Threonine-124 and phenylalanine-448 in *Citrobacter freundii* tyrosine phenol-lyase are necessary for activity with L-tyrosine

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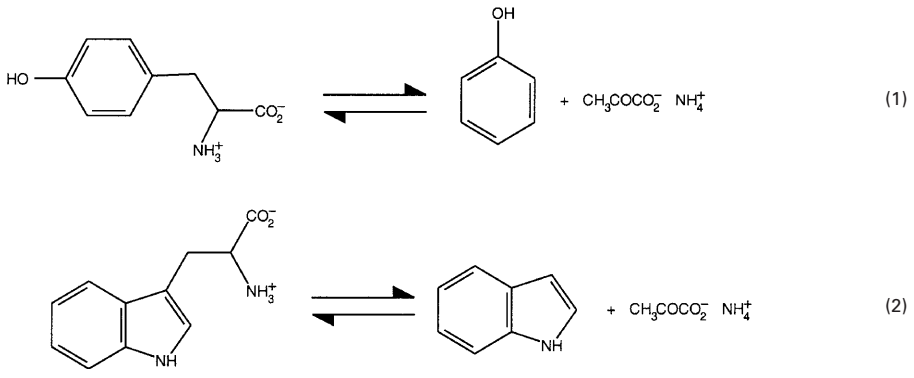
Thr-124 and Phe-448 are located in the active site of *Citrobacter freundii* tyrosine phenol-lyase (TPL) near the phenol ring of a bound substrate analogue, 3-(4′-hydroxyphenyl)propionic acid [Sundararaju, Antson, Phillips, Demidkina, Barbolina, Gollnick, Dodson and Wilson (1997) Biochemistry **36**, 6502–6510]. Thr-124 is replaced by Asp and Phe-448 is replaced by His in the crystal structure of a structurally similar enzyme, *Proteus vulgaris* tryptophan indole-lyase, which has 50% identical residues. Hence, Thr-124 and Phe-448 in TPL were mutated to Ala or Asp, and His, respectively, in order to probe the role of these residues in the reaction specificity for L-Tyr. These mutant enzymes have little or no β-elimination activity with L-Tyr or 3-fluoro-L-Tyr as a substrate, but retain significant elimination activity with *S*-(*o*-nitrophenyl)-L-cysteine, *S*-alkyl-L-cysteines and β-chloroalanine. Furthermore, the binding of L-Tyr and other non-substrate amino acids is not significantly affected by the mutations. The mutant TPLs form intermediates in rapid-scanning stopped-flow experiments with L-Phe, L-Tyr and L-Trp, similar to those seen with wild-type TPL. These results demonstrate that Thr-124 and Phe-448 are necessary for the reaction specificity of TPL for L-Tyr, and probably play a role in the elimination stage of the reaction mechanism. Thr-124 is within hydrogen-bonding distance of the phenolic group of the bound substrate, and may help to orientate the ring for β-elimination to occur. Phe-448 may be important to allow the formation of the closed conformation during the reaction.

Key words: β-elimination, mutagenesis, pyridoxal 5′-phosphate, stopped-flow kinetics, tryptophan indole-lyase.

INTRODUCTION

Tyrosine phenol-lyase (TPL; EC 4.1.99.2) is a pyridoxal 5′-phosphate (PLP)-dependent enzyme which catalyses the β-elimination reaction of L-Tyr to form phenol and ammonium pyruvate [1] (see reaction 1 in Scheme 1). TPL can also catalyse the elimination reactions of a wide range of amino acids with suitable leaving groups on the β-carbon *in vitro*, including *S*-(*o*-nitrophenyl)-L-cysteine [2], *S*-alkyl-L-cysteines [1], β-chloro-L-alanine [1], L-serine [1] and *O*-acyl-L-serines [2]. We have previously reported the X-ray crystallographic structure of the apo form of TPL [3], and of the holoenzyme complexed with the substrate analogue 3-(4′-hydroxyphenyl)propionic acid [4]. Examination of the residues in the substrate-binding site of *Citrobacter freundii* TPL shows that Arg-381, Phe-448 and Thr-124 are in the pocket where the phenol ring of the substrate binds (Figure 1). Mutagenic and kinetic analyses demonstrated previously that Arg-381 is required for the L-Tyr substrate specificity



Scheme 1 Reactions of TPL and Trpase

Reaction 1: β-elimination reaction of L-Tyr to form phenol and ammonium pyruvate catalysed by TPL. Reaction 2: Trpase catalyses the similar cleavage of L-Trp to indole and ammonium pyruvate.

Abbreviations used: TPL, tyrosine phenol-lyase; Trpase, tryptophan indole-lyase; SOPC, *S*-(*o*-nitrophenyl)-L-cysteine; PLP, pyridoxal 5′-phosphate.

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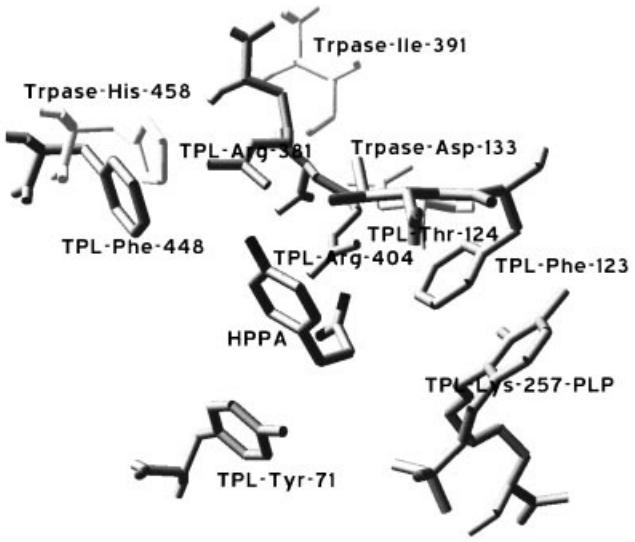

Figure 1 Active site of *C. freundii* TPL, showing the positions of Asp-133, Ile-391 and His-458 of *P. vulgaris* Trpase (pale grey)

