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Ironing out Their Differences: Dissecting the Structural Determinants of a Phenylalanine Aminomutase and Ammonia Lyase

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Supporting Information

ABSTRACT: Deciphering the structural features that functionally separate ammonia lyases from aminomutases is of interest because it may allow for the engineering of more efficient aminomutases for the synthesis of unnatural amino acids (e.g., β-amino acids). However, this has proved to be a major challenge that involves understanding the factors that influence their activity and regioselectivity differences. Herein, we report evidence of a structural determinant that dictates the activity differences between a phenylalanine ammonia lyase (PAL) and aminomutase (PAM). An inner loop region that closes the active sites of both PAM and PAL was mutated within PAM (PAM residues 77−97) in a stepwise approach to study the effects when the equivalent residue(s) found in the PAL loop were introduced into the PAM loop. Almost all of the single loop mutations triggered a lyase phenotype in PAM. Experimental and computational evidence suggest that the induced lyase features result from inner loop mobility enhancements, which are possibly caused by a 310-helix cluster, flanking α-helices, and hydrophobic interactions. These findings pinpoint the inner loop as a structural determinant of the lyase and mutase activities of PAM.

The diverse bioactivities of β-amino acid compounds include antimicrobial, cytotoxic, and anticancer effects.1−4 As a result, enzymatic routes using ammonia lyases (AL) and aminomutases (AM) have been explored for the preparation of optically pure α- and β-amino acids (Scheme 1).5 The phenylalanine AL (PAL) deaminates α-Phe to give trans-cinnamic acid (t-CA) and ammonia release while the phenylalanine AM (PAM) catalyzes a 2,3-amine shift that reversibly interconverts α-Phe to β-Phe. The catalytic mechanisms of ALs and AMs acting on aromatic compounds involve the electrophilic 4-methylideneimidazole-5-one (MIO) cofactor that is derived from an autocatalytic condensation of a Thr/Ala-Ser-Gly motif.6 Most recent mechanistic studies favor a MIO-amine adduct intermediate, which reversibly shuttles the amino group during the 2,3-amine shift in the mutase reaction and liberates the α-amino group from α-Phe in the lyase reaction with concomitant formation of t-CA.5−8

The application of wild-type (Wt) PAM for the synthesis of β-amino acids is hindered by low reaction rates and the mixture of α-Phe and β-Phe generated from the asymmetric synthetic route.9 Likewise, the α-regioselectivity of Wt PAL precludes its use to synthesize β-amino acids. In light of these drawbacks, we previously engineered Taxus chinensis PAM (TcPAM) to function as a β-lyase via two different approaches: (1) structure- and mechanism-guided alteration of the enzyme’s regioselectivity in the amination reaction10 and (2) loop engineering to

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improve the enzyme’s lyase reaction rates.9 This latter work demonstrated that lyase activity could be engineered into TcPAM after making the active-site inner loop more flexible by removing a loop-stabilizing salt bridge (Arg92-Glu542) via an R92S mutation. The analogous salt bridge is not present in the 3D structure of TcPAM that contains an open inner loop conformation. However, PcPAM could possibly still form a similar loop-stabilizing salt bridge between Arg122 and Asp580 when the loop is closed. Both residues are at equivalent positions in the primary sequence (Supporting Information Figure S1a) and Asp580 occupies the same 3D position as Glu542 in TcPAM (Supporting Information Figure S1b). The sequence alignment in Supporting Information Figure S1a shows that the included PAMs prefer Glu as a salt bridge contact with Arg, whereas PALs prefer Asp. Although it is a subtle structural difference of only one carbon atom, this could be a partial determinant of mutase vs lyase activities. Nevertheless, the salt-bridge likely controls the inner loop conformation in both enzymes and other mutase/lyase structural determinants disrupt it to enforce the open conformation. Based on this observation, it is still not clear how PAL achieves higher reaction rates through a seemingly more flexible or open loop conformation.11,12 Therefore, we addressed the question: what are the structural determinants of PAM and PAL that could foster the transformation of PAM to a PAL?

