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Remodeling of Conformational Dynamics Enhances Catalytic Activities of M1 Zinc-metallopeptidases from Lanthipeptide Biosynthesis

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ABSTRACT
Lanthipeptides are an important group of natural products with diverse biological functions, and their biosynthesis requires the removal of N-terminal leader peptides (LPs) by designated proteases. LanP<sub>M1</sub> enzymes, a subgroup of M1 zinc-metallopeptidases, are recently identified as bifunctional proteases with both endo- and aminopeptidase activities to remove LPs of class III and class IV lanthipeptides. Herein, we report the biochemical and structural characterization of EryP as the LanP<sub>M1</sub> enzyme from the biosynthesis of class III lanthipeptide erythreapeptin. We determined X-ray crystal structures of EryP in three conformational states, the open, intermediate and closed states and identified a unique inter-domain Ca binding site as a regulatory element to modulate its domain dynamics and proteolytic activity. Inspired by the regulatory Ca binding, we develop a strategy to engineer LanP<sub>M1</sub> enzymes for enhanced catalytic activities by strengthening inter-domain associations and driving the conformational equilibrium toward their closed forms.

INTRODUCTION
M1 family zinc-metallopeptidases are widely distributed in many species and regulate a diverse range of biological processes. For instance, <i>Escherichia coli</i> (E. coli) aminopeptidase N (ePepN) is involved in ATP-dependent downstream processing during cytosolic protein degradation. 2, 3 <i>Thermoplasma acidophilum</i> tricorn interacting factor F3 (F3) acts downstream of the proteasome to produce di- and tripeptides. 4 Human endosomal insulin-regulated aminopeptidase (IRAP) is a key regulator in endocytic trafficking and signaling, 5 while the endoplasmic reticulum aminopeptidases (ERAP1 and ERAP2) are responsible for antigen-trimming. 6, 7 Recently, a new biological function of M1 metallopeptidases is revealed in the biosynthesis of bacteria-derived natural product lanthipeptides, a subfamily of ribosomally synthesized and...
posttranslationally modified peptides (RiPPs).

As the most studied and rapidly growing family of RiPPs, lanthipeptides are featured with diverse structures and biological activities, including antimicrobial, anti-fungal, anti-virus and antiallodynic properties. The precursor peptide of a lanthipeptide is usually composed of an N-terminal leader peptide and a C-terminal core peptide, which undergoes enzymatic cyclization during biosynthesis. The N-terminal leader peptide plays important roles during the biogenesis of lanthipeptides as recognition elements for modification enzymes, as secretion signals during the exportation or for self-immunity. The removal of leader peptides is often the last but essential step to produce the mature lanthipeptides. If additional modification is required on the N-terminus of lanthipeptides, such as in the biosynthesis of lipolanthines and goadvionins, precise and efficient removal of the N-terminal leader peptide becomes even more essential.

A subgroup of M1 zinc-metallopeptidases, named LanP\textsubscript{M1}, function as key proteases to remove the N-terminal leader peptides (LPs) of class III and class IV lanthipeptides during their maturation. AplP from the biosynthesis of class III lanthipeptide NAI-112 represents the first example of LanP\textsubscript{M1} proteases and removes the leader peptide of precursor peptide AplA after cyclization and glycosylation at its core peptide. As M1 metallopeptidases usually prefer linear peptides that are shorter than 20 amino acids, LanP\textsubscript{M1} enzymes therefore represent a rare subgroup of M1 metallopeptidases that cleave large cyclic peptide substrates (> 50 amino acid long). Although the endopeptidase and exopeptidase activities of ePepN remain debatable, it is generally observed that M1 metallopeptidases only act on the N-terminus of polypeptides and exhibit no endopeptidase activity. Intriguingly, LanP\textsubscript{M1} proteases possess dual enzymatic activities and remove the LP of precursor peptides through a two-stage process: first, LanP\textsubscript{M1} cleaves a major N-terminal portion of the LP as an endopeptidase; second, LanP\textsubscript{M1} trims off the remaining overhang residues as an aminopeptidase to produce the mature natural products. The mechanism that LanP\textsubscript{M1} enzymes switch between these two functions remains unknown.
Fig. 1 | LanP<sub>M1</sub> enzymes in lanthipeptide biosynthesis. a, Bifunctional LanP<sub>M1</sub> enzymes remove LP of lanthipeptides through a two-step process. b, EryP partially removes the LP of cyclized EryA peptide (EryA<sub>cyc</sub>) by generating erythreapeptin congeners. The coding gene of EryP is located outside the erythreapeptin BGC.

LanP<sub>M1</sub> enzymes are usually highly active as endopeptidases, but significantly less efficient as aminopeptidases during the processing of LP overhang residues. The inefficient aminopeptidase activities of LanP<sub>M1</sub> enzymes often result in incomplete LP removal both in vitro and in vivo (Fig. 1a), which impedes the heterologous production and bioengineering of corresponding lanthipeptides. Thus, to understand the enzymology underlining the dual functionality of LanP<sub>M1</sub> proteases and to engineer LanP<sub>M1</sub> enzymes with improved aminopeptidase activities for lanthipeptide production are highly appealing. Herein, we report the biochemical and structural characterization of EryP, which is the LanP<sub>M1</sub> enzyme from the biosynthesis of class III lanthipeptide erythreapeptin. We determined crystal structures of EryP in three conformational states, the open, intermediate and closed states, by X-ray diffraction and identified a unique inter-domain Ca binding site, which functions as a regulatory element by modulating domain dynamics and the activity of the enzyme. Inspired by the effect of the regulatory Ca binding, we have developed a practical strategy to acquire engineered LanP<sub>M1</sub> mutants with enhanced aminopeptidase activities by strengthening inter-domain associations of LanP<sub>M1</sub> enzymes and driving the conformational equilibrium toward their closed forms.

RESULTS

EryP is a bifunctional LanP<sub>M1</sub> protease for leader peptide removal in erythreapeptin biosynthesis. To obtain mechanistic understanding of LanP<sub>M1</sub> enzymes, we focused on EryP from the biosynthesis of class III lanthipeptide erythreapeptin, which is encoded in the genome of its producing strain Saccharopolyspora erythraea NRRL 2338 but
located far outside the erythreapeptin BGC (Fig. 1b, Supplementary Fig. 1). EryP was expressed heterologously as a His<sub>6</sub>-tagged fusion protein from *E. coli* BL21 (DE3) and purified by immobilized metal affinity chromatography as a monomer in solution (Supplementary Fig. 2). His<sub>6</sub>-EryP cleaved EryA leader peptide (EryA<sub>LP</sub>) by generating fragments EryA<sub>LP(-15)</sub> and EryA<sub>LP(-21)</sub> as major products, indicating its function as an endopeptidase (Fig. 2a). In addition, His<sub>6</sub>-EryP exhibited aminopeptidase activity by hydrolyzing amino acid para-nitroanilide (pNA) derivatives, including Ala-pNA, Pro-pNA and Leu-pNA, therefore confirming the dual proteolytic activities of EryP for EryA<sub>LP</sub> processing (Fig. 2b, Supplementary Fig. 3).

