The Role of Glutamic Acid-69 in the Activation of *Citrobacter freundii* Tyrosine Phenol-Lyase by Monovalent Cations†

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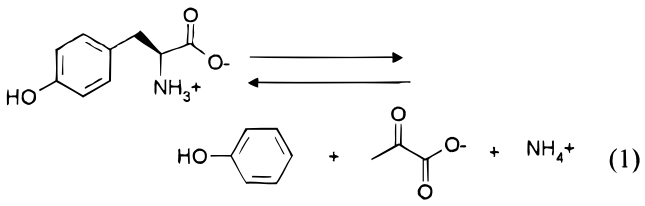
ABSTRACT: Tyrosine phenol-lyase (TPL) from *Citrobacter freundii* is activated about 30-fold by monovalent cations, the most effective being K^+, NH_4^+, and Rb^+. Previous X-ray crystal structure analysis has demonstrated that the monovalent cation binding site is located at the interface between subunits, with ligands contributed by the carbonyl oxygens of Gly52 and Asn262 from one chain and monodentate ligation by one of the ϵ-oxygens of Glu69 from another chain [Antson, A. A., Demidkina, T. V., Gollnick, P., Dauter, Z., Von Tersch, R. L., Long, J., Berezhnoy, S. N., Phillips, R. S., Harutyunyan, E. H., and Wilson, K. S. (1993) *Biochemistry 32*, 4195]. We have studied the effect of mutation of Glu69 to glutamine (E69Q) and aspartate (E69D) to determine the role of Glu69 in the activation of TPL. E69Q TPL is activated by K^+, NH_4^+, and Rb^+, with K_D values similar to wild-type TPL, indicating that the negative charge on Glu69 is not necessary for cation binding and activation. In contrast, E69D TPL exhibits very low basal activity and only weak activation by monovalent cations, even though monovalent cations are capable of binding, indicating that the geometry of the monovalent cation binding site is critical for activation. Rapid-scanning stopped-flow kinetic studies of wild-type TPL show that the activating effect of the cation is seen in an acceleration of rates of quinonoid intermediate formation (30−50-fold) and of phenol elimination. Similar rapid-scanning stopped-flow results were obtained with E69Q TPL; however, E69D TPL shows only a 4-fold increase in the rate of quinonoid intermediate formation with K^+. Preincubation of TPL with monovalent cations is necessary to observe the rate acceleration in stopped flow kinetic experiments, suggesting that the activation of TPL by monovalent cations is a slow process. In agreement with this conclusion, a slow increase ($k < 0.5\ s^{-1}$) in fluorescence intensity ($\lambda_{ex} = 420$ nm, $\lambda_{em} = 505$ nm) is observed when wild-type and E69Q TPL are mixed with K^+, Rb^+, and NH_4^+ but not Li^+ or Na^+. E69D TPL shows no change in fluorescence under these conditions. High concentrations (>100 mM) of all monovalent cations result in inhibition of wild-type TPL. This inhibition is probably due to cation binding to the ES complex to form a complex that releases pyruvate slowly.

Monovalent cations play a important role in many biological processes, for example, in the generation, transmission, and conduction of the nerve impulse, in the conservation of the osmotic balance of the cell, and the work of the muscles. An increasing number of enzymes have been found to be specifically activated by monovalent cations (*1*, *2*). Pyridoxal 5′-phosphate (PLP)[1] containing enzymes perform a variety of reactions in the metabolic pathways of amino acids, including transamination, β- and γ-elimination and replacement, decarboxylation, and racemization. Tyrosine phenol-lyase (TPL, [EC 4.1.99.2]) is a PLP-dependent enzyme that catalyzes the β-elimination reaction of L-tyrosine to produce phenol and ammonium pyruvate (eq 1).



(1)

TPL is markedly stimulated by some monovalent cations, which were proposed to stabilize the active conformation (*3*, *4*). It has been found for TPL that the holoenzyme with a protonated nitrogen atom in the internal aldimine is the catalytically active form (*4*). In addition to L-tyrosine, L- and D-serine, *S*-methyl-L-cysteine (*3*), *S*-ethyl-L-cysteine, *S*-(*o*-nitrophenyl)-L-cysteine, *O*-benzoyl-L-serine (*5*), and β-chloro-L-alanine also act as substrates for β-elimination, with the formation of ammonium pyruvate. TPL also catalyzes the synthesis of L-tyrosine and related amino acids (*6*, *7*), β-replacement reactions (*8*, *9*), and racemization of alanine (*8*, *10*).

The molecule of TPL consists of four chemically identical subunits (*11*), each with molecular mass of 51.4 kDa, and

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

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binds 4 mol of PLP/tetramer. We have previously reported the X-ray crystal structure of the apoenzyme of TPL (*12*) and the crystal structure of TPL with a substrate analogue 3-(4′-hydroxyphenyl)propionic acid (*13*). The latter crystal structure was obtained in the presence of Cs^+, and it was found that Glu69 forms part of the monovalent cation binding site. In the present work, to determine the role of Glu69 of TPL in monovalent cation binding and catalysis, we performed the mutation of this residue to glutamate and aspartate by site-directed mutagenesis. The reactions of the wild-type and mutant proteins were measured with steady-state and pre-steady-state kinetic methods in the absence and presence of various monovalent cations. The results of these studies are presented herein.

MATERIALS AND METHODS

Materials. Triethanolamine, LiCl, KCl, RbCl, NaCl, CsCl, and NH_4Cl were from Fisher Scientific (USA). Lactate dehydrogenase from rabbit muscle, PLP, and NADH were purchased from United States Biochemical Co., as was L-tyrosine. *S*-(*o*-Nitrophenyl)-L-cysteine (SOPC) was prepared as previously described (*14*). Octyl-Sepharose CL-4B and Sephacryl S-2000 were obtained from Pharmacia. Glutamate-pyruvate transaminase was obtained from Sigma.

Site-Directed Mutagenesis. Plasmid pTZTPL, which contains the *tpl* gene from *Citrobacter freundii* (*12*), was used to transform *Escherichia coli* CJ236 (*dut*$^-$ *ung*$^-$). Uracil-containing single stranded DNA of this plasmid was then prepared as previously described by Chen et al. (*15*). Site-directed mutagenesis was performed by the method of Kunkel (*16*) using the Muta-Gene in vitro mutagenesis kit from Bio-Rad. The mutagenic oligonucleotides used were GCGGGCAGCCAAAACTTCTA for the Glu → Gln mutation and GCGGGCAGCGACAACTTCTA for the Glu → Asp mutation. Clones obtained after mutagenesis were screened by sequencing the gene in the mutated region using an Applied Biosystem 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. in ref *12*) was used for sequencing the region containing the desired mutation. The resulting plasmid containing the Glu69 to Gln mutation was designated pTZTPL E69Q and the plasmid containing the Glu69 to Asp mutation was designated pTZTPL E69D. *E. coli* SVS370 cells were used as the host for the plasmid, since these cells are *tna*A$^-$, and thus do not produce Trpase, which would interfere with the use of SOPC (*17*) for TPL assay in cell extracts. The cells were grown and the enzymes were purified as previously described (*15*).

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 for Trpase (*18*). 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. Enzyme activity during purification was routinely measured with 0.6 mM SOPC in 50 mM potassium phosphate, pH 8.0, at 25 °C (*5*), 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 that produces 1 μmol of product/min. Alanine racemase activity of TPL was measured with D-alanine as substrate using a coupled assay with glutamate-pyruvate transaminase and lactate dehydrogenase as previously described (*10*).

Effect of Monovalent Cations. The effect of monovalent cations was determined using the same coupled assay described above, except that 0.05 M triethanolamine hydrochloride buffer (pH 8) was used instead of potassium phosphate buffer, pH 8. The enzyme solutions were passed through two consecutive gel filtration columns (PD-10, Pharmacia) equilibrated with 0.05 M triethanolamine hydrochloride buffer (pH 8) prior to use in the steady-state kinetic studies to remove all traces of inorganic monovalent cations.

Protein Determination. Protein was determined by the method of Bradford (*19*), with purified TPL as a standard. The concentration of purified TPL was determined from the absorbance at 278 nm ($E^{1\%} = 8.37$) (*20*) assuming a subunit molecular mass of 51.4 kDa (*12*). 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} (*21*).

