation of the quinonoid intermediates with E69D/K256R TPL (data not shown), in contrast to wild-type TPL, which shows dramatic effects of monovalent cations on the rates of formation of quinonoid intermediates, as much as 80-fold (*4*). The corresponding rapid-scanning stopped-flow data obtained with K256H and K256R TPLs are included in the Supporting Information. The reactions of these mutant TPLs are also faster than those of K256A TPL. There is only a small effect, about 2-fold, of K^+ on the rate constants of the reactions of K256R TPL with Ala, Met, and Phe (data not shown).

DISCUSSION

A large number of enzymes have been found to require monovalent cations for maximum activity (*25*, *26*). Some of these enzymes include pyruvate kinase (*27*), IMP dehydrogenase (*28*), methylamine dehydrogenase *(29*), and tryptophan synthase (*30*−*33*). The ligation environment of the monovalent cations in enzymes is remarkably diverse. Although one might expect to find an alkali metal ion in an environment rich in oxyanions, due to the positive charge, often the ligation environment is primarily or exclusively neutral amide carbonyl oxygens. In the case of methylamine dehydrogenase, the π-cloud of a phenylalanine side chain serves as a ligand to a monovalent cation (*34*). Even within the aminotransferase family of PLP-dependent enzymes, with very similar folds, the monovalent cation binding sites vary significantly. The activating monovalent cation site in dialkylglycine decarboxylase has aspartate carboxylate and serine hydroxyl ligands, as well as amide carbonyls and water, and is at a subunit interface near the active site, but in a different location than TPL and Trpase (*35*). TPL and Trpase exhibit very similar three-dimensional structures, with about 40% sequence identity, and they show similar activation by monovalent cations, K^+ or NH_4^+, while Na^+ does not activate (*3*, *4*, *7*). Since the K_d for K^+ activation is 3 mM, which is much less than the intracellular [K^+] in bacteria (100−150 mM), TPL should be activated constantly, and thus the cation plays a structural rather than regulatory role. Both enzymes have a monovalent cation binding site at the monomer−monomer interface of the dimer, with a glutamate γ-carboxylate oxygen as a ligand (*5*, *6*, *10*). Two amide carbonyl oxygens from the peptide backbone provide additional ligands, and the remaining coordination sites are filled by three water molecules (*5*, *6*, *10*). In both TPL and Trpase, there is a highly conserved lysine residue which precedes the PLP-binding lysine in the sequence and which is not found in any other member of the aminotransferase family. This lysine, Lys-256 in TPL, forms a hydrogen bond to one of the waters coordinated to the cation (Figure 1). To our knowledge, a lysine residue has not been shown to participate in monovalent cation binding in other proteins. Thus, we were interested in the role of this lysine in monvalent cation binding, activation, and catalysis by TPL.

Mutation of Lys-256 to Ala or His results in very low activity with all substrates (Table 1), and these mutant enzymes are not activated by Li^+, Na^+, K^+, Rb^+, or NH_4^+. Furthermore, these mutant TPLs do not exhibit any fluorescence emission from the PLP confactor (Figure 5), which was previously found to be increased by monovalent cation activation (*4*). Thus, Lys-256 is apparently essential for monovalent cation binding and activation of TPL. The hydrogen bond between Lys-256 and the structural water on the cation must make a significant contribution to the binding of the cation and/or activation of the enzyme. However, K256R TPL has modest activity and is strongly activated by K^+ (Figure 2), albeit with binding about 5-fold weaker than wild-type TPL. Furthermore, K256R TPL shows fluorescence emission from the PLP cofactor (Figure 5). Thus, arginine partially replaces lysine, suggesting that the guanidinium side chain can also form a hydrogen bond to the structural water. Lys-256 provides a direct pathway for transmission of structural changes between the monovalent cation site and the PLP-binding site, linked to Lys-257 (Figure 1). Additional communication between the monovalent cation site and the active site likely comes from Glu-69, through Tyr-71, which is essential for activity with L-Tyr (*36*). Both TPL and Trpase have a conserved lysine residue immediately preceding the PLP-binding lysines (Lys-257 in TPL and Lys-270 in Trpase) in the sequence. Some years ago, before any structures of TPL and Trpase were available, we prepared the K269R mutant *E. coli* Trpase, and we found that it had reduced activity (*37*), similar to that of K256R TPL, and diminished ability to form quinonoid complexes with inhibitors and substrates. We did not examine K269R Trpase for monovalent cation activation at that time, because we had no evidence that the Lys-269 was located in the monovalent cation site. The structure of the active site of