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**Fig. 2 | Bifunctional protease EryP processes both EryA<sub>LP</sub> and AplA<sub>cyc</sub> peptides.**

*a*, MS spectrum of EryA<sub>LP</sub> peptide cleaved by EryP at multiple sites as an endopeptidase. EryA<sub>LP</sub>: $M_{\text{calc.}}=2876.18$ Da, $M_{\text{obs.}}=2876.60$ Da; EryA<sub>LP(-15)</sub>: $M_{\text{calc.}}=1544.62$ Da, $M_{\text{obs.}}=1544.76$ Da; EryA<sub>LP(-21)</sub>: $M_{\text{calc.}}=2271.98$ Da, $M_{\text{obs.}}=2272.13$ Da. 

*b*, EryP hydrolyzes amino acid-pNA derivatives as an aminopeptidase. Error bars indicate standard deviation of three independent replicates. 

*c*, Schematic illustration of EryP removing the LP of AplA<sub>cyc</sub> peptide through a two-stage process. 

d, EryP completely removed the leader peptide of AplA<sub>cyc</sub> by generating AplA<sub>cyc-CP</sub> as the final product. AplA<sub>cyc-CP</sub>: $M_{\text{calc.}}^*=2242.95$ Da, $M_{\text{obs.}}^*=2243.42$ Da; AplA<sub>cyc-CP(-3)</sub>: $M_{\text{calc.}}^*=2584.12$ Da, $M_{\text{obs.}}^*=2584.76$ Da; AplA<sub>cyc-CP(-6)</sub>: $M_{\text{calc.}}^*=2929.24$ Da, $M_{\text{obs.}}^*=2928.94$ Da. Labels * and # represent sodium and potassium adducts of corresponding peptides, respectively.
Next, we examined the proteolytic activity of EryP toward cyclized lanthipeptide substrates. Since cyclized EryA peptide (EryAcyc, Fig. 1b), the native substrate of EryP, was not accessible, we employed cyclized AplA, the precursor peptide of NAI-112, as an alternative substrate. Cyclized AplA (AplAcyc) shares structural similarity with EryAcyc in the leader peptide and the core peptide, therefore serving as a good substrate mimic (Fig. 2c, Supplementary Fig. 4). As expected, EryP cleaved AplAcyc at multiple sites in the segment of AplALP peptide after 90 min incubation by generating AplAcyc-CP(-6) as the detectable core peptide product (Supplementary Fig. 5). Prolonged incubation led to stepwise removal of overhang residues in AplAcyc-CP(-6) and eventually yielded AplPcyc-CP as the final product (Fig. 2d). These results indicated that EryP possesses relaxed substrate specificity and is capable of completely removing LPs of class III lanthipeptides other than its native substrate. It is noteworthy that as an endopeptidase, EryP exhibited distinct preference of cleavage sites in EryALP and AplALP: it cleaved EryALP at the N-terminus of ELQ and APN motifs but preferred the C-terminus of ELQ and S-A-(S/T) motifs towards AplALP, indicating that the site-selectivity of EryP for endoproteolytic cleavage might be substrate-dependent (Fig. 2a and Supplementary Fig. 5).

| Peptides | K_D/μM |
|----------|--------|
| EryP | EryP_E802R | AplP |
| EryALP | 40 ± 6 | 32 ± 14 | N.D. |
| AplAcyc | 0.50 ± 0.11 | 0.57 ± 0.08 | 0.36 ± 0.18 |
| AplALP | 8.0 ± 2.7 | 4.0 ± 0.6 | 2.3±0.5 |
| AplAcyc-CP | 720±18 | N.D. | N.D. |

Table 1 | Binding affinities of EryP, EryP_{E802R} and AplP with peptide substrates. N.D. = not determined

To understand the substrate recognition mechanism of EryP, we performed microscale thermophoresis (MST) to determine the binding affinity between EryP and peptide substrates. Results showed that EryP binds to EryALP with a K_D of (40.8±5.8) μM, whereas its binding with EryALP(-15) is too weak to measure (Table 1). EryP binds to full length AplAcyc and AplALP with K_D values of (0.503±0.109) μM and (7.98±2.72) μM, respectively, indicating that the cyclized AplA core peptide contributes to binding with EryP. Indeed, EryP binds to AplAcyc-CP with a weak but measurable K_D of (721±18) μM. Similarly, AplP binds to full length AplAcyc with a K_D ~9-fold lower than that of AplALP (Table 1). Therefore, the substrate recognition of EryP and AplP primarily depends on their binding with leader peptides, whereas core peptides act as complementary binding elements.

Overall structure and active-site architecture of EryP. Three sets of EryP crystal structures were determined in distinct conformations, which are postulated as closed, intermediate and open states, with resolution limits from 1.80-2.66 Å (Fig. 3a). The structure of the closed conformation was solved by single isomorphous replacement with anomalous scattering from a Sederivative (SAD), and was used as a model to solve the other two structures by molecular replacement. Similar
to tricorn-interacting factor F3 (TIFF3) and ePepN of the M1 metalloprotease family, EryP folds into four distinct domains: domain-I (residues 1-201), domain-II (residues 202–456), domain-III (residues 457-552) and domain-IV (residues 553-860) (Fig. 3a). The barrel-like N-terminal domain-I is packed by a central 12-stranded β-sheet and two smaller antiparallel β-sheets. The following catalytic domain-II adopts a thermolysin-like fold characterized by an α/β lobe on the top of an α-helices lobe. A Zn ion binding motif, HEXXH(X)_{18}E, which is highly conserved among M1 metallopeptidases, is located at the deep groove between the two lobes (Supplementary Fig. 6). Domain-III presents a barrel-like structure formed by eight β-strands. The C-terminal domain-IV consists of 16 α-helices that are organized in an antiparallel manner by forming a bowl-shaped structure, which might regulate the accessibility of the catalytic pocket in domain-II.⁶

In the closed state of EryP, a zinc atom resides at the bottom of active-site cleft in catalytic domain and is coordinated by NE2 atoms of two histidine residues (H306 and H310) and OD atoms of E329 (Fig. 3b, Supplementary Fig. 6).²⁰ A catalytic water molecule lies immediately (2.0 Å) next to the Zn ion, resulting in a distorted trigonal-pyramidal coordination network (Fig. 3b). The presence of a catalytic water molecule in the active site prior to peptide substrate binding is consistent with previous studies of M1 metallopeptidases, such as thermolysin, and is considered to be essential for their enzymatic functions.²⁰ Residue E307 locates 3.3 Å from the water molecule and might participate in catalytic solvent activation as a general base. The highly conserved Y392 resides in the catalytic pocket and might participate in the proteolysis by stabilizing the tetrahedral intermediate formed by water addition to the substrate scissile amide bond.²¹ Both endopeptidase and aminopeptidase activities of EryP₃₀₇Q and EryP₃₉₂F were abolished, confirming the essential role of these residues for catalysis (Supplementary Fig. 7).