Even though PAM and PAL have nearly identical active sites, PAL generally exhibits no mutase activity, with a few exceptions.7 Despite that the substrate selectivity has been altered for both PAM and PAL,10,13–16 prior engineering studies have failed to convert an AM to an AL via strict mutase → lyase (M-L) residue mutations.17 The TcPAM and PcPAM 3D structures are very similar to an overall homotetrameric fold predominated by α-helical structures. Each monomer of a head-to-tail dimer contains three domains, which were referred to as the MIO-, core, and shielding domains by Ritter et al.9,11–13 Two shielding, or N-terminal, domains combine in the homotetrameric enzyme to create an arch over the active site that ultimately confines substrate access to a narrow tunnel.11 This narrow tunnel is gated by an inner and outer loop, which were recently implicated in dictating mutase vs lyase activity.2,9 The active-site inner loop, which contains the catalytic base Tyr, appears more rigid in PAM and more open or flexible in PAL (Figure 1a). The rigidity of this loop in PAM is considered crucial for sequestering the t-CA and MIO-amine adduct in the active site to promote readdition of the amino-group to either the α- or β-carbon positions of t-CA. Considering these structural differences between TcPAM and PcPAM, the inner loop appears to be a key structural determinant that influences mutase and lyase activity. However, to our knowledge, no published work exists that precisely defines which M-L residue mutations could influence an AM to acquire lyase-like properties. Therefore, we explored mutations that influence lyase vs mutase activity, which provided new insight into the structural determinants of AMs and ALs.

## RESULTS AND DISCUSSION

### Structural Differences between PAM and PAL Inner Loops.

To investigate the factors that differentiate an AM from an AL, we compared both the 3D structures (Figure 1a) of T. canadensis (TcPAM) and PcPAM and the amino acid sequences of the inner loops (Figure 1b) from TcPAM (residues 77–97) and PcPAM (residues 107–127). The TcPAM structure was chosen since it has a substrate bound in the active site. TcPAM (PDB: 2YII) has 97% sequence identity with TcPAM and also

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**Figure 1.** Structural differences between PAM and PAL. (a) Superimposed 3D inner loop structures of PcPAM (PDB: 1W27, orange) and TcPAM (PDB: 3NZ4, green). The catalytic tyrosines are indicated. The inner loop of TcPAM is crystallized in the closed conformation compared to a more open conformation in PcPAM. (b) Inner loop sequence alignment (Geneious) of TcPAM and PcPAM. Targeted M-L mutations (numbered according to PAM) that show differences in hydrophobicity below each residue (red = hydrophobic; blue = hydrophilic). (c) 3D structural model of the inner active-site loop (gray) of TcPAM with t-CA bound in the active site. Targeted M-L mutations are labeled red, along with substrate, t-CA, in magenta and the catalytic components in yellow (MIO internal cofactor, Tyr80, and Arg325).
crystallized with a closed inner loop. From the structural alignment, it is apparent that the inner loop acts as a lid and adopts a relatively closed structure in TcPAM$^{13}$ (PDB: 3NZ4) compared to the open conformation in P:\PAL$^{11}$ (PDB: 1W27).

In light of this observation and recent studies,$^{7,9,18}$ we hypothesized that the inner loop is a key mutase-lyase determinant that promotes the chemically challenging 2,3-amine shift when closed and causes a lyase reaction when open.

It is apparent that the active-site inner loop of TcPAM harbors more hydrophobic amino acids than P:\PAL (Figure 1b,c). For instance, TcPAM contains Ala77, Ile79, Cys89, and Leu97 whereas P:\PAL contains Thr107, Ser109, Thr119, and Gly127 at the corresponding positions. These hydrophobic residues may become exposed to solvent when the inner loop opens, causing inner loop mobility differences. Therefore, we hypothesized that these hydrophobicity differences within the inner loop may ultimately influence lyase vs mutase activity.

**Modification of PAM Inner Loop Properties.** To test our hypothesis, single (A77T, I79S, C89T, and L97G) and combinatory M-L mutants of TcPAM were constructed with reduced hydrophobicity of the inner loop. If high inner loop mobility indeed promotes lyase behavior, the M-L mutants should exhibit a higher activity with the native substrates as well as higher lyase/mutase activity ratios.

