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The structural interpretations of residue Ser^{297} in catalytic efficiency of *Escherichia coli* phenylalanine aminotransferase

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Abstract

Escherichia coli phenylalanine aminotransferase (ecPheAT) catalyzes the biosynthesis of phenylalanine and tyrosine. The crystal structure of ecPheAT was determined in our previous study. The comparison of the 3-D structure of several aminotransferases revealed that the residue at position 297 plays an important role in enzyme function. Analysis of activities and kinetic parameters of wild type and mutant ecPheATs suggested that the residue Ser^{297} was structurally selected for better catalytic efficiency. Computational modeling of ecPheAT mutants further suggested that Ser in position 297 could make ecPheAT easy with change of conformation from open form to closed form.

Keywords: Phenylalanine aminotransferase; *tyrB* gene; Structural role; Catalytic efficiency

1. Introduction

Phenylalanine aminotransferase (PheAT, EC 2.6.1.57), also called aromatic aminotransferase (ArAT) or tyrosine aminotransferase (TyrAT), is a pyridoxal 5′-phosphate (PLP)-dependent enzyme and is a key enzyme involved in the biosynthesis of phenylalanine and tyrosine. *Escherichia coli* PheAT (ecPheAT) possesses a broad substrate specificity and catalyzes the transamination reaction using either aromatic or dicarboxylic amino acid as the amino donor [1]. The enzyme is valuable in industry to produce some aromatic amino acids and their analogues, such as homophenylalanine, a starting material for synthesizing angiotension-converting enzyme inhibitor [2].

The crystal structure of ecPheAT was determined in our previous study [3]. A comparison of the crystal structure of *E. coli* PheAT with that of *Paracoccus denitrificans* ArAT [4] revealed that the residue at position 297 of both enzymes might play an important role in enzyme function. In ecPheAT, the residue Ser^{297} situated near the active center and was located in the vicinity of substrate-binding site (Figs. 1 and 3). Alignment of amino acid sequences of *E. coli* PheAT [5], *Salmonella typhimurium* ArAT [6], *P. denitrificans* ArAT [7], human cytosolic aspartate aminotransferase (AspAT) [8], pig cytoplasmic AspAT [9], chicken mitochondrial AspAT [10], and *E. coli* AspAT (ecAspAT) [11] indicated that the residue at position 297 was Ser, Phe, or Asn (Fig. 2). The alignment showed that Asn was conserved in AspATs, but not in pdArAT or ecPheAT (Fig. 2). However, pdArAT was found to have higher activity for aromatic substrates than other aminotransferase. This intrigued us to investigate the role of residue 297 of the enzyme. In this study, residue Ser^{297} of ecPheAT was substituted with other amino acids by site-directed mutagenesis. The resulting mutant enzymes were examined for their catalytic parameters. Computational modeling of these mutants was also performed to correlate the effect of mutated residues with enzyme activity.

2. Materials and methods

2.1. Strains and chemicals

E. coli DH5α was obtained from the Culture Collection and Research Center of FIRDI (Hsinchu, Taiwan) for

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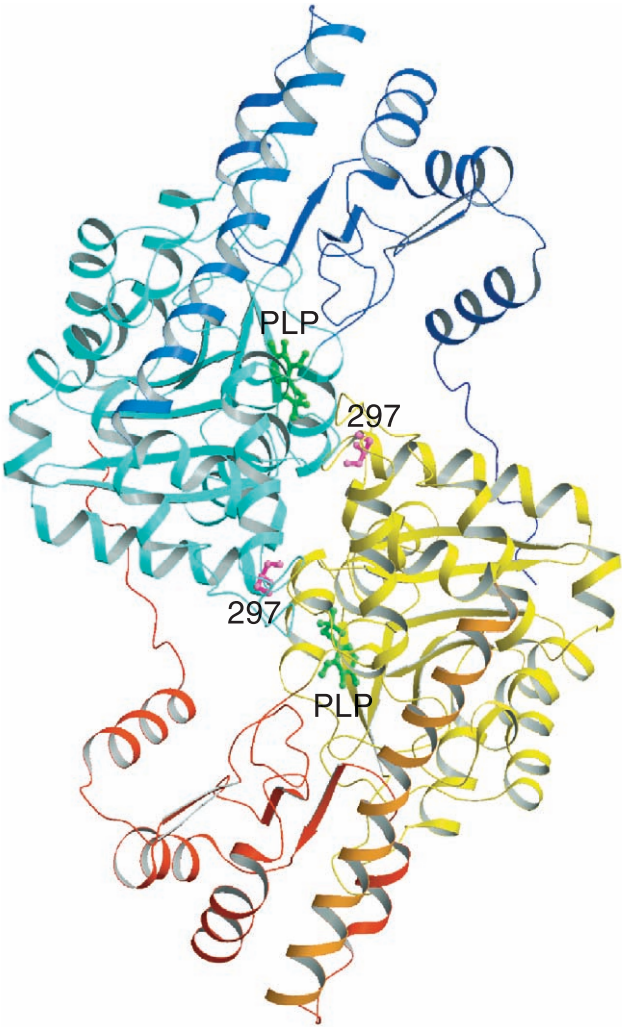