The interactions between short peptide substrates and the reaction center were visualized by docking a dipeptide Leu-Glu into the active site of EryP in the closed state (Supplementary Fig. 8). The highly conserved G₂₇₂ÂM₂₇₅N motif in EryP binds to the dipeptide substrate through multiple hydrogen bonds in the docking model. In particular, residue E275 directly binds to the amino group at the N-terminus of the dipeptide with additional binding from residues E132 and E329 (Supplementary Fig. 8). Furthermore, this docking model suggests that both S₁ and S₁’ pockets are spacious to accommodate amino acid side chains of various sizes in peptide substrates, allowing the enzyme to sequentially cleave various residues from the leader of EryA (Supplementary Fig. 8).
Fig. 3 | Overall structure and the metal ion binding sites of EryP. 

a, Three sets of crystal structures of EryP. The four domains of EryP are shown in forest green (domain-I), slate (catalytic domain-II), pink (domain-III), and orange (domain-IV). The catalytic Zn atom is shown as a yellow sphere, and the Ca atom is shown as a red sphere in the closed state. 

b, Superposition of the active site of EryP in closed (forest green), intermediate (slate) and open (orange) states. The Zn atom and the catalytic water molecule are shown in yellow and forest green, respectively, in the closed state. 

c, Superposition of closed, intermediate and open states of EryP in ribbon style. The interdomain angle $\theta$ is defined as the angle of the centers of mass of domain-I and domain-II, domain-III, and domain-IV. The torsion angle $\phi$ is measured between the center of mass of domain-I and domain-II, the residues 513 and 551 in domain-III, and the center of mass of domain-IV. The angles were measured in UCSF chimera. 

d, The inter-domain Ca ion binding site in the closed state of EryP. 

Comparative analysis of various conformations of EryP. Conformational flexibility is a common feature among four-domain M1 metallopeptidases, including ePepN and ERAP1. Among the three sets of EryP crystal structures, domain-I and domain-II are well aligned and share an interface of 36.0-44.3 Å$^2$ in area (RMSD from 0.155-0.313 Å) (Fig. 3c). Domain-III serves as a hinge that allows the domain-IV to move toward or away from the Zn-binding active site in domain-
II. Scissor motions of domain-IV (0.76 to 1.72 degrees) around domain-III (0.61 to 11.70 degrees of rotation) were observed in the intermediate and open states (Fig. 3c). In comparison with the closed conformation, the areas of domain interface between domain-II with domain-III and domain-IV are reduced from 878.7 to 851.8-876.7 Å² and from 1334.8 to 1129.6-476.2Å², respectively, in intermediate and open states. The EryP structure in the open state has an approximate 6852 Å³ internal cavity volume between domain-II and domain-IV as calculated by CASTp 3.0, which is highly accessible to solvent. This cavity of EryP is significantly larger than known M1 metallopeptidases in their open conformations. For example, ERAP1 accepts long linear peptide substrates and has a cavity of 5080 Å³ between its domain-II and IV in its open conformation. The exceptionally large internal cavity of EryP may facilitate the recruitment of large cyclized peptide substrate during catalysis, as AplA cyc-CP alone has an estimated volume of ~ 2300 Å³. In the closed conformation, the volume of this cavity significantly shrinks to 1531 Å³, and the cavity is fully encompassed by the domain rims in the closed state. Despite the significant conformation alteration among three EryP structural sets, critical active-site residues did not display significant structural differences (Fig. 3b). Specifically, residues Y392 in the closed and open states of EryP are in almost identical position. This is in contrast with several four-domain M1 metallopeptidases, such as ERAP1, in which the catalytic tyrosine residue orients closer toward the Zn center to participate amide hydrolysis in its open conformation than that in its closed conformation.

Calcium ion enhances inter-domain interactions and the aminopeptidase activity of EryP. In the closed state of EryP, a calcium ion is located about 12.1 Å from the Zn ion through coordination by residues E112, E132 and D135 from domain-I, residues E384 from domain-II and E802 from domain-IV (Fig. 3d). Such a Ca ion is missing in the open and intermediate states, which is in accordance with the shift of residue E802 away from E112 and D135 (Supplementary Fig. 9). In addition, the side chain of E384 orients in an opposite direction in the open state in comparison with that in the closed state, and the loop of residues 384-390 could not be modeled in the intermediate conformation likely due to its high flexibility (Supplementary Fig. 9). Together, these data suggest that the Ca ion binding site might function as a regulatory element that mediates the domain dynamics, particularly relative positions of domain-IV to domain-I and II.

To understand the role of Ca ion in mediating the tri-domain interaction, we performed 200 ns classical molecular dynamics (MD) simulations based on the crystal structure of EryP in the closed state with or without Ca²⁺ ion bound. Two-dimensional descriptors were selected to describe the motion of domain-IV relative to domain-I and II: d₁ is the distance between the CA atoms of E802 and E112, and d₂ is the distance between the CA atoms of E802 and E384 (Supplementary Fig. 10). Considering that d₁ and d₂ in the closed state of EryP is 10.95 Å and 9.22 Å, respectively, snapshots are marked as closed conformations if d₁ ≤ 10.95 Å and d₂ ≤ 9.22 Å; otherwise, conformational transformation is suggested to occur from closed states to an intermediate or open state. Results indicate that with Ca²⁺ ion bound, EryP tends to adopt closed conformations with domain-IV in close distance with domain-I and II (Fig. 4a). When the Ca²⁺ ion was removed from
EryP, a significantly wider distribution of conformations was observed, a portion of which deviated from the closed conformation (Fig. 4a). When Leu-pNA, the favorite aminopeptidase substrate of EryP, was docked into the active site to mimic the substrate-bound EryP, the binding of Ca ion showed an even stronger effect on locking EryP in the closed conformations (Fig. 4b). Collectively, these data suggest that by bridging residues from domain-I, II and IV, the Ca ion shifts the conformational equilibrium of EryP toward the closed conformation.

Next, we evaluated the impact of Ca ions on the proteolytic activity of EryP. Elevation of Ca$^{2+}$ concentration from 0 to 1.0 mM in assay buffer increased catalytic efficiency of EryP toward amino acid-pNA derivatives by 3-5 folds (Fig. 4c, Supplementary Fig. 3). In addition, both endopeptidase and aminopeptidase activities of EryP toward peptide substrates EryA$_{LP}$ and AplA$_{cyc-CP(-4)}$, respectively, were significantly improved by the addition of CaCl$_2$ in reaction buffers (Fig. 4d and 4e), although the binding affinity was not altered (Supplementary Fig. 11). To confirm that the response of EryP activity to Ca ions is resulted from the interdomain Ca$^{2+}$ binding site, E384 was mutated into an Ala residue. EryP$_{E384A}$ mutant retains the same level of aminopeptidase activity toward Ala-pNA as EryP, however, was no longer responsive to the change of Ca$^{2+}$ concentration (Supplementary Fig. 12). Collectively, our results demonstrate that although not strictly required for activity, the interdomain Ca$^{2+}$ binding site provides a level adjusting mechanism to stabilize the closed conformation of EryP, which consequently enhances its aminopeptidase activity.
Fig. 4 | The Ca ion binding site regulates the conformational equilibrium and catalytic activity of EryP. a, Distances $d_1$ and $d_2$ in EryP and EryP$_{E802R}$ with or without Ca ions. b, Distances $d_1$ and $d_2$ in EryP-(Leu-pNA) and EryP$_{E802R}$-(Leu-pNA) complexes with or without Ca ions. Data were collected from 20000 snapshots during 200 ns MD simulations. c, Relative aminopeptidase activity of EryP in the presence or absence of Ca ions toward three amino acid-pNA derivatives. Error bars indicate standard deviation of three independent replicates. d, EryP displays enhanced aminopeptidase activity toward peptide substrate AplA$_{cyc-CP(-4)}$ in the presence of CaCl$_2$. AplA$_{cyc-CP(-3)}$: $M_{\text{calc.}}^*=2584.12$ Da, $M_{\text{obs.}}^*=2584.77$ Da; AplA$_{cyc-CP(-4)}$: $M_{\text{calc.}}^*=2699.15$ Da, $M_{\text{obs.}}^*=2700.05$ Da. * and # represents the sodium and potassium adducts of peptides in MS, respectively. e, EryP displays enhanced endopeptidase activity toward EryA$_{LP}$ in the presence of CaCl$_2$. EryA$_{LP}$: $M_{\text{calc.}}=2876.18$ Da, $M_{\text{obs.}}=2876.63$ Da; EryA$_{LP}(-15)$: $M_{\text{calc.}}=1544.62$ Da, $M_{\text{obs.}}=1544.27$ Da; EryA$_{LP}(-21)$: $M_{\text{calc.}}=2271.98$ Da, $M_{\text{obs.}}=2271.45$ Da.

