Enzymatic characterization of non-ribosomal peptide synthetases condensation domains using an engineered adenylation domain with an enlarged substrate-binding pocket

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Research objective

Non-ribosomal peptide synthetases (NRPSs) are large multifunctional proteins that synthesize peptide natural products, or their derivatives thereof, with considerable structural and functional diversity. NRPS products include the antitumor agent, bleomycin; the antibiotic, vancomycin; and the immunosuppressant, cyclosporine A. Each NRPS module is responsible for the activation, loading, and condensation of a single amino acid. A standard NRPS module comprises three enzyme domains: the adenylation (A), thiolation (T; also known as the peptidyl carrier protein), and condensation (C) domains. The A-domain catalyzes the formation of an aminoacyl-adenylate (aminoacyl-AMP) intermediate using adenosine triphosphate (ATP) and Mg^{2+}. Next, the aminoacyl-AMP intermediate undergoes a nucleophilic attack by the thiol group of the 4′-phosphopantetheine moiety of the *holo*-T domain, forming the aminoacyl-S-T species. Finally, the C domain catalyzes peptide bond formation between the two aminoacyl-S-T species in the upstream and downstream NRPS modules. The A-domain serves as an entry point for an amino acid in the NRPS machinery and is therefore an attractive target for the reprogramming the NRPS machinery for the biosynthesis of non-native peptides. Furthermore, although the C domain plays an important role within the NRPS machinery, it remains poorly understood relative to other NRPS domains. In this study, we conducted enzymatic characterization of NRPS condensation domains using an engineered aryl acid adenylation domain with an enlarged substrate -binding pocket.

Methods

1) Biochemical characterization of an engineered aryl acid adenylation domain with an enlarged substrate-binding pocket.

EntE is part of a three-module NRPS assembly line involving EntB, EntE, and EntF and is responsible for the production of the siderophore enterobactin in *Escherichia coli*. EntE is a standalone aryl acid A-domain that catalyzes the activation of 2,3-dihydroxybenzoic acid (DHB) and transfers it to the aryl carrier protein (ArCP) domain of EntB. Recently, we have described an engineered aryl acid A-domain with an enlarged substrate-binding site capable

of accepting a wide range of non-native aryl acids functionalized with nitro, cyano, chloro, bromo, and iodo groups (Fig. 1).[1), 2)] The structures of EntE N235G in complex with 3-cyanobenzoic acid-AMS and 2-nitrobenzoic acid-AMS demonstrated that the N235G substitution enlarged the substrate-binding pocket by approximately 25 $Å^3$ compared with those of wild-type EntE (Figs. 1A and 1B).[1),3)] On the basis of these results, we designed and synthesized a large number of disubstituted benzoic acid derivatives. Subsequently, we evaluated the substrate profiles of the wild-type EntE and the N235G variant toward 31 benzoic acid (BA) derivatives with different modifications at the 2- and 3-positions using a non-radioactive high-throughput malachite green colorimetric assay. Furthermore, we determined the kinetic parameters of the adenylation reaction catalyzed by wild-type EntE and the N235G variant.