Nearly all single loop mutants showed an increase in the deamination rates of (S)-α-Phe and (R)-β-Phe (Figure 2). The L97G mutant showed the greatest enhancements in both the deamination and amination reaction rates with 16-fold and ∼2-fold increases, respectively. Although A77T and C89T mutants gave similar improvements in the deamination rates (∼2-fold increases), the C89T mutant exhibited a decrease in the amination rate while the A77T mutant displayed a slight increase. Since the L97G mutation causes the most drastic decrease in hydrophobicity among the M-L single mutations, this likely explains the associated activity improvements through enhanced inner loop mobility. Specifically, Leu97 appears to have hydrophobic interactions (Supporting Information Table S1) with three other inner loop residues (Ala73, Glu74, Val82) and Ala223 to sequester the active site. Leu97 also lies at a junction between the C-terminus of the inner loop and the subsequent α-helix. It is plausible that the L97G mutation induces the highest loop mobility among the single loop mutations by ablating multiple hydrophobic interactions, causing more backbone flexibility at this pivotal position. To probe which property—flexibility or hydrophobicity—is possibly more influential at residue 97, Ser (hydrophilic) and Pro (neutral, rigid) residues were introduced at this position. The PAM L97S and L97P mutants exhibited approximately the same improvements in all reaction rates and L/M activity ratios relative to Wt, except for a nearly 3-fold increase in the amination rate by the L97P mutant (Supporting Information Figure S2). The L97G improvements still dominate in the deamination reaction rates. These findings suggest an apparent balance between side chain steric, hydrophobicity, and backbone flexibility at position 97 that influences reaction rates and activity ratios of PAM.

In order to seek out additive effects on enzyme activity from a combination of loop mutations, double loop mutants were generated that contained the superior L97G mutation and one of the three other targeted M-L mutations (Figure 2). PAM L97G/A77T showed the highest reaction rates by maintaining similar rate enhancements for β-Phe deamination relative to PAM L97G. However, the other two activities (α-Phe deamination and t-CA amination) did not surpass those of PAM L97G. A triple loop mutant (PAM L97G/A77T/C89T) containing all beneficial mutations did show enhancement in all rates relative to Wt PAM, albeit not as much improvement as the double loop mutants. Overall, no additive effects on activity were observed among the M-L mutations since the L97G mutation prevailed as the most active variant with all substrates tested.

A more extensive sequence alignment (Supporting Information Figure S1a) shows that lyases prefer Ser and mutases prefer Ile at position 79 when homologous sequences are aligned for each enzyme class. Interestingly, the activities of the I79S mutant reveal a detrimental effect when a lyase residue (Ser) is introduced at position 79. The negative effect from the I79S mutation was amplified in a double loop mutant (PAM L97G/I79S) and rendered the quadruple mutant (PAM L97G/A77T/I79S/C89T) mostly inactive. Based on a 3DM19 structural alignment database of MIO enzymes (102 3D structures used to generate structure/sequence alignments of 2159 sequences), an analysis of correlated mutations using the Comulater$^{20}$ tool do not show any synergistic relationships between any of the inner loop residues targeted in this study. From the crystal structure of Wt PAM (PDB: 2YII), the Ile79 has hydrophobic interactions (Supporting Information Table S1) with the inner loop residues Ala77 and Val82. Interacting also with Leu97, Val82 seems as a nodal connection in the hydrophobic network that stabilizes the inner loop. From this, we hypothesize that the I79S mutation disrupts the structural integrity of the inner loop and prevents a productive orientation of Tyr80 by removing hydrophobic interactions. A similar loop disruption by Bartsch et al. was implicated in the PAM R93S mutant, wherein the intraloop salt bridge between Asp78 and Arg93 was removed and resulted in decreased amination activity along with disordered inner loops in the crystal structure.$^7$

