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Mechanism of Integrated β -Lactam Formation by a Nonribosomal Peptide Synthetase during Antibiotic Synthesis

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Abstract

Modular nonribosomal peptide synthetases (NRPSs) are large, multidomain engines of bioactive natural product biosynthesis that function as molecular “assembly lines” in which monomer units are selectively bound, modified, and linked in a specific order and number dictated by their mega-enzyme templates. Recently, a condensation domain in an NRPS was discovered to carry out the synthesis of an integrated β -lactam ring from a substrate seryl residue during antibiotic biosynthesis. We report here a series of experiments supporting a mechanism that involves C–N bond formation by stepwise elimination/addition reactions followed by canonical NRPS-catalyzed amide bond synthesis to achieve β -lactam formation. Partitioning of reactive intermediates formed during the multistep catalytic cycle provided insight into the ability of the NRPS to overcome the reversibility of corresponding reactions in solution and enforce directionality during synthesis.

In the thiotemplate model of nonribosomal peptide (NRPS) synthesis, an amino acid is specifically recognized by the adenylation (A) domain of each module and activated by ATP for transfer to its intramodular peptidyl-carrier protein (PCP). Post-translational phosphopantetheinylation of the PCP provides an ~ 18 Å “arm” to present the upstream “donor” amino acid or growing acylpeptide thioester intermediate to the condensation (C) domain of the downstream module for peptide bond formation with, and concomitant transfer to, the intramodular PCP bearing its “acceptor” amino acid.^{1–4} The donor and acceptor sites comprise two subdomains of the C domain, which can be visualized as an elongated valley that localizes the reacting partners delivered by their respective mobile phosphopantetheine arms. At the center resides a conserved HHxxxDG catalytic motif.⁵ The second His (bold) has been considered a general base or, more simply, to orient the acceptor for facile peptide bond formation.^{6–8}

A previously unknown dimension to C domain catalysis was recently discovered to act in the biosynthesis of the monocyclic β -lactam antibiotic nocardicin A [1 (Figure 1A)]. The

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Author Contributions

C.A.T. and D.H.L. designed the study, analyzed and discussed the results, and prepared the manuscript. D.H.L. performed reported syntheses and biochemical experiments.

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b00411. Materials and methods, Figures S1–S9, and ¹H NMR and mass spectroscopy traces of relevant compounds (PDF)

Importantly, a modified catalytic motif exists in C₅ of NocB, which harbors an additional His residue directly N-terminal to the C domain HHxxxDG active site signature, that is, H⁷⁹⁰HH⁷⁹²xxxDG. As depicted in Figure 2B, a mechanism was proposed in which the additional His was invoked to cause β -elimination of hydroxide (water) to give the electrophilic dehydroalanyl tetrapeptide thioester 9 linked to PCP₄ and bound in the donor site of C₅. β -Addition then of L-Hpg delivered on PCP₅ gives C–N bond formation.¹² Conventional peptide bond synthesis mediated by the remaining His pair completes β -lactam formation and transfer of the now modified pentapeptide to PCP₅ for final epimerization and hydrolytic release of pro-nocardicin G in the TE domain.¹¹ This proposed mechanism was supported by similar utilization of the dehydroalanyl tetrapeptide 9 to give product formation and by mutational analyses of the active site histidines.¹² Specific labeling and kinetic experiments are reported here to more rigorously interrogate this exceptional biosynthetic process and to gather support for the overall proposed mechanism.

Our ability to fully reconstitute the catalytic activities of the C domain in M5-TE by supplying tetrapeptides linked to PCP₄ in trans opened the way to detailed investigation of the key product-forming steps performed by NocB (Figure 2B).¹² Especially striking in the course of the latter experiments was the absence of detectable dehydroalanyl tetrapeptide either linked to PCP₄ 9 or released from the carrier protein during even complete reaction of the PCP₄-linked seryl tetrapeptide 8. In a more aggressive attempt to observe the proposed PCP₄-dehydroalanyl tetrapeptide intermediate, first ATP and then ATP and L-Hpg were withdrawn from the reaction components with the hope that 9 could be observed. No dehydroalanyl species was detected, and no consumption of the seryl tetrapeptide substrate took place.

The possibility was considered at this point that β -elimination does in fact occur but is masked by rehydration of the highly electrophilic dehydroalanyl tetrapeptide-PCP₄ thioester before its release back into the medium.¹² The possibility that such strict microscopic reversibility could play a part was tested by performing the reconstitution reaction in 50% ¹⁸O buffer. No accumulation of the heavy oxygen isotope ($m/z +2$ Da) could be seen in the unreacted seryl tetrapeptide-PCP₄ by ESI-MS analysis (Figure S1). These data suggest strongly that both the donor tetrapeptide and the acceptor L-Hpg must be present in the C domain active site before elimination to the dehydroalanyl intermediate 9 takes place. Once elimination of hydroxide (water) occurs, the forward commitment to synthesis is strong.

