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

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SUPPORTING INFORMATION

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Figure S1. Sequence and structural comparisons of regions that include inter-loop salt bridge contacts in TcPAM.¹

a) Amino acid sequence alignment of MIO enzymes homologous to TcPAM and PcPAL. The targeted M-L mutations within the inner loop are noted with boxed numbers that correspond with TcPAM residue numbers. The residue positions (Arg92, Glu542) involved in inter-loop salt-bridge contacts in TcPAM are indicated with 'sb.'

b) Superposition of 3D crystal structures of TcPAM (green) and PcPAL (gray) that illustrates orientation or side-chain differences at residue positions involved in an inter-loop salt bridge in TcPAM.

Figure S2. Activity comparisons between Wt PAM and position 97 mutants. **Primary y-axis:** Specific activity comparisons between Wt PAM and single loop mutants to probe position 97. **Secondary y-axis:** Lyase/mutase (L/M) activity ratio comparisons with substrate, (R)-β-phenylalanine.

Figure S3. Thermal unfolding profiles of Wt PAM and the relevant loop mutants assayed in the temperature-activity experiments (Figure 4). Each profile represents a mean $\pm$ SEM ($n = 6$) representing triplicate measurements at two different enzyme concentrations.

a

b

Figure S4. Umbrella sampling simulations. **a)** Full-range PMF profiles for the umbrella-sampling simulations of the PAM systems in this study. Up to 2 nm of the MIO-Tyr80 distance was deemed as the loop opening process (**inset**), whereas beyond 2 nm was considered a distance where globally-induced unfolding initiated from the pulled inner loop. The full range of pulling/umbrella-sampling simulations covered up to ~5.5 nm. **b)** Histograms of the configurations within the 47 umbrella sampling windows utilized in this study. Supplementary to **Figure 5b**.

Figure S5. Changes in solvent accessible surface area (SASA) during resting state simulations of the inner loop for each PAM system. All values are given as a percent change relative to Wt PAM, averaged over time. Supplementary to Figure S5d.

Table S1. Summary of hydrophobic interactions for selected inner loop residues^a

Inner loop residue	Atom	Residue	Atom	Distance [Å]
Leu97 ^b	C δ_1	Ala73	C β	3.73
		Glu74	C γ	4.23
		Ala223	C β	4.54
	C δ_2	Ala73	C β	3.88
		Val82	C γ_1	5.13
Ile79 ^b	C δ_1	Ala77 ^b	C β	3.94
		Val82	C β	4.09
	C β		C γ_2	

^a based on crystal structure of TcPAM (PDB: 2YII²)

^b targeted mutation sites

Table S2. Specific activity comparisons of Wt TcPAM and loop mutants with substrates. Supplementary to Figure 2.

PAM variant	substrates								
	α -phenylalanine			β -phenylalanine			<i>trans</i> -cinnamic acid		
	avg. S.A. ^a	$\pm$	s.e.m. ^b	avg. S.A.	$\pm$	s.e.m.	avg. S.A.	$\pm$	s.e.m.
Wt	9.0	$\pm$	0.2	14	$\pm$	1	16	$\pm$	0.3
	avg. S.A. _{rel} ^c	$\pm$	s.e.m.	avg. S.A. _{rel}	$\pm$	s.e.m.	avg. S.A. _{rel}	$\pm$	s.e.m.
Wt	1.00	$\pm$	0.02	1.00	$\pm$	0.08	1.00	$\pm$	0.02
L97G	13	$\pm$	3	17	$\pm$	4	1.6	$\pm$	0.04
A77T	1.8	$\pm$	0.2	2.3	$\pm$	0.2	1.1	$\pm$	0.03
I79S*	0.6	$\pm$	0.4	0.9	$\pm$	0.1	0.0	$\pm$	0
C89T	1.3	$\pm$	0.2	2.8	$\pm$	0.6	0.597	$\pm$	0.007
L97G/A77T	7	$\pm$	2	14	$\pm$	1	0.53	$\pm$	0.01
L97G/I79S*	0.13	$\pm$	0.03	0.24	$\pm$	0.05	0.0	$\pm$	0
L97G/C89T	3.5	$\pm$	0.4	8.0	$\pm$	0.8	1.34	$\pm$	0.05
L97G/A77T/C89T	2.2	$\pm$	0.2	5.4	$\pm$	0.5	1.05	$\pm$	0.02
L97G/A77T/I79S/C89T*	0.2	$\pm$	0.1	0.09	$\pm$	0.04	0.0	$\pm$	0.0
	$\alpha:\beta$ ^d								
	L/M ^e								
	L/M _{rel} ^f								
Wt	1.05			1.05			1.05		
L97G	1.24			1.7			1.6		
A77T	1.04			2.3			1.1		
I79S*	n.d.			0.9			0.0		
C89T	1.27			2.8			0.597		
L97G/A77T	1.14			14			0.53		
L97G/I79S*	n.d.			0.24			0.0		
L97G/C89T	1.55			8.0			1.34		
L97G/A77T/C89T	1.52			5.4			1.05		
L97G/A77T/I79S/C89T*	n.d.			0.09			0.0		

