Mutation of Aryl Binding Pocket Residues Results in an Unexpected Activity Switch in an Oryza sativa Tyrosine Aminomutase

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Supporting Information

ABSTRACT: A 3,5-dihydro-5-methylidene-4H-imidazol-4-one (MIO)-dependent tyrosine aminomutase (TAM) isolated from the rice plant Oryza sativa (OsTAM) makes β-tyrosine (75%) and p-coumarate (25%) from α-tyrosine. OsTAM is the first TAM to have, although slight, native phenylalanine aminomutase (PAM) activity (3% relative to TAM activity). The active sites of OsTAM and a TcPAM from Taxus plants differ by only two residues (Y125 and N446 of OsTAM vs C107 and K427 of TcPAM) positioned similarly near the aryl ring of their substrates. The kinetic parameters and substrate selectivity were measured for OsTAM single mutants Y125C and N446K OsTAM and double mutant Y125C/N446K OsTAM. Compared with OsTAM, each single mutant was slower at converting α-tyrosine to its β-isomer and p-coumarate; the double mutant did not produce any detectable product. Each mutant bound α-phenylalanine ~9-fold better than did OsTAM, suggesting that the mutations made the catalysts more selective for phenylalanine. The total turnover rate (kat) of each mutant for converting α-phenylalanine to both β-phenylalanine and cinnamate was ~4-fold greater than the OsTAM rate for making β-phenylalanine and cinnamate. This switch in catalytic activity from an MIO tyrosine aminomutase (TAM) to a phenylalanine ammonia lyase (PAL) with a change of only two active site side chains suggests that these residues not only play a central role in substrate selectivity but, in part, also set the intrinsic reactivity of OsTAM.

Members of a 3,5-dihydro-5-methylidene-4H-imidazol-4-one (MIO)-dependent class 1 lyase-like family are found in organisms from four of the six kingdoms of life. The MIO is formed post-translationally and autocatalytically by condensation of an amino acid triad, (Ala/Thr/Ser/Cys/Ser-Gly) (Figure 1A), and functions as an α/β-unsaturated electrophile that facilitates αβ-elimination of the NH₂/H pair from β-aryl-α-amino acids. Members of this family include aminomutases that isomerize phenylalanine and tyrosine (PAM) and TAM substrates to transfer the amino group from Cα to Cβ to form β-amino acids and re-form the catalytic MIO. Of the characterized MIO-dependent enzymes, several are tyrosine aminomutases from Myxococcus fulvus (MfTAM), Myxococcus sm. Mx-B0 (MxTAM), Actinomadura madurae (AmTAM), and Streptomyces sp. ATCC 53650 (KedY). This study looks at an MIO-dependent tyrosine aminomutase from Oryza sativa Japanese rice (OsTAM) isomizes (2S)-α- to (3R)-β-tyrosine catalyzed with 94% enantiomeric excess (ee) (Figure 2), while also producing an abnormally larger amount (25%) of p-coumarate as a byproduct versus the amount made by other MIO-reliant TAM enzymes. It is interesting to note that the CoA thioester of p-coumarate is known to be bacteriostatic and thus may provide this function in rice in addition to the role it plays in lignin and flavonoid biosynthesis. The high β-tyrosine enantiosel ectivity catalyzed by OsTAM resembles that of a Taxus canadensis phenylalanine aminomutase (TcPAM) from plants, which makes (3R)-β-phenylalanine with exceptionally high enantiosel ectivity (~100% ee). Three active site residues of MIO-dependent enzymes have been