To examine the effect on lyase/mutase activity ratios (L/M) of the loop mutations, we followed the rates of product formation (t-CA/α-Phe) with (R)-β-Phe as substrate (Figure 2). The
From these rates, shifts in the activity ratios relative to Wt PAM were calculated \((L/M_{\text{rel}}; \text{Supporting Information Table S2})\). The single loop mutants (L97G, C89T) that exhibited rate enhancements also generated \(L/M_{\text{rel}}\) activity ratios of about 5. Variants L97G/C89T and L97G/A77T/C89T showed the most drastic increases with an \(L/M_{\text{rel}}\) of \(\sim 72\) and 79, respectively. The apparent synergy between residues 89 and 97 seems to play a crucial role in controlling lyase vs mutase activity. However, there is no direct correlation between improvements in the deamination rates of \((R)-\beta\)-Phe and \(L/M\) activity ratios. For example, PAM L97G exhibits the highest rate enhancements but shows similar improvements as PAM C89T in \(L/M\) activity ratios. Likewise, the aforementioned double and triple mutants show the largest increases in \(L/M\) activity ratios but do not surpass PAM L97G in rate improvements. This suggests that although lyase activity can be improved through certain M-L loop mutations, these mutations increase \(L/M\) activity ratios through mitigating mutase product formation at different efficiencies. Overall, it appears that changes to inner loop mobility of PAM are coupled to alterations in reaction rates and lyase/mutase activity ratios. The above results indicate that predominant lyase activity is likely caused by enhanced loop flexibility from the M-L mutations, as a more flexible inner loop hinders the efficiency of the 2,3-amine shift during the mutase reaction.

**Cosolvent and Temperature Effects on PAM M-L Mutants.** Cosolvents can increase enzyme activity by changing conformational flexibility and active site accessibility, among other biophysical alterations.21−27 After demonstrating that most M-L mutations improve lyase activity through a purportedly more mobile inner loop, we assessed whether reduced hydrophobicity within the inner loop influences the cosolvent effects on activity. We expected that the propensity of loop opening would be mitigated in cosolvent, leading to decreases in lyase activity due to a more closed loop. For Wt PAM and the M-L variants that exhibited activity enhancements in Figure 2, the lyase activities with \((R)-\beta\)-Phe were tested in the presence of the cosolvent DMF (Figure 3 and Supporting Information Table S3). Although all single loop mutants and Wt PAM showed an increase (\(\sim 1.5−3.5\)-fold) in activity with cosolvent present, the combinatory loop mutants showed a decrease in activity. Overall, an apparent trend supports our previous hypothesis and shows that as the hydrophobicity of the inner loop is reduced, the beneficial effects of cosolvent diminish.

Previously, Chesters et al. elegantly demonstrated a temperature switch between mutase and lyase activity after evaluating Wt activity differences between a bacterial PAM and PAL at various temperatures.7 We analyzed the most interesting M-L loop mutants in the same manner in order to unravel any effects M-L mutations may have on thermal switches in activity. From the temperature−activity profiles (Figure 4a and Figure 4b) further confirm the aforementioned disconnect between activity improvements and \(L/M\) activity ratios. For example, the activities of Wt PAM increased with temperature, whereas PAM L97G/C89T showed the opposite trend with an \(\sim 65\)-fold less

![Figure 3](image3.png) **Figure 3.** Cosolvent effects on specific activity. The bars indicate fold-difference of activity (negative = decreased activity; positive = increased activity) with \((R)-\beta\)-Phe for each PAM M-L mutant relative to activity with no cosolvent present (30% DMF). Bars indicate mean ± SEM, \(n = 2\).

![Figure 4](image4.png) **Figure 4.** Temperature effects on activity of PAM M-L mutants. (a) Temperature effects on mutase vs lyase activity ratios. Mutase/lyase (bottom) and lyase/mutase (top) activity ratios are indicated for the deamination reaction with \((R)-\beta\)-Phe. (b) Total specific activity (mutase plus lyase) for each PAM mutant at varied temperatures. All bars indicate mean ± SEM, \(n = 2\).
total specific activity than Wt at 70 °C. Since these PAM variants exhibited similar thermal unfolding profiles (Supporting Information Figure S3), the activity differences are not caused by different enzyme thermostabilities but are proposed to be related to inner loop mobility differences among Wt PAM and M-L mutants.

