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Substrate specificity of the cypemycin decarboxylase CypD

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ABSTRACT

The linaridin antibiotic cypemycin is a ribosomal synthesized and post-translationally modified peptide (RiPP) that possesses potent activity against mouse leukemia cells. This peptide natural product contains an *S*-[(*Z*)-2-aminovinyl]-D-cysteine (AviCys) moiety in the C-terminus. Formation of AviCys moiety requires an oxidative decarboxylation of the C-terminal Cys of the precursor peptide CypA, and this process is catalyzed by a flavin-containing protein CypD. In this work, we tested CypD substrate specificity with a series of synthetic oligopeptides. We show that most of the N-terminal sequence of CypA is not required for CypD activity, and the C-terminal three residues serve as the minimal structural element for enzyme recognition. We also show that CypD tolerates various substrates with modified C-termini, allowing for the generation of four novel cypemycin variants with modified AviCys moiety by site direct mutagenesis of the precursor peptide CypA. Our study demonstrates the relaxed substrate specificity of CypD and lays a foundation for future bioengineering of AviCys-containing natural products.

1. Introduction

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a major class of natural products as revealed by the genome sequencing efforts in the past decade [1,2]. These compounds exist in all three domains of life and possess vast structural and biological diversity, and their biosynthesis involves highly diverse biochemistries [3–7]. Among these peptide natural products are linaridins, defined as linear dehydrated (arid) peptides [8]. As with most RiPPs, linaridins are produced from a precursor peptide, of which an N-terminal leader peptide is finally removed by proteolysis, and a C-terminal core region is posttranslationally modified to the mature product. Although only three members of linaridin family have been structurally characterized [8–10], a recent genome mining study showed that this RiPP family is widespread in nature and the members are structurally diverse [11].

Cypemycin is a prototypical member of the linaridin family produced by *Streptomyces* sp. OH-4156. This compound exhibits potent in vitro activity against mouse leukemia cells, and a narrow-spectrum antibiotic activity against *Micrococcus luteus* [12,13]. Derived from the precursor peptide CypA, cypemycin comprises 21 amino acid (aa) residues, including a N,N-dimethylalanine [14], two *allo*-isoleucines, four dehydrobutyrine residues, and an *S*-[(*Z*)-2-aminovinyl]-D-cysteine (AviCys) moiety (Fig. 1A). The presence of multiple dehydroamino acids (i.e. dehydrobutyrine or dehydroalanine) is usually an important characteristic of lanthipeptides, a large and well-studied class of RiPPs [15–17]. However, biosynthesis of cypemycin dehydroamino acids involves a unique set of enzymes with unclear functions and mechanisms [8,11]. AviCys moieties are also found in several lanthipeptides such as epidermin, mersacidin, NAI-107 [18,19], and microvionine [20]. The flavoproteins responsible for the biosynthesis of lanthipeptide AviCys moieties are generically termed LanDs (e.g. EpiD and MrsD are involved in the biosynthesis of epidermin and mersacidin, respectively) [17]. A flavoprotein CypD is involved in cypemycin biosynthesis (Fig. 1B), but this protein appears to be divergent from LanD enzymes, as it shares no sequence similarity with EpiD and MrsD.

In contrast to many enzymes involved in RiPP biosynthesis that are leader peptide-dependent, LanD-catalyzed Cys decarboxylation does not require the presence of leader peptide. Kupke et al. showed that epidermin decarboxylase EpiD is capable of decarboxylating a variety of oligopeptide substrates, and the minimal sequence for enzyme recognition is the C-terminal four residues of the precursor peptide EpiA [21]. EpiD exhibits remarkable substrate tolerance and acts on various oligopeptides containing a C-terminal Cys. In contrast, the substrate specificity of mersacidin decarboxylase MrsD appears to be strict, as it did not act on a synthetic octa-peptide corresponding to the MrsA C-terminal sequence [22]. Similar to MrsD, a recent study indicated that the NAI-107 decarboxylase MibD did not show appreciable substrate tolerance [19]. These studies suggested that the substrate specificity of Cys decarboxylases is diverse in different biosynthetic systems.

2. Material and methods

2.1. Chemicals, biochemicals, plasmids and strains

This information is provided in the Supplementary Information.