Fig. 1. Ribbon diagram of the overall folding of the open-form ecPheAT (PDB entry code 3TAT). The residue Ser^{297} (pink) and PLP molecules (green) are shown in a ball-and-stick model. The substrate-binding site is located next to the PLP at the interface of the small (blue) and large (cyan) domains of one subunit of the enzyme, and the large domain (yellow) of the opposite subunit.

preparation and construction of plasmids. *E. coli* TY103 (an *aspC* and *tyrB* deficiency strain) [12] was used for overproduction of wild type and mutant PheATs. Plasmid pCYB1 and restriction enzymes were purchased from New England Biolabs (Beverley, MA). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. DNA manipulation and protein purification

The *E. coli tyrB* gene coding for the phenylalanine aminotransferase [5] was subcloned from pTC3 [1] into the plasmid pCYB1 between sites of *Nde*I and *Xho*I to generate the plasmid pCY3. PCR overlap-extension mutagenesis technique [13] was used to construct the site-specific mutants. The *tyrB* gene mutants were confirmed by DNA sequencing. The wild type and the mutated *tyrB* genes were transformed into *E. coli* TY103 for overexpression. Wild type and mutant PheATs were then purified to homogeneity according to the method described previously [3].

2.3. Enzyme assay

The PheAT activity was determined by a modified malate dehydrogenase (MDH) coupling method [14]. Activity was assayed in 1 ml reaction mixture containing 8 mM of phenylpyruvate, 100 mM of L-aspartate, 5 mg/ml bovine serum albumin (BSA), 0.15 mM of NADH, 5 U of maleate dehydrogenase, 100 mM KCl and 50 mM HEPES buffer (pH 8.0). One unit (U) of PheAT activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of oxaloacetate per minute at 25 °C.

2.4. Computational modeling

The PDB coordinates of 3TAT were used as a template for the substrate-free open form. By employing the program O [15], the side chain of Ser^{297} was substituted with either of alanine, cysteine, asparagine, or phenylalanine, and adjusted for most likely orientation. The model was then subjected to energy minimization using X-PLOR [16], with restraints against deviations from the initial coordinates. For the closed form of the enzyme, the model coordinates of the hexamutant AspAT structure (1AHX from PDB) [17] were employed as a template for the polypeptide backbone. The starting model had the open conformation of 3TAT, but during energy minimization, the backbone atoms were tethered to mimic those of the 1AHX model. The regions of residues 65–67, whose

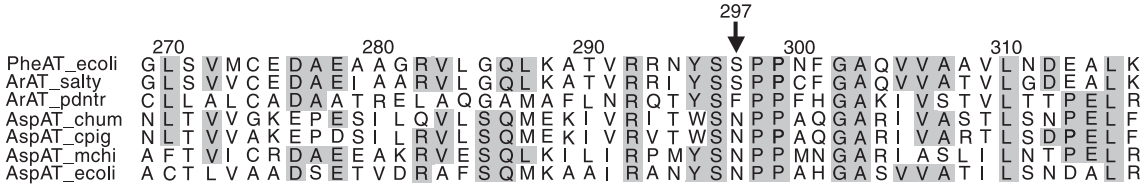

Fig. 2. Amino acid sequence alignment of PheAT from *E. coli* (ecoli) [5], ArATs from *S. typhimutium* (salty) [6] and *P. denitrificans* (pdntr) [7], and AspATs from human cytosol (chum) [8], pig cytosol (cpig) [9], chicken mitochondria (mchi) [10], and *E. coli* (ecoli) [11]. The arrow indicates position 297.