Rational engineering of inter-domain interactions enhances aminopeptidase activities of EryP. The removal of LP overhang residues is the rate-limiting and often incomplete step during the biosynthesis of class III lanthipeptides, and
therefore LanP1 proteases with enhanced aminopeptidase activities are highly desired.\textsuperscript{12, 15, 16} The correlation between structural dynamics and enzymatic activities of EryP provides an opportunity to create an engineered EryP mutant with improved aminopeptidase activity by stabilizing the tri-domain association. The Ca binding residues in EryP provide a promising engineering site to incorporate mutations for enhanced inter-domain network. Based on the \textit{closed} structure of EryP, we replaced the Ca\textsuperscript{2+} coordinating residue E802 from domain-IV with an arginine (R) residue, which potentially forms charge-charge interactions with residues E112 and E384 to strengthen the association between domain-I, II and IV. To confirm the engineering effect, we determined the crystal structure of EryP\textsubscript{E802R} in 1.77 Å resolution (Supplementary Fig. 13). In line with our expectation, the overall structure of EryP\textsubscript{E802R} displays a more compact conformation than EryP (inter-domain angle $\theta=48.18$, and the torsion angle $\phi=27.77$) mainly due to the newly formed interactions introduced by the E802R mutation (Fig. 5a). Specifically, residue R802 inserts into the cleft of domain II and forms a salt-bridge with residue E384, as well as cation-π interaction with residue F387 from domain II (Fig. 5b; Supplementary Fig. 13). Additional interaction between R802 and T133 from domain I was observed, which pulls domain I closer to domain IV and increases their domain interface from 58.2 to 352.3 Å\textsuperscript{2} (Fig. 5a). MD simulation based on the structure of EryP\textsubscript{E802R} showed that domain-IV of EryP\textsubscript{E802R} is in significantly closer contact with domain-I and II than that of EryP, and conformations of EryP\textsubscript{E802R} are largely maintained in the \textit{closed} state (Fig. 4a, b). Together, these data support that the E802R mutation strengthens the interactions between domain IV and domain I & II and drives the conformational distribution of EryP\textsubscript{E802R} toward a compacted \textit{closed} state.

Despite the conformational changes altered by the E802R mutation, the majority of active site residues in the catalytic pocket of EryP\textsubscript{E802R} reside almost identically as in EryP with the exception of Y392, which displays a significant shift with the phenolic oxygen atom approaching the Zn ion center, shortening their distance from 8.6 to 4.1 Å (Fig. 5c). Y392 is proposed to stabilize the tetrahedral intermediate during enzymatic amide hydrolysis (Supplementary Fig. 14), and a closer distance between the Zn ion and Y392 might facilitate the hydrolytic reaction.\textsuperscript{21, 26, 28} We next examined whether the proteolytic activity of EryP\textsubscript{E802R} was improved by the conformational remodeling. By Michaelis-Menten analysis, EryP\textsubscript{E802R} exhibited aminopeptidase activity toward amino acid-pNA substrates with $k_{\text{cat}}/K_{m}$ values increased by ~800-fold and $K_{m}$ values lowered by 7-60 folds, indicating that both substrate binding and catalytic efficiency are improved (Fig. 5d; Supplementary Fig. 15). Meanwhile, the catalytic activity of EryP\textsubscript{E802R} is no longer responsive to the alteration of Ca ions in the reaction buffer, supporting the notion that the E802R mutation is a reasonable design which could replace the enhancement effect induced by Ca binding (Supplementary Fig. 15). Importantly, EryP\textsubscript{E802R} showed significantly higher aminopeptidase activity towards peptide Apla\textsubscript{Ayc-cP(-4)} than EryP during the removal of the N-terminal Asp residue (Fig. 5e). Together, our data showed that the E802R mutation stabilizes the overall structure of EryP\textsubscript{E802R} in a more compact \textit{closed} conformation than EryP, which leads to improved aminopeptidase activity.
To further validate the origin of the enhanced aminopeptidase activity of EryP<sub>E802R</sub>, we performed MD analysis of the substrate binding of EryP and EryP<sub>E802R</sub> to Leu-pNA (Supplementary Fig. 16). Typically, three major factors govern the course of amide bond hydrolysis: the activation of an amide bond by Zn ion, the nucleophilic attack by the hydrolytic water and the stabilization of the resulting tetrahedron intermediate. MD analysis indicated that the average distance between the Zn ion and the amide oxygen of Leu-pNA bound in EryP<sub>E802R</sub> was ~1.5 Å shorter than that in EryP, suggesting a stronger amide activation and stabilization of the tetrahedron intermediate (Supplementary Fig. 16). In addition, the hydrolytic water is ~0.5 Å closer to the amide α-carbon, which increases the likelihood of nucleophilic attack to occur. Finally, the phenolic oxygen of Y392 displays an average distance of 2.0 Å from the amide oxygen, which is ~2.0 Å shorter than that in EryP, leading to a significantly stronger stabilization effect of the tetrahedron intermediate. These results show that the active site constellation of EryP<sub>E802R</sub> is more optimized for amide hydrolysis than that of EryP.

**Fig. 5** | The E802R mutation enhanced the inter-domain interaction and the catalytic activity of EryP. a, Structural comparison between EryP and EryP<sub>E802R</sub>. b, The remodeled inter-domain hydrogen bonding network introduced by the E802R mutation. c, Comparison of active site residues in EryP and EryP<sub>E802R</sub>. d, The aminopeptidase activity toward Ala-pNA and Leu-pNA was significantly enhanced. Error bars indicate standard deviation of three independent replicates. e, The aminopeptidase activity toward Apl<sub>cyc</sub>-CP(-4) peptide was significantly enhanced. Assay conditions: 20 mM Tris, pH 8.0, 100 μM Apl<sub>cyc</sub> peptide, 1.0 μM EryP or EryP<sub>E802R</sub> at 37 °C for 90 min. Apl<sub>cyc</sub>-CP(-3): <i>M<sub>calc.</sub></i> = 2584.12 Da.
\[ M_{\text{obs.}}^\ast=2584.69 \text{ Da}; \quad M_{\text{calc.}}^\ast=2699.15 \text{ Da}; \quad M_{\text{obs.}}^\#=2699.94 \text{ Da}. \]

* and # represents the sodium and potassium adducts of peptides in MS, respectively.

