Fermentation production of new polyketide compounds by engineered biosynthesis concerning protein-protein interaction

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Research objective

Polyketide compounds, including macrolides and macrolactam antibiotics, with antimicrobial and antitumor activities are synthesized by polyketide synthases (PKSs). PKSs are multidomain enzymes that catalyze the elongation of the polyketide chain tethered to the carrier protein (CP) domain via a thioester bond (Fig. 1). The acyltransferase (AT) domain of PKSs recognizes malonyl-CoA or methylmalonyl-CoA as extender units and transfers the acyl group to cognate CP, generating malonyl-CP or methylmalonyl-CP. The β-ketosynthase (KS) domain catalyzes a Claisen-type condensation between the elongated polyketide chain on the upstream CP domain and the alkylmalonyl-CP on the downstream CP domain. The resulting β-ketoacyl-CP is then modified by β-ketoreductase (KR), dehydratase (DH), and/or enoyl reductase (ER) domains, producing polyketide chains with β-hydroxy groups, α,β-unsaturated moieties, or fully reduced acyl groups. Starter units such as acetyl or propyl groups are also selected by the AT domain. Additionally, aminoacyl groups can serve as starter units, where the amino group participates in macrocyclization to yield macrolactams. Typically, adenylation enzymes (A domains) recognize specific amino acids, activate them, and attach them to the cognate CP to form aminoacyl-CP. Thus, both AT and A domains play key roles in determining the structure of the polyketide backbone. The exchange of the AT and A domains with those of different substrate specificities has been explored as a strategy for generating polyketide analogs. However, the success rate is generally low, likely because the substituted AT or A domains do not effectively recognize the unnatural CP domains required to transfer the alkylmalonyl or aminoacyl groups. This suggests that natural protein-protein interactions (PPIs) between the AT/A and CP domains are crucial for the efficient transfer of both the extender and starter units. Therefore,

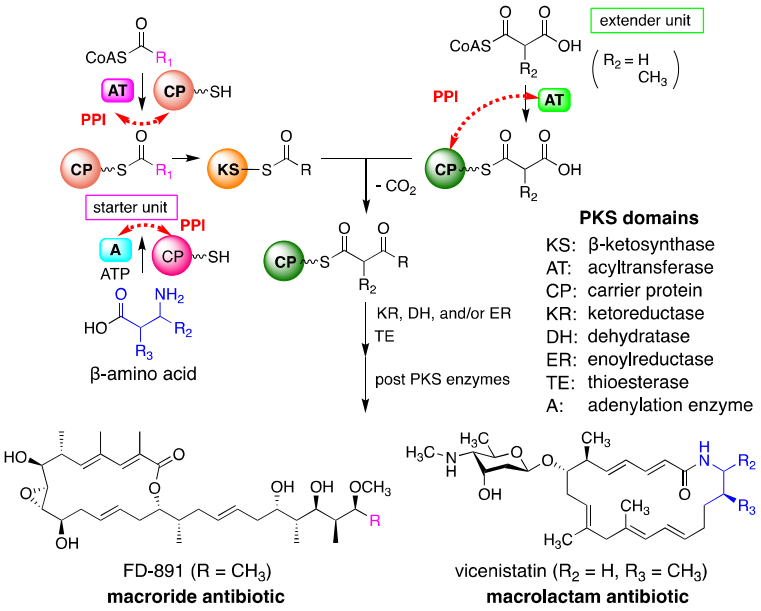

Fig. 1. Summary of polyketide biosynthesis

in domain-exchange experiments, proper manipulation is essential to preserve these PPIs.

In this study, we aimed to deepen our understanding of the natural PPIs between the AT/A and CP domains to facilitate successful domain exchange experiments, ultimately enabling the production of novel polyketide analogs through fermentation.

Methods

We have been involved in biosynthetic studies of the macrolide antibiotic FD-891, focusing on acyltransferase (AT) domain exchange to produce novel FD-891 analogs. Additionally, we have investigated the biosynthesis of macrolactam antibiotics that incorporate β-amino acids at the starter unit of the polyketide skeleton, employing mutasynthesis to generate novel macrolactam analogs. In this study, we used the AT and adenylation (A) domains from macrolide and macrolactam biosynthetic gene clusters to examine natural protein-protein interactions (PPIs) with their corresponding carrier protein (CP) domains. To evaluate these interactions, we utilized a crosslinking reaction involving modified CPs containing pantetheine mimics with electrophilic functional groups, such as α-bromoacetyl (Fig. 2). We designed and synthesized new pantetheine mimics featuring propanediamine (C3Br), butanediamine (C4Br), and hexanediamine (C6Br) linkers instead of the traditional ethylenediamine (C2Br) linkers. These mimics have been used to modify the apo forms of CPs, along with coenzyme A biosynthetic enzymes and phosphopantetheinyltransferases, to create *crypto*-CPs. Additionally, we mutated the conserved aspartate residue in the amino acid adenylation domains to cysteine, allowing the thiol group to nucleophilically attack the α-bromoacetyl group, forming a covalent bond in the cross-linking reaction—if the CP and A domains interact properly.