The PLP rings of both structures were superimposed. The conserved residues of *P. vulgaris* Trpase have been omitted for clarity. These data are from Protein Data Bank files 2TPL and 1AX4. HPPA, 3-(4′-hydroxyphenyl)propionic acid.

of TPL [4]. Comparison of the X-ray structure of *Proteus vulgaris* tryptophan indole-lyase (Trpase; also known as tryptophanase; EC 4.1.99.1) [5], an enzyme which catalyses the similar cleavage of L-Trp to indole and ammonium pyruvate (Scheme 1, reaction 2), shows that Thr-124 of TPL corresponds to Asp-133 of Trpase, and Phe-448 of TPL corresponds to His-458 of Trpase (Figure 1). The sequence alignment of known TPL sequences with those of Trpase shows that Thr-124 is invariably substituted by aspartate, and Phe-448 is invariably substituted by histidine (Figure 2). The proposed mechanisms of TPL and Trpase are presented in Scheme 2. Both TPL and Trpase bind their respective substrates to form similar external aldimine intermediates, which are subsequently converted into quinonoid intermediates by removal of the α-proton. Tautomerization of the aromatic rings, assisted by catalytic bases (B_2 in Scheme 2) identified in our previous pH-dependent kinetic studies [6,7], is then required for elimination to proceed to form the α-aminoacylate intermediate. It has been proposed that the tautomerization and elimination stages provide the high substrate specificity of these enzymes towards their physiological substrates [8]. Therefore, in the present work, we performed site-directed mutagenesis of Thr-124 of TPL to Asp and Ala, and of Phe-448 to His, to determine the role of these residues. The results suggest that Thr-124 and Phe-448 play significant roles in the reaction specificity of TPL for L-Tyr.

MATERIALS AND METHODS

Materials

L-Tyrosine, *S*-ethyl-L-cysteine, L-serine and 2-mercaptoethanol were obtained from Sigma. *S*-Methyl-L-cysteine and indole were purchased from Aldrich. *S*-(*o*-Nitrophenyl)-L-cysteine (SOPC) was prepared as described previously [9]. Lactate dehydrogenase from rabbit muscle, L-tryptophan, PLP and NADH were purchased from United States Biochemical Co. All other chemicals and reagents were of analytical grade.

Sequence alignment

Sequence alignments were performed with the GCG package of programs [10], using GAP for pairwise alignments and PILEUP for the global alignment of TPL and Trpase sequences, using the default parameters. Homologues were identified using BLAST to search the microbial databases available on the National Library of Medicine web server (http://www.ncbi.nlm.nih.gov/entrez).

Site-directed mutagenesis

Site-directed mutagenesis to replace Phe-448 of TPL with His (to produce F448H TPL) was performed on plasmid pTZTPL by the modified method of Kunkel [11] using a MutaGene kit from Bio-Rad, as described previously [4]. The mutagenic oligonucleotide was 5′-CAGCTCCGTCACTTTACTGCA-3′. The presence of the mutation was 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 (Athens, GA, U.S.A.). The resulting plasmid containing the single F448H mutation was designated pTZTPL F448H.

The oligonucleotide-directed site-specific mutagenesis to replace Thr-124 with Ala or Asp (to produce mutants T124A TPL and T124D TPL) and to prepare the double mutant T124D/F448H was performed by the method of Taylor et al. [12] using the Sculptor *in vitro* mutagenesis kit from Amersham Biosciences. The single-stranded DNA was prepared with the aid of M13K07 helper phage [13]. To perform the required T124A and T124D replacements the mutagenetic oligonucleotides 5′-GAATATGTATTTCGCTACTACCCG-3′ and 5′-GAATATGTATTTCGATACTACCCGTTAC-3′ were prepared. The resulting plasmids containing the required changes were screened by dideoxy sequencing [14] using a Sequenase kit (United States Biochemical Co.) in the mutation region. The resulting plasmids carrying the T124A and T124D mutations were designated pTZTPL T124A and pTZTPL T124D. To prepare the F448H/T124D double mutation, single-stranded DNA was prepared from plasmid pTZTPL T124D, and mutagenesis was performed with the mutagenetic oligonucleotide 5′-CGAAGCAGCTCCGTCACTTTACTGCACGC-3′. The resulting plasmid carrying TPL with T124D/F448H mutations was designated pTZTPL F448H/T124D. *Escherichia coli* SVS370 cells were used as the host for the plasmids, since these cells are *tna*A and thus do not produce Trpase (there is a transposon, *tn*5, in the genomic *tna*A gene that codes for Trpase), which could potentially interfere with the measurement of any possible Trpase activity of wild-type and mutant TPL.

Purification of enzymes

E. coli SVS370 cells containing pTZTPL with the wild-type and mutant TPL genes were grown and the enzyme was purified as described previously [15].

Enzyme assays

Steady-state kinetic experiments were performed using a Cary 1E UV/visible spectrometer equipped with a 6×6 thermoelectric cell compartment. The β-elimination reactions of wild-type and mutant TPL with L-Tyr and *S*-alkyl-L-cysteines were measured using the coupled assay with lactate dehydrogenase and NADH, measured at 340 nm ($\Delta\epsilon = -6.22 \times 10^3$ M$^{-1} \cdot$ cm^{-1}), as described previously by Morino and Snell [16] for Trpase. Reaction mixtures contained, in a total volume of 0.6 ml, 50 mM potassium phosphate, pH 8.0, 5 mM 2-mercaptoethanol, 50 μM PLP,