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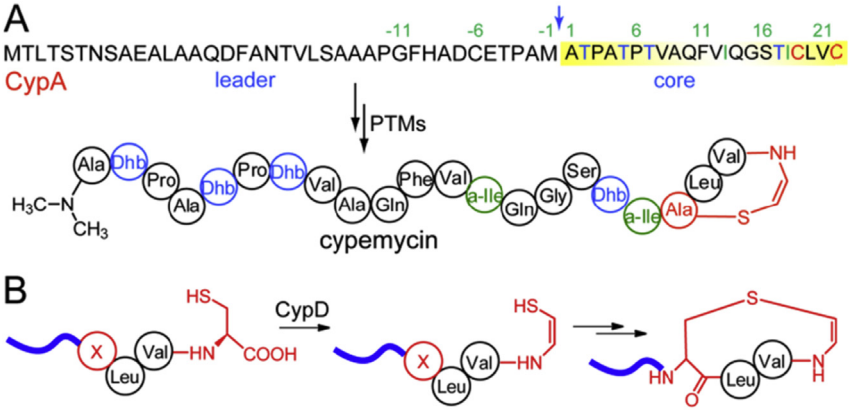

Fig. 1. Biosynthesis of cypemycin. (A) Structure of cypemycin. The amino acid sequence of the precursor peptide CypA is shown. Negative numbers represent the position of amino acids within the leader peptide with respect to the first amino acid in the core region (highlighted in yellow). Dehydrobutyrine and AviCys moiety are shown in blue and red. Cypemycin also contains two alloisoleucine (a-Ile) residues that are shown in green. PTM represents post-translational modifications. (B) The CypD-catalyzed oxidative decarboxylation of the C-terminal Cys, a key step in the formation of the cypemycin AviCys moiety. The blue line represents the peptide chain, and X represents either the CypA Cys19 or a dehydroalanine residue generated by dithiolation of Cys19.

2.2. Protein expression and purification

The construct pET-CypD (see Supplementary Information) for over-expressing CypD was transformed in to *E. coli* BL21 (DE3) cells. A single colony transformant was used to inoculate a 5 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 1 L of LB medium containing 50 μg/mL kanamycin. Cells were grown at 37 °C and 220 rpm to an OD_{600} ~ 0.6–0.8, and then IPTG was added to a final concentration of 0.5 mM. After additional 24 h of Incubation at 18 °C and 150 rpm, the cells were harvested by centrifugation at 4000 × g for 15 min at 20 °C. The pellet was used directly for protein purification or stored at −80 °C upon further use.

The cell pellet collected by centrifugation was re-suspended in 20 mL lysis buffer (50 mM Tris, 200 mM NaCl, and 10% glycerol, pH 8.0), and was lysed by sonication on ice. Cell debris was removed via centrifugation at 21000 × g for 30 min at 4 °C. The supernatant flowed through a column filled with 5 mL Ni-NTA resin pre-equilibrated with the lysis buffer. The desired elution fractions were combined and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit, and the concentrated protein solution was desalted using a DG-10 column (Bio-Rad) pre-equilibrated with the elution buffer (50 mM Tris, 25 mM NaCl, and 10% (v/v) glycerol, pH 8.0). The protein fraction was collected and concentrated, analyzed by SDS-PAGE (10% Tris-glycine gel), and was used directly for in vitro assay or stored at −80 °C upon further use. Protein concentration was determined using Bradford assaywith bovine serum albumin (BSA) as a standard.

2.3. Enzyme assays of CypD

The typical CypD reactions were performed in 200 μl mixture, containing 100 μM peptide substrates and 20 μM enzyme in 50 mM Tris-HCl buffer (pH 7.5). The assays were carried out at room temperature (25 °C) for 2 h before quenching by addition of trichloroacetic acid (TFA) to a final concentration of 5% (v/v). After removal of the protein precipitates by centrifugation, the supernatant was subjected to liquid chromatography coupled with high resolution mass spectrometry (LC-HR-MS) analysis.

