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Priming Type II Polyketide Synthases via a Type II Nonribosomal Peptide Synthetase Mechanism

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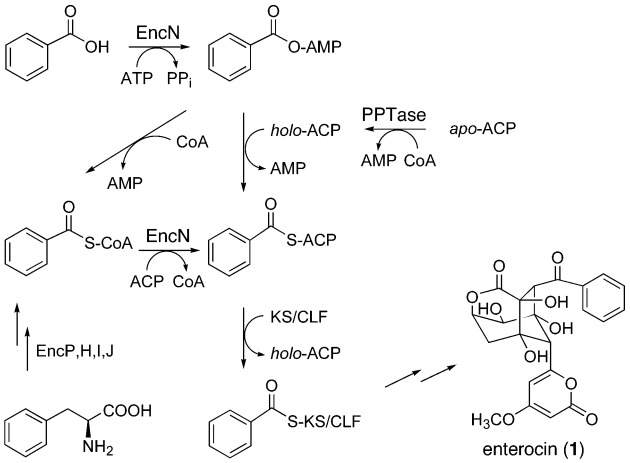
Nature has evolved several strategies for the activation and transfer of carboxylated substrates in the biosynthesis of polyketide metabolites.[1] Polyketide synthases (PKSs) utilize a wide assortment of starter units, such as short-chain (branched) fatty acids, various alicyclic and aromatic acids, and amino acids, in the assembly of their products. Often, the composition of the primer unit provides important structural and biological features to the natural product. For the most part, starter units derivatized as coenzyme A (CoA) thioesters are selected and attached to a specific acyl carrier protein (ACP) by a dedicated acyltransferase. This pathway may involve additional proteins, such as the modified ketosynthase (KS) subunit KSQ[2] or the fatty acid synthase FabH homologue KSIII,[3] to facilitate the priming reaction. While modular type I and iterative type II PKSs share these acyltransferase-mediated priming pathways, some type I PKSs, such as the rifamycin synthetase, utilize a very different strategy employing a nonribosomal peptide synthetase (NRPS)-like mechanism.[4] In this scenario, the starter unit containing a free carboxylic acid group is activated to its AMP derivative by an adenylase (A) before loading onto a phosphopantetheinylated *holo*-ACP in a CoA-independent reaction. These two proteins form a didomain located at the N-terminus of the type I modular PKS megasynthase. Here we report that the enterocin type II PKS utilizes a related process involving the monofunctional proteins EncN, which functions as a benzoate:ACP ligase, and the versatile ACP EncC for the activation and transfer of the aroyl acid. This unprecedented type II PKS priming mechanism is flexible, as shown in combination with the actinorhodin (act) and tetracenomycin (tcm) PKS systems.

We previously reported that the bacterium, *Streptomyces maritimus*, harbors two independent biosynthetic pathways to the enterocin (**1**) PKS starter unit benzoyl-CoA, an endogenous pathway from the amino acid L-phenylalanine and an exogenous pathway from supplemental benzoic acid (Scheme 1).[5] The latter pathway is catalyzed by EncN, which we formerly characterized in vivo[5e] and in vitro[5f] as a benzoate:CoA ligase. In the presence of ATP and Mg^{2+}, EncN activates benzoic acid by forming the aroyl-AMP intermediate, which then reacts with the nucleophilic thiol group of CoA to form benzoyl-CoA (Scheme 1). To test whether EncN additionally has ACP ligase activity, much like an NRPS-associated A domain, we prepared a series of recombinant ACPs from type II PKSs, including those from the enterocin (EncC), actinorhodin (ActI-ORF3), and tetracenomycin (TcmM) PKS complexes. We constructed the bifunctional pHIS8-based expression plasmid pBM58 to generate *holo*-ACPs in vivo by coexpressing the octahistidyl-tagged ACPs with the *Streptomyces verticillus* ATCC15003 phosphopantetheinyl transferase (PPTase) Svp protein in *Escherichia coli*.[6] MALDI-TOF MS was used to verify the authenticity

Scheme 1. Biosynthesis of Enterocin (**1**) in *S. maritimus* from L-Phenylalanine and Benzoic Acid



and purity of the *apo* and *holo* forms of the ACPs (Table 1). EncN-catalyzed transfer of [7-^{14}C]benzoic acid to *holo*-EncC was shown to be ATP-dependent, yet CoA-independent, as evidenced by SDS−PAGE analysis of the precipitated proteins followed by autoradiography (Figure 1). This reaction required EncC to be phosphopantetheinylated, as the *apo* protein was not active. Moreover, [7-^{14}C]benzoate was transferred by EncN to the actinorhodin and tetracenomycin ACPs, thereby demonstrating broad ACP tolerance (Figure 1). In each case, the authenticity of the benzoyl-ACPs was confirmed by MALDI-TOF MS with unlabeled benzoic acid, which showed the anticipated mass shift of 104 amu (Table 1). Further structural support of benzoyl-EncC was generated from its independent enzymatic synthesis from *apo*-EncC and benzoyl-CoA with the PPTase Svp followed by HPLC−MS analysis, which showed that the two samples were chromatographically and atomically identical.