**Engineering of inter-domain interactions as a general strategy to modulate the activity of LanPM1 enzymes.** The successful engineering of EryP by remodeling the inter-domain interaction prompted us to examine the general applicability of this strategy for other LanPM1 enzymes. AplP, the protease from NAI-112 biosynthesis and the first example of LanPM1 proteases, was selected as a model. Based on the high sequence similarities between EryP and AplP (49.5%), we built a model of AplP using EryP structure (closed) as a template to analyze the potential domain interface. Referring to the T133-E384-R802 inter-domain hydrogen bonding network in EryP\(_{E802R}\), we speculated that the incorporation of two Glu residues and an Arg residue at positions 98, 368 and 779 of AplP might also introduce charge-charge interactions and strengthen the inter-domain association (Supplementary Fig. 17). Thus, we prepared AplP\(_{R98E-A368E-A779R}\) and evaluated its catalytic activities. Gratifyingly, the aminopeptidase activity of AplP\(_{R98E-A368E-A779R}\) was enhanced by 2.6-fold compared with AplP toward Ala-\(p\)NA (Supplementary Fig. 18). Furthermore, AplP\(_{R98E-A368E-A779R}\) displayed significantly higher endopeptidase activity toward its native peptide substrate AplA\(_{cyc}\) than wild-type AplP, but with altered preference to cleavage sites (Fig. 6a). During prolonged incubation, AplP generated AplA\(_{cyc-CP(-4)}\) as the major product but no further trimming was detected, indicating that residue Glu(-4) is a challenging residue to remove (Fig. 6b). In contrast, AplP\(_{R98E-A368E-A779R}\) exhibited enhanced aminopeptidase activity by overcoming residue Glu(-4) and further produced AplA\(_{cyc-CP(-1)}\) as a major product. Therefore, we have constructed an AplP mutant with improved proteolytic activity for the removal of AplA leader peptide through rational introduction of bridging residues that strengthen the inter-domain interaction, which might serve as a general strategy to engineer LanPM1 proteases for improved efficiency during leader removal of corresponding lanthipeptides.
Fig. 6 | **a.** **ApIP**$_{R98E-A368E-A779R}$ cleaves ApIA$_{cyc}$ with improved endopeptidase activity

**b.** **ApIP**$_{R98E-A368E-A779R}$ displays improved aminopeptidase activity during prolonged incubation

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**DISCUSSION**

A molecular ruler model has been proposed to explain the substrate preference toward peptides of certain length and sequence for four-domain M1 metallopeptidases ERAP1/ ERAP2 and IRAP.$^{14,24,30}$ The C-termini of peptide substrates
bind to a regulatory site in ERAP1 and induce allosteric activation of ERAP1 by shifting its conformation toward the closed form. In contrast, the presence of peptide substrates has little impact on the hydrolytic efficiency of EryP toward Ala-\(p\)NA under assay conditions (Supplementary Fig. 19). In addition, the inter-domain cavity in the closed form of EryP is \(~1530\ \text{Å}^3\), which is not sufficient to fully accommodate the cyclized core peptide of AplA peptide \((~2300\ \text{Å}^3)\). These data implicate that EryP might have distinct mechanisms of substrate recognition and enzyme activation. Although EryP binds to full length EryA\(_{LP}\) and AplA\(_{LP}\) peptides (Table 1), it shows no measurable binding with endoproteolytic products EryA\(_{LP(-15)}\)(-1) and AplA\(_{LP(-6)}\)(-1) and displays no aminopeptidase activity toward them even after long time incubation (Supplementary Fig. 20). However, EryP could recognize and trim the C-terminal products with cyclized core peptide \((\text{AplA}_{\text{cyc-CP(-6)}})\), suggesting that the core peptide plays a critical role to facilitate substrate recognition by EryP during the second phase of leader removal. Incubation of EryP and AplA\(_{LP(-6)}\) in \textit{trans} with AplA\(_{\text{cyc-CP}}\) peptide did not lead to improved aminoproteolytic removal of N-terminal residues from AplA\(_{LP(-6)}\). Together, these data indicate that the core peptide AplA\(_{\text{cyc-CP}}\) functions as a substrate recognition unit to deliver the AplA\(_{LP(-6)}\) segment to EryP, instead of as an allosteric activator. Therefore, we propose that the apparent low aminopeptidase activity of EryP during the second phase of leader removal is primarily due to the low binding affinity to peptide substrates.

Three sets of EryP crystal structures, the open, intermediate and closed states, demonstrate that domain-IV are highly dynamic with large-scale motions relative to domain-I and II (Fig. 3a). The dynamic domain motion is a critical evolutionary adaptation for M1 aminopeptidases to act as efficient trimming enzymes and regulates the catalytic efficiency indirectly. A unique inter-domain Ca binding site was visualized in the closed state of EryP, which bridges coordinating residues from domain-I, II and IV, thereby stabilizing the inter-domain association. Unlike thermolysin,\(^1\) the Ca binding is not strictly required for the folding or proteolytic activity of EryP, and mutations of Ca binding site have no negative impact on its activity (Supplementary Fig. 12). However, the binding of Ca ions drives the conformational equilibrium of EryP toward the \textit{close} state and enhanced its aminopeptidase activity. Thus, this Ca binding site appears to be the key regulatory element with direct impact on its aminopeptidase activity. The direct correlation between the dynamic conformational changes and enzymatic activity is further demonstrated by the \(E_{\text{802R}}\) mutant with a more compact and stabilized \textit{closed} structure and aminopeptidase activity enhanced by over 700-fold compared with EryP. This result could be rationalized by considering that the long-range attractive electrostatic forces introduced by the E802R mutation facilitates the domain motion. Therefore, our observation strongly supports the proposal from previous studies that the conformational closure of M1 metallopeptidases is mandatory for optimal aminopeptidase activity, while the reciprocal open-close transition promotes the substrate binding and product release.\(^1,\)\(^2,\)\(^2\) Importantly, elucidation of the Ca binding site as the conformational regulatory element of LanP\(_{M1}\) enzymes allows their engineering for improved aminopeptidase activity. The successful construction of EryP\(_{E802R}\) and AplP\(_{R98E-A368E-A779R}\) with improved activities support our working
principle, providing a practical engineering strategy for improved LanP$_{M1}$ enzymes for lanthipeptide production.

Metallopeptidases with endopeptidase activities, such as astacin and human matrix metalloproteinase-1, contain a catalytic groove lying between the helices bundle and Greek key-like beta sheet to accommodate peptide substrates. However, the N-terminal cleft of the substrate binding groove in M1 family metallopeptidases is largely blocked by domain I, although preserves space to lodge the substrate N-terminus. In comparison to other M1 metallopeptidases, EryP has a relatively elongated groove extending from the catalytic site with potential binding subsites to accommodate longer N-terminal fragment of peptide substrates. This enlarged peptide binding site might facilitate EryP to achieve recognition of leader peptides and perform endoproteolytic cleavage. The V(L/Q)E(I/L)(Q/L)EL motif in EryA$_{LP}$ and AplA$_{LP}$, which is highly conserved in class III lanthipeptide leader peptides for substrate recognition by lanthipeptide synthetases, appears to be important for LanP$_{M1}$ enzymes. Major endoproteolytic cleavage sites by EryP enzymes often reside in the middle or to the C-terminal to this conserved site (Fig. 2a; Supplementary Fig. 5), suggesting that this motif might associate to the substrate binding groove around the active site of EryP. Nevertheless, how LanP$_{M1}$ enzymes switch functions between endo- and aminopeptidases requires further investigation.