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Examining in previous studies for their effect on substrate selectivity. For example, one study looked to change SgTAM (Figure 3A) to a functionally similar phenylalanine aminomutase by changing His93 to Phe, guided by a Phe residue positioned similarly in a distantly related plant PAL (Figures 3C and 4A). Surprisingly, the SgTAM His93Phe mutant was not an active aminomutase with either α-phenylalanine or α-tyrosine. Formation of an acrylate byproduct (cinnamate or coumarate) was not described in the SgTAM mutant study. Following a similar mutation strategy in an earlier study, RsTAL from *Rhodobacter sphaeroides* was successfully converted to a functionally equivalent PAL after a His89Phe mutation had been introduced. Following this recurring theme, another study changed the specificity of a TcPAM from phenylalanine to tyrosine (TcTAM) by a straightforward mutation of Cys107 (Figure 3B) to His or Ser, found in positions similarly located in other characterized TAMs (Figures 3A and 4D). Overall, several studies of MIO-based aminomutases and ammonia lyases have shown that the residue in line with the para carbon of the phenylpropanoid substrate (e.g., Cys107 in TcPAM) (Figures 3B and 4C) generally is a histidine. This residue is seemingly key for controlling substrate selectivity and less so for defining the intrinsic aminomutase or lyase activity.

Comparing the crystal structure of TcPAM with a model of OsTAM shows that their active sites differ by only two residues (Tyr125 and Asn446 in OsTAM vs Cys107 and Lys427 in TcPAM) near the para carbon of the bound substrate (Figure 3B,D). More unusual is that OsTAM, unlike other TAMs, does not contain a signature histidine residue in this binding region. Conserved residues Leu126 (OsTAM) and Leu108 (TcPAM) are in the aryl ring binding triad (Figure 3B,D). Thus, we hypothesized that OsTAM could be converted to a highly enantioselective, functionally similar OsPAM by mutation of its active site to match that of TcPAM. Herein, we describe an unexpected change in function from a tyrosine aminomutase to a phenylalanine ammonia lyase, caused by these minimal, non-catalytic active site alterations. We also highlight the substrate preference, kinetic parameters, and product distribution of a double mutant (Y125C/N446K) and two single mutants (Y125C and N446K) of OsTAM.

## METHODS

### Chemicals.

(3R)-β-Tyrosine and (3R)-β-phenylalanine were obtained from Peptech (Burlington, MA). All other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO) and used without further purification, unless noted otherwise.

### OsTAM Mutations.

Point mutations of the *ostam* gene were generated by the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene) using the buffers, enzymes, and dNTP mix provided. To make each single *ostam* mutant, the following forward and reverse primer pairs were used separately (mutated bases are underlined): Y125C, 5′-GAA CTG ATC GTG TGT CTG AAC GCT GCC GGC-3′ and 5′-GCC GTC GTT CAG ACA AGC GAT CAG TTC-3′; N446K, 5′-GAC TAT GCC TTT AAG GGT GCG GAA GTG-3′ and 5′-CAC TCC CGC ACC CTT AAA GCC ATA GTC-3′. The pOsTAM plasmid containing the wild-type *ostam* cDNA was used as the template for polymerase chain reaction (PCR) amplification using Phusion HF polymerase (New England Biolabs, Ipswich, MA) under the following protocol: 95 °C for 5 min, followed by 18 cycles of 95 °C for 30 s, 58 °C for 1 min, and 68 °C for 8.5 min, with a final elongation step of 68 °C for 7 min. Each PCR single-mutant product plasmid (pY125C-*ostam* and pN446K-*ostam*) was digested with DpnI (New England Biolabs) at 37 °C for 1 h. The resultant pN446K-*ostam* plasmid was used as the template in another round of PCR with the Y125C primer set to provide the pY125C/N446K-*ostam* double-mutant plasmid. Each mutant plasmid was then used to transform DH5α *Escherichia coli* (Invitrogen, Thermo Life Sciences, Grand Island, NY). The resulting colonies were inoculated in starter cultures and grown for DNA purification (Wizard Plus SV Minipreps DNA Purification System, Promega, Fitchburg, WI), and DNA sequencing was performed to verify the *ostam* point mutations at the Michigan State University Research Technology Support Facility.

