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The role of substrate strain in the mechanism of the carbon–carbon lyases

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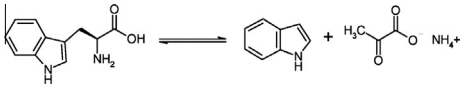
ABSTRACT

The carbon–carbon lyases, tryptophan indole lyase (TIL) and tyrosine phenol-lyase (TPL) are bacterial enzymes which catalyze the reversible elimination of indole and phenol from L-tryptophan and L-tyrosine, respectively. These PLP-dependent enzymes show high sequence homology (~40% identity) and both form homotetrameric structures. Steady state kinetic studies with both enzymes show that an active site base is essential for activity, and α-deuterated substrates exhibit modest primary isotope effects on k_{cat} and k_{cat}/K_m, suggesting that substrate deprotonation is partially rate-limiting. Pre-steady state kinetics with TPL and TIL show rapid formation of external aldimine intermediates, followed by deprotonation to give quinonoid intermediates absorbing at about 500 nm. In the presence of phenol and indole analogues, 4-hydroxypyridine and benzimidazole, the quinonoid intermediates of TPL and TIL decay to aminoacrylate intermediates, with λ_{max} at about 340 nm. Surprisingly, there are significant kinetic isotope effects on both formation and subsequent decay of the quinonoid intermediates when α-deuterated substrates are used. The crystal structure of TPL with a bound competitive inhibitor, 4-hydroxyphenylpropionate, identified several essential catalytic residues: Tyr-71, Thr-124, Arg-381, and Phe-448. The active sites of TIL and TPL are highly conserved with the exceptions of these residues: Arg-381(TPL)/Ile-396 (TIL); Thr-124 (TPL)/Asp-137 (TIL), and Phe-448 (TPL)/His-463 (TIL). Mutagenesis of these residues results in dramatic decreases in catalytic activity without changing substrate specificity. The conserved tyrosine, Tyr-71 (TPL)/Tyr-74 (TIL) is essential for elimination activity with both enzymes, and likely plays a role as a proton donor to the leaving group. Mutation of Arg-381 and Thr-124 of TPL to alanine results in very low but measurable catalytic activity. Crystallography of Y71F and F448H TPL with 3-fluoro-L-tyrosine bound demonstrated that there are two quinonoid structures, relaxed and tense. In the relaxed structure, the substrate aromatic ring is in plane with the C_β–C_γ bond, but in the tense structure, the substrate aromatic ring is about 20° out of plane with the C_β–C_γ bond. In the tense structure, hydrogen bonds are formed between the substrate OH and the guanidinium of Arg-381 and the OH of Thr-124, and the phenyl rings of Phe-448 and 449 provide steric strain. Based on the effects of mutagenesis, the substrate strain is estimated to contribute about 10^8 to TPL catalysis. Thus, the mechanisms of TPL and TIL require both substrate strain and acid/base catalysis, and substrate strain is probably responsible for the very high substrate specificity of TPL and TIL.

1. Introduction

The carbon–carbon lyases, tryptophan indole-lyase (TIL, or tryptophanase, EC 4.1.99.1) and tyrosine phenol-lyase (TPL, or β-tyrosinase, EC 4.1.99.2) catalyze the reversible hydrolytic cleavage of L-tryptophan and L-tyrosine, respectively (Eqs. (1) and (2)). In addition



(1)

Abbreviations: TIL, tryptophan indole-lyase (tryptophanase), EC 4.1.99.1; TPL, tyrosine phenol-lyase (β-tyrosinase), EC 4.1.99.2; KIE, kinetic isotope effect; MVC, monovalent cation.

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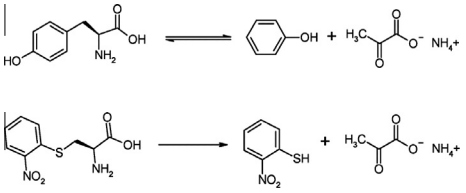

(2)

(3)

to their physiological substrates, both enzymes can catalyze the irreversible elimination of S-(*o*-nitrophenyl)-L-cysteine [1,2] (Eq. (3)) as well as other L-amino acids with leaving groups containing O, N, S, or Cl on the β-carbon. These alternative substrates in vitro include L-serine, O-alkyl and O-acyl L-serines, L-cysteine, S-alkyl L-cysteines, and β-chloro-L-alanine [3,4]. However, the enzymes are absolutely specific for their respective physiological substrates.

The formation of indole by bacterial putrefaction of meat and in feces, through the activity of TIL, was first described by Bopp in 1849 [5]. This observation led to the naming of tryptophan as the as-yet-unidentified source of indole in proteins by Neumeister in 1890 [6]. Tryptophan was first isolated from casein by Hopkins and Cole in 1901 [7], followed by the demonstration that tryptophan was in fact the source of indole formed by bacteria in 1903 [8]. Indole formation from tryptophan is a characteristic property of enteric bacteria, especially *Escherichia coli,* which inhabit the gut of animals [9]. In contrast, TPL was not recognized as an enzyme activity until it was reported in *Bacterium coli phenologenes* by Kakihara and Ichihara in 1953 [10]. Despite early studies that found the enzymes in only a few strains, recent advances in bacterial genomics show that both TIL and TPL genes are widely distributed in bacteria. Although most microorganisms have either TIL or TPL, a number of bacteria contain genes encoding for both enzymes in their genomes. The physiological role of these enzymes remained unclear for many years, and it was assumed that they functioned solely in a catabolic role to degrade excess amino acids for energy. However, the indole or phenol product does not undergo further metabolism in the bacteria which express these enzymes, and these products accumulate to relatively high (mM) concentrations in the growth media, which is inconsistent with a purely catabolic function. It has been found recently that indole is a signalling molecule in *E. coli* involved in regulation of biofilm formation [11–13], pathogenicity [14], plasmid stability [15] and antibiotic resistance [16]. In contrast, the physiological function of TPL, if there is one besides catabolism, remains as yet unknown.