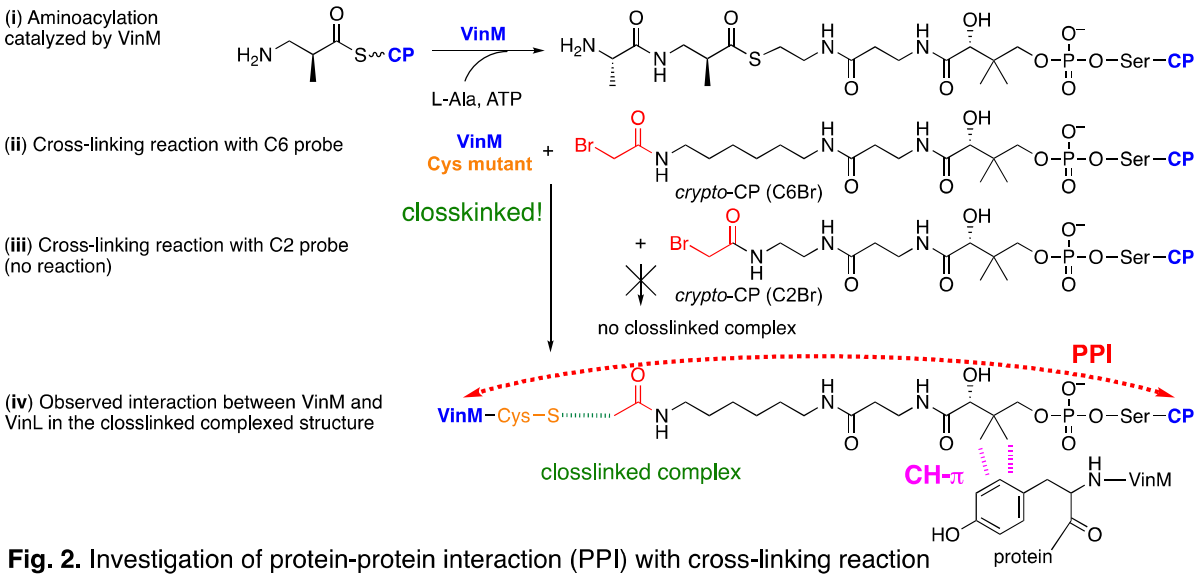

Fig. 2. Investigation of protein-protein interaction (PPI) with cross-linking reaction

Results

As a result, we identified a significant recognition mechanism during the formation of

dipeptidyl-CP from β-aminoacyl-CP by a unique adenylation enzyme involved in macrolactam biosynthesis (Fig. 2).[1] In the biosynthesis of macrolactams that utilize a β-amino acid as the starter unit of the polyketide skeleton, the pathway-specific β-amino acid-selective adenylation enzyme produces β-aminoacyl-CP. This compound is subsequently aminoacylated by another adenylation enzyme to form dipeptidyl-CP (Fig. 2-i). This second amide bond-forming adenylation enzyme specifically recognizes its natural substrate, β-aminoacyl-CP, and distinguishes it from nonacylated holo-CP and other molecules. To investigate the recognition mechanism, we performed crosslinking reactions using various *crypto*-CPs and adenylation enzymes from the vicenistatin biosynthesis pathway. We found that C6Br *crypto*-CP efficiently crosslinked with the enzyme, whereas C2Br did not (Fig. 2 ii and iii). In addition, we crystallized the cross-linked complex and determined its structure using X-ray crystallography. The structure revealed distinct protein-protein interactions (PPIs) between the amide bond-forming enzyme and the CP, including hydrophobic contacts, salt bridges, and hydrogen bonds. Mutational analysis of the key amino acid residues confirmed the importance of these PPIs. The C6 linker was found to optimally position the electrophilic α-bromoacetyl group for reaction with the cysteine residue introduced at the active site of the adenylation enzyme. This suggests that the length of β-aminoacyl-CP is crucial for its interaction with the alanyl adenylate intermediate at the enzyme's active site. Interestingly, we discovered a unique CH–π interaction between the dimethyl moiety of pantetheine and a conserved Tyr residue in this family of adenylation enzymes (Fig. 2-iv). This unexpected interaction appears to be critical for positioning the β-amino group of β-aminoacyl-CP.

Conclusion

The PPI between the A domain and the CP domain is crucial for the selective ligation, as the A domain recognizes β-aminoacyl-CP. Additionally, we discovered that the pantetheine arm linked to the CP domain interacted with a unique amino acid residue in the A domain. This finding suggests that, in AT/A domain exchange experiments aimed at producing unnatural polyketide analogs, it may be necessary to select an appropriate acylated pantetheine in addition to considering the PPI with the CP domain. To address this, we are investigating various interactions between acylated CPs and AT/A domains to enable rational domain exchange with the ultimate aim of producing the designed polyketide compounds through fermentation.

References

(1) Miyanaga, A., Nagata, K., Nakajima, J., Chisuga, T., Kudo, F., and Eguchi, T. (2023) Structural Basis of Amide-Forming Adenylation Enzyme VinM in Vicenistatin Biosynthesis, *ACS Chem. Biol.* **18:** 2343-2348.