In conclusion, we report the biochemical and structural characterization of bifunctional M1 zinc-metallopeptidase EryP. Crystal structures of EryP in different states provide insights into the conformational dynamics of the rate-limiting protease LanP$_{M1}$ enzymes in lanthipeptide biosynthesis. An unprecedented inter-domain Ca binding site is revealed as an effector mechanism for inter-domain association and aminopeptidase activity. These results not only highlight the importance of the conformation dynamics in M1 metallopeptidase function but also provide valuable insights with respect to understanding their trimming mechanism. Importantly, strengthening the inter-domain interactions through rationally designing bridging residues generates LanP$_{M1}$ variants with improved aminopeptidase activities, which hold potential application in the bioengineering and production of lanthipeptides. This successful engineering effort could potentially guide site-specific protein engineering on other M1 metallopeptidases to improve their catalytic efficiency.

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Author contributions

H.W. and R.B. initiated and directed this study together with W.W.. W.S., C.Z. and Y.W. prepared enzymes and peptides and performed biochemical assays. C.Z. performed structural biology experiments. W.W. performed computational studies on protein dynamics. All authors participate in the data analysis and manuscript preparation.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper online.

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Data availability

All crystal structures have been deposited in the PDB database under accession number 7V9N for EryP\textsubscript{closed}, 7V9P for EryP\textsubscript{intermediate}, 7V9Q for EryP\textsubscript{open} and 7V9O for EryP\textsubscript{E802R}.
Methods

General methods. Polymerase chain reactions (PCR) were carried out on a C1000 Touch™ thermal cycler (Bio-Rad). DNA sequencing was performed by the Genscript Biotech, using appropriate primers. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was carried out on Bruker UltraFlextreme. Liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) was carried out and processed using a Triple TOF 4600 System (AB Sciex) equipped with a Prominence Ultra-Fast Liquid Chromatography (UFLC) system (Shimadzu). UV-Vis spectrometry was recorded by Cary 300 (Agilent Technologies). Conditions for all ESI-MS and MS/MS were set as follows: nebulizer gas: 55 psi; heater gas: 55 psi; curtain gas: 35 psi; drying temperature: 550 °C; ion spray voltage: 5500 V; declustering potential: 100 V; collision energy: 35 V (positive); collision energy spread: 10 V. The mass range and accumulation time are 400-4000 m/z, 250 ms for ESI-MS and 100-2000 m/z, 100 ms for MS/MS, respectively. Collision-induced dissociation (CID) was performed for fragmentation of the respective peptide ions. Calibration solutions purchased from AB SCIEX were used for instrument calibration, and high resolution was chosen in the ESI+ mode.

Materials. All oligonucleotides were purchased from Genscript Biotech (Nanjing, China). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA). Phanta® Max Master Mix was purchased from Vazyme Biotech (Nanjing, China). Media components for bacterial cultures were purchased from Thermo Fisher (Waltham, MA, USA). Chemicals were purchased from Aladdin Reagent (Shanghai, China) or from Sigma-Aldrich (Schnelldorf, Germany) unless noted otherwise. Endoprotease GluC was purchased from Roche Biosciences (Basel, Switzerland). *E. coli* DH5α was used as the host for cloning and plasmid propagation, and *E. coli* BL21 (DE3) was used as a host for expression of proteins and peptides.

General nomenclature. In this report, we use the standardized nomenclature recommended by the lanthipeptide community in 2020 (*Nat. Prod. Rep.* 2020, 38, 130-239). The numbering of residues in lanthipeptide precursor peptides starts with the first residue of the core peptide. Residues in the leader peptide are indicated with negative numbers counting back from the junction between the leader peptide and core peptide.

Molecular cloning of *aplA*, *aplKC*, *aplP* and *eryP* genes. Plasmids containing target genes were synthesized by Genscript Biotech and PCR amplified by 30 cycles of denaturing (95 °C for 30 s), annealing (65 °C for 30 s), and extending (72 °C, 1 min/kb) using high fidelity Phanta® DNA Polymerase. Amplifications were confirmed by 2% agarose gel electrophoresis, and the PCR products were purified using an Omega Biotech Cycle Pure Kit. The target DNA fragment and pRSFDuet-1 vector were digested in separate reactions containing 1X NEB buffer (New England Biolabs) with selected pair of restriction enzymes for 2 h at 37 °C. The digested products were purified by agarose gel electrophoresis, and the DNA fragments were extracted from the gel using an Omega Biotech Gel Extraction Kit. The resulting DNA products were
ligated at 16 °C for 12 h in 1X T4 DNA Ligase buffer with T4 DNA Ligase (0.7 U/ µL). *E. coli* DH5α cells were
transformed with 2.5 µL of the ligation product by heat shock, and cells were plated on LB-kanamycin agar plates and
grown for 15 h at 37 °C. Several colonies were picked and used to inoculate separate 5 mL cultures of LB-kanamycin
medium. The cultures were grown at 37 °C for 12 h, and plasmids were isolated using an Omega Biotech Plasmid Mini
Kit. The sequences of the resulting plasmid products were confirmed by DNA sequencing.

**Mutagenesis of AplP and EryP.** Mutagenesis of AplP and EryP was carried out by a two-stage protocol, which starts with
the generation of primers using two parallel asymmetric PCRs, followed by an exponential whole-plasmid amplification.
PCR amplification was performed by 30 cycles of denaturing (95 °C for 30 s), annealing (65 °C for 30 s), and extending
(72 °C, 1 min/kb) using high fidelity Phanta® DNA Polymerase and confirmed by 1% agarose gel electrophoresis. PCR
products were purified using an Omega Biotech Cycle Pure Kit. The target DNA fragment was digested by DpnI in NEB
buffer (New England Biolabs) for 3 h at 37 °C. *E. coli* DH5α cells were transformed with 2.5 µL of the digested product
by heat shock, plated on LB-agar plates and grown for 15 h at 37 °C. Single colonies were picked to inoculate separate 5
mL cultures of LB-chloramphenicol medium, which were grown at 37 °C for 12 h. Plasmids were isolated using an Omega
Biotech Plasmid Mini Kit. The sequences of the resulting plasmid products were confirmed by DNA sequencing.

**Overexpression and purification of AplA_{cyc} peptide.** *E. coli* BL21(DE3) cells were transformed with pRSFDuet-1-His_{6}-
AplA and pACYCDuet-1- His_{6}-AplKC and plated on a Luria broth (LB) agar plate containing 50 mg/L of kanamycin and
35 mg/L of chloramphenicol. A single colony was used to inoculate a 5 mL culture of LB supplemented with 50 mg/L of
kanamycin and 35 mg/L of chloramphenicol at 37°C for 12 h. The culture was used to inoculate 4 L of LB containing 50
mg/L of kanamycin and 35 mg/L of chloramphenicol. Cells were grown at 37 °C to OD_{600}=0.6-0.8, cooled to 16 °C before
IPTG was added to a final concentration of 0.2 mM. The resulting cultures were grown for another 20 h, harvested and
processed following the purification procedure described in the previous section.