2. Structure of TIL and TPL

TIL and TPL are highly homologous (~40% sequence identity) and are members of the aminotransferase superfamily of pyridoxal-5′-phosphate (PLP) dependent enzymes. The enzymes form tetramers, as a dimer of dimers, with one PLP bound to each monomer (Fig. 1), and the active site is formed at the monomer–monomer interface of a dimer [17,18]. Both enzymes require a monovalent cation (MVC), either K^+, NH_4^+, Rb^+ or Cs^+ for activity, with Na^+ and Li^+ giving little or no activity [19,20]. The cation is bound by the γ-carboxylate of a conserved glutamate residue (Glu-69 in *Citrobacter freundii* TPL), peptide backbone carbonyl oxygens, and waters, and is located about 10 Å from the PLP [18,21]. The active site residues involved in PLP binding are highly conserved for both TIL and TPL (Fig. 2). The PLP is bound to the internal aldimine by a lysine ε-amino group (Lys-257 in *C. freundii* TPL) and the protonated pyridine ring forms an ionic/hydrogen bond with an aspartate β-carboxylate (Asp-214 in *C. freundii* TPL), as is found in all members of the aminotransferase superfamily. This aspartate is necessary for TPL activity, since D214A TPL has no activity with L-tyrosine or 3-F-L-tyrosine [22]. The X-ray crystal structure of mutant D214A TPL revealed that the interaction of Asp-214 with the pyridine ring nitrogen supports C_α–H-acidity of the external aldimine and the strained conformation of the internal aldimine [23]. It was proposed that in the aminotransferase superfamily the strictly conservative aspartate residue provides both the electron sink properties of the cofactor and an acceleration of the transaldimination stage. A unique feature of TIL and TPL is another strictly conserved lysine (Lys-256 in *C. freundii* TPL) immediately preceding the PLP-binding lysine, and hydrogen bonded to a water molecule bound to the MVC. This lysine appears to play a role in the activation of these enzymes by MVCs [24]. The residues that contact the substrate directly include a conserved tyrosine (Tyr-

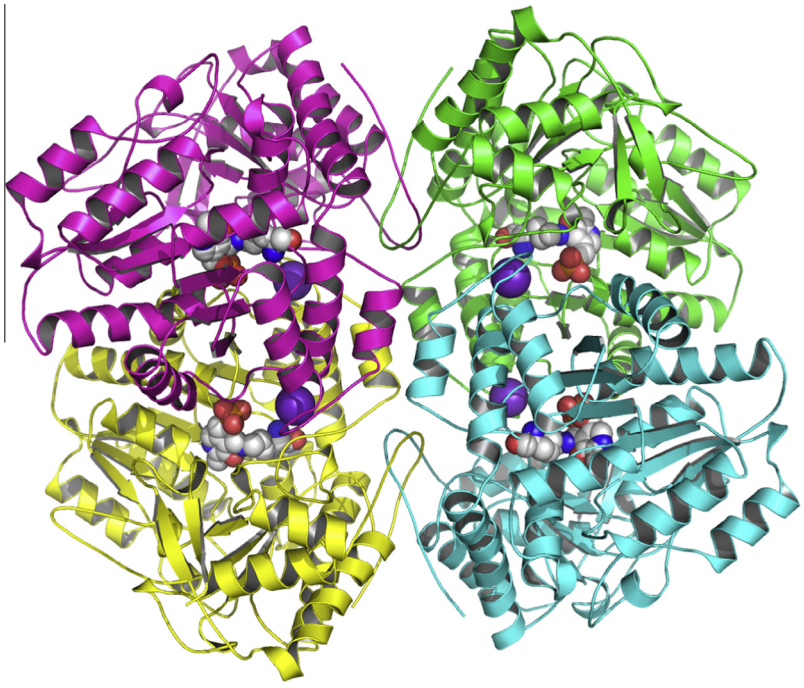

Fig. 1. Structure of *P. vulgaris* TIL (pdb file 1AX4). The enzyme is a tetramer, with a PLP (spheres with CPK colors) and Cs^+ (purple sphere) bound to each monomer.