Table 1
The specific activity of wild type and mutant ecPheATs

Enzyme	Specific activity[a] (U/mg)	Relative activity[b] (%)
Wild type	21.5 ± 2.0	100.0
S297F	0.8 ± 0.1	3.7
S297R	0.6 ± 0.1	2.8
S297L	0.5 ± 0.1	2.3
S297M	2.0 ± 0.2	9.3
S297C	23.1 ± 2.8	107.4
S297N	12.0 ± 1.7	55.8
S297A	16.3 ± 1.7	75.8

[a] One unit (U) of *E. coli* PheAT activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of oxaloacetate per minute at 25 °C. The assay condition was described in text.
[b] The specific activity of wild type ecPheAT was taken as 100%.

length are different in AspAT and PheAT, were not included in the restraints. The substrate analogue of hydrocinnamate (HCA) was not used in the progress of computational modeling.

3. Results and discussion

3.1. Analysis of activities and kinetic parameters of ecPheAT mutants

Seven mutants, S297F, S297L, S297R, S297N, S297M, S297A, and S297C, were constructed by PCR overlap-extension mutagenesis. Enzyme activity analysis for the crude extracts of each mutants showed that the activities of S297F, S297R, and S297L mutants were dramatically decreased to about 3% of that of the wild-type enzyme. The activity of S297M, S297N, and S297A were also decreased but retained about 9%, 56%, and 76% of original activity of the wild-type enzyme, respectively, whereas the S297C mutant had almost the same activity as that of the wild-type enzyme (Table 1). Kinetic analysis of the latter three mutants showed an increase in K_m for aspartate, but the K_m for phenylpyruvate was essentially unchanged. These mutants also showed a lower catalytic efficiency as compared with that of the wild type ecPheAT for the transamination reaction of phenylpyruvate and L-aspartate (Table 2). These data suggested that Ser^{297} of *E. coli* PheAT could have selected for better catalytic efficiency in the anabolic pathway of aromatic amino acids.

3.2. The effect of substituting Ser with Phe at position 297 on ecPheAt activity

The high-resolution three-dimensional structure of pdArAT provided strong evidence indicating that the residue Phe at position 297 of the enzyme can play a role on the catalytic activity, although it was not directly involved in substrate binding [4]. In the comparative study between pdArAT and ecAspAT, Okamoto et al. suggested that the residues Asn^{142} and Ser^{296*} (* indicates the residue being located on the other subunit) have a high degree of freedom, which could facilitate the movement of Arg^{292*} in accepting the incoming hydrophilic dicarboxylic substrate or hydrophobic aromatic substrate [4]. When the side chain of Arg^{292*} moved to the direction of active site, it facilitated the interaction with dicarboxylic substrate; on the other hand, when it moved away from the active site, it left more space for accommodating aromatic substrate.

The key factor that affected the freedom of residue Ser^{296*} was the adjacent residue 297*. In ecAspAT, residue Asn^{297*} provided Ser^{296*} with a hydrophilic environment and modulated the movement of Ser^{296*}. In pdArAT, Phe^{297*} was functional, because the residue Ile^{17}, Leu^{18}, and Leu^{73*} moved slightly outward to accommodate the phenyl side chain, which provided Ser^{296*} with a hydrophobic environment and facilitated its conformational change (Fig. 3). However, the side chain of Ser^{297*} in ecPheAT was shorter in comparison with that of Asn^{297*} in ecAspAT; hence, the hydrophilicity around Ser^{296*} of ecPheAT had to be lowered to ensure the freedom for Ser^{296*}. In this study, we found that mutant S297F of ecPheAT was practically inactive (Table 1), whereas in pdArAT, Phe^{297*} played an important role in function. In ecPheAT, we also noted that the substitution of Ser^{297*} with Phe hindered not only the freedom of movement of residue Ser^{296*} but also the binding of substrates. These indicated that other structural elements in the active center of ecPheAT could have put selective pressure on Ser at position 297 for better catalytic efficiency of the enzyme.