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hflutna      EREKGLDRSKMVVFSNYFFDTTQGHSQINGATVRNVYIKEAFDTTAKHPFKGNFDLEKLE
pmoltotna    EQEKGLDRSKMVVFSNYFFDTTQGHSQINGATVRNVYTKEAFDTSVNADFKGDFDLEKLE
ecoli        EQEKGLDRSKMVAFSNYFFDTTQGHSQINGCTVRNVYIKEAFDTGVRYDFKGNFDLEGLE
vcholera     EKEKGLDRSKMVALSNYFFDTTQGHTQINCCVAKNVYTEEAFDTGVKADFKGNFDLEKLE
pvulgaris    QKEGK..AKNPVFISNFHFDTTAAHVELNGCKAINIVTEKAFDSETYDDWKGDFDIKKLK
pinconstans  QEQGG..AKKPVFISNFHFDTTAAHVELNHCKAINIVTEKAYDSDTYDDWKGNFDIQKLK
entaer       ....K..SAHPVFISNFHFDTTAAHVELNGAKAINVVTPKAFDTTSWYDWKGNFDIDLLK
sttna1       GM.........YVLSNMFFDTTRGHVQLGGRP.VDLLIDVPTEEY..HPFKGNMDTERLE
sttna2       GM.........YVLSNMFFDTTRGHVQLAGRP.IDLLLDVPTEEY..HPFKGNMDTARLE
cfrseq       GQ.........YVAGNMYFTTTRYHQEKNGAVFVDIVRDEAHDAGLNIAFKGDIDLKKLQ
eherbicola   GQ.........YVAGNMYFTTTRFHQEKNGATFVDIVRDEAHDASLNLPFKGDIDLNKLA
pmoltotpl    GD.........YVPGNMYFTTTRAHQERNGATFVDIIIDEAHDSQIDLPFKGNVDVKKLQ
sttpl        GD.........YIPGNMYFTTTRTHQELQGGTFVDVIIDEAHDPQANHPFKGNVDIAKFE
pgingtna     GD.........VLPGNSHFDTTKGHIEYRRAFAPDCTIDEAADTQIELPFKGNMDLNKLE
apernixtna   .....RRRNARIVPANTHFDTGRAVILNQGGVPLDLPSPQA.SRREAYPFKGDIDVARLE
rhodocaptna  .....FGGKGRRIPSNTHFDTTRGNIEASGATGDDLVIAEGKDPQNLHPFKGNMDLARLE
consensus              .. .* .*.*.........  ........ ..    ..**..*. ...
             121......130.......140.......150.......160.......170........

hflutna      LLRLTVPRATYTQTHMDFIIEAFQKVKENAENI.KGLTFTYEPKV..LRHFTARLKEVE~
pmoltotna    LLRLTIPRATYTQTHMDFIIEAFKQVKENAENI.KGLTFTYEPKV..LRHFTAR~~~~~~
ecoli        LLRLTIPRATYTQTHMDFIIEAFKHVKENAANI.KGLTFTYEPKV..LRHFTAKLKEV*~
vcholera     LLRLTIPRATYTQTHMDFIIEAFEKVKANARNV.KGLEFTYEPPV..LRHFTARLKEKA~
pvulgaris    FMRLTIARRVYTNDHMDYIADALIGLKEKFATL.KGLEFEYEPPV..LRHFTARLKPIE*
pinconstans  FMRLTIARRVYTNDHMDYIADALIGLKDKFATL.KGLDFEYEPPV..LRHFTARLKPIK~
entaer       LLRLTIPRRVYTNDHMDYIADALIAVKARAATI.KGLTFTYEPPV..LRHFVARLKPVK*
sttna1       FTRLAIPRRVYTNLHLEDVAETVINAFQKREEI.RGVKFAREPKV..LRHFTAWFDPA*~
sttna2       FTRLAIPRRVYTNLHLEDVAETVINAFQKREQI.RGVKFTREPKV..LRHFTAHFDLV*~
cfrseq       TVRLTIPRRVYTYAHMDVVADGIIKLYQHKEDI.RGLKFIYEPKQ..LRFFTARFDYI*~
eherbicola   TVRLTIPRRVYTYAHMDVVADGIIKLYQHKEDI.RGLTFVYEPKQ..LRFFTARFDFI*~
pmoltotpl    LVRLTIPRRVYTYAHLDHVADTIIKLFKHRDDI.KGLDMVYEPKL..LRFFTARFE~~~~
sttpl        LVRLTIPRRVYTDRHMDVVAYSVKHLWKERDTI.RGLRMVYEPPT..LRFFTARFEPIS*
pgingtna     LLRLAIPRRTYTNNHMDVIAAAVKNVYDRRESITRGYVITYENPI..MRHFTVELE~~~~
apernixtna   LLRLAVPRRTYTNSHMEYVAASLARLLREGRRKVKGLRVVKEPRIKGIRHFLAELEPIEP
rhodocaptna  LVRLAFPRRTYTQSHADYIVEAFEELAAT.KDALRGYRIVKEPKL..MRHFTCRFEKL~~
consensus    ..**...*..**. *........ .    . .  .*. . .*...  .*.*..... .
             421......430.......440.......450.......460.......470........
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Figure 2 Partial sequence alignment of TPL, Trpase and similar sequences from various microbial sources