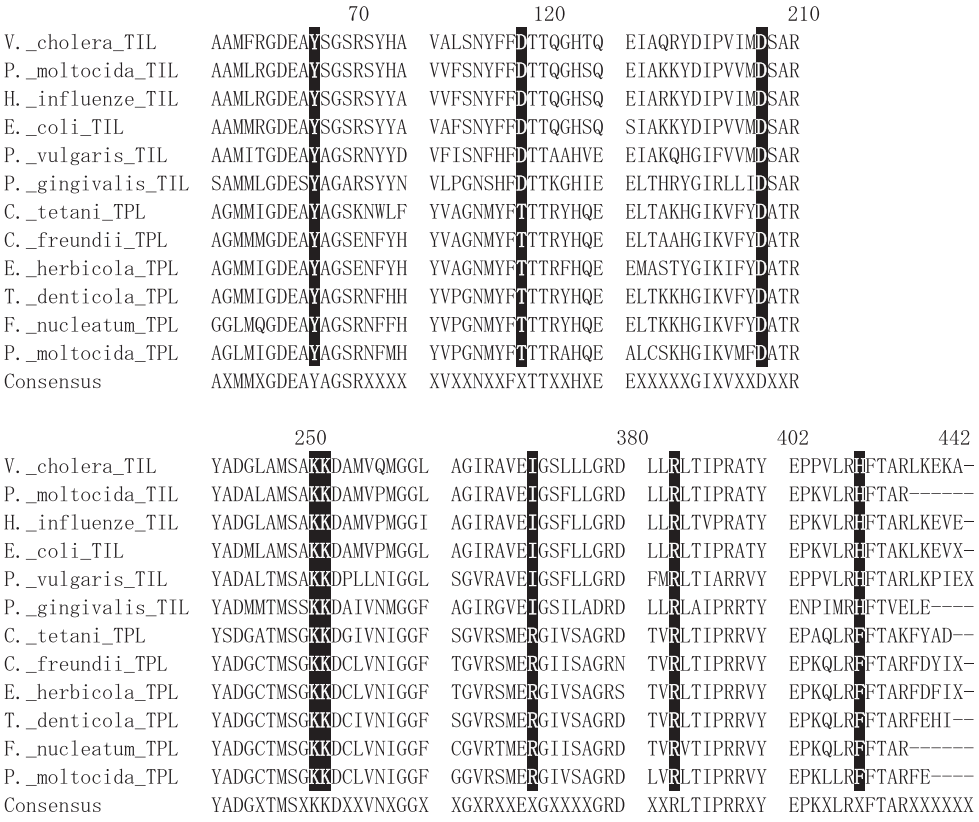

Fig. 2. Sequence alignments of TPL and TIL. The sequence numbers are from *C. freundii* TPL.

71 in *C. freundii* TPL, Tyr-74 in *E. coli* TIL), a threonine (Thr-124 in *C. freundii* TPL) or aspartate (Asp-137 in *E. coli* TIL), an arginine (Arg-381 in *C. freundii* TPL) or isoleucine (Ile-396 in *E. coli* TIL), and a phenylalanine (Phe-448 in *C. freundii* TPL) or histidine (His-463 in *E. coli* TIL) (Fig. 3). Furthermore, there is a strictly conserved phenylalanine in both TPL (Phe-449) and TIL (Phe-459) that is in close contact with the bound substrate (Fig. 3). The active site tyrosine is essential for activity, as Y71F mutant TPL and Y74F mutant TIL show no detectable elimination activity (i.e., $<10^{-5}$ that of wild-type) with their physiological substrates [22,25,26], although both have significant activity with SOPC and other non-physiological substrates with good leaving groups. Although TIL and TPL show strict substrate specificity for their physiological substrates, mutation of Thr-124, Arg-381 and Phe-448 in TPL to the corresponding residues of TIL, aspartate, isoleucine and histidine, respectively, resulted in loss of TPL activity, retention of SOPC elimination activity, but no detectable TIL activity [21,27].

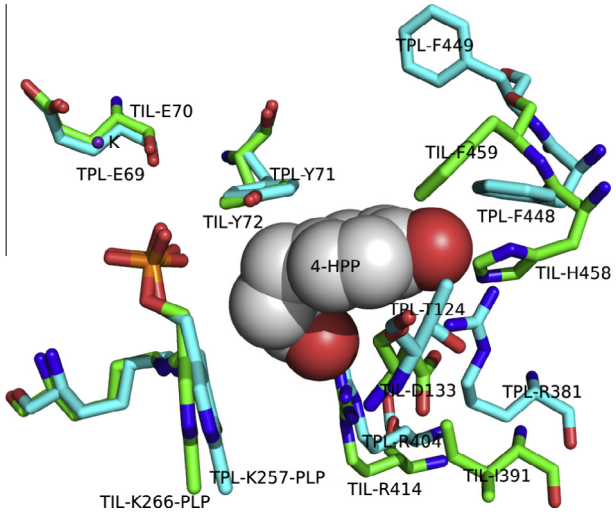

Fig. 3. Active site of *C. freundii* TPL-4HPP complex, overlaid on *P. vulgaris* TIL. The TPL residues are shown in cyan, and the TIL residues are shown in green.

3. Steady-state kinetics

The pH dependence of k_{cat}/K_m of *E. coli* TIL for tryptophan exhibits 2 basic groups, with pK_as of 6.0 and 7.6 [28]. The pH dependence of K_i for oxindolyl-L-alanine, an inhibitor which is an analogue of the proposed indolenine intermediate, also exhibits two basic pK_as of 6.0 and 7.6. In contrast, the pH dependence of k_{cat}/K_m for S-methyl-L-cysteine and the pH dependence of K_i for L-alanine show only the pK_a of 7.6. Thus, the base with pK_a of 7.6 is involved in the deprotonation of the α-carbon of substrates, and the base with pK_a of 6.0 activates the indole ring of the tryptophan substrate for elimination. Mutation of His-463 to phenylalanine in *E. coli* TIL results in a 1000-fold decrease in tryptophan elimination activity and loss of the pK_a of 6.0 in the pH dependence of k_{cat}/K_m, suggesting that His-463 is that base [25]. In contrast, k_{cat} is pH independent, demonstrating that only the correctly protonated form of the enzyme binds the substrate, and the enzyme-substrate complex does not undergo protonation. There is a pH-independent primary isotope effect on k_{cat} ($^{D}k_{cat} = 2.5$) and k_{cat}/K_m ($^{D}k_{cat}/K_m = 2.8$) for α-[^{2}H]-L-tryptophan, indicating that a step (or steps) involving transfer of the α-proton is partially rate-limiting [28]. The TIL reaction shows pD-independent solvent isotope effects in D_2O ($^{D_2O}k_{cat} = 3.8$ and $^{D_2O}k_{cat}/K_m = 2.8$), and the substrate isotope effect is reduced in D_2O ($^{D}k_{cat} = 1.25$ and $^{D}k_{cat}/K_m = 1.82$), suggesting that the steady-state solvent and substrate isotope effects are on different steps. The proton inventory for the reaction of TIL is concave downward, indicating that multiple waters are involved in the transition state of the solvent sensitive step [29].