Table 2
Kinetic parameters of ecPheAT mutants[a]

Mutants	k_{cat} (s^{-1})	K_m (mM)		k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)	
		PhePyr	Asp	PhePyr	Asp
Wild type	154.68 ± 7.06	0.019 ± 0.002	4.33 ± 0.44	8.1×10^3	35.7
S297N	160.19 ± 8.92	0.070 ± 0.009	11.40 ± 1.95	2.3×10^3	11.9
S297A	246.79 ± 10.69	0.048 ± 0.005	59.30 ± 4.40	5.1×10^3	4.2
S297C	210.14 ± 9.60	0.034 ± 0.004	16.67 ± 0.75	6.2×10^3	12.6

[a] The kinetic parameters of PheAT-catalyzed transamination reaction of phenylpyruvate (PhePyr) and aspartate (Asp) under steady state conditions were measured using malate dehydrogenase-coupling assay in 50 mM HEPES buffer, pH 8.0, containing 100 mM KCl at 25 °C with various concentrations of L-aspartate (2.5–650 mM) and phenylpyruvate (0.01–3 mM).

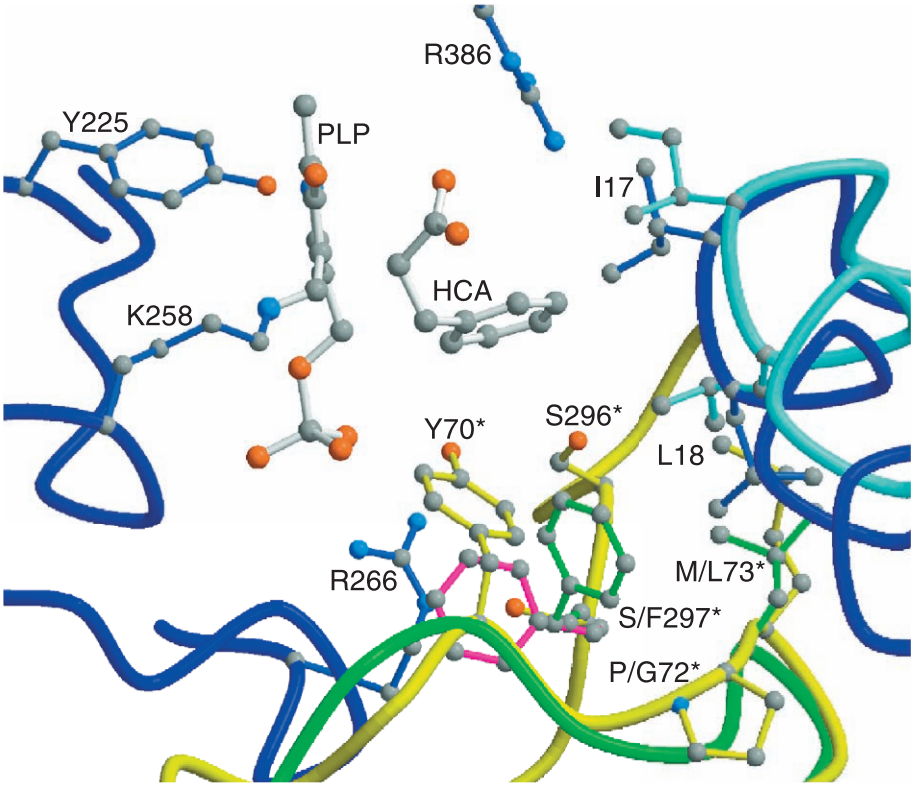

Fig. 3. Computational modeling of the active center of S297F PheAT. The polypeptide backbone and side chain bonds are shown in blue and yellow for different subunits, while the PLP group and the HCA molecule are shown in white. This model of ecPheAT in closed conformation is superimposed with that of pdArAT, shown in cyan and green. The substitution of Ser with Phe in position 297 is shown in pink.