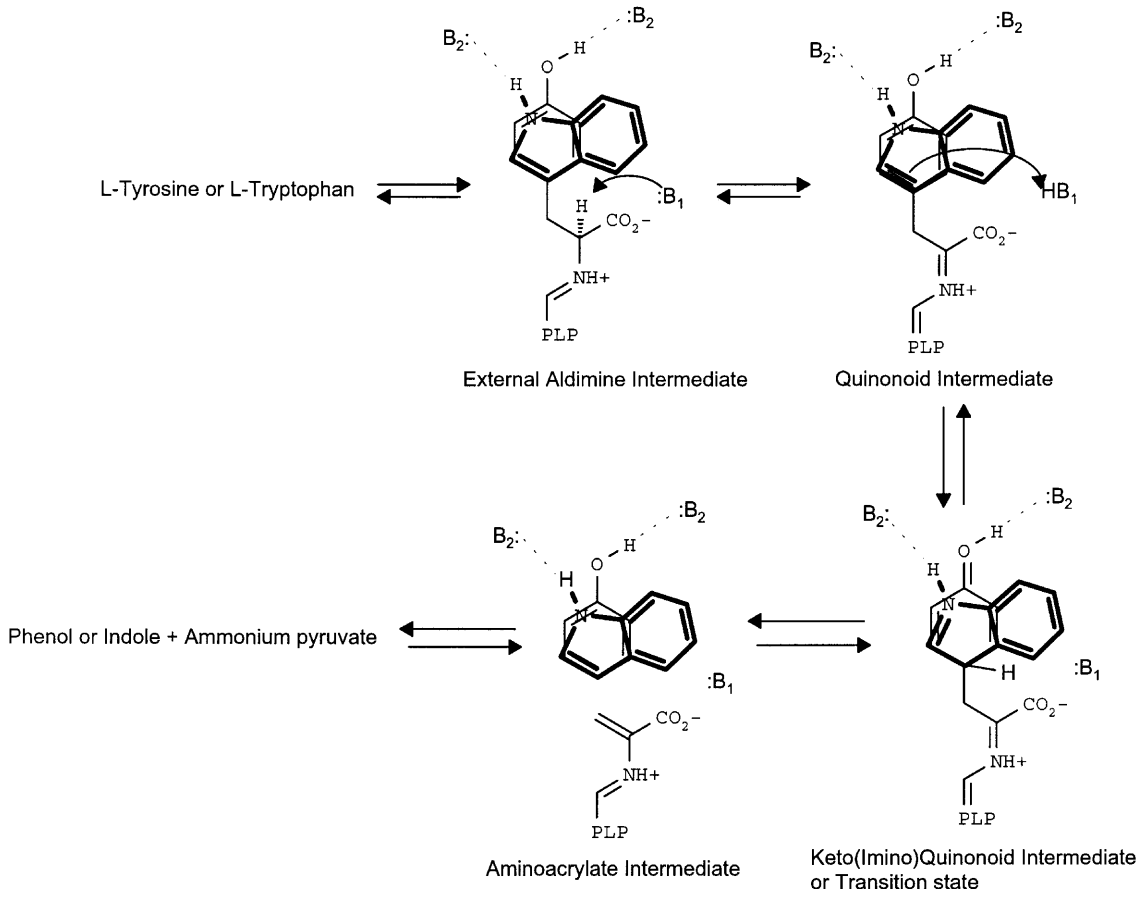
hflutna, *Haemophilus influenzae* Trpase; pmoltotna, *Pasteurella moltocida* Trpase; ecoli, *E. coli* Trpase; vcholera, *Vibrio cholerae* Trpase; pvulgaris, *P. vulgaris* Trpase; pinconstans, *Proteus inconstans* Trpase; entaer, *Enterobacter aerogenes* Trpase; sttna, *Symbiobacterium thermophilum* Trpase; cfrseq, *C. freundii* TPL; eherbicola, *Erwinia herbicola* TPL; pmoltotpl, *P. molotocida* TPL; sttpl, *Symbiobacterium thermophilum* TPL; pgingtna, *Porphyromonas gingivalis* Trpase; apernixtna, *Aeropyrum pernix* Trpase; rhodocaptna, *Rhodobacter capsulatus* Trpase. Residues corresponding to Thr-124 and Phe-448 of *C. freundii* TPL are shown in bold. Asterisks indicate completely conserved residues.

0.2 mM NADH, 0.02 mg of lactate dehydrogenase and various amounts of amino acid substrate at 30 °C. The reaction was initiated by the addition of enzyme solution. This assay can detect rates as low as 1×10^{-4} s^{-1} with 1 mg/ml enzyme. The very low β-elimination activity for L-Tyr of the F448H mutant enzyme could not be detected by the spectrophotometric assay, but was measured by HPLC analysis using a C_{18} column (4.1 mm × 250 mm), at 1 ml/min, running a gradient of methanol (20–80 %) over 30 min in 50 mM sodium phosphate buffer (pH 3.0). The elution of compounds was monitored by UV absorbance at 220 nm. This assay can detect TPL activity in a 1 mg/ml solution as low as 1×10^{-5} s^{-1}. Enzyme activity during purification was measured routinely with 0.6 mM SOPC in 50 mM potassium phosphate, pH 8.0, at 25 °C [2], following the decrease in absorbance at 370 nm ($\Delta\epsilon = -1.86 \times 10^3$ M$^{-1} \cdot$ cm^{-1}). A unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of product/min. Determination of the kinetic parameters for SOPC was performed at 30 °C in 50 mM potassium phosphate, pH 8.0, 5 mM 2-mercaptoethanol and 50 μM PLP, with various amounts of SOPC and appropriate dilutions of wild-type and mutant TPL.

The Trpase activity of wild-type and mutant TPL was measured by modification of a previously published procedure using Ehrlich's reagent [17]. Reaction mixtures containing 1 mg of enzyme with 2 mM Trp were incubated for 1 h at 37 °C in 200 μl of 50 mM potassium phosphate buffer, pH 7.8, and the reaction was stopped by addition of 200 μl of 0.5 M perchloric acid. The precipitated protein was removed by centrifugation. Formation of indole was then measured by addition of 300 μl of the supernatant solution to an equal volume of freshly prepared Ehrlich's reagent [17]. After incubation at 20 °C for 30 min, the absorbance was read at 571 nm. Standards were also prepared containing 1–10 nmol of indole.

Protein determination

Protein was determined in crude extracts by the method of Bradford [18], with purified TPL as a standard. The concentration of purified TPL was determined from the absorbance at 278 nm ($\epsilon = 3.47 \times 10^4$ M$^{-1} \cdot$ cm^{-1}) [19]. The PLP content was determined by measuring the spectrum of the enzyme in 0.1 M NaOH, assuming $\epsilon_{390} = 6600$ M$^{-1} \cdot$ cm^{-1} [20].



Scheme 2 Comparison of reaction mechanisms of TPL and Trpase

B_1, B_2 and HB_1 refer to proposed basic and acidic catalytic groups in the reactions.

Table 1 Steady-state kinetic parameters for α,β-elimination reactions catalysed by wild-type and mutant TPL

ND, not detected; the limit of detection for the spectrophotometric assay coupling pyruvate formation with NADH consumption using lactate dehydrogenase is 1×10^{-4} s^{-1}, and the detection limit for the HPLC assay is 1×10^{-5} s^{-1}, assuming an enzyme concentration of 1 mg/ml. –, Not determined.