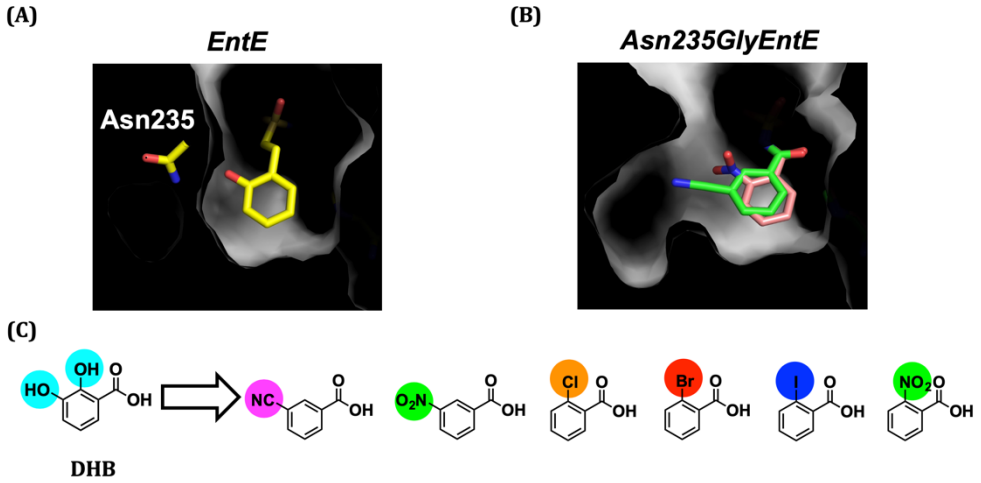

Figure 1. Structural analysis of the N235G variant. (A) The substrate-binding pocket of the EntE–EntB (ArCP) complex (PDB ID: 3RG2) bound with a Sal-based vinyl sulfonamide inhibitor. The Asn235 side chain and Sal-based vinyl sulfonamide inhibitor are shown as stick models. (B) Structures of non-hydrolysable aryl-AMP analogues, 3-cyanoBA-AMS and 2-nitroBA- AMS. The 3-cyanoBA-AMS and 2-nitroBA-AMS molecules are shown as stick models. C) Aryl acid substrates accepted by the N235G variant.

2) Biochemical characterization of NRPS condensation domains using an engineered aryl acid adenylation domain with an enlarged substrate-binding pocket.

To reconstitute the *E. coli* enterobactin biosynthetic pathway *in vitro*, we expressed His-tagged wild-type EntE, the N235G variant, EntB, and EntF and purified these by affinity chromatography. Next, we performed *in vitro* reconstruction of the enterobactin biosynthetic pathway by including the components EntE, EntB, EntF, DHB, L-Ser, and ATP. Finally, using the N235G variant and non-native aryl acid substrates, we analyzed the production of enterobactin derivatives and their shunt products using high-performance liquid

chromatography (HPLC) and liquid chromatography/mass spectrometory (LC/MS).

Results

1) Biochemical characterization of an engineered aryl acid adenylation domain with an enlarged substrate-binding pocket.

The substrate profile of the N235G variant demonstrated that it was capable of accepting 20 of the 31 BA derivatives. This is noteworthy because of the wild-type EntE showed no enzymatic activity toward these aryl acids.

2) Biochemical characterization of the NRPS condensation domains using an engineered aryl acid adenylation domain with an enlarged substrate-binding pocket.

We successfully detected enterobactin and shunt products by HPLC and LC/MS. The optimized conditions were as follows: EntE (1 μM), EntB (10 μM), EntF (2 μM), DHB (1 mM), L-Ser (2 mM), and ATP (10 mM). Furthermore, we confirmed the biosynthesis of enterobactin using the N235G variant. We investigated the production of enterobactin derivatives using the N235G variant and non-native aryl acids to elucidate the substrate specificity of the C domain of EntF.

Conclusion

In this study, we described the limitations and scope of the substrate specificity of the N235G variant. We demonstrated that the N235G variant could accept a wide range of benzoic acid derivatives with different modifications at the 2- and 3-positions. Furthermore, we reconstructed the enterobactin biosynthetic pathway using the N235G variant. Studies on the biosynthesis of enterobactin derivatives are currently underway.

References

1）Ishikawa, F., Miyanaga, A., Kitayama, H., Nakamura, S., Nakanishi, I., Kudo, F., Eguchi, T., and Tanabe, G. (2019) An engineered aryl acid adenylation domain with an enlarged substrate binding pocket. *Angew. Chem. Int. Ed.* **58:** 6906-6910.

2）Ishikawa, F., Nohara, M., Nakamura, S., Nakanishi, I., and Tanabe, G. (2020) Precise probing of residue roles by NRPS code swapping: mutation, enzymatic characterization, modeling, and substrate promiscuity of aryl acid adenylation domains. *Biochemistry* **5:** 351-363.

3）Ishikawa, F., Kitayama, H., Nakamura, S., Takashima, K., Nakanishi, I., and Tanabe, G. (2021) Activity, binding, and modeling studies of a reprogrammed aryl acid adenylation domain with an enlarged substrate binding pocket. *Chem. Pharm. Bull.* **69:** 222-225.