To secure additional support for the proposed mechanism depicted in Figure 2B, a technically more demanding series of experiments was undertaken with L-seryl tetrapeptide-PCP₄ specifically labeled with deuterium (>95%) at the α -carbon (10 in Figure 3A; synthesis described in the Supporting Information). As hoped, parallel incubations of the [α -¹H]- and [α -²H]seryl tetrapeptides-PCP₄ 8 and 10, respectively, in a 100:1 ratio with M5-TE and excess ATP with L-Hpg proceeded at different rates in keeping with a primary kinetic isotope effect (KIE) (Figure 3B). Such a KIE is consistent with removal of the seryl α -hydrogen (deuterium) in a partially rate-limiting step, that is, in accord with the proposed β -elimination to form the dehydroalanyl intermediate 9 and further supported by the observation of only hydrogen at the corresponding carbon of the product, pro-nocardicin G. The reverse experiment with unlabeled tetrapeptide 8 in D₂O medium gave high level of incorporation (>95% per site) of two deuterium atoms (Figure S2) as expected for this β -elimination/addition process and C-terminal epimerization by the TE, as previously established.¹¹

During the early portion of the reaction, tetrapeptide-PCP₄ was presented in a high stoichiometric excess relative to its in trans M5-TE reaction partner, to approximate well Michaelis-Menten steady-state conditions. During the later portion of the reaction, of course, this kinetic approximation breaks down. Nonetheless, an apparent k_H/k_D of ~ 2 at 15 min of reaction decreased to ~ 1.5 over the course of the experiment. That is, at intermediate time points the apparent rate of the initially ²H-labeled substrate increased relative to that of the unlabeled control, an observation most readily accommodated by exchange of the seryl α -deuterium for hydrogen from the medium in partial competition with the overall rate of β -lactam formation. Such behavior is well-known in the organic chemistry and enzymology of the carbonyl group where concerted E2 elimination competes with prior reversible deprotonation/reprotonation at the α -carbon accompanied by stepwise β -elimination, or the E1cb mechanism.¹³ If such a process were acting during β -lactam synthesis in M5, one would expect the gradual accumulation of hydrogen at the seryl α -carbon in unreacted substrate 10 during the course of reaction and a steady diminution of the observed KIE as, indeed, the following experiments established.

Prior experiments had shown that serial mutation of the unusual active site His triad in C5 to Ala blocked all synthesis, a result consistent with their proposed importance for catalysis.¹² The observation of an apparent E1cb mechanism led us to reexamine these mutational studies in greater depth. Gln was substituted for Ala as it is a better isostere of His and bears a side chain of similar polarity.¹⁴ When the M5*H790Q, M5*H791Q, and M5*H792Q site-

specific mutants in the C domain of M5-TE were examined as before in the fully reconstituted catalytic system, M5*H790Q and M5*H792Q were again inactive, but M5*H791Q gave pro-nocardicin G (3) in contrast to the M5*H791A mutant, albeit at a level reduced from that of the wild type (Figure 4A). We were heartened by this result as His at this position in C domain active sites is thought to play a structural, not catalytic, role in peptide bond formation.¹⁵

In an attempt to further investigate the roles of the active site His triad, the M5*H790Q, M5*H791Q, and M5*H792Q mutants were incubated with dehydroalanyl intermediate 9 in the presence of L-Hpg and ATP. Pro-nocardicin G production was not detected from any of the mutants (Figure S3), including M5*H791Q, which produced pro-nocardicin G in the presence of seryl tetrapeptide 8, as described above. This result may be explained by the high reactivity of the dehydroalanyl intermediate 9, which has been previously reported¹² to interfere with efficient conversion to pro-nocardicin G by wild-type M5. Given the reduced level of enzymatic catalysis of M5*H791Q, any pro-nocardicin G production is likely at undetectable levels.

To examine the hypothesized loss of deuterium from [α -²H]seryl tetrapeptide-PCP₄ 10 during the course of reaction to pro-nocardicin G, aliquots were withdrawn upon initiation of reaction and after 6 h. Each was treated with cysteamine (10 mM, 4 °C) to cleave the unreacted tetrapeptide from PCP₄ as the rearranged amide disulfide 11 having a readily protonated primary amine favorable for sensitive ESI-MS analysis (Figure 3A).¹⁶ Exchange was detected from wild-type M5-TE and M5*H791Q, but not from M5*H790Q and M5*H792Q in keeping with their behavior in the overall synthesis observed above (Figure 4B and Figures S4–S8). To further distinguish an E1cb mechanism from concerted E2 elimination, the possibility of rehydration of the dehydroalanyl tetrapeptide and release back into the medium (see Figure S1) was re-examined in 18O buffer using the higher analytical sensitivity of the cysteamine cleavage assay. While exchange of hydrogen into the unreacted [α -²H]seryl tetrapeptide-PCP₄ 10 was seen as before, no ¹⁸O incorporation could be detected (Figure S9). We interpret these findings to mean that both the donor and acceptor sites must be occupied before even reversible hydrogen exchange of the seryl peptide can take place.