	Wild-type TPL		T124D TPL		T124A TPL		F448H TPL		T124D/F448H TPL	
Substrate	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} · s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} · s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} · s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} · s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} · s^{-1})
L-Tyr	3.5	1.75×10^4	ND	ND	4.24×10^{-3}	4.24	3.4×10^{-5}	0.37	ND	ND
3-Fluoro-L-Tyr	1.4	1.4×10^4	ND	ND	3.8×10^{-3}	4.3	–	–	ND	ND
SOPC	9.7	4.6×10^4	12.6	4.7×10^4	10.2	2.0×10^4	0.6	3.3×10^3	0.35	2.4×10^3
S-Methyl-L-Cys	5.4	3.4×10^3	0.4	1.07×10^3	1.1	1.7×10^2	1.0	6.9×10^2	0.34	5.0×10^2
S-Ethyl-L-Cys	1.1	4.9×10^2	0.24	1.41×10^3	0.87	1.36×10^3	0.27	1.7×10^2	0.3	8.6×10^2
S-Benzyl-L-Cys	0.63	5.3×10^3	0.29	1.81×10^3	0.69	1.86×10^3	–	–	0.44	3.7×10^2
β-Chloro-L-Ala	6.0	2.8×10^3	0.81	1.23×10^3	2.75	1.4×10^3	–	–	–	–

Stopped-flow reactions

Stopped-flow experiments were carried out using an RSM-1000 instrument from OLIS, equipped with a stopped-flow mixing chamber. The stopped-flow mixer has a 10 mm path length and a dead time of approx. 2 ms. Absorbance spectra were collected after flow stop over the wavelength range from 320 to 560 nm at a rate of 1000 spectra · s^{-1}. Enzymes for stopped-flow measurements were preincubated with excess PLP at 30 °C for 30 min, then passed through a PD-10 gel-filtration column (Pharmacia) equilibrated with 0.05 M potassium phosphate, pH 7.8, to remove excess PLP. The reactions were performed at 25 °C with the syringes immersed in a bath controlled by an external circulating Lauda water bath.

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 (eqn 1):

$$V = k_{cat} \cdot [E] \cdot [S]/(K_m + [S]) \quad (1)$$

using the HYPER FORTRAN program of Cleland [21]. In eqn (1), k_{cat} is the turnover number of the enzyme, [S] is the substrate concentration, [E] is the total enzyme concentration and K_m is the Michaelis constant. The output of HYPER provides S.E.M. values for the calculated parameters, and the kinetic parameters reported in Table 1 have S.E.M. values of less than $\pm 10\%$. Stopped-flow data were analysed by global analysis of all spectra at all wavelengths using the Global Works software provided by OLIS [22]. Stopped-flow data for formation of quinonoid intermediates at various ligand concentrations were fitted to eqn (2) [23]:

$$k_{obs} = 1/\tau = k_1[S]/(K_d + [S]) + k_{-1} \quad (2)$$

where k_1 is the rate constant for formation of the quinonoid intermediate (Scheme 2), τ is the relaxation time, k_{-1} is the rate constant for its re-protonation to return to the external aldimine, and K_d is an equilibrium constant for the rapid binding step preceeding the slow observed reaction. Eqn (2) is derived for the mechanism shown in eqn (3):

$$E + S \underset{\text{fast}}{\overset{K_d}{\rightleftharpoons}} EA \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} EQ \quad (3)$$

where EQ is the quinonoid intermediate (Scheme 2) and EA is the external aldimine intermediate (Scheme 2).

Table 2 Kinetic isotope effects (KIE) on the reactions of wild-type and mutant TPL with suitable substrates

$^{D}k_{cat}$ refers to the deuterium isotope effect on k_{cat}.

Substrates compared	Solvents compared	KIE ($^{D}k_{cat}$ value)	
		Wild-type TPL	T124A TPL
3-Fluoro-L-Tyr and 3-fluoro- L-[α-^{2}H]Tyr	H_2O	4.1 ± 0.2	4.22 ± 0.2
3-Fluoro-L-Tyr	H_2O and ^2H_2O	1.7 ± 0.2	2.38 ± 0.17
3-Fluoro-L-Tyr and 3-fluoro-L-[β,β-$^2H_2$]Tyr	H_2O	1.1 ± 0.04	1.16 ± 0.25

Table 3 K_i values of aromatic amino acids for wild-type and mutant TPL

Enzyme	K_i (mM)		
	L-Tyr	L-Trp	L-Phe
Wild-type TPL	0.2 ± 0.02*	7.0 ± 0.9	2.0 ± 0.1
T124D	0.96 ± 0.095	3.3 ± 0.3	0.62 ± 0.04
T124A	1.0 ± 0.1*	–	3.7 ± 0.4
F448H	0.092 ± 0.016	5.8 ± 0.7	2.6 ± 0.2
T124D/F448H	0.7 ± 0.1	7.8 ± 1.3	2.8 ± 0.3

* L-Tyr was used as a substrate.

RESULTS

Design and characterization of mutant TPLs

We mutated Thr-124 to Asp and Phe-448 to His in TPL to determine the role of these residues in the substrate specificity of TPL for L-Tyr. In addition, the double mutant, T142D/F448H TPL, and another single mutant, T124A TPL, were prepared. The presence of the desired mutations, and the absence of other mutations, was confirmed by automated DNA sequencing in the region of the expected mutation. The PLP content of the mutant enzymes was also found to be 1 mol/subunit, as was shown previously for wild-type TPL [24].