Stopped-Flow Measurements. Before the rapid kinetic experiments were performed, the stock enzyme was incubated with 1 mM PLP for 1 h at 30 °C and then separated from excess PLP and monovalent cations by passage through two consecutive short desalting columns (PD-10, Pharmacia) equilibrated with 50 mM triethanolamine hydrochloride buffer, pH 8.0. Rapid-scanning stopped-flow kinetic data were obtained with an RSM-1000 instrument from OLIS, Inc. For absorbance measurements, the observation cell has a 10-mm path length, and a 150 W Xe lamp was used as the light source, with a 0.2-mm slit in the scan disk and 0.6-mm exit slit on the monochromator. For fluorescence measurement, the observation cell has a 4-mm path length, and a 450 W Xe lamp was used for excitation, with a 6-mm entrance slit, a 3.6-mm exit slit, and a 1-mm slit in the scan disk of the emission monochromator. This instrument has a dead time of ca. 2 ms and is capable of collecting either absorbance or fluorescence emission spectra in the visible region from 300 to 600 nm at 1 kHz. The enzyme solutions in 50 mM triethanolamine hydrochloride buffer, pH 8.0, were mixed with 2 mM L-tyrosine, and the absorbance changes were followed for either 1 s or 1 min, in consecutive shots, to observe both fast and slow processes. The rate constants for quinonoid intermediate formation were then evaluated by exponential fitting of the absorbance changes at 500 nm using the LMFIT or SIFIT programs provided by OLIS. The validity of the fitting was evaluated by the standard deviation or the Durbin−Watson value (*22*). Typically, rate constants were estimated to a standard deviation of less than 5%. Fluorescence measurements were performed by mixing TPL solutions in 0.05 M triethanolamine hydrochloride, pH 8.0, with solutions of various cations in the same buffer. The fluorescence spectra and rate constants in Figure 5 were obtained by robust global analysis of the rapid-scanning fluorescence data using the program supplied by OLIS (*23*).

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

Table 1: Effects of Monovalent Cations on Wild-Type and E69Q Mutant Tyrosine Phenol-Lyase[a]

	wild-type			E69Q		
cation	K_A (mM)	K_I (mM)	activtn (fold)	K_A (mM)	K_I (mM)	activtn (fold)
Li^+	30 ± 6	240 ± 50	14	16 ± 6	680 ± 370	6
K^+	2.1 ± 0.6	410 ± 120	32	3.4 ± 1.0	270 ± 80	24
Rb^+	1.4 ± 0.2	770 ± 140	24	0.9 ± 0.3	300 ± 110	25
Cs^+	40 ± 14	380 ± 170	20	19 ± 6.0	460 ± 200	8
NH_4^+	0.1 ± 0.02	170 ± 30	28	0.2 ± 0.07	250 ± 80	24

[a] Reactions were performed at 25 °C in 0.05 M triethanolamine hydrochloride, pH 8.0, containing 2 mM L-tyrosine, 0.1 mM NADH, 20 μg/mL LDH, and varying amounts of the cations.

(eq 2) using a hyperbolic regression program. In eq 2, S is the substrate, E is the total enzyme concentration, and K_m is the Michaelis constant. The monovalent cation activation data were fit to eq 3, where C^+ is the cation, K_A is the binding constant for activation, and K_I is the binding constant for inhibition. The monovalent cation inhibition data of the alanine racemase activity were fit to eq 4, where C^+ is the cation, V_{max} is the maximum activity, and V_{min} is the minimum activity in the presence of the cation, and K_I is the apparent binding constant for inhibition. For pre-steady-state kinetic studies, the effects of concentration on rate constants for formation of quinonoid intermediates were evaluated by the use of eq 5 (*24*), where k_f is the rate constant for the formation of the quinonoid intermediate, k_r is the rate constant for the reprotonation, K_d is the binding constant for the amino acid, and [L] is the concentration of the amino acid.

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

$$V = V_{max}[C^+]K_I/([C^+]+K_A)([C^+] + K_I) \quad (3)$$

$$V = V_{max}K_I/([C^+]+K_I) + V_{min} \quad (4)$$

$$k_{obs} = k_f[L]/(K_d + [L]) + k_r \quad (5)$$

RESULTS

Activation and Inhibition of Wild-Type, E69Q, and E69D Tyrosine Phenol-Lyase by Monovalent Cations. Tyrosine phenol-lyase has been known for some time to require monovalent cations to exhibit maximum elimination activity (*4*). The most effective cations were reported previously to be K^+, Rb^+, and NH_4^+ (*4*, *8*). We have qualitatively confirmed these results with wild-type TPL. The physiological substrate, L-tyrosine, was used for measuring activation in our experiments since it exhibits greater response to monovalent cations than does the in vitro substrate, SOPC. However, we have found a significantly greater extent of activation of TPL by monovalent cations in our experiments (as much as 30-fold, see Table 1 and Figure 1) than has been previously reported, ranging between 2−10-fold activation (*4*, *8*). We believe that this is due to our stringent efforts to eliminate all sources of contamination of our solutions with the ubiquitous monovalent cations, NH_4^+ and K^+. Thus, our k_{cat} value for the fully activated enzyme is in good agreement with previous results, but our basal k_{cat} value observed in the absence of monovalent cations is considerably less than that previously reported (Table 2). In agreement with previous investigations, we also found that K^+, Rb^+, and NH_4^+ are the most effective activators, with Cs^+ considerably less effective, while Na^+ shows negligible activation (Figure 1A). It is notable that NH_4^+ activates TPL at considerably lower concentrations than does K^+ or Rb^+ (Figure 1 and Table 1). However, in contrast to previous reports (*4*), we have found that Li^+ is also a weak activator of TPL at much higher concentrations (Figure 1A and Table 1). It should be noted that for *E. coli* Trpase, which has high sequence homology to TPL (*12*) and which has a very similar three-dimensional structure, Li^+ and Na^+ are reported to be weakly activating, with SOPC as substrate (*25*). We found that activation of TPL by monovalent cations increases k_{cat} but does not significantly affect the K_m for L-tyrosine, as was previously reported (*4*). Divalent alkaline earth cations examined (Mg^{2+}, Ca^{2+}, Ba^{2+}, Sr^{2+}) do not activate *C. freundii*

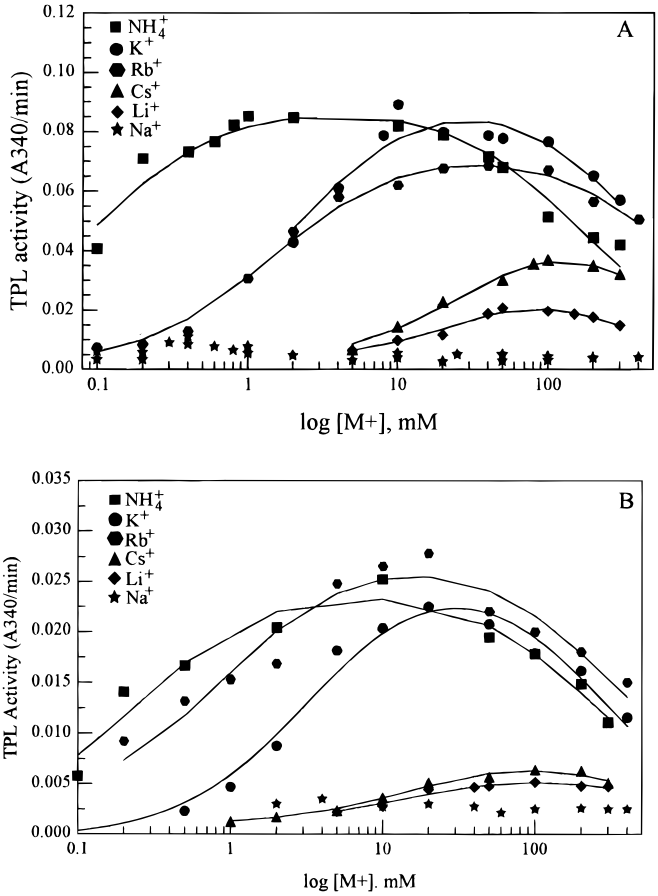


FIGURE 1: Effects of monovalent cations on steady-state activity of wild-type and mutant TPL. Circles, K^+; squares, NH_4^+; hexagons, Rb^+; triangles, Cs^+; diamonds, Li^+; stars, Na^+. Lines are calculated from eq 3 using the parameters given in Table 1. (A) Wild-type TPL. (B) E69Q TPL.

