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

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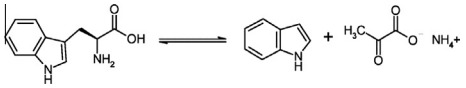
ABSTRACT

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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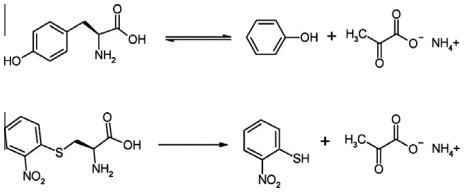

(2)

(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-

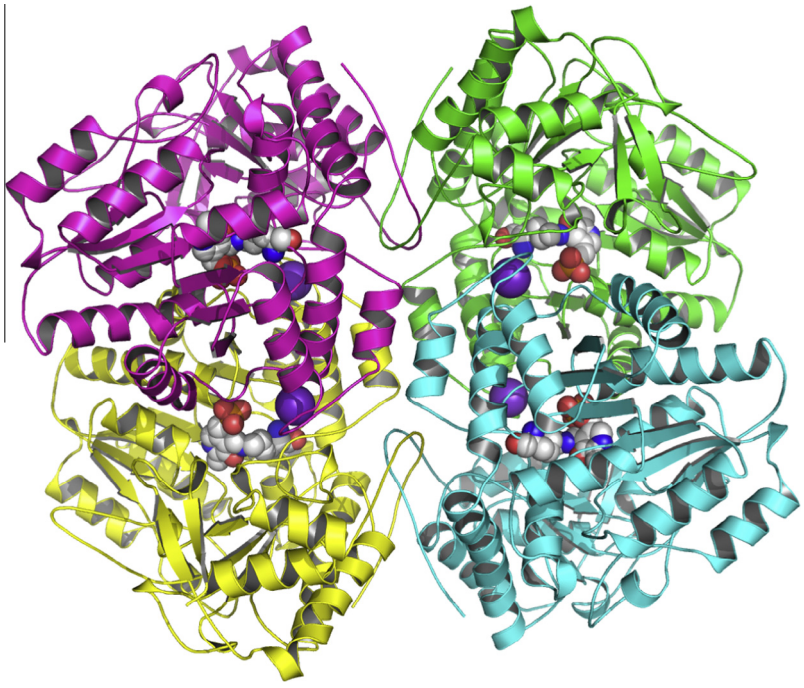

Fig. 1. Structure of *P. vulgaris* TIL (pdb file 1AX4). The enzyme is a tetramer, with a PLP (spheres with CPK colors) and Cs^+ (purple sphere) bound to each monomer.