^a avg. S.A. = average specific activity

^b s.e.m. = standard error of the mean

^c average of change in S.A. relative to Wt, $n = 3$

^d regioselectivity ratio

^e lyase/mutase activity ratio

^f L/M relative to Wt

Table S3. Cosolvent effects on specific activity. Supplementary to **Figure 3**.

PAM variant	S.A.	avg.	±	s.e.m. ^c
	[mU/mg] ^a	S.A. _{rel} ^b		
Wt	16.5	3.4	±	0.1
L97G	163.5	1.31	±	0.08
A77T	29.0	3.44	±	0.02
C89T	59.4	2.30	±	0.02
L97G/A77T	163.0	-1.15	±	0.04
L97G/C89T	94.0	-1.59	±	0.06
L97G/A77T/C89T	63.8	-1.95	±	0.04

^a S.A. = specific activity with 5 mM (*R*)-β-Phe

^b average relative change in S.A. due to 30 % DMF, fold-increase/-decrease

^c s.e.m. = standard error of the mean

Table S4. Temperature effects on lyase vs. mutase activity ratios. Supplementary to **Figure 4a**.

PAM variant	Temp. [°C]	L/M, (M/L) activity ratios		
		avg. ratio ^a	±	s.e.m. ^b
Wt	15	(100)	±	30
	30	(4.6)	±	0.2
	40	(2.55)	±	0.01
	50	(2.00)	±	0.01
	60	(1.26)	±	0.02
	70	1.71	±	0.02
L97G	15	(2.18)	±	0.04
	30	(1.412)	±	0.006
	40	1.155	±	0.006
	50	2.021	±	0.007
	60	4.813	±	0.004
	70	15.43	±	0
L97G/C89T	15	7	±	2
	30	8.1	±	0.6
	40	13	±	0
	50	24.2	±	0.2
	60	48	±	5
	70 ^c	--	±	--

^a average of activity ratios for lyase (L) and mutase (M) activities
(positive ratios = L/M ; negative ratios = M/L)

^b s.e.m. = standard error of the mean, *n* = 2

^c 100% lyase activity, no detectable mutase activity

Note: ratios comprise of initial rates of product formation (lyase product, *t*-CA; mutase product, α-Phe) with (*R*)-β-Phe (5 mM) as substrate

Table S5. X-ray crystallography data collection and refinement statistics.

	PAM L97G	PAM L97G/C89T
Beam line	ID23-1	ID23-1
Data collection		
Resolution (Å) ^a	47.45–2.20 (2.32–2.20)	47.38–1.95 (2.06–1.95)
Space group	C2	C2
Cell dimensions		
a, b, c (Å)	181.5, 76.49, 120.42	181.5, 76.2, 120.5
β (°)	120.0	120.4
Monomers per asymmetric unit	2	2
R _{merge} ^b	0.072 (0.40)	0.065 (0.44)
Wavelength (Å)	0.9999	0.9999
I/σ (I)	12.0 (3.0)	13.1 (2.6)
Completeness (%)	99.8 (99.9)	99.8 (99.9)
No. unique reflections	72574 (10588)	103115 (15002)
Multiplicity	3.4 (3.5)	3.4 (3.5)
Refinement		
Resolution (Å)	2.20	1.95
R _{work} ^c	0.21	0.19
R _{free} ^d	0.25	0.22
R.m.s. deviations		
Bond lengths (Å)	0.01	0.008
Bond angles (°)	1.36	1.21
Ramachandran (Molprobit)		
Favored (%)	96.8	98.1
Outlier (%)	0.1	0.0
PDB entry	4V2R	4V2Q