### Subcloning, Expression, and Purification of Mutant OsTAM.

Mutant plasmids were used to transform *E. coli* BL21(DE3) cells with the pY125C-*ostam*, pN446K-*ostam*, and pY125C/N446K-*ostam* double-mutant plasmids expressing the Y125C-OsTAM, N446K-OsTAM, and Y125C/N446K-OsTAM mutant enzymes, respectively. Cells were grown in 3 L of Luria-Bertani (LB) medium supplemented with kanamycin (50 μg/mL). Overexpression of the OsTAM cognates was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) (125 μM), and the cells were grown at 18 °C for 18 h. The cells were
harvested by centrifugation, and the resulting pellet was resuspended in 50 mL of lysis buffer [50 mM sodium phosphate containing 300 mM NaCl, 10 mM imidazole, and 5% (v/v) glycerol (pH 8.0)]. The cells were lysed by sonication (Misonix Sonicator, Farmingdale, NY), and the cellular debris was removed by centrifugation. The crude lysate was added to a nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography column (Qiagen, Valencia, CA) pre-equilibrated with wash buffer [50 mM sodium phosphate containing 300 mM NaCl, 10 mM imidazole, and 5% (v/v) glycerol (pH 8.0)], and each mutant OsTAM expressed with a His6 epitope was purified according to the protocol described by the manufacturer. Y125C-, N446K-, and Y125C/N446K-OsTAM (76 kDa) eluted separately in 250 mM imidazole fractions, were concentrated, and were buffer exchanged against 50 mM sodium phosphate containing 5% (v/v) glycerol (pH 8.0) using a Centriprep...
Kinetic data were acquired. Aliquots (250 μL) of protein concentration at 29 °C were tested for activity with α-Tyrosine.

**Table 1. Kinetic Parameters of Wild-Type and Mutant OsTAM Enzymes**

<table>
<thead>
<tr>
<th>OsTAM enzyme</th>
<th>$K_{M}$ (mM)</th>
<th>$k_{cat}^{rel}$ ($×10^{-3}$ s$^{-1}$)$^{a}$</th>
<th>$k_{cat}^{tot}$ ($×10^{3}$ s$^{-1}$)$^{b}$</th>
<th>$k_{cat}/K_{M}$ ($×10^{3}$ s$^{-1}$ $M^{-1}$)</th>
<th>$k_{cat}$</th>
<th>$V_{max}$ ($×10^{-3}$ s$^{-1}$)</th>
<th>$V_{max}$</th>
<th>$S$</th>
<th>$S_{o}$</th>
<th>$S_{o}/S$</th>
<th>$k_{cat}$</th>
<th>$V_{max}$</th>
<th>$V_{max}$</th>
<th>$S$</th>
<th>$S_{o}$</th>
<th>$S_{o}/S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type A</td>
<td>0.54 (0.03)</td>
<td>5.0 (0.014)</td>
<td>1.7 (0.003)</td>
<td>75.25</td>
<td>6.7</td>
<td>100</td>
<td>12.4</td>
<td>100</td>
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<tr>
<td>Y125C B</td>
<td>0.51 (0.06)</td>
<td>0.053 (0.003)</td>
<td>0.18 (0.007)</td>
<td>22.78</td>
<td>0.23</td>
<td>3.4</td>
<td>0.45</td>
<td>3.6</td>
<td>---</td>
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<tr>
<td>N446K C</td>
<td>0.64 (0.08)</td>
<td>0.0021 (0.0002)</td>
<td>0.086 (0.007)</td>
<td>2.98</td>
<td>0.088</td>
<td>1.3</td>
<td>0.133</td>
<td>1.1</td>
<td>---</td>
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</tr>
<tr>
<td>Y125C/N446K D</td>
<td>ND$^c$</td>
<td>ND$^c$</td>
<td>ND$^c$</td>
<td>---</td>
<td>---</td>
<td>---</td>
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$^a$k$_{cat}$ of enzyme for β-tyrosine. $^b$k$_{cat}$ of enzyme for coumarate. $^c$Ratio of β-amino acid to acrylate product. $^d$Total turnover of enzyme for β-amino acid and acrylate products. $^e$Relative total turnover. $^f$Not detected; limit of detection estimated to be 0.000075 × 10$^{-3}$ s$^{-1}$. $^g$Nonlinear regression of the turnover rate of OsTAM for α-phenylalanine did not reach saturation. Thus, apparent $K_{M}$ and $k_{cat}$ values were estimated from a linear regression (Lineweaver–Burk) plot. $^h$k$_{cat}$ of enzyme for β-tyrosine. $^i$k$_{cat}$ of enzyme for coumarate. Standard error given in parentheses.