[7-^{14}C]Benzoyl-CoA, enzymatically prepared from [7-^{14}C]benzoic acid and CoA and separated from unreacted benzoate by acid−base extraction, was next tested as a substrate for the EncN-catalyzed transfer of the benzoyl unit to the *holo*-ACP. To our surprise, [7-^{14}C]benzoyl-EncC was produced in an ATP-dependent manner (Figure 1). However, it does not appear as if ATP is consumed for energy as AMP and pyrophosphate could separately be substituted for ATP (see Table S2, Supporting Information). ATP may thus act as an allosteric modulator to promote EncN into a catalytically active conformation. This transacylase activity is indeed enzyme-dependent as in the absence of EncN there was no transthiolation reaction (Figure 1).

To evaluate the transfer of the benzoyl unit from the ACP to the active site of the enterocin KS EncA (Scheme 1), we next prepared the recombinant EncA-B heterodimer in *Streptomyces lividans*

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Table 1. Observed (obs.) and Calculated (calcd) Masses of ACPs Measured by MALDI-TOF Mass Spectrometry

	apo-ACP		*holo*-ACP		*benzoyl*-ACP	
	obs.	calcd	obs.	calcd	obs.	calcd
EncC[a]	11802	11811	12155	12151	12256 (12262[b])	12255
ActI-Orf3[a]	11806	11811	12152	12151	12256	12255
TcmM	11646	11646	11987	11986	12091	12090

[a] EncC and ActI-Orf3 have different molecular compositions, but coincidentally have similar molecular masses. [b] Benzoyl-EncC was also generated from *apo*-EncC and benzoyl-CoA with purified Svp.

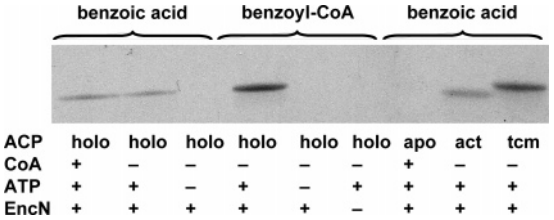

Figure 1. Coenzyme A-independent loading of benzoate to *holo*-ACPs. Autoradiograph of a protein gel resolved by SDS−PAGE (5−15%); the presence or absence of ACP (*holo*-EncC, *apo*-EncC, *holo*-ActI-Orf3, and *holo*-TcmM are designated as holo, apo, act, and tcm, respectively), CoA, ATP, and EncN is indicated in the chart, while the addition of [7-^{14}C]-benzoic acid or [7-^{14}C]benzoyl-CoA is designated above each lane.

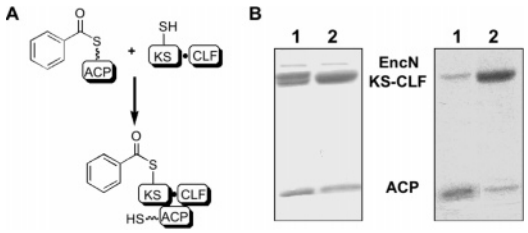

Figure 2. Transfer of [7-^{14}C]benzoyl-EncC (ACP) to the enterocin and actinorhodin KS-CLF heterodimers. (A) The sulfidryl groups of the phosphopantetheinyl group of the ACP and the active-site cysteine residue of the KS are depicted. (B) SDS−PAGE analysis of the reaction mixture described in Figure 1, lane 2 with the addition of the actinorhodin KS-CLF (lane 1) and the enterocin KS-CLF (lane 2) and the autoradiography thereof (left and right panels, respectively).

TK24 via the thiostrepton-inducible *Streptomyces* expression plasmid pXY200.[7] Hexahistidyl tags were placed on the N-terminus of EncA and the C-terminus of the chain length factor (CLF) EncB to facilitate their co-purification by Ni^{2+}-affinity chromatography over a Hi-Trap metal chelate column. MALDI-TOF MS analysis revealed EncA (46 765 Da; 46 934 Da calcd) and EncB (43 629 Da; 43 796 Da calcd) in roughly equal ratios. Incubation with [7-^{14}C]benzoyl-EncC resulted in the transfer of the radiolabeled benzoate from the enterocin ACP to the KS (Figure 2), thereby confirming the role of EncC in the priming reaction. EncC is the lone enterocin ACP and participates not only in the priming reaction with benzoate but also in the seven extension reactions with malonate, as shown by heterologous expression of the enterocin PKS.[8]

We next tested the applicability of the unprecedented enterocin PKS aroyl priming reaction on the actinorhodin PKS, which is primed in vivo with acetate via the decarboxylation of malonate ligated to ACP.[2] Since the enterocin ACP can be substituted for the actinorhodin ACP in polyketide assembly (Xiang, L.; Moore, B. S., unpublished observations), we prepared [7-^{14}C]benzoyl-EncC in situ as described above and then incubated it with the purified actinorhodin KS-CLF heterodimer[9] prepared in recombinant form in a manner similar to that for the enterocin enzymes. The transfer of the benzoyl unit via the enterocin ACP to the actinorhodin KS was clearly evident by SDS−PAGE autoradiography (Figure 2). This result is consistent with earlier observations in the actinorhodin system in which synthetic acyl-ACPs were chain extended by the KS-CLF heterodimer to yield new polyketides by in vitro directed biosynthesis.[10]

In conclusion, we have demonstrated that the enterocin PKS utilizes a novel priming mechanism employing an NRPS-like pathway involving the dissociable enzymes EncN and EncC.[11] This pathway resembles the way in which hybrid type I NRPS-PKS systems activate their carboxylic acid starter units on A-PCP loading didomains for PKS processing. Thus despite the different structural organization of modular and dissociable PKSs, nature has evolved a similar strategy to activate and transfer carboxylic acid priming molecules to their cognate KSs.

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Supporting Information Available: Full experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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