^a Values in parentheses are for the highest resolution shell.^b $R_{\text{merge}} = \sum_i \sum_j |I(h_i) - \langle I(h) \rangle| / \sum_i \sum_j \langle I(h) \rangle$, where I is the observed intensity and $\langle I \rangle$ is the average intensity of multiple observations of symmetry-related reflections.^c $R_{\text{work}} = \sum_h |F_{\text{obs}} - F_{\text{calc}}| / \sum_h |F_{\text{obs}}|$, where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes, respectively.^d R_{free} is calculated as R_{work} using 5% of all reflections randomly chosen and excluded from structure factor calculation and refinement.**Table S6.** Summary of the MIO–Tyr80 COM distances observed at the energy minima of the PMF profiles and at the resting state of simulated PAM variants. Supplementary to Figure 5c.

PAM system	PMF		Resting	
	MIO–Tyr80 distance [nm]	s.d. ^a	MIO–Tyr80 distance [nm]	s.d. ^b
Wt	1.27	0.03	1.27	0.02
L97G	1.31	0.03	1.32	0.02
L97G/C89T	1.39	0.04	1.29	0.03
L97G/A77T/I79S/C89T	1.41	0.03	1.36	0.03

^a standard deviation from 1 simulation of 20 ns^b standard deviation from 47 independent simulations (20 ns) at different random initial velocities

Supplementary Methods and Text

1. Protein expression and purification. Wt PAM and mutants were expressed and purified according to Wu *et al.*, with the following exceptions.³ The expression culture was induced with arabinose at 0.02% (w/v) and then incubated for 48h at 17°C. Gravity-flow chromatography using a 25 mL column (Bio-Rad) and 1 mL of Cobalt resin (Talon) was used for purification. The column was pre-equilibrated with the re-suspension buffer described by Wu *et al.*. The lysate was incubated (1 h, 4°C) with the binding resin using an orbital shaker. The bound resin was washed with re-suspension buffer (24 mL) and then eluted with elution buffer (4 mL). The elution fraction was concentrated down to ~ 500 μ L with a centrifugal filter unit (Amicon, catalog # UFC903024) and exchanged with storage buffer through three rounds of dilution (14 mL storage buffer) and concentration (down to ~ 500 μ L) using the same centrifugal filter unit (4,000 rpm). Total protein content was measured using the Uptima BC assay protein quantification kit (Interchim) and yielded 5–10 mg mL⁻¹ of purified enzyme.

2. HPLC analysis of lyase/mutase activity ratios. For the mutase vs. lyase activity measurements (Figure 2) at a single temperature (30°C, 300 rpm using 1.5 mL tube shaker (Eppendorf Thermomixer comfort)) using reverse-phase HPLC, the deamination conversions of (*R*)- β -phenylalanine were measured by detecting the elution analytes at λ_{210} . The reaction mixture (2 mL) contained Tris-HCl buffer (20 mM, pH 8.5, 50 mM NaCl), purified PAM enzyme variant (100 μ g mL⁻¹ final conc.), and 5 mM substrate. For the amination reaction using *trans*-cinnamic acid (1mM) as substrate, ammonia solution (6 M, pH 10) was added to the substrate, followed by addition of purified PAM enzyme variant (500 μ g mL⁻¹ final conc) to give a final reaction volume of 2 mL. At each time point (5, 10, 15, 20, 25, 30, 40, 60, 120, and 1,900 min) for both amination and deamination reactions, 100 μ L samples were taken and heat inactivated for 5 min at 96°C to denature the enzyme. After centrifugation (10 min, 16,000 g, 4°C), the supernatant was analyzed using an Alltech C18 3 μ column (Ridderkerk; 3 μ m, 100 mm x 4.6 mm) to separate α -phenylalanine, β -phenylalanine, and cinnamic acid. K-phosphate buffer (50 mM, pH 3, eluent A) was used for stationary phase and HPLC-grade acetonitrile (eluent B) was used as the mobile phase. The flow rate was 1 mL min⁻¹. The 18 min elution gradient was programmed as follows: start at 97% A for 7 min, 2 min to 94% A, 2.5 min to 30% A, 30% A for 1 min, 0.5 min to 97% A, 5 min at 97% A. The concentrations of α -phenylalanine, β -phenylalanine, and cinnamic acid were calculated from the peak areas based on corresponding standard curves.