centrifugal filter (30K molecular weight cutoff, Millipore). The purity of the OsTAM mutants (60, 90, and 70%, respectively) was judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with Coomassie Blue staining, using a Kodak Gel Logic 100 Imaging System (Figures S1–S3 of the Supporting Information). The concentration of each mutant enzyme was estimated to be 2.1 mg/mL (corrected for purity) on a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE).

**Activity of OsTAM Mutants Tested with α-Tyrosine and α-Phenylalanine.** Y125C-OsTAM, N446K-OsTAM, and Y125C/N446K-OsTAM (0.7 mg of each) were separately incubated for 18 h with α-tyrosine or α-phenylalanine (2 mM) in assay buffer [50 mM sodium phosphate and 5% (v/v) glycerol (pH 8.0)]. The product mixture containing amino acids and acrylates was treated with pyridine (2 × 0.6 mmol) and ethyl chloroformate (2 × 0.5 mmol) in one pot and stirred for 5 min each time. For assays incubated with α-tyrosine, the resulting 4′-O-2-N- and 4′-O-3-N-di(ethoxycarbonyl) derivatives of the α- and β-tyrosine, respectively, and any ethyl 4′-O-ethoxycarbonyl derivatives of p-coumarate were extracted into diethyl ether (3 × 1 mL). The organic layer was separated and removed under a stream of nitrogen. The resultant residue was resuspended in a 3:1 ethyl acetate/methanol mixture. For assays incubated with α-phenylalanine, the resulting 2-N- and 3-N-ethoxycarbonyl derivatives of the α- and β-phenylalanine, respectively, and cinnamonate were extracted into diethyl ether (3 × 1 mL); the organic layer was separated and removed under a stream of nitrogen. The residue was resuspended in a 3:1 ethyl acetate/methanol mixture and titrated with diazomethane until a yellow color persisted. The derivatives were analyzed by gas chromatography and mass spectrometry (GC–EI-MS). The derivatized biosynthetic products had retention times and fragment ion abundances (Figures S8–S20 of the Supporting Information) identical to those of authentic standards derivatized equivalently.

**Kinetic Parameters of OsTAM Mutants with α-Tyrosine.** Y125C- and N446K-OsTAM (0.36 mg) were incubated separately in 3 mL of assay buffer containing (2S)-α-tyrosine to establish linearity with respect to time at a fixed protein concentration at 29 °C. Note that the Y125C/N446K-OsTAM double mutant was not active with α-tyrosine, so no kinetic data were acquired. Aliquots (250 μL) were withdrawn from each at 1-h interval over 12 h. The products were derivatized and quantified as described above, and steady state conditions were determined. To calculate the kinetic constants, the (2S)-α-tyrosine concentration was varied (0.1–1.5 mM) in separate assays under steady state conditions. The resultant aryl alanine and aryl acrylate products were derivatized and quantified as described earlier. Kinetic parameters ($K_{M}$ and $k_{cat}$) were determined from Michaelis–Menten plots (Figures S21 and S22 of the Supporting Information). OriginPro 9.0 was used to perform nonlinear curve fitting. Although $K_{M}$ is not a true dissociation constant, in this study it will serve as a means of comparing interactions of enzymes with different substrates.