Table 2: Kinetic Parameters for L-Tyrosine with Wild-Type and Mutant Tyrosine Phenol-Lyases in the Presence and Absence of Monovalent Cations[a]

	wild-type		E69Q		E69D	
cation	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})
+ 50 mM K^+	1.8	8.8×10^3	1.2	5.0×10^3	6×10^{-3}	3
none	0.07	3.2×10^2	0.06	2×10^2	1.4×10^{-3}	5

[a] Reactions were performed at 25 °C in 0.05 M triethanolamine hydrochloride, pH 8.0, with 0.1 mM NADH and 20 μg/mL LDH.

wild-type or mutant TPL, despite a previous report that TPL from *Clostridium tetanomorphum* is activated by Mg^{2+} (*26*).

Surprisingly, E69Q TPL exhibits β-elimination activity (Table 2) and response to monovalent cations (Figure 1B and Table 1) very similar to that of wild-type TPL. This indicates that the presence of the negative charge on Glu69 is not critical for cation binding and activation. Furthermore, the K_A values for the highly activating cations, NH_4^+, K^+, and Rb^+, are almost identical for wild-type and E69Q TPL (Table 1). A modest difference can be seen in selectivity for E69Q TPL for Li^+ and Cs^+ (Table 1). In contrast, E69D TPL has very low activity (Table 2), and even in the presence of 50 mM K^+, it exhibits only about 0.4% of the activity of activated wild-type TPL. Maximal activation of no more than 4-fold is seen for E69D TPL even with the best activating cations.

At higher concentrations of monovalent cations, there is a marked inhibition of wild-type TPL, with a maximal plateau of activity reached between 10 and 50 mM for K^+ (Figure 1A). Sodium is the only cation previously reported to be an inhibitor of TPL, and Li^+ was reported to be neither an activator nor an inhibitor (*4*). However, we found that Li^+ is also a weak inhibitor at much higher concentrations than previously examined (Figure 1A). In contrast to previous reports, we have found that Na^+ does not significantly inhibit TPL in the absence of other cations (see Figure 1, stars). At [Na^+] below 1 mM, there is possibly a very slight activation (less than 2-fold), and then the activity returns to the basal level and does not decrease further even at 300 mM Na^+ (Figure 1A). In our experiments, inhibition by Na^+ is observable only if TPL is previously partially activated by preincubation with low concentrations (e.g., 1 mM) of good activators such as K^+ in the solution. The monovalent cation inhibition of TPL at high concentration is not simply an effect of high ionic strength, since it shows specificity for the various cations (Figure 1A and Table 1). To obtain the K_A and K_I for the cations, the data were fitted to eq 3, and the results are presented in Table 1. Similar results were observed for E69Q TPL at high cation concentrations (Figure 1B and Table 1).

Effects of Monovalent Cations on Alanine Racemase Activity of Wild-Type and Mutant TPL. In contrast to the β-elimination activity, the alanine racemase activity of wild-type TPL is not activated by monovalent cations but rather is strongly inhibited by K^+ at relatively low concentrations (Figure 2). Similar results were obtained with K^+ for E69Q and E69D TPL (Figure 2) and with wild-type TPL with NH_4^+ and Na^+ (data not shown). The apparent K_I values for K^+ with wild-type, E69Q, and E69D TPL from fitting to eq 4 are 39.5 ± 8.1, 19.1 ± 6.3, and 29.0 ± 5.6 mM, respectively. These values are considerably larger than the K_A values for activation of TPL; however, the racemase activity is likely due to a different conformation of enzyme than the β-elimination activity (see Discussion). In the absence of monovalent cations, E69Q and E69D mutant TPL have alanine racemase activities of 65 and 40%, respectively, of wild-type TPL. E69D TPL is less subject to inhibition by monovalent cations than wild-type or E69Q TPL (Figure 2), so at high [K^+] E69D TPL has racemase activity comparable or even higher than wild-type or E69Q TPL. Thus, mutations of Glu69 have much less effect on the alanine racemase activity than on the β-elimination activity of TPL.

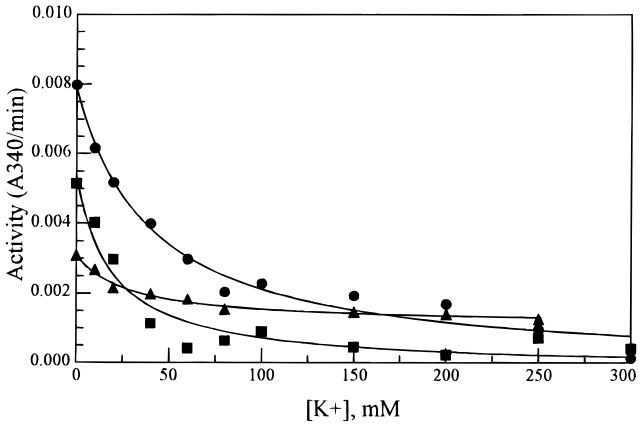


FIGURE 2: Effect of K^+ on alanine racemase activity of wild-type and mutant TPL. Circles, wild-type TPL; squares, E69Q TPL; triangles, E69D TPL.

Effects of Monovalent Cations on Pre-Steady-State Kinetics of Wild-Type TPL Reactions. When wild-type TPL in triethanolamine hydrochloride, pH 8.0, containing 50 mM K^+, is mixed with 2 mM L-tyrosine, there is a rapid reaction resulting in the formation of a quinonoid intermediate, with λ_{max} at 500 nm and with rather low steady-state absorbance (Figure 3A). This is also accompanied by a small increase in the absorbance of the 420 nm peak attributed to the external aldimine (Figure 3A). Very similar results have been reported previously by Muro et al. (*20*) and by Phillips et al. (*27*) in potassium phosphate buffer. The absorbance increase at 500 nm is biphasic (see inset, Figure 3A), with $1/\tau_1 = 105\ s^{-1}$ for the fast phase, and $1/\tau_2 = 2\ s^{-1}$ for the slow phase (Table 3). Results virtually identical to those in Figure 3A are also obtained with either 50 mM NH_4^+ or Rb^+ (Table 3). In contrast, when TPL is mixed with 2 mM L-tyrosine in triethanolamine hydrochloride, pH 8.0, without any activating monovalent cation present, the reaction is remarkably different and complex (Figure 4A). The fastest phase of the absorbance increase at 500 nm is no longer observed, but instead a slow biphasic increase in absorbance at 500 nm is observed ($1/\tau_1 = 1.4\ s^{-1}$ and $1/\tau_2 = 0.16\ s^{-1}$) followed by a slower decrease in absorbance over a period of 60 s to finally reach a steady-state level (Figure 3B, inset). The 420-nm peak increases in absorbance as the 500-nm peak declines in the approach to steady state. It was not possible to obtain satisfactory first-order fits of the phase of absorbance decrease with the data shown. There are no clear isosbestic points in the spectra, indicating that a number of overlapping absorbing species contribute to the spectra (Figure 4A). The absorbance peak at 500 nm reaches a maximum at about 20 s after mixing and is about 10-fold higher for these reactions than for those performed in the presence of activating cations.

Similiar results were seen with L-phenylalanine in the absence of activating cations, except that there is no slow phase of decreasing absorbance at 500 nm (data not shown). This result suggest that the phase of decreasing absorbance in the reaction of L-tyrosine is due to phenol elimination. For L-phenylalanine, the rate constant of the fast phase of the absorbance increase at 500 nm changes with concentration in a hyperbolic manner (data not shown). Fitting the concentration dependence to eq 5 gives a K_D of 10.4 ± 1.9 mM, $k_f = 2.2\ s^{-1}$, and $k_r = 0.8\ s^{-1}$ for L-phenylalanine. This K_D is very similar to that seen in the presence of potassium