M-L Mutations Alter Reaction Kinetics of PAM. As the L97G and L97G/C89T mutations gave the highest increase in activity and L/M ratios, respectively, the kinetic constants for the lyase activity (i.e., t-CA detection) were determined for these M-L loop mutants (Table 1). Both PAM mutants showed slight to major improvements (up to 13-fold increase) in the $k_{cat}$ values for all three substrates, relative to Wt PAM. The $k_{cat}$ improvements for PAM L97G are most prominent (~12-fold higher than the double mutant). For the aminated substrates, all $K_M$ values increased ~7-11-fold relative to Wt PAM. This resulted in a small increase (~1.2-fold) in catalytic efficiency for the L97G mutant with (R)-b-Phe and decreased catalytic efficiencies for the rest. Since no major alterations in $K_M$ values with t-CA were observed, the amination catalytic efficiencies for both mutants increased ~2-fold while maintaining Wt enantioselectivity (footnote b, Table 1). Regioselectivity was shifted toward a-preference (Supporting Information Table S2). Overall, the activity (Figures 2-4) and kinetics (Table 1) results emphasize the marked effects on reaction rates caused by the L97G mutation and that the inner loop is an essential structural determinant between PAM and PAL.

<table>
<thead>
<tr>
<th>substrate</th>
<th>PAM variant</th>
<th>$k_{cat}$ [s$^{-1}$]</th>
<th>$K_M$ [mM]</th>
<th>$K_M$ [mM]</th>
<th>$k_{cat}/K_M$ [mM$^{-1}$s$^{-1}$]</th>
<th>$k_{cat}/K_M$ [mM$^{-1}$s$^{-1}$]</th>
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</thead>
<tbody>
<tr>
<td>t-CA$^a$</td>
<td>Wt</td>
<td>0.073 ± 0.003</td>
<td>2.7 ± 0.3</td>
<td>0.027 ± 0.004</td>
<td>1.5</td>
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<tr>
<td></td>
<td>L97G</td>
<td>0.129 ± 0.004</td>
<td>2.8 ± 0.2</td>
<td>0.046 ± 0.005</td>
<td>1.7</td>
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<tr>
<td></td>
<td>L97G/C89T</td>
<td>0.092 ± 0.004</td>
<td>2.2 ± 0.3</td>
<td>0.041 ± 0.007</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>(S)-a-Phe</td>
<td>Wt</td>
<td>0.0032 ± 0.0001</td>
<td>0.008 ± 0.002</td>
<td>0.4 ± 0.1</td>
<td>0.7</td>
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<tr>
<td></td>
<td>L97G</td>
<td>0.0184 ± 0.0005</td>
<td>0.067 ± 0.008</td>
<td>0.28 ± 0.04</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L97G/C89T</td>
<td>0.0088 ± 0.0002</td>
<td>0.058 ± 0.006</td>
<td>0.15 ± 0.02</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>(R)-b-Phe</td>
<td>Wt</td>
<td>0.0092 ± 0.0003</td>
<td>0.028 ± 0.004</td>
<td>0.32 ± 0.06</td>
<td>0.4</td>
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<tr>
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<td>L97G</td>
<td>0.117 ± 0.005</td>
<td>0.31 ± 0.05</td>
<td>0.38 ± 0.08</td>
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<tr>
<td></td>
<td>L97G/C89T</td>
<td>0.062 ± 0.002</td>
<td>0.29 ± 0.03</td>
<td>0.21 ± 0.03</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

$^a$kcat > 99% for the amination products, (S)-a-Phe and (R)-b-Phe. $^b$Fold-difference relative to Wt PAM.