**Kinetic Parameters of OsTAM Mutants with α-Phenylalanine.** Y125C-, N446K-, and Y125C/N446K-OsTAM (0.18 mg) were incubated separately in 3 mL of assay buffer containing (2S)-α-phenylalanine to establish linearity with respect to time at a fixed protein concentration at 29 °C. Aliquots (250 μL) were withdrawn from each at 1 h intervals over 12 h. The products were derivatized and quantified as described above, and steady state conditions were determined. To calculate the kinetic constants, the (2S)-α-phenylalanine concentration was varied (0.1–3 mM) in separate assays under steady state conditions. The resultant aryl alanine and aryl acrylate products were derivatized and quantified as described above. Kinetic parameters ($K_{M}$ and $k_{cat}$) were determined from Michaelis–Menten or Lineweaver–Burk plots (Figures S23–S26 of the Supporting Information). OriginPro 9.0 was used to perform nonlinear curve fitting.

**RESULTS AND DISCUSSION**

**Activity of OsTAM Mutants.** On the way to making the Y125C/N446K-OsTAM double mutant, we cloned, expressed, and tested the progenitor single mutants Y125C- and N446K-OsTAM for activity with α-tyrosine and α-phenylalanine.

**Y125C-OsTAM Mutant.** Compared to OsTAM, Y125C-OsTAM turned over α-tyrosine to p-coumarate (Table 1F) in order of magnitude slower (Table 1E). While the $K_{M}$ values of both Y125C-OsTAM and OsTAM for α-tyrosine were similar (Table 1A,B), the $k_{cat}$ ($k_{cat}^{tot} + k_{cat}^{rel}$) was drastically reduced (29-fold) for the mutant. Besides the decreased rate of turnover, the product ratios were inverted; Y125C-OsTAM produced a 2.78 β-tyrosine:coumarate ratio compared to a reciprocal 7:5:25 ratio catalyzed by OsTAM. These data suggest...
that loss of the 4′-hydroxyl group of the Tyr125 single mutant negatively affects substrate turnover and the ability of OsTAM to retain the coumarate intermediate for the NH2 rebound on the reaction pathway. Further, the catalytic efficiency of Y125C-OsTAM for α-phenylalanine (Table 1F) was ~3-fold higher than that for α-tyrosine (Table 1B). These data support that the Y → C mutation changed the substrate selectivity preference towards α-phenylalanine.

The relative catalytic efficiency of Y125C-OsTAM, dominated by 9-fold lower $K_m$ than that of OsTAM for α-phenylalanine (Table 1E,F). This data estimates that the Cys125 replacement and the original N446 of Y125C-OsTAM binds phenylalanine better than does the Y125/N446 diad of OsTAM. Despite its higher catalytic efficiency over OsTAM, Y125C-OsTAM disproportionately makes more cinnaamate (97%) as a mixture with 3% β-phenylalanine (Table 1F). To compare, OsTAM makes a slightly more balanced distribution of cinnaamate (70%) to β-phenylalanine (30%) (Table 1E). In summary, the naturally occurring Y125C-OsTAM is catalytically higher than that of the wild-type enzyme nearly 100-fold compared to that of the wild-type enzyme.

Y125C/N446K-OsTAM Mutant. The relative catalytic rate of N446K-OsTAM (Table 1C) for α-tyrosine was 2.6-fold slower than the rate of Y125C-OsTAM (Table 1B) and astounding 76-fold slower than that of OsTAM (Table 1A). The relative catalytic efficiency (kcat/Km) of N446K-OsTAM dropped a mere ~3-fold compared to that of Y125C-OsTAM and by nearly 100-fold compared to that of the wild-type enzyme (Table 1). The $K_m$ values of Y125C-OsTAM, Y125C-OsTAM, and OsTAM (Table 1A-C) are similar for tyrosine, yet it is clear that the N446K mutation affects the catalytically competent conformation of α-tyrosine. The ratio of product made by N446K-OsTAM from α-tyrosine was 2:98 β-tyrosine:p-coumarate (Table 1C). The predominant production of p-coumarate again supported the idea that the N446 residue was responsible, in part, for holding the acylate intermediate within the active site long enough for the isomerization to advance.