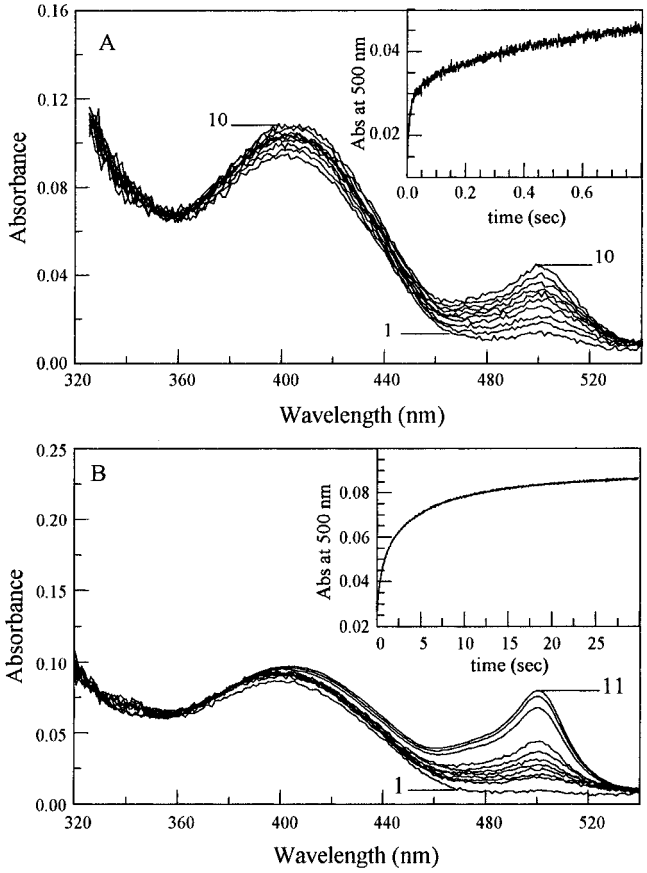


FIGURE 3: Pre-steady-state kinetics of wild-type and E69Q TPL in the presence of K^+. (A) Reaction of wild-type TPL (1 mg/mL, 19.4 μM) in 0.05 M triethanolamine hydrochloride, pH 8.0, containing 50 mM KCl, with 2 mM L-tyrosine. Spectra are shown at the following times: (1) 0.001; (2) 0.003; (3) 0.005; (4) 0.010; (5) 0.020; (6) 0.040; (7) 0.080; (8) 0.160; (9) 0.320; (10) 0.640 s. Inset shows the time course of the absorbance at 500 nm. (B) Reaction of E69Q TPL (1 mg/mL, 19.4 μM) in 0.05 M triethanolamine hydrochloride, pH 8.0, containing 50 mM KCl, with 2 mM L-tyrosine. Spectra are shown at the following times: (1) 0.001; (2) 0.010; (3) 0.020; (4) 0.040; (5) 0.080; (6) 0.16; (7) 0.32; (8) 0.64; (9) 4; (10) 8; (11) 12 s. Inset shows the time course of the absorbance at 500 nm.

(9.1 mM), suggesting that the equilibrium for formation of the external aldimine is not affected by cations, although the value of k_f and k_r are reduced about 8-fold from those observed in potassium phosphate buffer (*15*).

Rapid-scanning stopped-flow spectra were also collected at 330 mM K^+, and the results are very similar to those shown in Figure 3A, except that the amplitude of the slow phase is further reduced (data not shown). The steady-state absorbance at 500 nm for the L-tyrosine quinonoid intermediate in the presence of 330 mM K^+ is slightly lower than in the presence of 50 mM K^+, although the rate constants for quinonoid intermediate formation are unaffected. Thus, high [K^+] does not significantly affect the rate of binding of substrates or of formation and breakdown of quinonoid intermediates.

Surprisingly, when the order of metal addition is changed, and cation-free TPL is mixed with a solution of 2 mM L-tyrosine containing 50 mM K^+, the rapid-scanning stopped-flow data are identical to those shown in Figure 4A obtained without any cation. The time course of the absorbance change at 500 nm under these conditions is identical to that obtained without any cation present. Rapid-scanning stopped-flow spectra obtained in the presence of Li^+ and Na^+ are also qualitatively similar to Figure 4A. There are two slow phases of absorbance increase at 500 nm followed by a slow decrease in absorbance, with rate constants similar to those of the cation-free reaction (Table 3). However, if Cs^+ is included, there is, in addition, a fast phase of absorbance increase at 500 nm, of low amplitude. Thus, when 100 mM Cs^+ is included in the reactions, there is a triphasic increase in absorbance at 500 nm ($1/\tau_1 = 28\ s^{-1}$; $1/\tau_2 = 1.6\ s^{-1}$; $1/\tau_3 = 0.14\ s^{-1}$), followed by a slow decrease in absorbance (Table 3).

Effects of Monovalent Cations on Pre-Steady-State Kinetics of E69Q TPL Reactions. When E69Q TPL in 0.05 M triethanolamine hydrochloride, pH 8.0, containing 0.05 M KCl is mixed with 2 mM L-tyrosine (Figure 3B), there is a fast phase ($1/\tau_1 = 63\ s^{-1}$) of increase in absorbance at 500 nm, similar to that seen with wild-type TPL, followed by a slower phase with $1/\tau_2 = 1.8\ s^{-1}$. However, in contrast to wild-type TPL, there is an additional slow phase of absorbance increase at 500 nm with $1/\tau_3 = 0.13\ s^{-1}$ (Table 3) and a relatively large amplitude (see inset, Figure 3B). As a result of this, E69Q TPL exhibits a larger steady-state absorbance at 500 nm with L-tyrosine in the presence of K^+ than does wild-type TPL (compare Figure 3, panels A and B). Results very similar to those in Figure 3B were obtained with E69Q TPL and either NH_4^+ or Rb^+ (data not shown). Reaction of E69Q TPL with L-tyrosine in the absence of cations (Figure 4B) or in the presence of Cs^+, Na^+, or Li^+ (data not shown) also gives similar results to those of wild-type TPL. Without cations present, or with Na^+ or Li^+, there is a biphasic increase in absorbance at 500 nm, with $1/\tau_1 = 1.3\ s^{-1}$ and $1/\tau_2 = 0.18\ s^{-1}$, followed by a slow decay in absorbance (Figure 4B). In the presence of 100 mM Cs^+, there is an additional fast phase of absorbance increase with low amplitude at 500 nm, with $1/\tau_1 = 30\ s^{-1}$, just as was seen with wild-type TPL (Table 3).

Effects of Monovalent Cations on Pre-Steady-State Kinetics of E69D TPL Reactions. Reaction of E69D TPL in 0.05 M triethanolamine hydrochloride, pH 8.0, containing 40 mM KCl with 2 mM L-tyrosine results in a 500-nm absorbance peak similar to that seen in wild-type and E69Q TPL. The absorbance increase is biphasic, with $1/\tau_1 = 1.3\ s^{-1}$ and $1/\tau_2 = 0.4\ s^{-1}$ (Table 3). When E69D TPL reacts with L-tyrosine without a monovalent cation present, the spectra are similar, but the rate constants for the two phases are reduced to $1/\tau_1 = 0.4\ s^{-1}$ and $1/\tau_2 = 0.04\ s^{-1}$. In contrast to wild-type and E69Q TPL, the reaction with E69D TPL in the absence of monovalent cations does not show a phase of decreasing absorbance at 500 nm.

Effects of Monovalent Cations on Fluorescence of TPL. Mixing of wild-type TPL in 0.05 M triethanolamine hydrochloride, pH 8.0, with 50 mM K^+ did not produce any significant difference in absorbance spectra for a period up to 30 s (data not shown). However, when wild-type TPL is mixed with 0.05 M triethanolamine hydrochloride, pH 8.0, containing no activating cations, there is a slow decrease in fluorescence emission at 505 nm, with 420 nm excitation, arising from the PLP internal aldimine, which occurs with $1/\tau = 0.027\ s^{-1}$ (compare spectrum 0 and dotted spectrum in Figure 5A). When wild-type TPL is mixed with 50 mM K^+, Rb^+, or NH_4^+, there is an increase in fluorescence