Activity of wild-type and mutant TPLs

The activities of wild-type, T124D, T124A, F448H and T124D/F448H mutant TPL were analysed with a number of different substrates. The steady-state kinetic parameters k_{cat} and k_{cat}/K_m are shown in Table 1. Comparison of k_{cat}/K_m values is the best measure of relative efficiency of mutant enzymes, since k_{cat}/K_m is the enzyme specificity constant [25]. The reactions of SOPC, *S*-alkyl-L-cysteines and β-chloroalanine are not greatly affected by any of the mutations, as all the mutant enzymes exhibited k_{cat}/K_m values within about an order of magnitude of wild-type TPL for these substrates. This indicates that these mutations do not interfere with the global formation of the active site of TPL, although the observed effects suggest that the mutations cause some minor structural changes. In contrast, T124D TPL did not display any detectable activity with L-Tyr or its close analogue, 3-fluoro-L-Tyr, and T124A and F448H TPL had very low, but measurable, activities with L-Tyr that were several orders of magnitude lower than that for wild-type TPL. The very low rate of phenol formation by F448H TPL was detectable only by HPLC, but could not be detected by the standard coupled spectrophotometric assay with lactate dehydrogenase. Thus the mutations affected activity towards L-Tyr to a much greater extent than activity with non-physiological substrates, SOPC, *S*-alkyl-L-cysteines and β-chloroalanine. Neither wild-type TPL nor the T124D, F448H and T124D/F448H mutants showed any significant Trpase activity with L-Trp, measured by Ehrlich's-reagent assay. Ehrlich's reagent can easily detect the formation of 1 nmol of indole. We did not detect any indole in 30 min assays containing 1 mg of protein. Thus the Trpase activity of TPL, if any, must be less than 33 pmol/min per mg.

T124A TPL exhibited the highest activity with L-Tyr of any of the mutant proteins studied. In order to determine whether there was a change in the mechanism for T124A TPL, solvent and substrate deuterium kinetic isotope effects were measured with 3-fluoro-L-Tyr and its deuterated analogues in water or ^2H_2O. The kinetic isotope effects were found to be similar to the respective isotope effects for the wild-type enzyme (Table 2), suggesting that there is no change in the rate-determining step caused by the Thr-to-Ala mutation.

Competitive inhibitors of wild-type and mutant TPL

A large number of natural amino acids are competitive inhibitors of TPL [8]. To evaluate the interaction of the mutant enzymes with L-Phe, L-Tyr and L-Trp, SOPC was used as a substrate. The K_i values for both wild-type and mutant enzymes are compared in Table 3. L-Tyr, L-Phe and L-Trp proved to be competitive inhibitors of SOPC elimination by both wild-type and mutant enzymes. The K_i values for these amino acids did not change significantly for the mutant enzymes. L-Tyr competitively inhibits β-elimination of SOPC by the mutant enzymes with K_i values similar to the K_m values of L-Tyr for the wild-type and T124A mutant enzyme. These results demonstrate that these active-site mutations do not have significant effects on the binding of amino acids.

Rapid-scanning stopped-flow reactions of wild-type and mutant TPL with amino acids

We examined the pre-steady state reactions of wild-type, T124A, T124D, F448H and T124D/F448H TPL with L-Phe, L-Tyr and

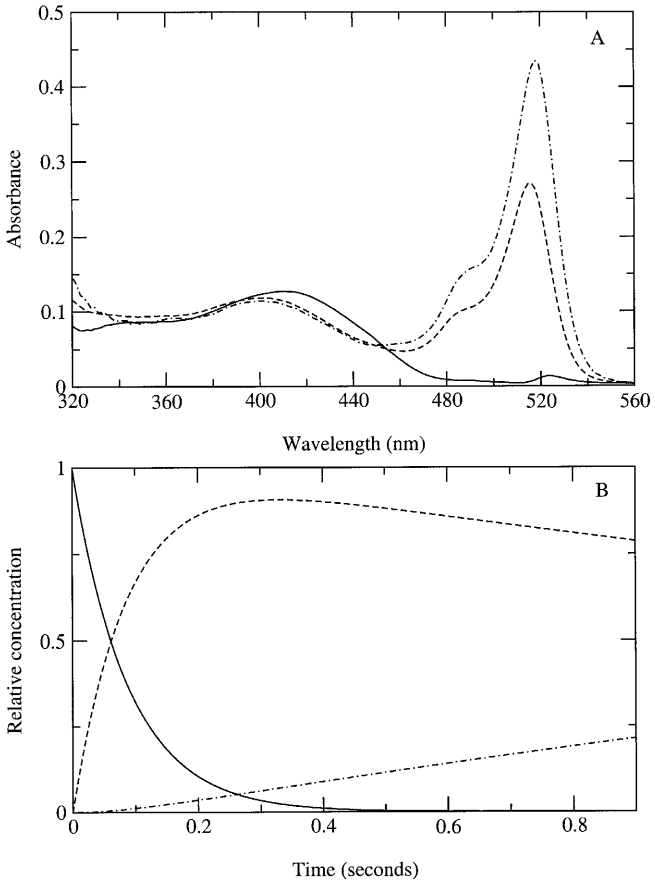


Figure 3 Rapid-scanning stopped-flow spectra of the reaction of F448H TPL with 2 mM L-Tyr

(**A**) Calculated spectra from global fitting of the reaction data. Solid line, initial spectrum; dashed line, intermediate spectrum; dashed and dotted line, final spectrum. (**B**) Time courses for the reaction. Solid line, initial spectrum; dashed line, intermediate spectrum; dashed and dotted line, final spectrum.

Table 4 Kinetic parameters for quinonoid intermediate formation and transamination from L-Phe

ND, could not be determined since there was no concentration-dependence of k_1. k_t is the rate constant for the transamination reaction.

Enzyme	K_d (mM)	k_1 (s^{-1})	k_{-1} (s^{-1})	k_t (s^{-1})
Wild-type TPL	9.1	16.2	6.4	8.0×10^{-4}
T124A	5.1 ± 5.2	10.1 ± 1.4	6.4 ± 2.1	5.3×10^{-5}
T124D	ND	17 ± 3	ND	0
F448H	ND	20 ± 1.0	ND	1.2×10^{-5}
T124D/F448H	ND	20 ± 1.0	ND	2.5×10^{-5}

L-Trp by rapid-scanning stopped-flow spectrophotometry. Wild-type TPL mixed with L-Tyr rapidly forms a quinonoid complex with a relatively weak absorbance peak at 500 nm [15,19]. At 2 mM L-Tyr, the rate constant for the formation of the 500 nm intermediate is approx. 80 s^{-1} [15,26]. Other non-substrate amino acids, such as L-Phe, L-Ala and D-Ala, form similar quinonoid complexes with absorbance peaks at 500 nm that are of considerably higher intensity [8,27,28]. Wild-type TPL also binds L-Trp, and forms a mixture of external aldimine and quinonoid intermediates with absorbance peaks at 420 and 500 nm [8]. At 20 mM L-Trp, this intermediate forms in a single exponential process, with $k_{obs} = 9.4$ s^{-1}.