TPL from *C. freundii* also exhibits two basic pK_as, with an average value of 7.8, in the pH dependence of k_{cat}/K_m, and k_{cat} is pH independent [30]. However, k_{cat} in TPL from *Erwinia herbicola* is pH-dependent for some substrates, in particular L-DOPA [31]. The TPL reaction shows primary isotope effects of about 3 on k_{cat} and 2 on k_{cat}/K_m for both enzymes, also suggesting that steps involving transfer of the α-proton are partially rate-limiting. The primary isotope effect increases to 5.4 and 3.8, respectively, for k_{cat} and k_{cat}/K_m with α-[^{2}H]-3-F-L-tyrosine with H343A TPL, compared to 3.9 and 2.2 for wild-type TPL [32]. Although not catalytically essential, since H343A TPL has k_{cat} and k_{cat}/K_m values ~10% those of wild-type TPL, this histidine residue is strictly conserved in all sequences of TPL and TIL. It is located only 3.8 Å from the conserved α-carboxylate binding arginine (Arg-404 in *C. freundii* TPL), so this histidine may assist in the conformational changes associated with substrate binding and catalysis.

The reactions of both enzymes show partial internal return (10–27%) of the α-proton to the indole or phenol products when α-deuterated substrates are cleaved [33–35]. These results, together with the retention of configuration at the β-carbon in the pyruvate product, suggested mechanisms for both enzymes with *syn*-elimination using a single acid/base catalytic group.

4. Pre-steady state kinetics

The spectroscopic properties of the PLP make it possible to observe directly the reaction intermediates in the mechanism of these enzymes. Under steady state conditions, the spectra of both TIL and TPL in the presence of substrates show mixtures of external aldimines, absorbing at about 420 nm, and quinonoid complexes, absorbing at about 500 nm. Mixing TIL with L-tryptophan in the stopped-flow spectrophotometer shows the rapid formation of the external aldimine in the dead time, followed by formation of the quinonoid intermediate with $1/\tau > 500\ s^{-1}$ (Fig. 4A and B) [36,37]. α-Deuteration of L-tryptophan results in a primary kinetic isotope effect of 3.6 on formation of the quinonoid intermediate, as expected. When benzimidazole, an isoelectronic indole analogue which is an uncompetitive inhibitor of TIL, is included in the stopped-flow reaction with L-tryptophan, the quinonoid intermediate decays, with a rate constant of 30 s^{-1}, to form a new species with an absorption peak at 340 nm (Fig. 4C and D) [37,38]. This spectrum is consistent with an α-aminoacrylate Schiff's base, formed after elimination of indole. A similar species was also seen in static spectra when TIL is incubated with ammonium pyruvate and benzimidazole. Mixing of this aminoacrylate complex with indole in the stopped flow spectrophotometer resulted in concentration dependent formation of a quinonoid complex, as expected for an aminoacrylate [37]. This assignment was further confirmed by rapid quench experiments, which demonstrated that indole is formed from L-tryptophan at about 30 s^{-1} in a stoichiometric burst, followed by the linear steady-state reaction [39]. When benzimidazole is included in stopped-flow reactions of TIL with α-[^{2}H]-L-tryptophan, not only is the expected primary kinetic isotope effect observed on the formation of the quinonoid complex, but also a KIE of 2.99 ± 0.30 is seen on the formation of the aminoacrylate [37]. This result is consistent with the internal return discussed above. Formation of the aminoacrylate intermediate from β,β-di-[^{2}H]-L-tryptophan showed a normal