Table 3: Pre-Steady-State Kinetic Parameters for Reaction of Wild-Type and Mutant TPL with L-Tyrosine[a]

enzyme	$1/\tau_1$ (s^{-1})	$1/\tau_2$ (s^{-1})	$1/\tau_3$ (s^{-1})
wild-type			
no cation		1.4 ± 0.1 (0.045)[b]	0.16 ± 0.03 (0.165)
50 mM Li^+		1.8 ± 0.1 (0.052)	0.20 ± 0.02 (0.115)
50 mM Na^+		1.5 ± 0.1 (0.072)	0.14 ± 0.05 (0.183)
50 mM K^+	83 ± 3 (0.017)	2.0 ± 0.1 (0.018)	
50 mM Rb^+	101 ± 4 (0.015)	2.0 ± 0.1 (0.021)	
100 mM Cs^+	28 ± 4 (0.018)	1.6 ± 0.1 (0.058)	0.14 ± 0.01 (0.075)
50 mM NH_4^+	101 ± 3 (0.019)	2.2 ± 0.1 (0.011)	
E69Q			
no cation		1.4 ± 0.1 (0.058)	0.19 ± 0.03 (0.212)
50 mM Li^+		2.4 ± 0.1 (0.094)	0.32 ± 0.01 (0.214)
50 mM Na^+		2.0 ± 0.1 (0.091)	0.23 ± 0.03 (0.255)
50 mM K^+	63 ± 6 (0.012)	1.8 ± 0.3 (0.031)	0.13 ± 0.07 (0.023)
50 mM Rb^+	57 ± 3 (0.010)	1.8 ± 0.1 (0.036)	0.11 ± 0.01 (0.027)
100 mM Cs^+	29 ± 1 (0.019)	1.7 ± 0.1 0.090)	0.18 ± 0.01 (0.100)
50 mM NH_4^+	73 ± 3 (0.016)	2.3 ± 0.1 (0.022)	0.12 ± 0.01 (0.021)
E69D			
no cation		0.43 ± 0.02 (0.13)	0.04 ± 0.002 (0.28)
50 mM K^+		0.96 ± 0.07 (0.11)	0.13 ± 0.01 (0.10)

[a] Reactions were performed with 19.5 μN enzyme in 0.05 M triethanolammonium chloride, pH 8.0 containing 2 mM L-tyrosine and the cation indicated. [b] Numbers in parentheses are the absorbances of each phase.

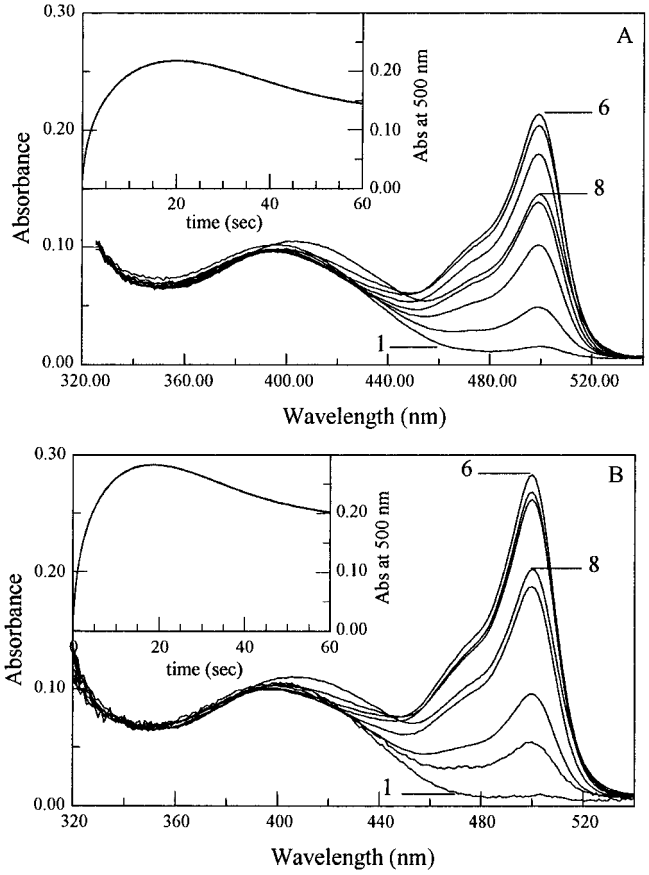


FIGURE 4: Pre-steady-state kinetics of wild-type and E69Q TPL in the absence of K^+. (A) Reaction of wild-type TPL (1 mg/mL, 19.4 μM) in 0.05 M triethanolamine hydrochloride, pH 8.0, with 2 mM L-tyrosine. Spectra are shown at the following times: (1) 0.001; (2) 0.50; (3) 2; (4) 4; (5) 8; (6) 15.5; (7) 30; (8) 60 s. Inset shows the time course of the absorbance at 500 nm. (B) Reaction of E69Q TPL (1 mg/mL, 19.4 μM) in 0.05 M triethanolamine hydrochloride, pH 8.0, with 2 mM L-tyrosine. Spectra are shown at the following times: (1) 0.001; (2) 0.4; (3) 1.1; (4) 4; (5) 8; (6) 19.2; (7) 30; (8) 60 s. Inset shows the time course of the absorbance at 500 nm.

intensity that occurs within the mixing time of the stopped-flow instrument (compare spectrum 0 and 1 in Figure 5A). Subsequently, there is a slower increase in fluorescence over about 60 s (inset, Figure 5A). This fluorescence increase gives a reasonable fit to a single exponential at low $[K^+]$ but fits better to a two exponential process at $[K^+]$ above 10 mM. The calculated spectra for the two phases are shown in Figure 5A, curves 2 and 3. The largest increase in fluorescence intensity is observed with NH_4^+, while K^+ and Rb^+ give about 50% smaller increases. All the activating monovalent cations show comparable rate constants for the fluorescence increase, and the observed rate constants for the slow phase show no significant variation with $[K^+]$ from 5 to 50 mM (Figure 5, panels B and C). The rate constant for the fast phase clearly decreases with $[K^+]$. There is insufficient data to fit these to a model, however. The total fluorescence change for wild-type TPL is saturable, showing an apparent K_d for K^+ of 17.5 ± 0.3 mM (Figure 5D). In contrast, no significant change in fluorescence intensity was seen when 50 mM Na^+ or 100 mM Li^+ was mixed with wild-type TPL. An increase in fluorescence intensity similar to Figure 5A was obtained upon mixing E69Q TPL with 50 mM K^+ (data not shown). However, there is no detectable change in fluorescence intensity when E69D TPL is mixed with 50 mM Na^+, Li^+, K^+, Rb^+, or NH_4^+.

DISCUSSION

An increasing number of enzymes have been found to require monovalent cations for maximum activity (*1*, *2*). Some of these enzymes include pyruvate kinase (*28*), inosine monophosphate dehydrogenase (*29*), and fructose 1,6-bisphosphatase (*30*). For most of these monovalent cation-dependent enzymes, K^+, Rb^+, and NH_4^+ are the best activators, while Na^+ and Li^+ are either less effective activators or inhibitors. The monovalent cation binding sites in these enzymes are generally composed primarily of carbonyl oxygen ligands from the peptide backbone and water, with occasional ligation contributed from oxygen in serine, threonine, aspartate, or glutamate side chains.

Because of the ubiquitous nature of these monovalent cations in common laboratory buffers, their role in the reactions of PLP-dependent enzymes was unappreciated for many years. However, the monovalent cation binding sites of a number of PLP-dependent enzymes have been identified