**Overexpression and purification of EryP and AplP enzymes.** *E. coli* BL21 (DE3) cells were transformed with pET-28a
plasmids containing genes encoding target enzymes. A single colony transformant was used to inoculate a 30 mL culture
of LB supplemented with 50 µg/mL kanamycin. The culture was grown at 37 °C for 12 h and was used to inoculate 3 L of
LB containing 50 µg/mL kanamycin, and cells were grown at 37 °C to OD_{600} = 0.6. The culture was incubated at 4 °C on
ice for 20 min, then IPTG was added to a final concentration of 0.2 mM and the culture was incubated at 18 °C for 20 h.
Cells were harvested by centrifugation at 12,000 ×g for 15 min at 4 °C, and the pellet was resuspended in 30 mL of start
buffer (20 mM Tris buffer, pH 8.0, 500 mM NaCl, 1.0 mM TCEP, 10% glycerol) and stored at −80 °C. All protein
purification steps were performed at 4 °C. The cell paste was suspended in start buffer and the cells were lysed using a
high pressure homogenizer (Avestin, Inc.). Cell debris was pelleted via centrifugation at 23,700 ×g for 20 min at 4 °C. The
supernatant was loaded onto a 5 mL HisTrap HP IMAC column charged with Ni^{2+} and equilibrated with start buffer. The
column was washed with 50 mL of buffer A (30 mM imidazole, 20 mM Tris, pH 7.5, 300 mM NaCl), and the protein was eluted using a linear gradient of 0-100% buffer B (200 mM imidazole, 20 mM Tris, pH 7.5, 300 mM NaCl) over 40 min at a 2 mL/min flow rate. UV absorbance (280 nm) was monitored and fractions were collected and analyzed by SDS-PAGE. The fractions containing target proteins were combined and concentrated using an Amicon Ultra-50 Centrifugal Filter Unit (30 kDa MWCO, Millipore). Gel filtration purification was used to further purify target proteins. The concentrated protein sample was injected onto an FPLC system (ÄKTA) equipped with an XK16 16/60 (GE Healthcare Life Sciences) column packed with SuperDex 75 resin previously equilibrated in 20 mM HEPES (pH 7.5), 200 mM KCl. The protein was eluted with a flow rate of 1.0 mL/min. Both UV (280 nm) and conductance were monitored and fractions were collected. Misfolded/aggregated protein was efficiently separated from soluble, correctly folded protein and the desired fractions were combined and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit. The resulting protein sample was stored at −80 °C. Protein concentration was determined using a Bradford Assay Kit (Pierce).

**Preparation of AplA<sub>cyc</sub>-CP(-4) peptide.** AplA<sub>cyc</sub> (100 μM) was digested by GluC (1.0 μM) in 50 mM Tris buffer, pH 8.0, at 37 °C for 12 h. The resulting AplA<sub>cyc</sub>-CP(-4) peptide was purified by HPLC.

**In vitro assay of EryP and EryP mutants cleaving EryA<sub>LP</sub>, AplA<sub>cyc</sub> and AplA<sub>cyc</sub>-CP(-4) peptides.** Typically, EryP or a EryP mutant (10 μM) was incubated with a peptide substrate (100 μM) in 20 mM Tris buffer, pH 8.0, in a 37 °C water bath. EryP enzymes were omitted in negative control experiments.

**In vitro assay of EryP cleaving EryA<sub>LP</sub>, AplA<sub>cyc</sub> and AplA<sub>cyc</sub>-CP(-4) in the presence of CaCl<sub>2</sub>.** Typically, EryP or a EryP mutant (10 μM) was incubated with EryA<sub>LP</sub> peptide (100 μM) in 20 mM Tris buffer, pH 8.0, with or without CaCl<sub>2</sub> (0.1 mM or 1.0 mM) in a 37 °C water bath. EryP enzymes were omitted in negative control experiments.

**Reaction kinetics of the hydrolysis of amino acid-pNA derivatives by EryP and AplP.** Typically, AplP, EryP (1.0 μM), inactive EryP mutants (1.0 μM) or EryP<sub>E802R</sub> (0.02 μM) was incubated with amino acid-pNA derivatives of varied concentrations in 20 mM Tris buffer, pH 8.0, at 37 °C (assay samples). AplP or EryP enzymes were omitted in control samples. The formation of product p-nitroanilide was measured by the absorbance at 405 nm (Abs<sub>pNA</sub>=Abs<sub>405nm_assay_sample</sub>-Abs<sub>405nm_control_sample</sub>). To investigate the impact of Ca ions on enzymatic activities, CaCl<sub>2</sub> of certain concentration was added in the assay and control buffers.

**Fluorescence-labeling of peptides.** FITC-labeling of AplA<sub>cyc</sub> peptide was performed in conditions: 90 mM phosphate buffer, pH 8.4, 45 μM peptide and 450 μM FITC for 12 h at room temperature and in dark conditions. Fluorescence-labeling of EryA<sub>LP</sub> and AplA<sub>LP</sub> peptides was performed in conditions: 20 mM phosphate buffer (EtOH:H<sub>2</sub>O=2:8, v/v), pH 7.4, 10 μM peptide, 20 μM fluorescein o-acrylate. The reaction was allowed to proceed for 1 h at room temperature in dark conditions. The desired FITC-peptide conjugates were purified by HPLC.

**Measurement of protein-peptide interactions by MST.** As a representative example, the binding affinity between EryP
and FITC-labeled EryA LP was measured using Monolith NT.115 Pico (Nanotemper Technologies). The EryA LP sample was diluted using MST buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween-20, 10 mM MgCl₂, 5% glycerol) until pretest fluorescence excitation range from 200-2000 for the most optimized response (~1.0 μM). To perform the MST assay, FITC-labeled EryA LP sample (10 μL) was first incubated with EryP (10 μL) of 16 different serial dilutions in the assay buffer for 5 min to allow binding. Samples were then loaded into Monolith NT.115 capillary (Nanotemper Technologies) and measured using 20% (Auto-detect) Nano – BLUE as excitation power and medium MST power. The measurement was repeated for three times. Data analysis was performed using Nanotemper affinity analysis software.

**Crystallization, data collection of EryP.** The purified protein was adjusted to about 9 mg/ml and subjected to screenings of different crystallization conditions at 18 ℃. For EryP in the closed state, crystals in the space group P4₁2₁2 were grown from hanging drops with 3 μL of reservoir solution (0.1 M calcium acetate; 0.1 M sodium acetate, pH 4.5; 10% w/v PEG 4000). The condition for the growth of Se-Met-labeled crystals is the same as that for the native crystals in the closed state. When co-crystallized with 2mM VLELQE peptide, which is conserved in the leader peptides of class III lanthipeptides, crystals of EryP in the intermediate state that belongs to the space group P12₁1 appeared after two months under the condition of 0.2 M NaCl, 0.1 M Tris, pH 8.0 and 20% w/v PEG 4000. When co-crystallized with 4mM cyclized-AplA, which contains the leader peptide and the cyclized core peptide, the open form crystals in the space group P2₁2₁2₁ were obtained by mixing 1.5 μL of sample solution and 1 μL of reservoir solution (0.1 M citric acid, pH 3.5; 25% w/v PEG3350) using the sitting method. The crystals of EryP₃₈₀₂₃ were grown in 0.2 M ammonium chloride, 20% w/v PEG 6000 and MES, pH 6.0. Prior to data collection, all the crystals were transferred to cryoprotectant solutions (20% v/v glycerol in corresponding crystallization solution) and flash-cooled in liquid nitrogen. X-ray data were collected at BL-17U1 or BL-18U1 station of the Shanghai Synchrotron Radiation Facility (SSRF), China.

