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Iterative Chain Elongation by a Pikromycin Monomodular Polyketide Synthase

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Polyketides are a diverse class of natural products that possess antibiotic, anticancer, and other biological activities.[1] Biosynthesis of polyketides and fatty acids share a common mechanism where condensation of simple carboxylic acids such as acetate and propionate are catalyzed by complex multienzyme systems, the polyketide synthases (PKSs) and fatty acid synthases (FASs), respectively.[2] In bacterial type II FAS and some type II PKS systems the keto-acid condensation and acyl transfer activities (e.g., malonyltransferase) are expressed as separate proteins that function iteratively until the elongated, processed intermediate is released from an acyl carrier protein (ACP). Eukaryotic type I FAS and PKS (e.g., fungal) as well as prokaryotic type I PKS systems combine up to six catalytic domains (including ketoacyl synthase (KS), acyltransferase (AT), dehydratase (DH), enoylreductase (ER), acyl carrier protein (ACP), and thioesterase (TE)) into multifunctional polypeptides. The type I FASs catalyze iterative condensation/reductive processing reactions to provide palmitic acid. Likewise, fungal type I PKSs are composed of a single multifunctional protein that catalyzes iterative chain elongation and processing, leading to polyketide products such as lovastatin.[3] In contrast, the modular design of bacterial type I PKSs mediates successive elongation where each distinct set of condensation/reductive processing activities or "modules" catalyzes a single elongation cycle before passing the intermediate chain to the next PKS polypeptide. Thus, it is the number of modules comprising a prokaryotic type I PKS that determines the length, functionality, and stereochemistry of the polyketide product.

The mechanistic basis for successive chain elongation in bacterial type I PKSs has been investigated using purified proteins of the 6-deoxyerythronolide B synthase (DEBS). "Docking" of the domains at the NH_2 and COOH ends of these bimodular proteins (DEBS1, -2, and -3) have been proposed to promote the sequential interaction of PKS modules and the channeling of polyketide chain intermediates.[4−7] Although modular PKSs (e.g., DEBS) have evolved to function successively, several recent reports have revealed an additional ability to perform polyketide biosynthesis iteratively. Iterative processing by a type I modular PKS was first observed in vivo by Leadlay and co-workers who determined that "stuttering" of the elongating chain intermediate by DEBS2 module 4 leads to the formation of a 16-membered ring macrolactone.[8] Interestingly, the recent identification of bacterial type I-like PKSs for calicheamicin[9] and C-1027[10] suggests that an iterative mechanism is responsible for synthesis of these polyketide-derived enediyne natural products.

The chain elongation/processing functions of modular PKSs have been studied in vitro using *N*-acetylcysteamine (NAC) diketide thioesters as substrates for native bimodular DEBS PKS proteins, as well as DEBS proteins that have been re-engineered and purified as nonnative monomodular enzymes.[4−7] This work has shown the ability of unnatural DEBS monomodules to catalyze a single chain elongation step, resulting in the production of tri- and tetraketide lactones.

The intriguing observation of in vivo stuttering as well as the in vitro analysis of an "unnatural" DEBS monomodular type I PKS motivated our effort to investigate an iterative function of the native monomodular type I PKSs from the pikromycin (Pik) PKS[11] of *Streptomyces venezuelae*, PikAIII (module 5) and PikAIV (module 6). We propose to define iterative function as a monomodular PKS that catalyzes a minimum of two condensation reactions. To explore this possibility in the Pik PKS, we sought to identify iterative activity using purified PikAIII and PikAIV proteins and exploit the observation that PKS modules may be able to perform "self-priming".[12] In a crude extract, DEBS3 was shown to produce a triketide lactone (TKL) (**1**) in the absence of an acceptable elongation intermediate or other DEBS proteins. In this case, TKL production was dependent on the decarboxylation of methylmalonyl-CoA (or methylmalonyl-ACP) and "priming" of a KS domain with a propionyl unit that could be extended by modules 5 and 6 of DEBS3.[12,13] Thus, we proceeded to investigate the self-priming ability of PikAIII with the expectation that iterative polyketide assembly would produce a triketide chain released by spontaneous lactonization, yielding TKL (**1**). Moreover, we reasoned that in vitro pairing of PikAIII and PikAIV enzymes would generate TKL (**2**) (Figure 1).

PikAIII and PikAIV were individually coexpressed in *Escherichia coli* BL21 (DE3) with the *Bacillus subtilis sfp* gene encoding a phosphopantetheine transferase[14] for posttranslational modification of the ACP domains. The PikAIII protein was engineered with a NH_2-6×HIS tag, and PikAIV was modified to contain a COOH-6×HIS tag. Both proteins were purified by Ni-affinity chromatography and were assayed with 2-[methyl-^{14}C]-methylmalonyl-CoA as the substrate. The reaction products, **1** and **2**, were visualized by radio-TLC and rigorously identified using synthetic standards. We found that PikAIII indeed operates iteratively as a single multifunctional protein as shown by its ability to synthesize TKL (**1**). Reaction of 2-[methyl-^{14}C]-methylmalonyl-CoA with PikAIII and PikAIV yielded TKL (**2**), consistent with our hypothesis that self-priming of PikAIII allows elongation by PikAIII and PikAIV. The rate of synthesis (k_{cat}) for **1** and **2** was determined to be 100-fold lower than the elongation rates ($k_{cat} = 0.005-0.01$ min^{-1}) observed for PikAIII and PikAIV primed with a *N*-acetylcysteamine (2*S*,3*R*)-2-methyl-3-hydroxypentanoic diketide thioester (B. Beck,