To compare, N446K-OsTAM turned over α-phenylalanine to 6:94 β-phenylalanine:cinnaamate ratio (Table 1G) at a rate far superior (16-fold greater kcat/Km) than it turned over tyrosine to its products (Table 1C). Thus, the altered N → K446 coupled with the natural Y125 residue not only enhanced specificity for phenylalanine (kcat/Km increased ~40-fold) over that for α-tyrosine but also affected the NH2 rebound, producing more acylate intermediate than of the β-amino acid, as seen for the Y125C mutant. Moreover, the 95-fold increase and the 95-fold increase in catalytic efficiency of N446K-OsTAM for phenylalanine and tyrosine, respectively, compared to that of OsTAM, show that the mutant is more compatible with a phenylpropanoid than a hydroxyphenylpropanoid. This selectivity preference was also seen for the Y125C mutant and for the double mutant, described next.

Y125C/N446K-OsTAM Mutant. The double mutant Y125C/N446K-OsTAM lost all of its detectable α-tyrosine activity and did not convert the 4′-hydroxyphenylalanine to either β-tyrosine or p-coumarate (Table 1D,H), even after 48 h. This exclusive phenylalanine selectivity, over tyrosine, is much like that of TcPAM, whose active site sequence guided the design of Y125C/N446K-OsTAM. Y125C/N446K-OsTAM converted α-phenylalanine to ~40-fold more cinnaamate than β-phenylalanine (Table 1H), with a total turnover rate similar to those of the single-mutant congeners (Table 1F,G). Using $K_m$ as an estimate of $K_d$, each OsTAM mutant bound α-phenylalanine approximately as well as OsTAM bound α-tyrosine (Table 1A-F,H). Thus, it appears that even though Y125C/N446K-OsTAM binds α-phenylalanine as sufficiently through its three binding contacts (the aryl ring binding pocket, the amino acid NH2–MIO adduct, and the salt bridge with R344) (Figure 4) like other MIO aminomutases,5,23,24 (e.g., see Figure 3A) the cinnaamate intermediate is not well-restrained by the active site after elimination of the NH2/H pair (see Figure 1B).

In summary, switching Y125 and N446 of the tyrosine-specific OsTAM to C125 and K446 (residue identity found in the TcPAM) changed OsTAM to a phenylalanine-specific catalyst. In addition, these mutations changed the aminomutase reactivity to that of an ammonia lyase. Thus, the Y125 and N446 residues of OsTAM are key to binding the tyrosine substrate and in part defining the reaction type (aminomutase or lyase). These residues near the 4′-hydroxy of the substrate likely interact through polar interactions and hydrogen bonding to control the capture time of the acylate intermediate. Moreover, characterized bacterial TAMs hypothetically use three or four polar active site residues, His-Ser-[Glu/Ala]-Tyr (underlined), to aid in binding their 4′-hydroxyphenylalanine substrate (Figures 3A and 4D). By contrast, OsTAM uses only two: Tyr125-Leu126-Asn446-Val450 (underlined) (Figures 3D and 3A). The reduced number of polar contacts with the tyrosine substrate may provide insight into why the proportion of p-coumarate release increased and why the $K_m$ of OsTAM was higher for tyrosine than those of previously characterized TAMs.