Table 2: Rate Constants for Wild-Type and Lys-256 Mutant TPLs

	wild type[a]		K256A		K256H		K256R		E69D/K256R	
ligand	$1/\tau_1$ (s^{-1})	$1/\tau_2$ (s^{-1})	$1/\tau_1$ (s^{-1})	$1/\tau_2$ (s^{-1})	$1/\tau_1$ (s^{-1})	$1/\tau_2$ (s^{-1})	$1/\tau_1$ (s^{-1})	$1/\tau_2$ (s^{-1})	$1/\tau_1$ (s^{-1})	$1/\tau_2$ (s^{-1})
L-Ala	1.8	nd[b]	0.14	0.057	0.97	0.056	1.19	0.057	0.96	0.090
L-Met	1.1	nd[b]	0.19	0.045	1.44	0.054	4.10	1.40	6.40	0.50
L-Phe	21.2	nd[b]	0.23	0.050	3.00	0.130	14.10	3.60	9.40	3.00

[a] Calculated from data in Table 5 of ref *11*. [b] Not determined.

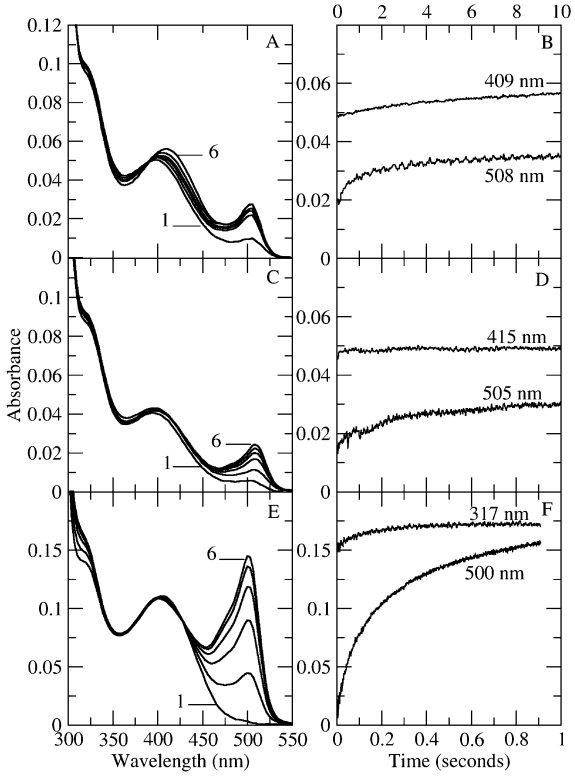

FIGURE 7: Rapid-scanning stopped-flow spectra of the reaction of E69D/K256R TPL with L-Ala, L-Met, and L-Phe in 0.05 M potassium phosphate, pH 8.0. (A) Reaction of 250 mM L-Ala. Scans are shown at 0.01, 0.16, 0.32, 0.48, 0.80, and 1.59 s. (B) Time courses of the reaction of 250 mM L-Ala at 409 and 508 nm. (C) Reaction of 50 mM L-Met. Scans are shown at 0.002, 0.106, 0.292, 0.872, 1.16, and 1.74 s. (D) Time courses of the reaction of 50 mM L-Met at 415 and 505 nm. (E) Reaction of 50 mM L-Phe. Scans are shown at 0.002, 0.045, 0.181, 0.362, 0.632, and 0.812 s. (F) Time courses of the reaction of 50 mM L-Phe at 317 and 500 nm.

