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

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Abstract

Benzoic acid priming of the enterocin and actinorhodin type II polyketide synthase complexes was accomplished *in vitro* via an unprecedented type II non-ribosomal peptide synthetase-like mechanism involving the benzoate:acyl carrier protein (ACP) ligase EncN and the ACP EncC. The transfer of the aryl acid to the ACP is ATP-dependent, yet coenzyme A-independent, as characterized with radiolabeled substrates and protein mass spectrometry. Subsequent transport of the ACP-bound aryl group to the native enterocin and the aberrant actinorhodin keto synthase-chain length factor heterodimers was further demonstrated, thereby demonstrating the potential of this biocatalyst for engineering diverse aryl-primed aromatic polyketide agents.

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 KSQ² or the fatty acid synthase FabH homolog KSIII³ 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 non-ribosomal 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 (I) 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 co-expressing 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 and purity of the *apo* and *holo* forms of the ACPs (Table 1). EncN-catalyzed transfer of [7-¹⁴C]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, [7-¹⁴C]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.

[7-¹⁴C]Benzoyl-CoA, enzymatically prepared from [7-¹⁴C]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, [7-¹⁴C] 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 data). 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 transthioation 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* 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 calc.) and EncB (43,629 Da, 43,796 Da calc.) in roughly equal ratios. Incubation with [7-¹⁴C]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 (L. Xiang, B. S. Moore, unpublished observations), we prepared [7-¹⁴C] benzoyl-EncC *in situ* as described above and then incubated it with the purified actinorhodin KS–CLF heterodimer⁹ prepared in recombinant form in a similar manner 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 in spite of 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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11. The *in vivo* relevance of this mechanism was probed and shown to require EncN, as the minimal expression of EncABCDN in *S. lividans* K4-114 supplemented with benzoic acid yields the benzoate-primed wailupemycins D-G (J. A. Kalaitzis, B.S.M., unpublished observations). We previously hypothesized that EncL was a benzoyl-CoA:EncC aroyltransferase,⁵ yet *in vivo* and *in vitro* experiments show that EncL is not required for enterocin biosynthesis and rather has malonyl-CoA:ACP acyltransferase activity (L. Xiang, M.I., B.S.M., unpublished observations).

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 [7-¹⁴C]benzoic acid or [7-¹⁴C]benzoyl-CoA is designated above each lane.

Figure 2.

Transfer of [7-¹⁴C]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).

Scheme 1.

Biosynthesis of enterocin (**1**) in "*S. maritimus*" from L-phenylalanine and benzoic acid.

Table 1 Observed (obs.) and calculated (calc.) masses of ACPs measured by MALDI-TOF mass spectrometry.

	<i>apo</i> -ACP		<i>holo</i> -ACP		<i>benzoyl</i> -ACP	
	obs.	calc.	obs.	calc.	obs.	calc.
EncC ^a	11,802	11,811	12,155	12,151	12,256	12,255
ActI-Orf3 ^a	11,806	11,811	12,152	12,151	(12,262 ^b)	12,255
TemM	11,646	11,646	11,987	11,986	12,091	12,090

^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.