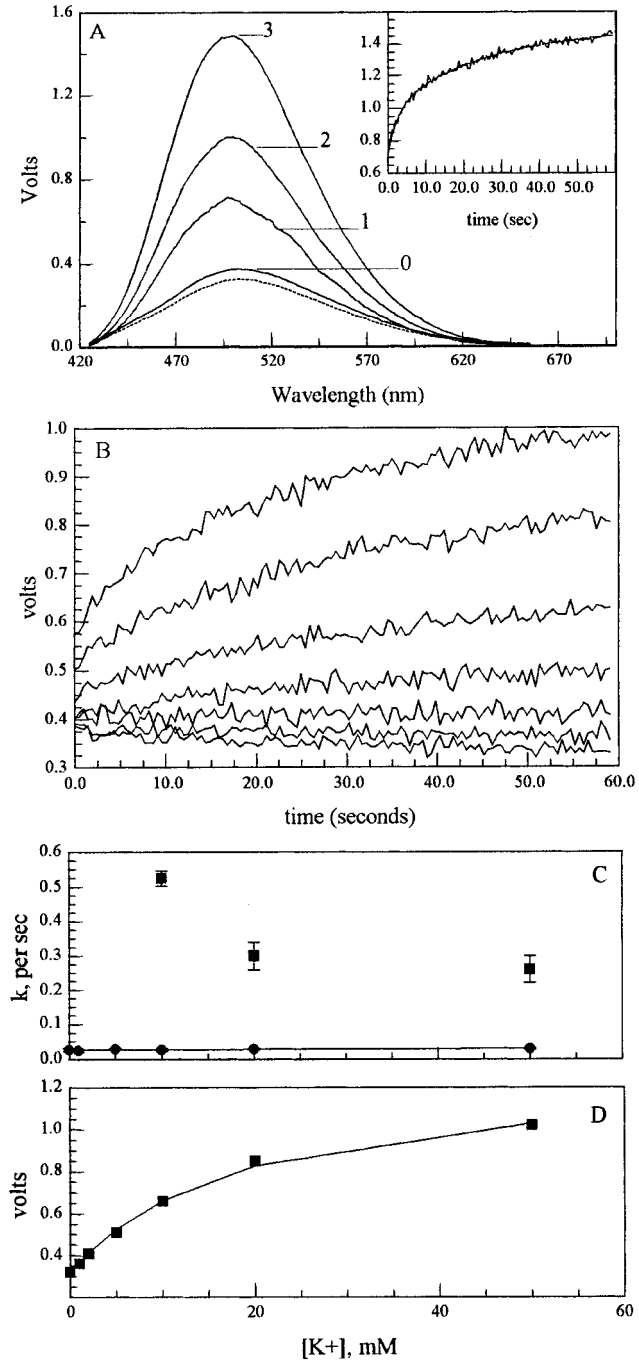


FIGURE 5: (A) Fluorescence emission spectra (λ_{ex} = 420 nm) of wild-type tyrosine phenol-lyase (1 mg/mL, 19.4 μM) before and after mixing in 0.05 M triethanolamine hydrochloride, pH 8.0, with 50 mM NH_4^+. Curve 0 is the calculated spectrum obtained in the absence of NH_4^+, immediately after 2-fold dilution with buffer in the stopped-flow instrument. The dotted spectrum is the final spectrum of the enzyme mixed with buffer. Curve 1 is the calculated spectrum obtained immediately after mixing with buffer containing 50 mM NH_4^+. Curve 2 is the calculated spectrum for the first observeable phase of fluorescence increase after mixing with 50 mM NH_4^+. Curve 3 is the calculated spectrum for the final phase of fluorescence increase after mixing with 50 mM NH_4^+. Inset: Time course of fluorescence change at 505 nm with 420 nm excitation upon mixing with 50 mM NH_4^+. The solid line is the calculated curve with $1/\tau_1 = 0.27\ s^{-1}$ and $1/\tau_2 = 0.030\ s^{-1}$. (B) Dependence of fluorescence changes on $[K^+]$. Curves are obtained with, from bottom to top, 0, 1, 2, 5, 10, 20, and 50 mM K^+. (C) Dependence of rate constants for fluorescence change on $[K^+]$. Squares, fast phase; circles, slow phase. (D) Concentration dependence of the total fluorescence increase on $[K^+]$. The solid line is the calculated curve with K_d = 17.5 mM.

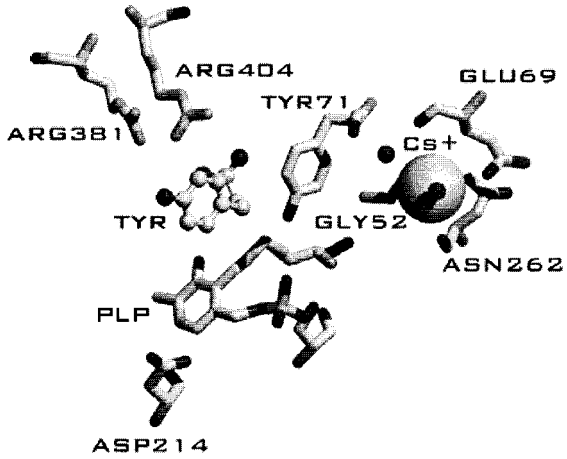


FIGURE 6: Structure of TPL showing the position of the bound Cs^+ cation, prepared from the coordinates of 2TPL.PDB. Small spheres near the Cs^+ are coordinated waters.

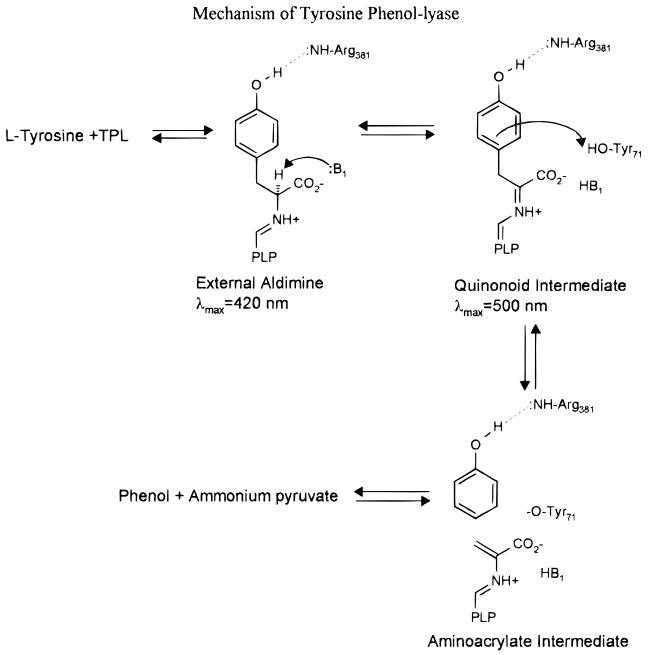
recently by X-ray crystallography. The PLP-dependent enzymes with crystal structures determined that require monovalent cations include dialkylglycine decarboxylase (*31*), tryptophan synthase (*32*), TPL (*13*), and Trpase (*33*). The structures of the cation binding sites in these PLP-dependent enzymes are remarkably diverse. In the structure of TPL (*13*), crystallized in the presence of Cs^+, the cation binding site is at the interface of two subunits in the dimer, with ligands contributed by the carbonyl oxygens of Gly52 and Asn262 from one chain and monodentate ligation by one of the ϵ-oxygens of Glu69 from another chain (Figure 6). The other coordination positions are occupied by water molecules. The cation site is adjacent to the active site, but the cation is not in direct contact with either substrate or PLP (Figure 6). However, Tyr71 forms a hydrogen bond with the phosphate group of PLP (*13*, *34*), suggesting that the mechanism of activation may involve a change in the structure of Glu69, which is transmitted to the PLP site by Tyr71. The structure of Trpase from *Proteus vulgaris* shows a K^+ binding site very similar to that of TPL (*28*). The activating monovalent cation site in dialkylglycine decarboxylase has an aspartate ligand and is also at a subunit interface near the active site but in a different location than TPL and Trpase (*31*). In contrast, the monovalent cation site in tryptophan synthase is not located at a subunit interface and is coordinated only to peptide backbone carbonyl oxygens and water molecules (*32*). Furthermore, Na^+ is as effective as K^+ or NH_4^+ for tryptophan synthase activation (*35*).

In the present work, we wanted to determine the role of Glu69 in the monovalent cation activation of TPL by mutation to Gln and Asp. These are conservative mutations, which either retain the negative charge but increase the ligand distance from the cation site (Asp), or retain the distance but remove the charge (Gln). We were surprised to find that the negative charge is apparently not required for either cation binding or activation, as E69Q TPL is activated by K^+, Rb^+, and NH_4^+ at concentrations comparable to that of wild-type TPL (Tables 1 and 2). This result is in contrast to studies of *S*-adenosyl methionine synthase, in which mutation of Glu42, a K^+ ligand, to Gln abolished monovalent cation activation (*36*). There are some minor differences in the monovalent cation specificity between wild-type and E69Q TPL (Table 1, Figure 1, panels A and B) In particular, Cs^+ and Li^+ bind

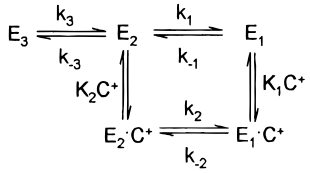
significantly better to E69Q TPL than wild-type TPL, although the level of activation is somewhat reduced (Table 1). These differences may reflect some minor difference in conformation caused by the mutation. In both wild-type and E69Q TPL, however, NH_4^+ is the most effective activator, with K_A values of about 0.1−0.2 mM, at least 10-fold lower than the other monovalent cations. This suggests that the tetrahedral geometry of NH_4^+ is ideally suited for binding to the cation site. It is likely that NH_4^+ forms hydrogen bonds to O^ϵ of Glu69 as well as the carbonyl oxygens of Gly52 and Asn262. These interactions must be stronger than the electrostatic interactions of the alkali metal cations with these ligands. In contrast, E69D TPL has very low elimination activity, even when strongly activating monovalent cations are present (Table 2), showing that the structure of the cation ligand is more critical than the presence of the charge for activation of the enzyme. The low activity of E69D TPL could be due to structural changes caused by the mutation, as was observed when a similar mutation was performed in Staphylococcal nuclease (*37*). However, monovalent cation binding apparently still occurs to E69D TPL, as evidenced by a 4-fold increase in steady-state elimination activity (Table 2) and a decrease in racemase activity (Figure 2), as well as changes in the pre-steady-state kinetic behavior (Table 3). Hence, our data suggest that Glu69 is the residue responsible for transmission of activation from the monovalent cation site to the active site.