2.4. Construction of the recombinant plasmids for expressing CypA mutants

PCR amplification was performed by using pHB-CypA-wt as the template [11], and a primer pair including a general primer CypA-For and a specific primer for each mutant (i.e. CypA-I20L-Rev, CypA-V21A-Rev, CypA-V21C-Rev, CypA-V21I-Rev for the I20L, V21A, V21C, V21I mutants of CypA, respectively, see Table S1 in SI for the primer sequence). The PCR products were digested with the restriction enzymes *Nde*I and HidIII, recovered by agarose gel electrophoresis, and ligated into the same restriction site of pIJ10257 [8], giving the plasmids that express the corresponding CypA mutants. The plasmids were confirmed by DNA sequencing at Genewiz Co. Ltd and RuiDi Biological Technology Co. Ltd.

2.5. Intergeneric conjugation between E. coli and Streptomyces

E. coli ET12567 derivatives containing the corresponding plasmids were grown to an OD_{600} of 0.4. Cells from a 20 mL culture were collected by centrifugation, washed twice with same volume of LB broth, and re-suspended in 2 mL of LB broth as the *E. coli* donor. Spores of *S. coelicolor* M1414 [8] (0.5 mL, 10^3-10^9/mL) stocked in 20% glycerol at −80 °C were washed twice with 0.5 mL of TES buffer (0.05 M, pH 8.0), re-suspended in 0.5 mL of TES buffer, and then incubated for 10 min at 50 °C (heat shock) to activate germination. The culture of spore suspension was incubated for 2–3 h at 37 °C after additional 0.5 mL of TSB broth was added. The cells were recovered and re-suspended in 0.5 mL of LB broth as the *Streptomyces* recipients. The donors (100 μl) and the recipients (100 μl) were mixed in different ratios and the resulting mixture was spreaded evenly onto a MS plates freshly supplemented with 10 mM $MgCl_2$. The plates were incubated at 28 °C for 20 h. After removal of most of the *E. coli* cells by washing the plate surface with sterile water, the plates were overlaid with 1 mL of LB containing nalidixic acid (final concentration, 50 μg/mL) and hygromycin (final concentration, 100 μg/mL) and were incubated at 28 °C for further 3–5 days. The exconjugants were then inoculated into a fresh TSB medium containing 100 μg/mL hygromycin, and the genotype of these strains were confirmed by DNA sequencing.

2.6. In vivo production of cypemycin variants

SPA medium (in grams per liter of solution: soluble starch, 20; beaf extract, 1; $MgSO_4$, 0.5; KNO_3, 1; NaCl, 0.5; K_2HPO_4, 0.5; agar, 20) was used for spore production. The spore suspension of the *Streptomyces* strains were inoculated into 5 mL TSB medium, and were grown at 30 °C and 180 rpm for 72 h. The resulting cultures were used to inoculate 50 mL SOC medium (in grams per liter of solution: tryptone 20, yeast extract 5, NaCl 0.5, KCl 0.19, $MgCl_2$ 0.95, glucose 3.6), which was grown at 30 °C and 180 rpm for 72 h. 3 mL of the resulting seed culture was used to inoculate 30 mL solid fermentation medium (in grams per liter of solution: glucose 5, yeast extract 30, KBr 10, KH_2PO_4 0.5, $MgSO_4$ 0.5 g, agar 20 g). The cells were grown at 30 °C and culture for 15–18 days, and the resulting solid culture was collected, cut into small pieces, and mixed with 3 x volume of acetone. After overnight incubation at room temperature, the resulting mixture was filtered. The filtrate was collected, evaporated to dryness under vacuum, and the residue was dissolved in methanol before HPLC or LC-HR-MS analysis.

Table 1
Determination of the substrate specificity of CypD. "+" and "-" denote decarboxylation was observed and not observed, respectively.

Peptide	Sequence	Activity	MS Spectrum
1	QGSTISLVC	+	Fig. S1
2	STISLVC	+	Fig. S2
3	ISLVC	+	Fig. S3
4	SLVC	+	Fig. S4
5	LVC	+	Fig. S5
6	VC	–	/
7	STISLVS	–	/
8	STISLAC	+	Fig. S6
9	STISLCC	+	Fig. S7
10	STISLIC	+	Fig. S8
11	STISLYC	–	/
12	STISLEC	–	/
13	STISLKC	–	/
14	STISIVC	+	Fig. S9
15	STISKVC	–	/
16	STISYVC	–	/
17	STIALVC	+	Fig. S10
18	STITLVC	–	/
19	STILLVC	–	/