Proteus vulgaris Trpase (PDB file 1AX4) is extremely similar to that of TPL (*38*), so it is likely that Lys-269 in *E. coli* Trpase is also involved in monovalent cation binding and activation.

We prepared E69D/K256R TPL with the hypothesis that the guanidinium group of the Arg would replace the monovalent cation and, thus, result in an activated enzyme which was no longer dependent on monovalent cations. Computer modeling showed that it was possible to form an ion pair with the guanidinium of Arg-256 and the carboxylate of Asp-69 (Figure 1, bottom). Previously, we demonstrated that E69D TPL is not activated by cations and has a low activity, about 0.1% of wild-type TPL, similar to the activity of the K256A and K256H mutant TPLs prepared in the present study. Our expectation was partially successful, in that E69D/K256R TPL has near wild-type activity, higher than either E69D or K256R TPL, with either SOPC or *S*-Et-L-Cys (Table 1). Similarly, a glutamate to lysine mutation in the cation binding site was shown to remove the monovalent cation dependence of pyruvate kinase (*39*). Surprisingly, we are unable to detect any activity of the double mutant TPL with L-Tyr, the natural substrate (Table 1). It is also interesting that E69D/K256R TPL does not show any fluorescence emission from the PLP (Figure 5).

The K256A, K256H, and K256R TPLs show weaker PLP binding than wild-type TPL (Figure 3). This is consistent with previous data with TPL and Trpase showing that PLP binding is affected by monovalent cations (*4*, *40*). Furthermore, monovalent and divalent cations affect the binding equilibrium of PLP to dialkylglycine decarboxylase (*41*). The double mutant TPL, E69D/K256R, shows no change in activity when PLP is added. Although the PLP content of the enzyme is low as isolated, about 0.4 mol/subunit, it takes relatively severe conditions (2 h at 37 °C with high [PLP]) to increase the PLP content to 1 mol/subunit. This finding suggests that the double mutation has altered the dynamics of the enzyme, and it does not open the active site readily to allow PLP binding. These data suggest that the monovalent cation in TPL plays a dynamic role in stabilizing protein conformations during catalysis (*26*), in addition to a structural role. This apparent "stiffness" of E69D/K256R TPL may be the reason that there is no observable activity with L-Tyr, if a significant conformational change is required during catalysis with L-Tyr, but not with *S*-Et-L-Cys or SOPC. Both *S*-Et-L-Cys and SOPC have much better leaving groups than L-Tyr, so their elimination reactions are less likely to be affected by conformational alterations. It is interesting to note that there is an orthologue of TPL from *Rhodopseudomonas palustris* CGA009 (Gene ID no. 2690118) which exhibits the Glu-69 to Asp and Lys-256 to Arg double mutation naturally, so there must be other compensating mutations which are necessary to restore the dynamics and the activity with L-Tyr.

All of the TPL mutants of Lys-256 form equilibrating mixtures of external aldimine and quinonoid intermediates with the competitive inhibitors, L-Ala, L-Met, and L-Phe (Figures 6 and 7). The formation of the quinonoid bands, with λ_{max} at about 500 nm, for all of the mutant proteins, especially with L-Phe, shows that Lys-256 is not the catalytic base responsible for α-deprotonation of substrates. This is consistent with our previous results on the K269R mutant Trpase (*36*). Thus, the primary function of Lys-256 in TPL is in monovalent cation binding and activation.

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SUPPORTING INFORMATION AVAILABLE

Rapid-scanning stopped-flow spectra of reactions of K256H and K256R TPLs with L-Ala, L-Met, and L-Phe. This material is available free of charge via the Internet at http://pubs.acs.org.

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