3.3. Structural interpretations of mutations at position 297 of ecPheAT

What is the reason that the substitution of Ser^{297} with other residues also made the catalytic efficiency of ecPheAT decrease? Computational modeling of the mutant enzymes showed their overall structures were basically identical to that of the wild type ecPheAT in the open form, substrate-free conformation; however, in the closed conformation, replacement of Ser^{297*} with other amino acids was found to

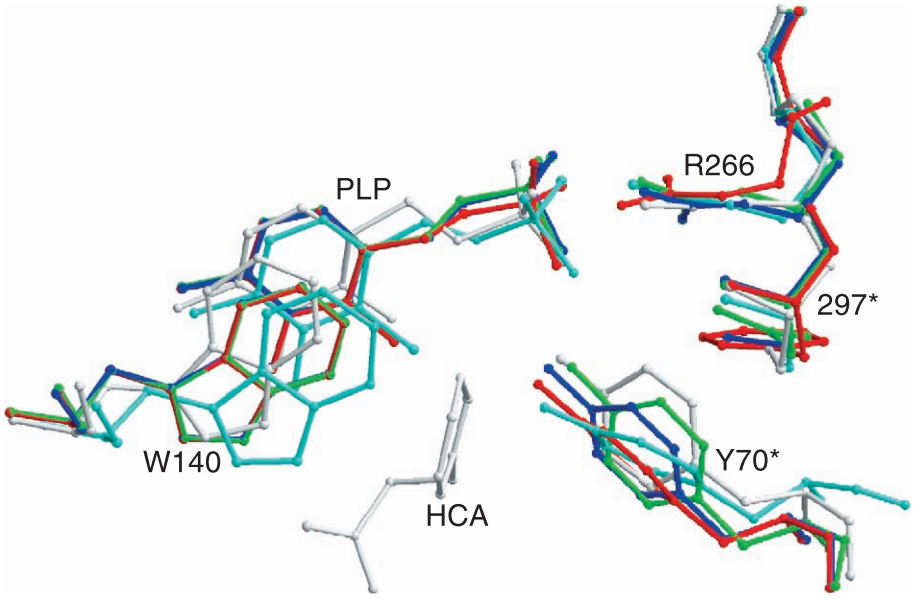

Fig. 4. The superimposition of the closed-form *E. coli* PheAT mutants, S297F (red), S297N (green), S297C (blue), and S297A (cyan), were modeled according to the structure of AspAT hexamutant (white), PDB entry code 1AHX, in the active site. The HCA molecule bound to the AspAT is also shown.

result in structural change around the active site and affected the capacity of substrate binding (Fig. 4). The side chain of residue 297* was sandwiched between Tyr^{70}* and Arg^{266}. These two residues retained exactly the original conformation in the S297A and S297C models, but were pushed slightly apart in the S297N and S297F models. The amide group of Asn and the phenyl group of Phe were stacked with the hydroxyphenyl group of Tyr^{70}* and the guanidinium group of Arg^{266}. The 1AHX structure with bound HCA showed that both side chains of Trp^{140} and Tyr^{70}* were displaced from the substrate-free positions, in order to accommodate the phenyl ring of HCA. The mutant enzymes should undergo similar conformational changes to bind aromatic substrates. For the mutants of S297A, S297C, and S297N, it appeared feasible to make such rearrangement. However, in the structure of mutant S297F, the side chain of Tyr^{70} would remain stacked with the phenyl ring of Phe^{297}*, and thus may interfere with substrate binding. These observations strongly supported the notion that although Ser^{297} of ecPheAT is not evolutionally conserved, it may be structurally selected for better catalytic efficiency of the enzyme.

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References

[1] S.-P. Wu, C. Liu, T.-S. Hwang, J.-T. Chen, H. Tsai, Improving the catalytic activity of phenylalanine aminotransferase of *E. coli* by site-specific mutation, Ann. N.Y. Acad. Sci. 864 (1998) 561–564.

[2] P.P. Taylor, D.P. Pantaleone, R.F. Senkpeil, I.G. Fotheringham, Novel biosynthetic approaches to the production of unnatural amino acids using transaminases, Trends Biotechnol. 16 (1998) 412–418.