It has been proposed previously that binding of monovalent cations to TPL affects a conformational equilibrium between low and high activity forms, with binding of K^+, NH_4^+, or Rb^+ favoring the high activity form (*4*). This model proposes that in the absence of activating cations, the equilibrium lies strongly toward the inactive form. The activated form, stabilized by monovalent cations, binds the substrate and catalyzes the reaction. This mechanism is consistent with the activation only affecting k_{cat} of L-tyrosine and not affecting K_m. Our fluorescence data are in general agreement with this model, as only activating cations cause the increase in the fluorescence emission of the internal aldimine. However, our data require more than a two-state model to explain the observed changes in fluorescence emission. The minimum model to explain these data is given by eq 6. The relaxation equations expected for this model are given in eq 7 and eq 8 (*38*). In this model, E_1 is the fully activated enzyme, E_2 and E_3 are low activity forms, and C^+ is the monovalent cation. In the absence of cations, there is a relaxation upon enzyme dilution to give a less fluorescent form, E_3 (compare Figure 5A, curve 0 and dotted line). This form may be the apoenzyme, since it is likely that PLP binding is weakened in the absence of activating monovalent cations, and dilution would favor PLP dissociation. At equilibrium, without monovalent cations present, the predominant forms of enzyme are E_2 and E_3. Monovalent cation binding clearly occurs rapidly upon mixing to form E_1C^+ and E_2C^+, with an associated increase in fluorescence emission intensity (Figure 5A, curve 1). The system then relaxes to the new equilibrium position where the fully active form, E_1C^+, is a dominant species, with even higher fluorescence intensity (Figure 5A, curve 2). The decrease in $1/\tau_1$ with increasing $[C^+]$ suggests that $k_1 + k_{-1}$ is somewhat larger than $k_2 + k_{-2}$. Finally, the relaxation between E_2 and E_3 is responsible for the slow increase in fluorescence, which

Scheme 1: Mechanism of Tyrosine Phenol-Lyase



is independent of $[C^+]$ (Figure 5C). The total increase in fluorescence emission intensity is saturable (Figure 5D), with an apparent K_D of 17.5 mM. This apparent constant also includes the equilibrium of E_2 and E_3, which is likely dependent on [PLP]. These experiments were performed with stoichiometric [PLP], whereas the activation assays were performed in the presence of a large excess of PLP.



(6)

$$1/\tau_1 = k_1K_2/(K_2 + [C^+]) + k_{-1}K_1/(K_1 + [C^+]) + k_2[C^+]/(K_2 + [C^+]) + k_{-2}[C^+]/(K_1 + [C^+]) \quad (7)$$

$$1/\tau_2 = k_3 + k_{-3} \quad (8)$$

The proposed mechanism of TPL based on previous work in our and other laboratories (*13*, *39*) is shown in Scheme 1. L-Tyrosine binds to TPL to form initially an external aldimine, absorbing at 420 nm, which is subsequently deprotonated at the α-carbon to form the quinonoid intermediate, with an absorbance peak at 500 nm. The pre-steady-state kinetic data show that the external aldimine forms from L-Tyr within the dead time (ca. 2 ms) of the stopped-flow instrument whether an activating monovalent cation is present or not. In addition, in the reaction of L-Phe, the K_d for external aldimine formation with wild-type TPL is almost identical with or without K^+, indicating that monovalent cations do not significantly affect the equilibrium for external aldimine formation. These results are in contrast to tryptophan synthase, where monovalent cations significantly increase the rate of external aldimine formation from L-serine (*35*).

In contrast to previous studies carried out with L-alanine (*4*, *10*), the absorbance due to the quinonoid intermediate is

much greater with L-tyrosine in the absence of cations or in the presence of Na^+, Li^+, or Cs^+ than in the presence of K^+, NH_4^+, or Rb^+. Although at first glance it seems inconsistent that the alanine racemase activity is inhibited (Figure 2) under the same conditions that promote quinonoid intermediate formation, we have previously shown that a conformational change of the quinonoid intermediate, not its formation, is rate-limiting for alanine racemization (*10*). Furthermore, we found that increasing $[K^+]$ decreases the intensity of the quinonoid band formed from D-alanine (*10*), and we proposed at that time that D-alanine binds to a different conformation than L-alanine. The present data also suggest that the conformation reactive with D-alanine is the one that has low activity for the β-elimination reaction. The pre-steady-state kinetic data show that the activating effect of the monovalent cations on TPL is primarily felt at the stage of quinonoid intermediate formation (Figures 3 and 4; Table 3). The activating monovalent cation must be present with the enzyme prior to mixing with substrate to see the effect of the activation in the pre-steady state. This result suggests that the activation of TPL is relatively slow as compared to substrate binding. In agreement with this conclusion, we found that the rate constant for the fluorescence increase at 505 nm upon mixing with activating cations (Figure 5C) is far slower ($1/\tau < 0.5$ s^{-1}) than the reaction with substrate to form an external aldimine complex ($1/\tau \geq 1000$ s^{-1}) or quinonoid intermediate ($1/\tau = 100$ s^{-1}). The formation of the 500-nm absorbance peak from L-tyrosine is at least biphasic under all conditions studied (Table 3). The fastest phase of formation, with $1/\tau = 60-100$ s^{-1}, of the quinonoid intermediate from L-tyrosine is only observed in the presence of K^+, NH_4^+, and Rb^+ (Table 3). This phase is the α-proton abstraction, since we previously reported that it shows a primary isotope effect of 6.0 with α-^{2}H-3-F-L-tyrosine in the presence of K^+ (*15*). The slower rate of deprotonation by the Cs^+ enzyme, $1/\tau$ about 30 s^{-1}, suggests that the active site structure is slightly different with Cs^+ bound in the monovalent cation site. The second phase, with $1/\tau$ about 2 s^{-1}, is observed under all conditions with both wild-type and E69Q TPL. This phase may represent the rate constant for α-proton abstraction by the low activity conformation. Hence, the formation of the quinonoid intermediate is increased about 30−50-fold by activation. The third phase of the reaction is much slower, with $1/\tau = 0.2$ s^{-1}, and is likely to represent a conformational equilibration between active and inactive enzyme forms. This proposal is supported by the fact that the rate constant for this phase is very similar to the rate constant of the fast phase of fluorescence change associated with activation. Cs^+ is unique in that it is the only cation for which all three phases are observed in the reaction of wild-type TPL. Although E69Q TPL is highly active in steady-state kinetic studies (Table 2), the stopped-flow data clearly shows that all three phases of the reaction at 500 nm are observed even in the presence of cations that activate wild-type TPL highly (Table 3). This suggests that the mutation of Glu69 to Gln affects somewhat the ability of the cation to influence the enzyme internal equilibrium of high and low activity conformations. The rate constants for quinonoid intermediate formation in the absence of monovalent cations (Table 3) are still faster than k_{cat} (Table 2), indicating that α-deprotonation is not rate-limiting.

Phenol elimination from the quinonoid intermediate is assisted by Tyr71 (*34*) and Arg381 (*13*) to give the α-aminoacrylate intermediate (Scheme 1), which then releases ammonium pyruvate. The slow phase of decreasing absorbance at 505 nm observed in the absence of cations is seen in the reactions of wild-type and E69Q TPL with L-tyrosine, suggesting that this may be due to slow phenol elimination. This decrease in absorbance of the quinonoid intermediate formed in the absence of activating monovalent cations is not seen with wild-type TPL reacting with L-Phe or with E69D TPL reacting with L-Tyr. Thus, the rate of elimination of phenol from the quinonoid intermediate is also increased by monovalent cation activation. However, because of the poor fit of the slow phase, it was not possible to quantify the extent of activation of the elimination step from these data. Tryptophan synthase was also shown to exhibit an increase in the rate constant for α-aminoacrylate intermediate formation in the presence of monovalent cations, Na^+ or NH_4^+ (*40*).