In conclusion, condensation domains mediate peptide bond formation and catalyze the assembly step that is essential to a diverse universe of NRPS products. Despite their critical role and an increasing number of C domain crystal structures,^{17,18} mechanistic understanding of this central process remains elusive.⁶ While presentation of “open” or “closed” orientations of a conserved “latch” that covers the active site are apparent,¹⁹ all C domains share a common fold seen earlier in acyltransferase enzymes that catalyze chemically analogous but simpler reactions in the superfamily involving coenzyme A (CoA) esters as the acyl donor; for example, chloramphenicol O-acetyltransferase,²⁰ histone acetyltransferase,²¹ and several others.²² Detailed steady-state kinetic analyses of these enzymes show they all proceed by way of ternary complex formation and an ordered bi-bi mechanism in which the acyl donor is first to bind and CoASH is the last product to depart the active site.²² Related isothermal titration calorimetry experiments clearly demonstrate binding of the donor substrate, but not the acceptor substrate, reinforcing the order of

binding established above and implying that acyl donor binding causes a conformational change that enables acceptor binding to occur, thus completing assembly of the ternary complex and presumably closure of the latch and initiation of reaction,²³ an overall view supported by small-angle X-ray scattering experiments.¹⁷

An opportunity to gain further insight into the dynamics of C domain function in NRPSs is presented by C₅ in NocB where a multistep, but experimentally factorable, synthetic process to create a β -lactam ring embedded in a growing peptide is incorporated into the catalytic program of conventional amide bond formation. The data support an E1cb mechanism and show that, apart from the initial α -carbon deprotonation of the PCP₄-bound seryl tetrapeptide 8, the remainder of the individually probed steps are rendered functionally irreversible and, in so doing, enforce forward synthesis and, thus, “directionality” to the overall assembly process. Correlation of distinct catalytic steps to protein conformational changes has been observed for A domains.²⁴ An especially well-studied case in the ANL superfamily of adenylating enzymes is pchlorobenzoate:CoA ligase.^{25–27} Here mutational, substrate analogue, and detailed kinetic analyses coupled to X-ray crystal structures yielded intimate views of substrate binding, activation, and reaction that could be correlated to protein conformational changes. The evidence points to a sequential kinetic mechanism, ternary substrate binding and chemistry partnered with coupled protein motions across NRPS synthetic domains that underlie directionality and are key to overall synthetic success.^{4,28,29}

Supplementary Material

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Figure 1.

NRPSs involved in nocardicin A biosynthesis. (A) Domain organization of the two nocardicin NRPSs, NocA and NocB. Comprised of a total of five modules, they have been found to activate and condense, in order, L-Hpg, L-Arg, L-Hpg (which is then epimerized to the D configuration), L-Ser, and L-Hpg. The direct product of the NRPSs has been identified as the β -lactam-containing pentapeptide pro-nocardicin G (3). (B) Representative tri- and pentapeptide thioester substrates containing the β -lactam ring. These substrates were recognized and processed by excised nocardicin thioesterase, in contrast to their linear peptide analogues, demonstrating that β -lactam formation must occur earlier on the NRPS.

Figure 2. Proposed mechanism of β -lactam formation. (A) Schematic of *in vitro* reconstitution of M5 activity with tetrapeptidyl-PCP₄ 8. (B) Proposed mechanism of β -lactam formation within the M5 condensation domain by way of dehydroalanyl intermediate 9.

Figure 3.

Mechanistic studies of C_5 -mediated β -lactam ring formation. (A) Schematic of incubation of tetrapeptidyl-PCP₄ 10 with holo-M5 and subsequent cysteamine cleavage of the tetrapeptide. Cleavage yields cysteamine adduct 11, which can then be subject to UPLC-MS analysis for determination of the extent of exchange of the deuterium label. Cysteamine experiments have demonstrated that elimination occurs by an E1cb mechanism. (B) Kinetic isotope effect observed during pro-nocardicin G production. Left: HPLC trace of pro-nocardicin G production when M5(wt) was incubated with 8 over a 6 h time period. Middle: HPLC trace of pro-nocardicin G production when M5(wt) was incubated with 10 over a 6 h time period. Right: Plot of pro-nocardicin G production as observed by HPLC. Higher concentrations are observed in the reaction with unlabeled 8 than in that with 10, demonstrating a kinetic isotope effect.

Figure 4.

Role of catalytic histidines in β -lactam formation. (A) HPLC traces of reactions of indicated M5 constructs incubated with tetrapeptidyl-PCP₄ 8. Pro-nocardicin G production was observed with M5(wt) and mutant M5*H791Q, but not with mutants M5*H790Q and M5*H792Q. (B) Exchange of deuterium at the α position of serine, as observed through cysteamine cleavage. Left: representative mass data, from M5(wt) reaction, to determine the extent of exchange at reaction initiation (t = 0 h) and at 6 h. In the case of the wild-type reaction, significant exchange was observed as demonstrated by the increase in the intensity of the 694.2797 [M + H⁺] peak. Right: summary of mass data from cysteamine cleavages in the absence of M5 (control) and presence of either M5(wt) or M5*H790Q, M5*H791Q, and M5*H792Q. Averages and standard deviations over three experiments are shown.