For the mutase vs. lyase activity measurements (Figure 4) at multiple temperatures (15, 30, 40, 50, 60, and 70°C) using the above reverse-phase HPLC system, the deamination conversion of (*R*)- β -phenylalanine to (*S*)- α -phenylalanine (mutase product) and *trans*-cinnamic acid (lyase product) was measured as described above, with a few modifications: (1) reaction volume, 1.4 mL; (2) sample time points (min.): 5, 10, 15, 20, 30, 60, 90, 120, 240, 480, and 1440; and (3) 500 rpm shaking.

3. Chiral HPLC analysis of amination products. The enantioselectivity of the relevant PAM loop mutants (footnote b, Table 1) was analyzed using a reverse phase HPLC chiral column (Daicel Chemical Ind. LTD.: Crownpak CR(+), 4mm x 150 mm, 5 μ m particle size) at -6°C with aqueous HClO₄, 8% MeOH, pH 2.0 as the mobile phase at a flow rate of 0.2 mL min⁻¹ isocratically for 140 min per run. A UV detector at 210 nm was used to measure the elution profiles of the analytes. The average retention times were: 41.1 min (*R*)- α -phenylalanine, 78.6 min (*S*)- α -phenylalanine, 97.5 min (*S*)- β -phenylalanine, and 119.3 min (*R*)- β -phenylalanine. The concentrations were calculated from the peak areas based on corresponding standard curves. The amination reactions (200 μ L total volume, 5 mM *t*-CA as substrate) for the relevant PAM loop mutants were performed at 30°C (300 rpm shaking using an Eppendorf ThermomixerC) and a 20 μ L sample was used for analysis. The remaining sample work-up was followed according to Wu *et al.*³

4. In silico methods: pulling and umbrella-sampling simulations. Subsequent to the experimental work, MD simulations using an umbrella-sampling approach were performed in order to estimate the free energy differences for disengaging the respective inner loops of various PAM loop mutants (Figure 5, Supplementary Figure S4), as well as quantifying the buried hydrophobic surface area (Figure S5), and secondary structural features within this loop region (Figure 6). Wt TcPAM was used as the starting model for all simulated PAM systems since its structure is known (PDB-ID: 2YH²), its gene template was used for mutagenesis, and complete structural information for all loops are available. The mutants considered in this *in silico* study are denoted as L97G, L97G/C89T and L97G/A77T/I79S/C89T. The Wt structure was used as a control. The N- and C-termini of each protein monomer was modeled in its charged forms. All other titratable amino acids were assigned their canonical state at physiological pH.

Parameters from the standard GROMOS96 53A6 force field were applied to all components of the simulated systems. The necessary parameters for the MIO cofactor were generated by the Automatic Topology Builder (ATB) website for the above-mentioned force field.⁴ Improper dihedrals were manually adjusted to assure ring planarity of the MIO cofactor. Short-range non-bonded interactions were cut off at 1.4 nm, with long-range electrostatics calculated using the particle mesh Ewald (PME) algorithm.⁵ Dispersion correction was applied to energy and pressure terms to account for truncation of van der Waals terms. Periodic boundary conditions were applied in all directions.