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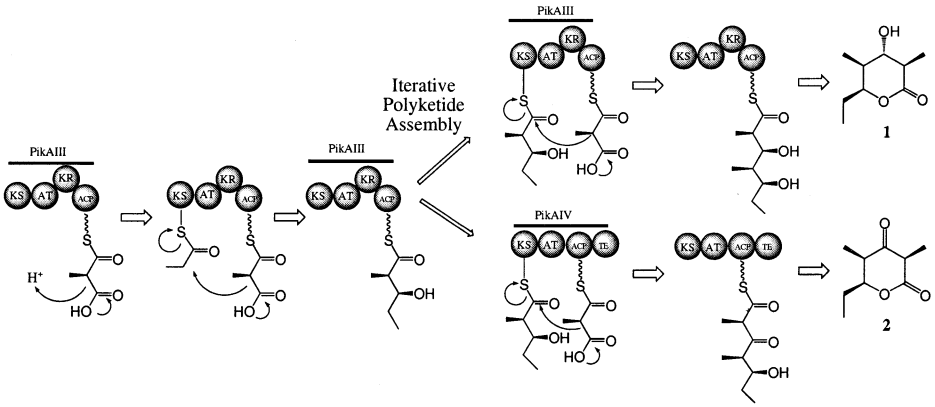

Figure 1. Proposed self-priming mechanism for triketide lactone formation by PikAIII and PikAIV.

unpublished results). As expected, PikAIV alone did not produce a TKL product because the enzyme lacks a KR domain required to generate the hydroxyl group necessary for nucleophilic attack leading to lactonization. Reaction of these proteins with radiolabeled [1-^{14}C]-propionyl-CoA and unlabeled methylmalonyl-CoA substrates failed to produce radiolabeled TKLs, indicating that the priming reaction requires enzymatic decarboxylation of methylmalonyl-CoA.

The processing of a polyketide chain intermediate by a PKS module has been shown to proceed through a homodimeric active site.[13,15,16] Accordingly, once primed with a propionyl unit, the first extension reaction leading to diketide formation by PikAIII would be predicted to follow the established pathway. However, the final extension reaction producing a triketide intermediate could occur through two possible mechanisms. Iterative cycling of the diketide from ACP_5 to the KS_5 domain on the same homodimer would precede condensation with a new methylmalonyl-CoA extender unit producing the triketide chain elongation intermediate. Alternatively, an inter-modular transfer could occur, whereby a PikAIII homodimer with a diketide attached to the ACP_5 domain reacts with a separate PikAIII homodimer loaded with methylmalonyl-CoA. To distinguish between these two mechanisms we reasoned that polyketide chain transfer between PikAIII and PikAIV would be the favored path for the diketide intermediate rather than inter-enzyme transfer between PikAIII homodimers. Supporting this assumption is the demonstration that docking domains between interacting modules ensure sequential interaction of PKS modules and channeling of substrates.[4−6] Additionally, the purified PikAIII protein contains an NH_2-6×HIS tag sequence (13 amino acids) that may interfere with the interaction between PikAIII homodimers, whereas the COOH-terminal location of the PikAIV HIS tag presumably does not interfere with PikAIII−PikAIV interaction.

When 2-[methyl-^{14}C]-methylmalonyl-CoA was reacted with PikAIII and PikAIV, only TKL (**2**) was produced. The exclusive synthesis of TKL (**2**) provides experimental evidence that a diketide intermediate formed by self-priming of PikAIII is preferentially elongated by PikAIV rather than being iteratively processed by PikAIII. However, when PikAIII was incubated with a mutant form of PikAIV with KS and ACP catalytic domains inactivated by site-directed mutagenesis, no inhibition of TKL (**1**) synthesis was observed. Since PikAIII would be expected to interact preferentially with PikAIV rather than another PikAIII homodimer, the synthesis of comparable amounts of TKL (**1**) by PikAIII alone and in the presence of inactive PikAIV is consistent with the proposed iterative processing of the diketide chain intermediate by PikAIII. Thus, the inability to transfer to a catalytically active module results in the iterative processing of the polyketide chain by PikAIII.

The finding that a type I monomodular PKS can perform iterative polyketide synthesis provides another evolutionary link between the diverse classes of polyketide biosynthetic systems. This characteristic may reflect the functional remnant of a progenitor FAS or PKS enzyme that has subsequently evolved into a bacterial type I modular system. Investigations focused on the evolution of these unique mechanisms of small-molecule assembly may provide new strategies to generate greater diversity of polyketide compounds through combinatorial biosynthesis.

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Supporting Information Available: Construction of overexpression vectors, protein purification methods, and assay conditions; radio-TLC data of TKL product formation as well as the synthetic schemes used to make reference compounds (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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