3. Results and discussion

The in vitro activity of CypD was previously reconstituted by Claesen and Bibb, showing that CypD catalyzes oxidative decarboxylation of the CypA Cys22 [8]. However, it is unclear whether the N-terminal sequence of CypA is required for CypD activity. To test this point, we synthesized a series of oligopeptides corresponding to the nine, seven, five, four, three, and two C-terminal sequence of CypA (peptide **1**–**6**, Table 1). Because we previously showed the C19S mutant of CypA was modified to cypemycin with only a slightly decreased efficiency [11], the C-terminal fourth Cys residue (corresponding to Cys19) was changed to Ser in all the oligopeptides to prevent formation of intramolecular disulfide bonds. Biochemical analyses with these peptides showed that peptides **1**–**5** were decarboxylated by CypD (Figs. S1–S5), whereas decarboxylation of **6** was not observed. These results suggest that the C-terminal three residues of CypA serve as the minimal structural element for CypD recognition.

As expected for all the Cys decarboxylases, peptide **7** in which the C-terminal Cys is changed to Ser, was not decarboxylated by CypD, suggesting that, similar to LanD enzymes, CypD is only able to act on Cys. We next tested the substrate tolerance on the penultimate C-terminal position. To this end, we synthesized peptides **8**–**11**, in which the penultimate Val was changed to Ala, Cys, Ile, and Tyr, respectively.