In order to generate equilibrated starting structures for the pulling simulations, each system (Wt and mutants) was placed in a dodecahedral box (with dimensions sufficient to satisfy the minimum image convention and provide space for the pulling of the loop) of simple point charge (SPC) water, to which 100 mM NaCl (including neutralizing counter-ions) was added to simulate physiological salinity.⁶ Following steepest descents minimization (50000 steps, or maximum force lower than $100 \text{ kJ mol}^{-1} \text{ nm}^{-2}$), each of the systems were equilibrated in two steps, with position restraints applied to the protein heavy atoms throughout. The first phase involved simulating for 100 ps under a constant volume (NVT) ensemble. The complete system was coupled to temperature coupling baths, and temperature was maintained at 298 K using the V-rescale coupling method.⁷ Following NVT equilibration, 500 ps of constant pressure (NPT) equilibration were performed, also using weak coupling to maintain pressure isotropically at 1.0 bar.⁸

Pre-pulling MD simulations were conducted for 10 ns in the presence of position restraints for all α -carbon atoms to assure side-chain equilibration but not backbone movements, especially of the inner loop regions. For this pre-pulling simulation, the Nosé-Hoover thermostat was used to maintain temperature, and the Parrinello-Rahman barostat was used to isotropically regulate pressure.⁹⁻¹¹ This combination of thermostat and barostat ensures that a true NPT ensemble is sampled. Structures from the end of each of these trajectories (Wt, L97G, L97G/C89T and L97G/A77T/I79S/C89T) were used as starting configurations for pulling simulations. Only the loops of chain A were submitted to the pulling protocol described here, while chains B–D were allowed to fully relax during the execution of the pulling. Following equilibration, restraints were removed from all protein chains except for the atoms forming the MIO cofactor of chain A, whose center of mass was used as reference for the pulling simulations. For each of the systems studied here, the center of mass of the catalytic residue Tyr 80 of chain A was pulled away from the MIO cofactor allowing full mobility in all directions over 500 ps, using a spring constant of $1000 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ and a pull rate of 0.01 nm ps^{-1} . A final center of mass (COM) distance between peptides A and B of approximately 5.5 nm was achieved.

From these trajectories, snapshots were taken to generate the starting configurations for the umbrella sampling windows.¹² A spacing of 0.1 nm COM separation was used, resulting in 47 windows. Each window was submitted to a 20 ns MD simulation in order to produce the production run for umbrella sampling analysis. All simulations were conducted using the GROMACS package, version 4.5.5.¹³ The analysis of the simulations was performed using the analysis tools provided in the GROMACS package. The DSSP database was used for secondary structure analyses and the `g_sas` utility in the GROMACS package was used for SASA calculations.^{14,15}

All molecular graphical presentations were created by VMD or UCSF Chimera packages.^{16,17} The potential of mean force (PMF) analysis of umbrella sampling runs was performed with the weighted histogram analysis method (WHAM), while its error analysis was conducted using a bootstrap method described previously.^{18,19} Statistical analyses were carried out utilizing ANOVA procedure together with Bonferroni's multiple-comparison test using GraphPad Prism version 6.0 for Mac, GraphPad Software (www.graphpad.com).

4a. Solvent accessible surface area (SASA) calculations. The total SASA % decrease ($-4 \pm 1\%$) for the quadruple mutant comprises drastic changes in both the hydrophilic ($4 \pm 2\%$ increase) and hydrophobic ($12 \pm 1\%$ decrease) SASA (**Figure S5**). Compared to the quadruple mutant, PAM L97G displayed a slightly less decrease ($-3 \pm 1\%$) in total SASA % that is mostly influenced by a decrease ($-6 \pm 1\%$) in hydrophobic SASA. The total SASA % change for the double loop mutant seems to be mostly dictated by the decreased hydrophobic SASA ($-3 \pm 2\%$).

4b. Secondary structure analyses of the PAM inner loop. **Figures 6 b–e** show that the percentage of residues in coil, α -helix, turn, β -bridge, 3_{10} -helix, bend, and β -ladder conformations per residue are present for the same inner loop region analyzed in panel A. From panel B, it is apparent that a Gly mutation at position 97 increased the amount of coil conformation at this specific point. Also, it is important to note that the Wt, L97G, and L97G/C89T systems contain 3_{10} -helix clusters (residues 87–89) that seem to be in equilibrium with turn and bend conformations. On the contrary, the quadruple mutant showed a significant loss of 3_{10} -helix conformation in this region. The cluster seems to be flanked by two extended portions of β -ladder-rich conformations (residues 84–86 and 89–93). PAM L97G and L97G/C89T show a small increase in β -ladder conformation in these portions, which possibly stabilizes the formation of the 3_{10} -helix cluster.

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