MD Simulations of PAM M-L Mutants. The possible effects of M-L mutations on inner loop dynamics were examined further using MD simulations. To investigate differences in inner loop flexibilities among selected mutants (L97G, L97G/C89T, and L97G/A77T/I79S/C89T) and Wt PAM, we calculated the potential of mean force (PMF) to open the inner loop by using an external pulling force applied to the Tyr80 center-of-mass (COM) and then measuring the distance between the COMs of the MIO cofactor and the catalytic residue Tyr80. For each system, loop opening simulations were carried out on monomer A in the homotetramer (Figure 5a), while the other 3 monomers were allowed to fully relax. Thus, the inner loops within the nonpulled monomers are referred to as the “resting state” and served as controls. The PMF was calculated with umbrella sampling, using 47 windows spaced 0.1 nm apart. Initially, chain A was pulled to generate the 47 starting configurations for the PMF calculation, as depicted in Figure 5a. For each window, a 20 ns simulation was performed. For the nonpulled monomers, random initial velocities were assigned for each window to generate 47 independent simulations of the inner loop at the “resting state”. The resulting PMF is shown in Figure 5b. We present the PMF data only up to 2 nm; at larger distances loop opening becomes unrealistic as it leads to protein unfolding (Supporting Information Figure S4a depicts full PMF profiles). The histograms of the configurations (Supporting Information Figure S4b) show sufficient conformational overlapping.

Based on the results, it is apparent that the M-L mutations alter inner loop dynamics not only during loop pulling simulations (Figure 5b) but also during the resting state simulations that had no external perturbations (Figure 5c). Overall, each mutant showed a broader minimum free energy well in the PMF profile compared to Wt, which suggests higher flexibility within the inner loops of the M-L mutants. The shallow profile of the quadruple mutant suggests a more flexible loop region compared to the other PAM loop variants, which may have caused the detrimental activity effects (cf. Figure 2). Interestingly, each mutant showed a statistically significant increment in the COM distance at the resting state of the loop (Figure 5c and Supporting Information Table S6), which is also indicative of a more open loop conformation for all the mutants compared to Wt.

Given our hypothesis that a putative driving force to stabilize the closed conformation of the PAM inner loop may involve hydrophobic interactions, solvent accessible surface area (SASA) calculations were applied to the resting state simulations of the inner loop. We observed an average decrease...
(−3 ± 1%) in the total SASA for all simulated PAM variants (Supporting Information Figure S5). The same apparent trend prevails in the hydrophilic/hydrophobic SASA ratios depicted in Figure 5d. As expected, the biggest change (117 ± 2% relative to Wt) is observed for the quadruple mutant, which has more hydrophilic residues in the inner loop than the other simulated PAM systems. The double mutant is most similar to Wt in both the hydrophilic/hydrophobic SASA ratio (2 ± 1% ratio difference) and the preferred MIO−Tyr80 COM distance during resting state simulations (Figure 5c). Thus, the inner loop of PAM L97G/C89T seems less flexible than that of the other PAM variants and this may explain its lower activity compared to the PAM L97G mutant (cf. Figures 2 and 4).

To explore other possible features affecting inner loop dynamics, a secondary structure analysis over the last 10 ns of the simulations of the resting state subunits was carried out using the DSSP database. This time frame was chosen in order to allow relaxation since the Wt structure was used to construct the mutant systems. Figure 6a shows the percentage of residues relative to Wt predicted to be in coil, α-helix, turn, β-bridge, 3_10-helix, bend, and β-ladder conformations within the inner loop region (residues 75−97) of the selected mutants. Both PAM L97G and L97G/C89T showed a significant increase in 3_10-helix and β-ladder %, while the single mutant exhibited the highest coil % among the PAM variants with improved activities. This suggests that a balance between backbone flexibility (i.e., coil) and secondary structure (i.e., 3_10-helix and β-ladder) within the inner loop may be a partial determinant for the induced lyase activity.

Subsequent analyses of secondary structure profiles (Figure 6b−e) of each residue within the inner loop show notable variations among the four PAM variants. These include a transient α-helix terminus at residue 97 and a 3_10-helix (residues 87−89) that is reinforced by two flanking β-ladders (residues 84−86 and 89−93). Based on Figure 7, which shows an overlay of inner loops from various PAM crystal structures and models, it seems that the 3_10-helix serves as a lid to promote substrate stabilization. The 3_10-helix is also present in the Anabaena variabilis PAL (AvPAL) double mutant crystal structure (PDB: 3CZO) that harbors a closed inner loop. This further supports our proposed role of 3_10-helix and suggests its necessity for a productive closed inner loop. While the 3_10-helix and β-ladder percentages are highest for the L97G and L97G/C89T mutants (Figure 6a), it is severely diminished in the...
quadruple mutant. Thus, the prevalence of these inner loop features seem to correlate with activity effects.