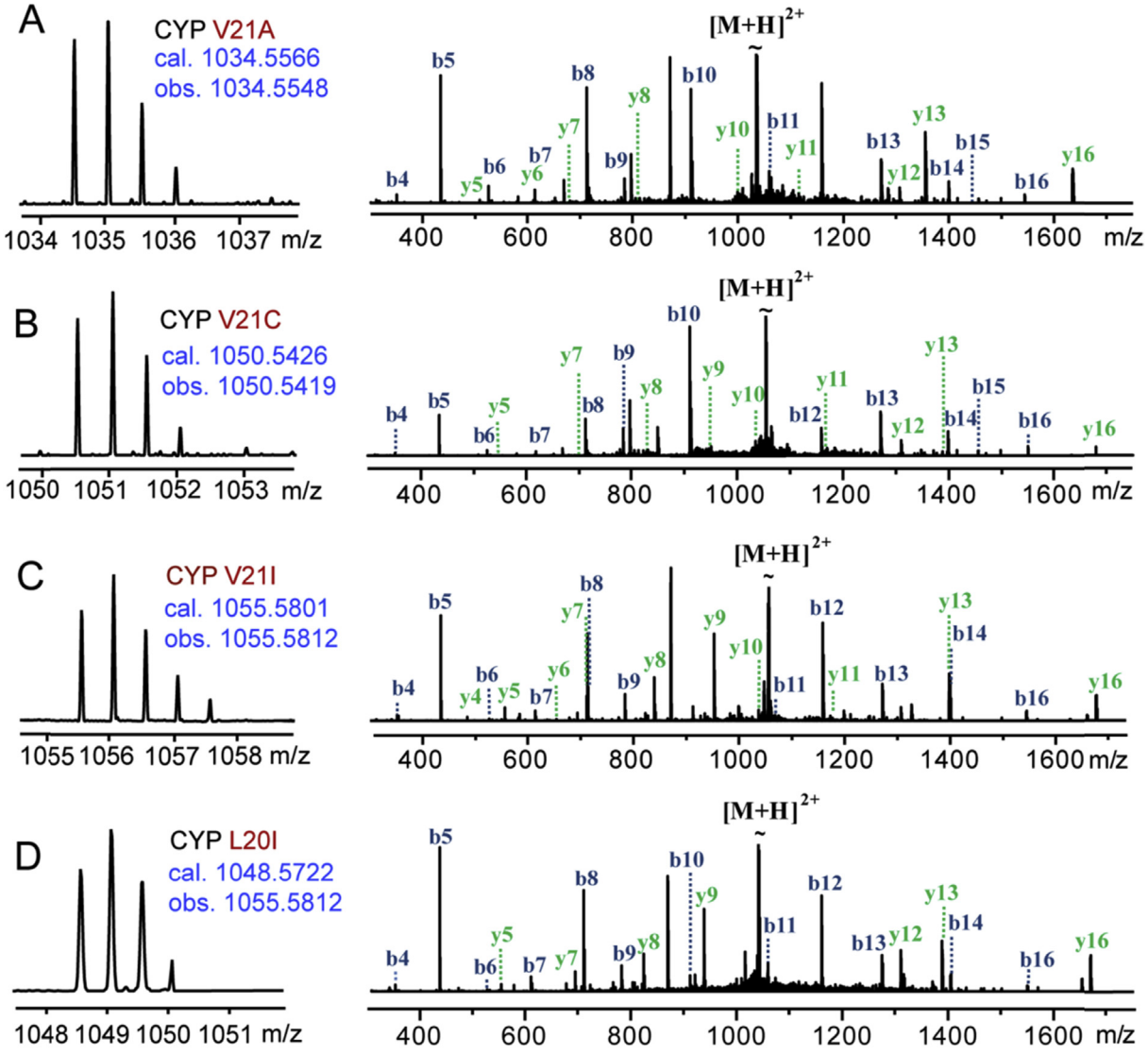

Fig. 2. LC-HR-MS characterization of (A) V21A, (B) V21C, (C) V21I, and (D) L21I variants of cypemycin. For the detailed HR-MS/MS spectra, see Figs. S11–S14.

Incubation of CypD with each of these substrate showed that peptides **8**, **9**, and **10** were decarboyxlated (Table 1 and Figs. S6–S8). However, peptide **11** was not decarboxylated by CypD, suggesting that the enzyme tolerates structural variation at the penultimate position to some extend, but does not accept large aromatic residue at this position. To test whether charged residues can be accepted at this position, we synthesized peptides **12** and **13**, in which the penultimate Val was changed to Glu and Lys, respectively. LC-HR-MS analysis of each reaction mixture with peptides **12** or **13** show that neither of these two peptides was decarboxylated, suggesting that CypD does not accept charged residues at the penultimate C-terminal position.

To test the substrate tolerance of CypD at the antepenultimate C-terminal position, we synthesized peptides **14–16**, in which the antepenultimate Leu was changed to Ile, Lys, and Tyr. Biochemical analysis showed that although peptide **14** was decarboxylated (Fig. S9), no decarboxylated product of peptide **15** and **16** were observed, suggesting neither charged residues nor large aromatic residues can be accepted by CypD.

We next tested the CypD activity with peptide **17–19**, in which the fourth Ser was changed to Ala, Thr, and Leu. LC-HR-MS analysis of each reaction mixture showed that although peptide **17** was decarboxylated (Fig. S10), no decarboxylation was found for peptide **18** and **19**. The fact that CypD does not act on peptide **18** is consistent with our previous engineering study, showing that although CypA C19S was converted to cypemycin, CypA C19T was not converted to the corresponding cypemycin variant [11].

The relaxed substrate specificity of CypD raises the possibility to modify the cypemycin AviCys moiety by biosynthetic engineering. To test this possibility, we utilized the heterologous expression system developed previously that uses *Streptomyces coelicolor* as a host for cypemycin production [8,11]. Based on the results presented above, we generated four plasmids by site-directed mutagenesis, which express the L20I, V21A, V21I, and V21C mutants of CypA, respectively. Each mutant-expressing plasmid was introduced into the *cypA*-knockout strain, and the resulting fermentation cultures were analyzed by LC-HR-MS analysis. This analysis showed that all the four expected cypemycin variants (L20I, V21A, V21I, and V21C) were produced from the corresponding recombinant strains, and each cypemycin variants was further validated by HR-MS/MS analysis (Fig. 2 and Figs. S11–14).

4. Conclusion

In summary, by testing CypD activity with a series of synthetic oligopeptides, our study revealed that most of the N-terminal sequence of CypA is not required for CypD activity, and the C-terminal three residues serve as the minimal structural element for enzyme recognition. Although peptides containing charged or aromatic residues at the C-terminal position were generally not acceptable by CypD, many other variations were modified by the enzyme, allowing for the generation of various novel cypemycin variants with varied AviCys moiety. Bioengineering of the AviCys-containing natural products certainly warrants future investigation because of their intriguing biological activities [18], and we expect this study will contribute to these endeavors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.synbio.2018.09.002.

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