Monovalent cations activate TPL at low concentrations but inhibit at higher concentrations (Figure 1, panels A and B). A similar biphasic response was seen previously in the effect of K^+ on the 500-nm absorbance of the complexes of TPL with D-alanine and L-alanine (*10*). The inhibition of TPL by Na^+ was reported previously (*4*). However, in our hands, the activity in the presence of Na^+ does not decrease below the basal level observed in the absence of cations, and inhibition by Na^+ is seen only after partial activation with low $[K^+]$. Our data show that this inhibition is a general phenomenon, as it is also seen with K^+, Rb^+, Li^+, Cs^+, and NH_4^+ at concentrations greater than 100 mM (Figure 1, panels A and B). The mechanism of the monovalent cation-dependent inhibition of TPL is of interest. One possibility could be an ordered mechanism for product and cation release. However, our pre-steady-state data indicate that the activating monovalent cation must bind to TPL prior to substrate and remain bound throughout the reaction cycle. Thus, a likely explanation for the inhibition seen at high concentrations of cations is that there is a low affinity site somewhere in the TPL structure that inhibits activity. A second inhibitory monovalent cation binding site was observed in the structure of dialkylglycine decarboxylase (*31*). A second cation binding site has not been observed in TPL structures, possibly because of the use of Cs^+ in the crystallization studies, which appears to have lower affinity for inhibition (Table 1). Since we found no significant effect of high $[C^+]$ on the rate of external aldimine and quinonoid intermediate formation in the pre-steady state, and the steady-state absorbance at 500 nm is similar at 50 mM and 330 mM $[K^+]$, implying that the rate of elimination is unaffected, it seems unlikely that the inhibiting effect of cations at high concentration is due to binding to a second cation site on the free enzyme. Thus, the inhibitory effect is most likely on the rate of pyruvate release. Since NH_4^+ is a product and also one of the most effective inhibitors (see Figure 1), the general inhibiting effect of monovalent cations may be due to product inhibition by binding at the active site. In contrast, the specific inhibitory effect of Na^+ in the presence of relatively low concentrations of activating cations is likely due to competition with other cations for the activator site, where Na^+ can apparently bind but does not alter the conformational equilibrium toward the active form.

Conclusion. Glu69 in TPL is the residue that is responsible for transmission of activation from the monovalent cation site to the active site in *C. freundii* TPL. The negative charge of Glu69 is not necessary for monovalent cation binding or activation. The monovalent cation activation of TPL is due at least in part to large increases in the rate constants for quinonoid intermediate formation and phenol elimination. The interconversion of high and low activity forms of TPL is a relatively slow process. High concentrations of cations inhibit TPL, possibly by product inhibition, reducing the rate of pyruvate release.

SUPPORTING INFORMATION AVAILABLE

Rapid-scanning stopped-flow spectra of wild-type, E69Q, and E69D TPL in the presence of various monovalent cations. Also, time courses of the pre-steady-state reaction with L-tyrosine at 500 nm with and without preincubation with K^+ are included. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Suelter, C. H. (1970) *Science 168*, 789.
2. Woehl, E. U., and Dunn, M. F. (1995) *Coord. Chem. Rev. 144*, 147.
3. Kumagai, H., Yamada, H., Matsui, H., Ohkishi, H., and Ogata, K. (1970) *J. Biol. Chem. 245*, 1767.
4. Myagkikh, I. V., and Demidkina, T. (1985) *Biokhim (Eng. Trans.) 19*, 555.
5. Phillips, R. S. (1987) *Arch. Biochem. Biophys. 256*, 302.
6. Yamada, H., Kumagai, H., Kashima, N., Torii, H., Enei, H., and Okumura, S. (1972) *Biochem. Biophys. Res. Commun. 46*, 370.
7. Enei, H., Yamashita, K., Okumura, S., and Yamada, H. (1973) *Agric. Biol. Chem. 37*, 485.
8. Kumagai, H., Yamada, H., Matsui, H., Ohkishi, H., and Ogata, K. (1970) *J. Biol. Chem. 245*, 1773.
9. Ueno, T., Fukami, H., Ohkishi, H., Kumagai, H., and Yamada, H. (1970) *Biochim. Biphys. Acta 206*, 476.
10. Chen, H., and Phillips, R. S. (1993) *Biochemistry 32*, 11591.
11. Kazakov, V. K., Tarusina, I. I., Myagkikh, I. V., and Demidkina, T. V. (1987) *Biokhimiya* (*Moscow*) *52*, 1319.
12. Antson, A. A., Demidkina, T. V., Gollnick, P., Dauter, Z., Von Tersch, R. L., Long, J., Berezhnoy, S. N., Phillips, R. S., Harutyunyan, E. H., and Wilson, K. S. (1993) *Biochemistry 32*, 419.
13. Sundararaju, B., Antson, A. A., Phillips, R. S., Demidkina, T. V., Barbolina, M. V., Gollnick, P., Dodson, G. G., and Wilson, K. S. (1997) *Biochemistry, 36*, 6502.
14. Dua, R. K., Taylor, E. W., and Phillips, R. S. (1993) *J. Am. Chem. Soc. 115*, 1264.
15. Chen, H., Gollnick, P., and Phillips, R. S. (1995) *Eur. J. Biochem. 229*, 540.
16. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488.
17. Suelter, C. H., Wang, J., and Snell, E. E. (1976) *FEBS Lett. 66*, 230.
18. Morino, Y., and Snell, E. E. (1970) *Methods Enzymol. 17A*, 439.
19. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
20. Muro, T., Nakatani, H., Hiromi, K., Kumagi, H., and Yamada, H. (1978) *J. Biochem. (Tokyo) 84*, 633.
21. Peterson, E. A., and Sober, H. A. (1954) *J. Am. Chem. Soc. 76*, 169.
22. Durbin, J., and Watson, G. S. (1970) *Biometrika 37*, 409.
23. Matheson, I. B. C. (1990) *Comput. Chem. 14*, 49−57.
24. Strickland, S., Palmer, G., and Massey, V. (1975) *J. Biol. Chem. 250*, 4048.
25. Suelter, C. H., and Snell, E. E. (1976) *J. Biol. Chem. 252*, 1852.
26. Brot, N., and Weissbach, H. (1970) *Methods Enzymol. 17A*, 642.
27. Phillips, R. S., Von Tersch, R. L., and Secundo, F. (1997) *Eur. J. Biochem. 244*, 658.
28. Laughlin, L. T., and Reed, G. H. (1997) *Arch. Biochem. Biophys. 348*, 262.
29. Kerr, K. M., Cahoon, M., Bosco, D. A., and Hedstrom, L. (2000) *Arch. Biochem. Biophys. 375*, 131.
30. Zhang, R., Villeret, V., Lipscomb, W. N., and Fromm, H. J. (1996) *Biochemistry 35*, 3038.
31. Toney, M. D., Hohenester, E., Cowan, S. W., and Jansonius, J. N. (1993) *Science 261*, 756.
32. Rhee, S., Parris, K. D., Ahmed, S. A., Miles, E. W., and Davies, D. R. (1996) *Biochemistry 35*, 4211.
33. Isupov, M. N., Antson, A. A., Dodson, E. J., Dodson, G. G., Dementieva, I. S., Zakomirdina, L. N., Wilson, K. S., Dauter, Z., Lebedev, A. A., and Harutyunyan, E. H. (1998) *J. Mol. Biol. 276*, 603.
34. Chen, H., Demidkina, T. V., and Phillips, R. S. (1995) *Biochemistry 34*, 12776.
35. Woehl, E., and Dunn, M. F. (1999) *Biochemistry 38*, 7118.
36. McQueney, M. S., and Markham, G. D. (1995) *J. Biol. Chem. 270*, 18277.
37. Libson, A. M., Gittis, A. G., and Lattman, E. E. (1994) *Biochemistry 33*, 8007.
38. Bernasconi, C. F. (1976) *Relaxation Kinetics*, Academic Press, New York.
39. Faleev, N., Ruvinov, S. B., Demidkina, T. V., Myagkikh, I. V., Gololobov, M. Y., Bakhmutov, V. I., and Belikov, V. M. (1988) *Eur. J. Biochem. 177*, 395.
40. Woehl, E., and Dunn, M. F. (1999) *Biochemistry 38*, 7131.

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