Lastly, an \( \alpha \)-helix shortening near position 97 is noticeable in the model structures for the single and quadruple mutants (Figure 7b,d). Moreover, both \( \alpha \)-helices flanking the inner loop are shortened in the single and double mutant crystal structures. A similar \( \alpha \)-helix shortening was also observed in crystal structures of the AvPAL reported by Wang et al., who postulated that a conformational switch in both \( \alpha \)-helices flanking the inner loop may regulate active site accessibility and effectively reaction kinetics.\(^1\) In light of these observations, we propose that residue 97 in PAM acts as a hinge in the inner loop that influences overall loop mobility by modulating the length of the adjoining \( \alpha \)-helix. Overall, the \( 3_{10} \)-helix and \( \beta \)-ladder features of the inner loop could influence substrate stabilization while a flanking \( \alpha \)-helix modulates the conformation of the inner loop. Together, these features possibly influence lyase vs mutase activity preferences.

**Conclusions.** This work provides novel and direct evidence that the active site inner loop is a partial structural determinant distinguishing PAM vs PAL activities. We propose that hydrophobic interactions may stabilize the closed conformation of the PAM inner loop and that reducing these interactions through M-L mutations renders the inner loop more flexible and inherits lyase features. After performing relevant alterations (A77T, I79S, C89T, L97G), a lyase phenotype was triggered in
**METHODS**

**Chemicals.** (R)-β-phenylalanine was purchased from both Peptech Corp. Both (S)-α-phenylalanine and trans-cinnamic acid were purchased from Sigma-Aldrich.

**Construction of PAM Loop Mutants.** The PAM loop mutants were constructed through site-directed mutagenesis of the pBAD-Wt PAM plasmid according to the QuikChange protocol (Stratagene). The PfuUltra Hotstart PCR Master Mix (Stratagene) was used for the PCR reaction and all primers used were ordered from Eurofins MWG.

**Protein Expression and Purification.** Wt PAM and mutants were expressed and purified according to Wu et al., with exceptions outlined in Supporting Information.

**X-ray Crystallography.** For crystallization, procedures were performed as described in Wu et al. Refinement statistics are provided in Supporting Information Table S5.

**Activity Measurements.** The deamination and amination reactions rates were determined according to Bartsch et al. The initial rates were fit to the substrate concentrations with the Michaelis–Menten equation. The kcat value was calculated using the molecular mass of one monomer (78 kDa).

**HPLC Analysis of Lyase/Mutase Activity Ratios and Enantioselectivities.** For the mutase vs lyase activity and enantioselectivity measurements, reverse-phase HPLC was applied according to prior reports with modifications outlined in Supporting Information.

**Thermostability Tests.** The melting temperature was measured by the fluorescence-based thermal stability method as described by Ericsson et al. The measurements were performed as described by van Leeuwen et al. in storage buffer (20 mM Tris-HCl, 50 mM NaCl, pH 8.5, 25% glycerol). The thermal unfolding profiles are found in the Supporting Information.

**MD Simulations.** Wt TaPAM was used as the starting model for all simulated PAM systems (PDB: 2YII10). Parameters were taken from the standard GROMOS96 53A6 force field. All simulations were conducted using the GROMACS package, version 4.5.5. For a more detailed protocol of the MD simulations, PMF calculations, and subsequent analyses, please refer to the Supporting Information.

**ASSOCIATED CONTENT**

**Supporting Information**

Supporting methods, figures, and tables in addition to supplementary text for SASA calculations and secondary structure analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

**Accession Codes**

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS**

AL, ammonia lyase; AM, aminomutase; PAM, phenylalanine aminomutase; PAL, phenylalanine ammonia lyase; MIO, 4-methylideneimidazole-5-one; M-L, mutase → lyase; t-CA, trans-cinnamic acid; DMF, dimethylformamide; MD, molecular
dynamics; COM, center of mass; SASA, solvent accessible surface area

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