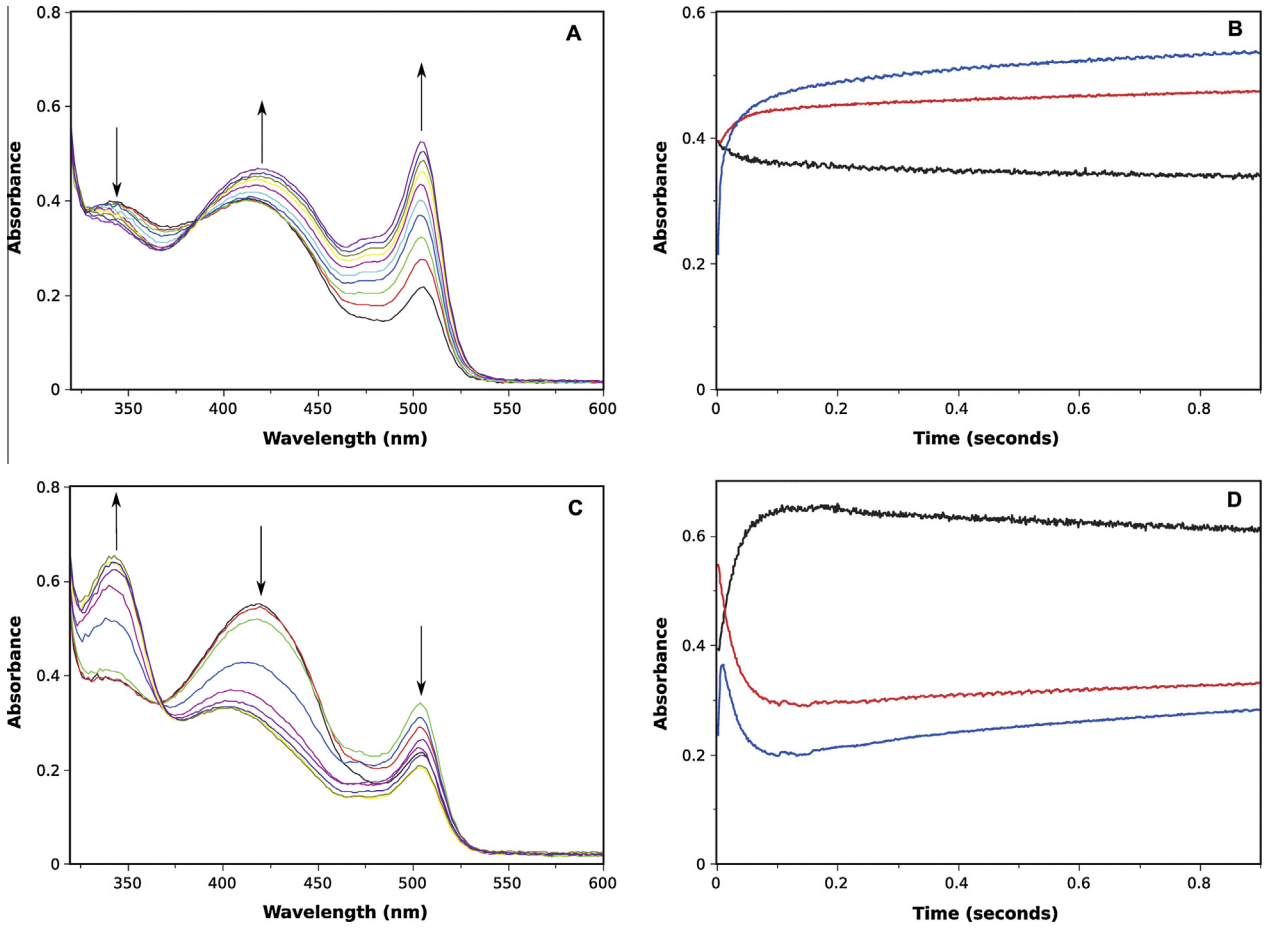

Fig. 4. Stopped-flow spectra of the reaction of TIL with L-tryptophan [36]. Panel A. Spectra of the TIL reaction with L-tryptophan. Panel B. Time courses of the reaction of TIL with L-tryptophan. Blue, 505 nm; Red, 420 nm; Black, 340 nm. Panel C. Spectra of the TIL reaction with L-tryptophan in the presence of benzimidazole. Panel D. Time courses of the reaction of TIL with L-tryptophan in the presence of benzimidazole. Blue, 505 nm; Red, 420 nm; Black, 340 nm.

secondary KIE of 1.17 ± 0.03, consistent with the C_β–C_γ bond cleavage. Unexpectedly, the reaction of α,β,β-tri-[^{2}H]-L-tryptophan exhibits a combined primary and secondary KIE of 4.42 ± 0.67 on aminoacrylate formation, suggesting that proton transfer to the 3-position of the indole product and C_β–C_3 bond cleavage is concerted [37]. Formation of the aminoacrylate intermediate also shows a solvent KIE of 1.79 ± 0.11 for L-tryptophan, and with α-[^{2}H]-L-tryptophan the KIE is increased to 4.30 ± 0.16 in D_2O, indicating that solvent participates in the transition state for elimination.

The reaction of TPL with L-tyrosine and 3-fluoro-L-tyrosine in the stopped-flow spectrophotometer also shows rapid formation of quinonoid intermediates ($k \sim 100\ s^{-1}$) with λ_{max} at 502 nm from external aldimines absorbing at 420 nm [40,41]. Addition of 4-hydroxypyridine, an uncompetitive inhibitor, in these reactions results in decay of the quinonoid–aldimine spectrum with rate constant of 1.4 s^{-1} and formation of an intermediate absorbing at 338 nm, assigned to an aminoacrylate intermediate [41]. The rate constant for formation of the aminoacrylate intermediate is similar to k_{cat} for TPL, ~2 s^{-1}, suggesting that elimination is rate-limiting for the reaction of tyrosine. As was found with TIL, isotope effects are seen with α-deuterated substrates, both on the formation of the quinonoid intermediate as well as the aminoacrylate intermediate, consistent with the internal return cited above.

Based on the steady-state and transient kinetics results, the acid–base catalysis mechanism of TIL and TPL is shown in Fig. 5. Binding of the respective amino acid substrate results in an external aldimine, which is deprotonated on the α-carbon by the active site lysine to give the quinonoid intermediate. Generally, *gem*-diamine intermediates are not observed in these transient kinetic studies, but in the reaction of TIL with homophenylalanine a transient intermediate was observed with λ_{max} at 340 nm which decayed to form the external aldimine [42]. This intermediate was also trapped in pre-steady state kinetics of the N185A mutant TPL reaction with 3-fluoro-L-tyrosine [43]. Proton transfer to the aromatic ring C_γ of the quinonoid complex, followed by C_β–C_γ cleavage, results in an aminoacrylate intermediate, which is protonated on C_β and released as iminopyruvate. The protonation of the aminoacrylates at C_β occurs on the same face as the elimination of the leaving group [32,34]. However, the mechanism in Fig. 5 does not explain the absolute specificity of the enzymes for their physiological substrates.

5. Crystallography of substrate and inhibitor complexes of TIL and TPL

Although *E. coli* TIL crystallizes readily, the crystals generally do not diffract well. Recently, structures of the *E. coli* TIL apoenzyme have been obtained [43,44]. In contrast, TIL from *Proteus vulgaris* gives good quality crystals, and the structure of the holoenzyme of wild type TIL [18] has been obtained at 2.1 Å resolution. However, no structures of substrate or inhibitor complexes of TIL have been obtained to date. *C. freundii* TPL crystallizes readily under a range of conditions, and a number of complexes of wild type and mutant TPLs with substrates and inhibitors have been obtained [17,21,45,46]. Thus, the TPL complexes provide the majority of the structural information on the mechanisms of the carbon–carbon lyases.