[3] T.-P. Ko, S.-P. Wu, W.-Z. Yang, H. Tsai, H.S. Yuan, Crystallization and preliminary crystallographic analysis of the *Escherichia coli* tyrosine aminotransferase, Acta Crystallogr. D55 (1999) 1474–1477.

[4] A. Okamoto, Y. Nakai, H. Hayashi, K. Hirotsu, H. Kagamiyama, Crystal structures of *Paracoccus denitrificans* aromatic amino acid aminotransferase: a substrate recognition site constructed by rearrangement of hydrogen bond network, J. Mol. Biol. 280 (1998) 443–461.

[5] S. Kuramitsu, K. Inoue, T. Ogawa, H. Ogawa, H. Kagamiyama, Aromatic amino acid aminotransferase of *Escherichia coli*: nucleotide sequence of the *tyrB* gene, Biochem. Biophys. Res. Commun. 133 (1985) 134–139.

[6] Y. Nakai, H. Hayashi, H. Kagamiyama, Cloning and characterization of the *tyrB* gene from *Salmonella typhimurium*, Biochim. Biophys. Acta 1308 (1996) 189–192.

[7] S. Oue, A. Okamoto, Y. Nakai, M. Nakahira, T. Shibatani, H. Hayashi, H. Kagamiyama, *Paracoccus denitrificans* aromatic amino acid aminotransferase: a model enzyme for the study of dual substrate recognition mechanism, J. Biochem. 121 (1997) 161–171.

[8] J.M. Doyle, M.E. Schinina, F. Bossa, S. Doonan, The amino acid sequence of cytosolic aspartate aminotransferase from human liver, Biochem. J. 270 (1990) 651–657.

[9] Y.A. Ovchinnikov, T.A. Egorov, N.A. Aldanova, M.Y. Feigina, V.M. Lipkin, N.G. Abdulaev, E.V. Grishin, A.P. Kiselev, N.N. Modyanov, A.E. Braunstein, O.L. Polyanovsky, V.V. Nosikov, The complete amino acid sequence of cytoplasmic aspartate aminotransferase from pig heart, FEBS Lett. 29 (1973) 31–34.

[10] S.V. Shlyapnikov, A.N. Myasnikov, E.S. Severin, M.A. Myagkova, Y.M. Torchinsky, A.E. Braunstein, Primary structure of cytoplasmic aspartate aminotransferase from chicken heart and its homology with pig heart isoenzymes, FEBS Lett. 106 (1979) 385–388.

[11] S. Kuramitsu, S. Okuno, T. Ogawa, H. Ogawa, H. Kagamiyama, Aspartate aminotransferase of *Escherichia coli*: nucleotide sequence of the *aspC* gene, J. Biochem. 97 (1985) 1259–1262.

[12] T. Yano, S. Kuramitsu, S. Tanase, Y. Morino, K. Hiromi, H. Kagamiyama, The role of His^{143} in the catalytic mechanism of *Escherichia coli* aspartate aminotransferase, J. Biol. Chem. 266 (1991) 6079–6085.

[13] S.N. Ho, H.D. Hunt, R.M. Horton, J.K. Pullen, L.R. Pease, Site-directed mutagenesis by overlap extension using the polymerase chain reaction, Gene 77 (1989) 51–59.

[14] H. Hayashi, K. Inoue, T. Nagata, S. Kuramitsu, H. Kagamiyama, *Escherichia coli* aromatic amino acid aminotransferase: characterization and comparison with aspartate aminotransferase, Biochemistry 32 (1993) 12229–12239.

[15] T.A. Jones, J.Y. Zou, S.W. Cowan, M. Kjeldgaard, Improved methods for binding protein models in electron density maps and the location of errors in these models, Acta Crystallogr., A 47 (1991) 110–119.

[16] A.T. Brunger, X-PLOR Version 3.1: A System For X-ray Crystallography and NMR, Yale Univ. Press, New Haven, CT, 1993.

[17] V.N. Malashkevich, J.J. Onuffer, J.F. Kirsch, J.N. Jansonius, Alternating arginine-modulated substrate specificity in an engineered tyrosine aminotransferase, Nat. Struct. Biol. 2 (1995) 548–553.