**Data processing and structure determination of EryP.** Diffraction images were integrated and scaled with the HKL2000 program package. The phase problem of Se-Met-labeled EryP was solved by using the single-wavelength anomalous dispersion (SAD) method, and nine Se sites were found by using the AutoSol tool of PHENIX suite. The native structures of EryP in different conformations were determined by molecular replacement using the Phaser-MR tool of PHENIX and the structure of Se-Met-labeled EryP was selected as a template. The structures were rebuilt with COOT and refined using the Refinement tool of PHENIX or CCP4 for several rounds. Data collection and refinement statistics are shown in Table S1. The structural figures were drawn with PyMOL (https://pymol.org/2/) and USCF Chimera X.

**Initial structural preparation for computational studies.** The initial structure of EryP with Ca²⁺ ion bound was based on the closed structure of EryP (PDB ID: 7V9N). The Ca ion was manually removed from the EryP closed structure to build an EryP structure without Ca²⁺ bound. The starting coordinate of the EryP₃₈₀₂₃ mutant were based on its crystal structure (PDB ID: 7V9O). The protonation states of charged residues were determined at constant pH 7.5 based on pKₐ.
calculations via the PROPKA program\(^\text{39}\) and the consideration of the local hydrogen bonding network. In models of EryP with/without Ca ion bound, residues His102, 182, 231, 249, 422, 489, 492, 512, 532, 659, 756, 774 and 797 were assigned as HIE; residues His306, 310 and 710 were set as HID; the rest His residues were HIP. In the model of EryP\(_{E802R}\), residues His102, 182, 306, 310, 512, 756, 710, 756 and 797 were assigned as HIE; residues His115, 231, 249, 422, 489, 492, 532, 659 and 774 were set as HID; the rest His residues were HIP. In above three models, all Asp and Glu residues were set as deprotonated status except for Asp315, while Lys and Arg residues were all set as protonated status. The force field parameters for the Zn center active site were prepared using the Metal Center Parameter Builder (MCPB.py)\(^\text{40}\) as implemented in Amber\(^\text{16\textsuperscript{41}}\), in which residues H306, H310, E329 and a catalytic water molecule were all treated in coordinate bonded way. Bond and angle force constants were derived using the Seminario method\(^\text{42}\), and point charge parameters for the electrostatic potentials were obtained using the ChgModB method. Each model was neutralized by adding Na\(^+\) ions and solvated into a truncated octahedron TIP3P\(^\text{43}\) water box with a 10 Å buffer distance on each side. These three models consisted of 84549, 84547 and 88052 atoms for the EryP bound with or without Ca\(^{2+}\) and EryP\(_{E802R}\), respectively.

**Molecular docking.** The GLIDE program\(^\text{44}\) was used to perform molecular docking of EryP with the LE peptide. The crystal structure of EryP in the closed state was minimized by using the OPLS3 force field and the ligand was prepared with the ‘LigPrep’ module. All docking procedures were calculated in the “Standard Precision” (SP) mode with default parameters. The final scoring was performed on energy-minimized poses and displayed as Glide scores. The best-docked pose with the lowest Glide score value was selected for further analysis.

To dock Leu-pNA into the active sites of EryP enzymes, 50000 snapshots uniformly distributed at equal intervals from the last 100 ns MD simulation (with time intervals of 2 ps) were picked up and divided into ten groups using hierarchical agglomerative (bottom-up) approach\(^\text{45}\). Leu-pNA, the favorite aminopeptidase substrate of EryP, was fully optimized at the B3LYP-D3/6-31+G(d) level of Gaussian 16 using the CPCM\(^\text{46\textsuperscript{-48}}\) model in water, and then docked into the active site of one representative group snapshot to mimic the native substrate-EryP complex. Molecular docking was performed using the Lamarckian genetic algorithm local search method in the AutoDock 4.2 and AutoDockTools-1.5.6\(^\text{49}\). The docking approach was employed on rigid-receptor conformation, while all the rotatable torsional bonds of Leu-pNA were set free. A grid box was centered on the Zn atom and its size was set to 40 Å × 40 Å × 40 Å points with a 0.375 Å spacing. A total of 500 independent docking runs were undertaken with a maximum energy evaluation of 2.5 × 10\(^7\). The obtained 500 docked conformations were clustered with 2.0 Å RMSD and ranked depending on an energy-based scoring function. The possible catalytically active binding modes were
selected as initial configurations to perform MD simulations of EryP and EryP\textsubscript{E802R} in complex with Leu-pNA according to scoring function and reasonable conformation.

**Molecular dynamics simulation.** All molecular dynamics (MD) simulations were performed by Amber 16 package\textsuperscript{41}. The MD pre-equilibrated EryP and EryP\textsubscript{E802R} structures and possible catalytic active binding modes of Leu-pNA were used as the starting conformations for MD simulations on the protein-ligand complexes. The partial charge of Leu-pNA was fitted with HF/6-31G(d) calculations and the restrained electrostatic potential (RESP)\textsuperscript{50, 51} protocol implemented by the Antechamber module in Amber 16 package\textsuperscript{41}. The force field parameters for Leu-pNA were adapted from the standard general amber force field 2.0 (gaff2)\textsuperscript{52} parameters, while the standard Amber14SB force field was applied to describe the protein. Here, the force field parameters for the Zn center active site were treated in a hybrid bonded/restrained nonbonded mode in which H306, H310 and E329 were treated in bonded way while a catalytic water molecule was in nonbonded way using MCPB.py\textsuperscript{40} module. Each system was initially neutralized with Na\textsuperscript{+} counter ions and solvated with explicit TIP3P\textsuperscript{43} water in a truncated octahedron box with a 10 Å buffer distance. The resulting system contained 90788 (EryP bound with Ca\textsuperscript{2+} and Leu-pNA), 89937 (EryP bound with Leu-pNA but no Ca\textsuperscript{2+}) and 87661 (EryP\textsubscript{E802R} bound with Leu-pNA but no Ca\textsuperscript{2+}) atoms, respectively.

Each system was equilibrated with a series of minimizations interspersed by short MD simulations during which restraints on the protein backbone heavy atoms were gradually released (with force constant of 10, 2, 0.1 and 0 kcal/(mol·Å\textsuperscript{2})), and then heated slowly from 0 to 300 K for 50 ps. Finally, the standard unrestrained 200 ns MD simulation with periodic boundary condition at 300 K and 1 atm was carried out. The pressure was maintained at 1 atm and coupled with isotropic position scaling. The temperature was controlled at 300 K with Berendsen thermostat method. Long-range electrostatic interactions were treated with particle mesh Ewald (PME)\textsuperscript{53} method and 12 Å cutoff was applied to both PME and van der Waals (vdW) interactions. Time step of 2 fs was employed along with SHAKE algorithm for hydrogen atoms, and periodic boundary condition was used. Each system was checked for stability (structure, energy, and temperature fluctuations) and convergence (root mean square deviations-RMSD of structures).

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