The structure of *C. freundii* TPL in complex with 4-hydroxyphenylpropionate (4HPP) was the first structure obtained with a bound ligand [21]. This compound is a competitive inhibitor of

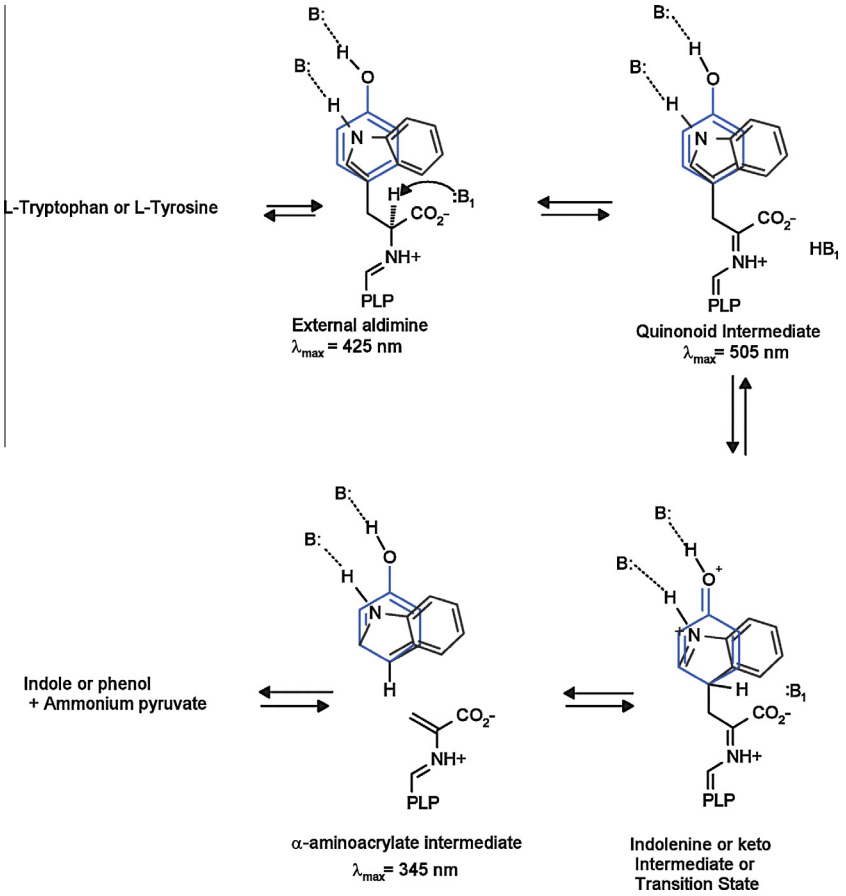

Fig. 5. Proposed acid–base catalysis mechanism of TPL and TIL.

TPL, and lacks an α-amino group, so it cannot form an external aldimine. Thus, this structure provides insight into the Michaelis complex which precedes external aldimine formation in TPL and TIL. There is very little change in the active site structure on 4HPP binding (Fig. 6a). Arg-404 forms an ion pair with the ligand carboxylate, and the phenolic OH of 4HPP is located 4.1 Å from Arg-381 and 4.9 Å from Thr-124. The phenol of Tyr-71 forms a hydrogen bond at 2.6 Å with a bound water, which is 2.7 Å from a PLP phosphate oxygen. The phenol of Tyr-291 is 2.7 Å, and the ε-amino group of Lys-256 is also 3.2 Å from this water, forming a distorted tetrahedron of hydrogen bonds.

Although no structures have been obtained of an external aldimine of TPL, there are a number of structures of quinonoid intermediates of wild-type TPL with L-alanine and L-methionine [45], and Y71F and F448H mutant TPL with 3-F-L-tyrosine [46]. The quinonoid intermediate structures with 3-F-L-tyrosine form either open (relaxed) or closed (tense) conformations. In the open conformation, the distances from the OH of the substrate to Thr-124 and Arg-381 have not changed significantly from those in the 4HPP complex (Fig. 6b). Phe-71 has moved toward the substrate to a position 4.0 Å from the bound water, and the ε-amino group of Lys-257 is now 5.4 Å from the water. In the closed conformation, a flexible loop covers the active site, and the distances between Thr-124, Arg-381 and the substrate OH are now 2.4 and 2.9 Å, respectively, due to expulsion of bridging waters (Fig. 6c). The ε-amino group of Lys-257 is now 3.8 Å from the bound water, and Tyr-291 has moved to 3.3 Å. Even more important, the aromatic ring of the substrate is bent out of the plane of the C_β–C_γ bond by about 20° in the closed conformation. In F448H mutant TPL, the histidine can form an additional hydrogen bond with the OH of the substrate in the closed conformation, while in Y71F TPL, Phe-448 moves within 3.0 Å of the substrate OH in the closed conformation. This distance is even closer when the substrate is planar, so there is steric strain introduced in the substrate in the closed conformation. Furthermore, the conserved Phe-449 in TPL (Phe-459 in *P. vulgaris* TIL) also comes within 3 Å of the substrate aromatic ring in the closed conformation (Fig. 7). This strain is relieved by bending the ring, resulting in partial pyramidalization of C_γ. This distortion decreases the resonance of the π-system, and hence increases the basicity of C_γ, which can be protonated by the OH of Tyr-71, now only 3.8 Å away. The strain thus distorts the substrate ground state structure toward the transition state geometry, moving it up the reaction coordinate and hence reducing the activation energy. The contribution of this substrate strain to catalysis can be estimated by the effects of mutagenesis of Thr-124 and Arg-381 on k_{cat}/K_m for elimination of phenol from L-tyrosine, since the short strong hydrogen bonds formed in the strained intermediate are needed to overcome the higher energy of the bent structure. T124A TPL has k_{cat}/K_m reduced by about 10^3, and R381A TPL has k_{cat}/K_m reduced by about 10^5 [27]. Thus, the ground state strain can be estimated to contribute at least 10^8 to catalysis by TPL.