Sequence Analysis of OsTAM with Other MIO-Dependent Enzymes. Comparison with Ammonia Lyase and Aminomutases. Aligning OsTAM with plant PALs (CpPAL, PtPAL, AtPAL, OsPAL, and ZmPAL) (Figure 4A) shows a high degree of sequence identity from 63% (for AtPAL from Arabidopsis thaliana) to 73% (for CpPAL from Cenchrous purpureus); the degree of sequence similarity ranged between 77 and 81%. It was surprising to find that OsTAM had low degrees of sequence identity (19–20%) and similarity (29–31%) with other TAMs (Figure 4D). These sequence comparisons infer that OsTAM likely arises from a close ancestral plant MIO-AL rather than from a plant MIO-AM. An earlier study found that OsTAM released an unusually elevated proportion of p-coumarate byproduct (25%), through its TAL activity.20 Other MIO aminomutases release less acrylate; for example, TcPAM releases 5–10% byproduct (thereby less AL activity) at 31 °C during its reaction at steady state.1,2,14 This reduced AL activity for TcPAM could correlate with its lower level of sequence overlap (41–43% identical and 59–62% similar) (Figure 4C) when compared with PAL sequences.

MUTATIONAL ANALYSES. Previous investigations looked to switch the activities of MIO-dependent aminomutases and ammonia lyases.39–41 One study showed how increasing the reaction temperature promoted ammonia lyase activity catalyzed by PaPAM isolated from Pantoaea agglomerans.41 A separate study switched a PAM to a PAL by mutating four residues (Leu97, Ala77, Ile79, and Cys89) of the inner loop structure of Taxus chinensis (TchPAM) [essentially identical (97%) in sequence to TcPAM from T. canadensis] to those in PaPAM (Gly127, Thr107, Ser109, and Thr119, respectively).30 The sequence of the same loop region of OsTAM is 54%
This triad is unique from the consensus sequence (Ser-His-aminomutase activity. The objective was to determine the (Tyr125-Leu126-Asn446) in the aryl ring binding pocket in to that made by TcPAM. OsPAM, by comparison, has a hydrophilic Thr97 residue and is thought to weaken the ability of the inner loop to retain the acrylate intermediate, thus preventing it from retaining p-coumarate as well as other MIO TAMS. We note that a likely paradox exists if the hydrophilic Thr97 residue of the inner loop of wild-type OsPAM is presumed to reduce the level of retention of the acrylate intermediate on the aminomutase pathway. Phenylalanine is an inherently weaker binding substrate for OsPAM (Table 1E) when the residues near the phenyl ring binding pocket are Y125 and N446. This weaker binding interaction combined with the ammonia lyase-like loop structure of OsPAM suggests that OsPAM should selectively release cinnamate before it recaptures NH3 to form β-phenylalanine. This was not the case observed here; OsPAM made a 70:30 mixture of cinnamate and β-phenylalanine from α-phenylalanine. It was also interesting to observe that the mutants, designed to more selectively bind a phenyl substrate, could not capture the cinnamate to complete the mutase reaction.

CONCLUSION

This study looked to improve our understanding of the determinants of MIO-dependent catalysts that define substrate selectivity, binding affinity, turnover rate, and ammonia lyase or aminomutase activity. The objective was to determine the effect on substrate selectivity after mutations of a triad (Tyr125-Leu126-Asn446) in the aryl ring binding pocket in OsPAM. This triad is unique from the consensus sequence (Ser-His-α-phenylalanine) conserved in all PALs and Tyr125C/N446K-Y125C/N446K-TcPAM exactly matched the OsPAM active site. We imagined that TAM would function chiefly as an OsPAM, but instead, we found that it principally catalyzed a PAL reaction at a rate similar to that of N446K-OsPAM, showing only 2% PAM reactivity. This investigation demonstrates a unique instance of mutating two binding pocket residues of a TAM to convert it into a PAL and thus provides critical insight into the role these key residues might play with those in the inner loop structure that caps the active site. These residues in combination with the loop structure likely increase the residence time of the acrylate intermediate in the active site for the NH2 rebound step.