F448H TPL reacts with L-Tyr and forms a spectrum with a prominent absorption peak with a λ_{max} value of 515 nm (Figure 3). The best global fit of the spectral data requires two rate constants, with k_{obs} values of 11.2 ± 0.1 and 0.28 ± 0.1 s^{-1}. With L-Trp, F448H TPL also behaves like wild-type TPL, forming a prominent peak at 500 nm in a single exponential process, with $k_{obs} = 2.1 \pm 0.1$ s^{-1} (results not shown). T124D TPL also reacts with L-Tyr and L-Trp to form equilibrating mixtures of external aldimine and quinonoid intermediates, with λ_{max} at 420 and 505 nm (results not shown). The reaction of T124D TPL is biphasic, with k_{obs} values of 4.8 ± 0.8 and 0.96 ± 0.4 s^{-1} for L-Tyr, and 11 ± 2.6 and 1.1 ± 0.33 s^{-1} for 3-fluoro-L-Tyr, while there is only a single phase for L-Trp, with $k_{obs} = 4.4 \pm 0.2$ s^{-1}. T124D/F448H TPL also forms similar mixtures of quinonoid and aldimine intermediates with L-Tyr and L-Trp (results not shown). These results show that the mutations do not significantly affect the ability of the enzyme to form aldimine and quinonoid intermediates with L-Tyr and L-Trp, despite the loss of β-elimination activity with L-Tyr.

The spectra of the mutant enzymes with L-Phe also exhibit a decrease in the internal aldimine absorption peak ($\lambda_{max} =$ 420 nm) and appearance of the quinonoid intermediate absorption peak ($\lambda_{max} = 500$ nm). The time course for quinonoid intermediate formation for the reaction of T124A TPL fits well to a single exponential process. The dependence of k_{obs} on L-Phe concentration is described by eqn (2). Thus the reaction obeys the kinetic mechanism shown in eqn (3). The calculated kinetic parameters are presented in Table 4, together with the analogous parameters for wild-type TPL. It is obvious that the mutations only slightly affect the α-deprotonation rate for L-Phe, while the re-protonation rate remains practically unchanged. For the reactions of T124D and F448H TPL with L-Phe, the kinetic curves were fitted by two exponential processes, and for both exponentials, no dependence on the concentrations of the substrates was found. The respective values were 17 ± 3 and 20 ± 1 s^{-1} for the fast phase, and 1.28 ± 0.25 and 7.3 ± 0.4 s^{-1} for the slow phase of the reaction with L-Phe. When α-deuterated L-Phe was used instead of L-Phe with T124D TPL, the rates were 7.5 ± 0.85 and 1.0 ± 0.2 s^{-1}. Thus a kinetic isotope effect of 2.3 was observed for the faster reaction, and consequently it was associated with the α-proton abstraction. The slower process did not exhibit a kinetic isotope effect and probably reflects a conformational change of the protein.

When excess PLP was not present in the reaction mixture, the 500 nm absorbance peak initially formed in the reaction with L-Phe decreased slowly, with a concomitant increase in absorbance at 325 nm (results not shown). This is due to an abortive transamination reaction, which previously has been demonstrated for wild-type TPL, that converts PLP and an amino acid into pyridoxamine 5′-phosphate and an α-ketoacid [28]. The decay of the 500 nm peak and formation of the 325 nm peak upon incubation of T124A, T124D, F448H and T124/F448H TPL with L-Phe have been used to calculate the rates of these reactions. These data are also presented in Table 4. The transamination rate was determined by protonation of the quinonoid intermediate at the 'wrong' C-4′ atom, instead of Cα. For T124A, F448H and T124D/F448H mutant TPL, this rate was decreased compared with wild-type TPL by a factor of 10–20. In contrast, there was no measurable decay of the quinonoid peak upon incubation of T124D with L-Phe. Thus these mutations result in a subtle conformational change in the quinonoid intermediate which retards the protonation of C-4′. For T124D TPL, the

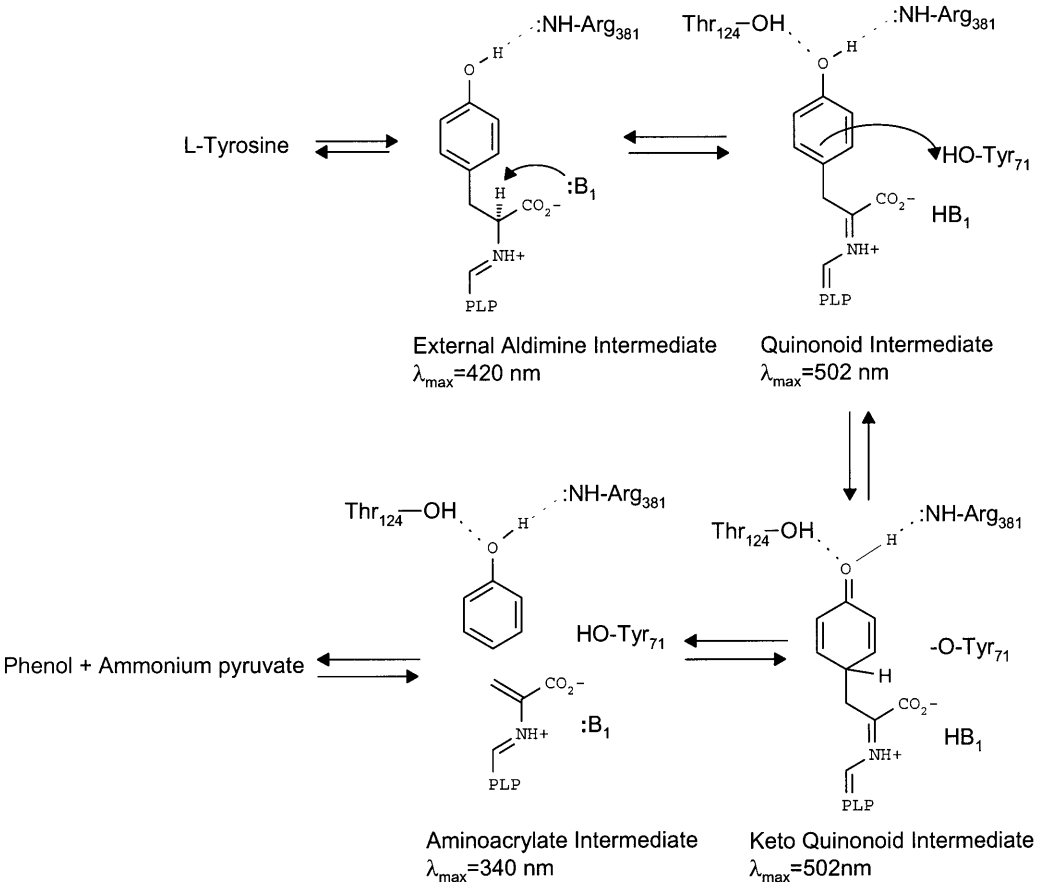
microenvironment of the quinonoid intermediate was evidently changed quite drastically, which completely prevented the transamination reaction.