It is likely that this substrate strain is the reason for the strict substrate specificity of TPL and TIL. The potent inhibition of TIL observed with substrate analogues, oxindolyl-L-alanine and 2,3-dihydro-L-tryptophan, is consistent with an indolenine intermediate, shown in Fig. 5 [47,48]. Bending the indole ring out of plane of the C_β–C_γ bond, as was seen with TPL, would pyramidalize the C_γ, raising the energy, increasing the basicity of the carbon and distorting the structure toward the indolenine intermediate geometry. If L-tryptophan is bound to TPL in place of L-tyrosine, Thr-124 and Arg-381 must not be geometrically positioned to stabilize a bent structure, and there is no detectable (i.e., rate $<10^{-5}$ that of L-tyrosine) elimination of indole. In contrast, His-463 and Asp-137 in *E. coli* TIL replace Phe-448 and Thr-124, while Arg-381 is substituted with Ile-396 (Fig. 2). Mutation of His-463 to Phe in *E. coli* TIL results in a 10^3-fold decrease in tryptophan elimination activity [25], while the corresponding H458A mutant in *P. vulgaris*

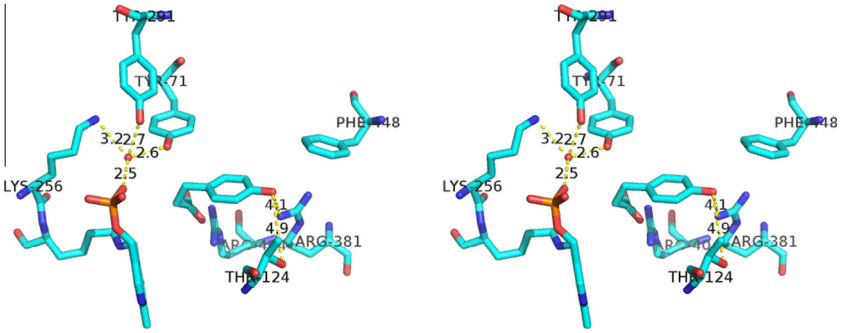

Fig. 6a. Stereo view of the active site of *C. freundii* TPL with 4HPP bound.

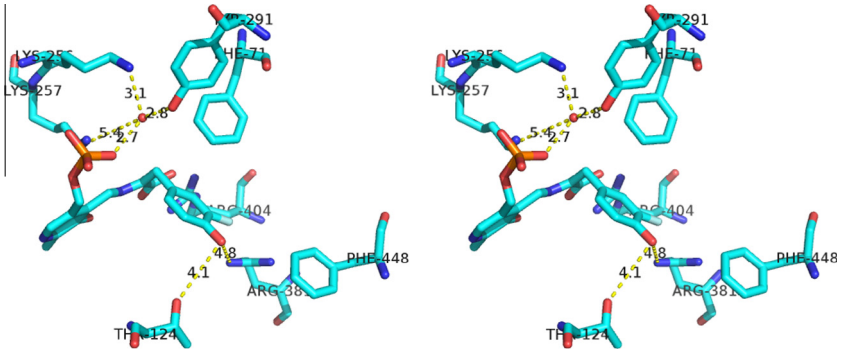

Fig. 6b. Stereo view of the active site of the relaxed conformation of Y71F TPL with 3-F-L-tyrosine bound.

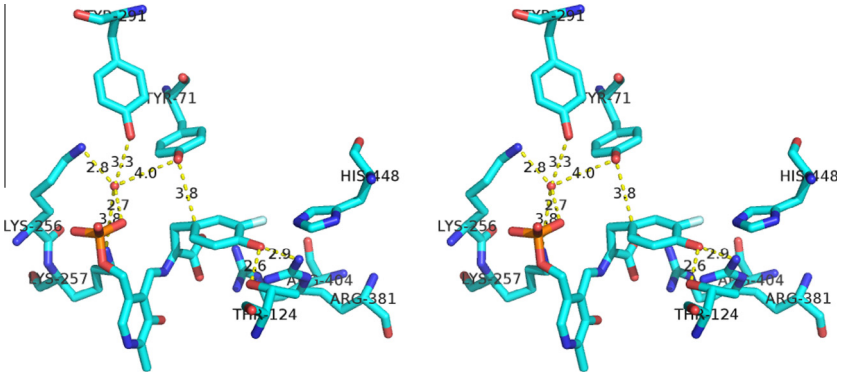

Fig. 6c. Stereo view of the active site of the tense conformation of F448H TPL with 3-F-L-tyrosine bound.

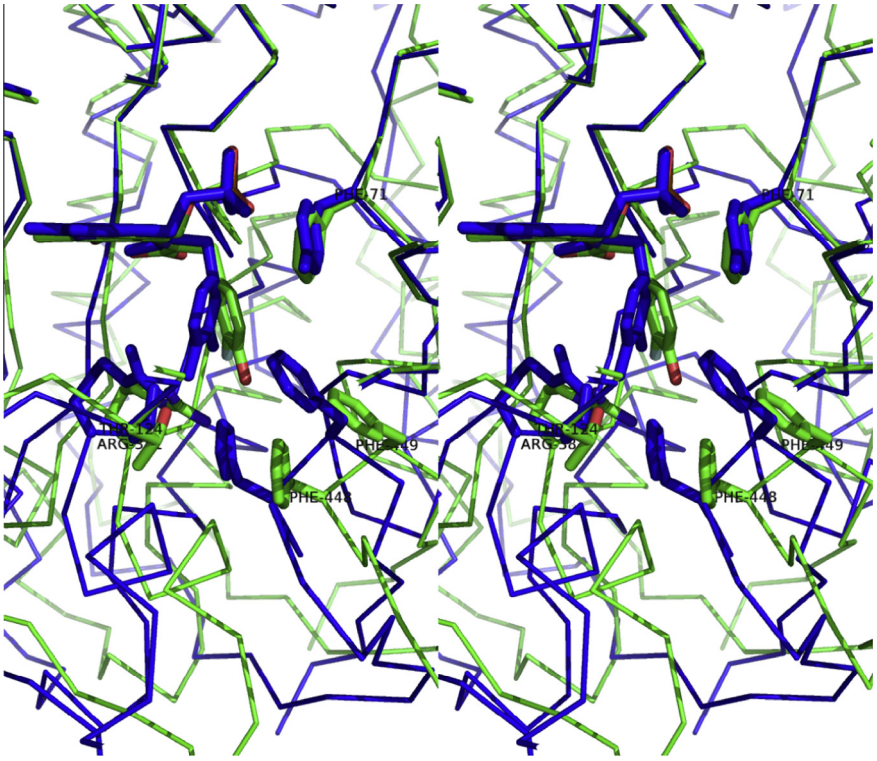

Fig. 7. Crossed-eye stereo view of the overlay of open and closed conformations of TPL with 3-F-L-tyrosine bound. Green: Open conformation of Y71F TPL quinonoid complex with 3-F-L-Tyr. Blue: Closed conformation of F448H TPL quinonoid complex with 3-F-L-Tyr.

TIL has about 1% activity [49]. Mutation of the homologous Asp-133 in *P. vulgaris* TIL has an even larger effect, as D133A TIL has no detectable activity [49]. Since the indole ring of the tryptophan substrate can only form one hydrogen bond through its NH, only one of these residues can directly form a hydrogen bond with the NH, and the other may hydrogen bond to the first. The larger effect of mutagenesis suggests that Asp-133 may be the residue that contacts the substrate. However, the imidazole of F448H TPL forms a hydrogen bond to the substrate, consistent with the histidine being capable of hydrogen bonding to the substrate in TIL. The effects of hydrostatic pressure on quinonoid intermediate formation from L-tryptophan and *E. coli* H463F TIL suggest that the indole ring is preorganized into the active conformation before α-deprotonation occurs [50]. As previously mentioned, mutation of the catalytic residues in TPL to those in TIL do not confer TIL activity. This suggests that there may be more remote changes in structure that are required for the appropriate conformational changes needed to introduce the strain required for catalysis.

The structure of the quinonoid complex of TPL with L-alanine and pyridine N-oxide mimics the next stage in the mechanism, the aminoacrylate-phenol product complex. In this structure, the oxygen of the pyridine N-oxide still forms hydrogen bonds with Thr-124 and Arg-381, with distances of 2.8 and 2.9 Å, respectively (Fig. 8). The OH of Tyr-71 is still 4 Å from the bound water, which is 2.6 Å from the PLP oxygen and the ε-amino group of Lys-256, and 3.3 Å from the phenol of Tyr-291. It should be noted that the pyridine-N-oxide lies on the opposite face of the PLP-L-alanine quinonoid complex from Lys-257; thus, the elimination occurs in an *anti* fashion, rather than *syn,* as was suggested by the internal return. This suggests that the elimination of indole from L-tryptophan catalyzed by TIL is also *anti*. Release of phenol or indole from TPL or TIL, respectively, at this stage gives the aminoacrylate intermediate. The aminoacrylates of both enzymes are protonated on the same face as the leaving group left from, based on chiral methyl group analysis of the pyruvate products [32,34]. Since the active site lysine (Lys-257 in *C. freundii* TPL, Lys-270 in

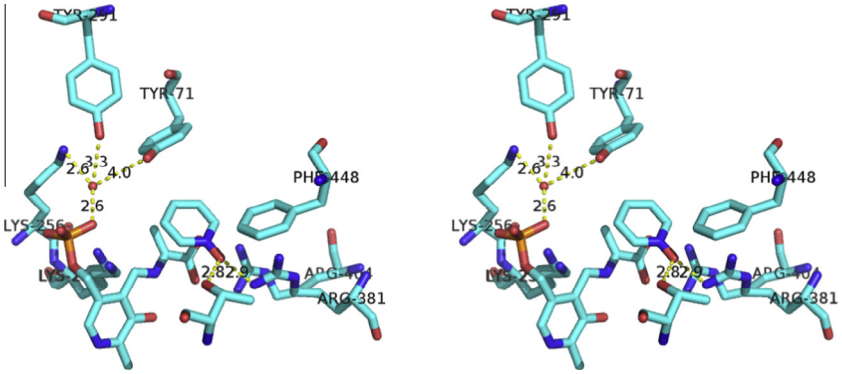

Fig. 8. Stereo view of the active site of the TPL quinonoid complex with L-alanine and pyridine N-oxide.

E. coli TIL) is located on the opposite face, it cannot serve as the proton donor to the aminoacrylate. However, the tyrosine (Tyr-71 in *C. freundii* TPL, Tyr-74 in *E. coli* TIL) which donated a proton to the leaving group is now in position to protonate C_β of the aminoacrylate (Fig. 8), concomitant with *anti*-addition of the lysine ε-amino group to the Schiff's base at C-4′. This results in a *gem*-diamine complex of iminopyruvate, which can release iminopyruvate, completing the catalytic reaction. Iminopyruvate has been shown to be the initial product of TIL, which then undergoes nonenzymatic hydrolysis to pyruvate and ammonium [51].

6. Conclusions

The elimination reactions of both TPL and TIL are expected to require acid–base catalysis to allow cleavage of a formally unactivated carbon–carbon bond. Kinetic and mutagenesis experiments provide evidence for the participation of acid–base catalysis in the mechanisms of both enzymes. However, the crystal structures of TPL complexes show that there is a significant contribution ($\sim 10^8$) of substrate strain to the rate acceleration, in addition to acid–base catalysis. This substrate strain is also likely for the mechanism of TIL, and probably is responsible for the strict substrate specificity of these two homologous enzymes.

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