DISCUSSION

Examination of the three-dimensional structure of TPL complexed with an inhibitor, 3-(4′-hydroxyphenyl)propionate, demonstrates that Arg-381, Arg-404, Phe-448, Thr-124 and Tyr-71 are in the substrate-binding site (Figure 1) [4]. Arg-404, which is conserved in all sequences of TPL and Trpase, binds the α-carboxylate group of the inhibitor (Figure 1) [4]. This arginine residue is also conserved in the structures of all members of the aminotransferase family [29], where it binds the α-carboxylate of amino acid substrates. Tyr-71, which plays a role in both PLP binding and proton transfer to the leaving group in TPL [30], is also conserved in the sequences of Trpase and aspartate aminotransferase [31]. Although Y71F mutant TPL is inactive for the Tyr elimination reaction [30], Y70F mutant aspartate aminotransferase has significant activity [31], indicating differences in the role of the conserved Tyr residue in the different proteins. The structural data show that the phenolic hydroxyl group of 3-(4′-hydroxyphenyl)propionate is close to the side chains of Arg-381 and Thr-124 (Figure 1) [4]. The sequence and structural comparison of TPL with Trpase also shows Asp in place of Thr-124 and His in place of Phe-448 (Figures 1 and 2). Kinetic analysis of R381A, R381I and R381V TPL previously demonstrated that Arg-381 is required for the Tyr substrate specificity of TPL [4]. Thus we have now performed mutagenesis of TPL to prepare the T124D, T124A, F448H and T124D/F448H TPL mutants.

All of these mutant enzymes have very little or no activity with L-Tyr, although they catalyse the elimination of SOPC, and they bind L-Tyr and other amino acids with similar binding affinity to that of wild-type TPL. This indicates that Phe-448 and Thr-124 play a key role in the Tyr reaction specificity. Furthermore, all of the mutant enzymes retain the ability to form quinonoid intermediates with absorption maxima at approx. 500 nm from L-Tyr, 3-fluoro-L-Tyr, L-Trp and L-Phe. It was proposed earlier that TPL discriminates between the physiological substrate, L-Tyr, and *o*-L-tyrosine, a competitive inhibitor of the β-elimination reaction, at the stage of the aromatic moiety elimination, by an interaction of some protein residue with the *p*-hydroxyl group of L-Tyr [32]. Thus it is possible that hydrogen bonding of the hydroxy group of Thr-124, the guanidine nitrogen of Arg-381 and the oxygen at the C-4 position of the ketoquinonoid intermediate may be required for proper orientation of the phenol ring for the β-elimination stage to occur (Scheme 3). The loss of this hydrogen-bonding capability in T124A TPL results in a dramatic decrease in elimination activity (Table 1). The Asp residue in T124D TPL would still be capable of hydrogen bonding, but as an acceptor rather than a donor; however, this evidently does not facilitate the reaction, since T124D TPL shows no detectable β-elimination activity with L-Tyr.

In the case of F448H TPL, it is less clear how the substitution of His for Phe can have such a dramatic effect on the elimination



Scheme 3 Mechanism of TPL showing the proposed roles of Tyr-71, Thr-124 and Arg-381 in the reaction mechanism

reaction of L-Tyr. In the three-dimensional structure of a complex modelling the ketoquinonoid intermediate, the TPL molecule is in a closed conformation (T. V. Demidkina and A. A. Antson, unpublished work). The subunit structure of TPL contains a small and large domain, with the active site situated between them [3,4]. In the closed conformation the small domain moves towards the large domain. Phe-448 is located on a flexible loop, which is part of the small domain, and which may be important to allow the closed active-site conformation. Possibly, the mutation to His changes the polarity of this loop enough to keep it from closing, since the protonated imidazole ring would be transferred from the aqueous medium to a highly hydrophobic environment in the closed conformation, but the imidazole would not interact with the substrate. In this regard, it is interesting to note that the corresponding H463F mutant Trpase of *E. coli* exhibits very low elimination activity with L-Trp, suggesting a specific role of the imidazole side chain in the catalytic mechanism [33]. To clarify the role of Phe-448 in the mechanism of TPL, it may be necessary to compare X-ray structures of the wild-type and F448H mutant TPL complexed with L-Tyr.

Finally, we note that sequences of TPL and Trpase show the motif of either Thr-124/Arg-381/Phe-448 for all known TPL sequences or the corresponding Asp/Leu(Ile)/His for all known Trpase sequences. Thus similar sequences in the genome databases of unknown activity cannot be assigned simply on the basis of overall sequence similarity, since all display a similar percentage of identical residues, but they can be readily assigned to TPL or Trpase with the use of these motifs. Thus the two sequences recently reported in the genome of *Pasteurella moltocida*, available from GenBank®, are assignable to TPL and Trpase respectively, whereas the sequences from *Porphyromonas gingivalis*, *Aeropyrum pernix* and *Rhodobacter capsulatus* can be assigned confidently to Trpase (Figure 2).

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