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Structure and Mechanism of Assembly Line Polyketide Synthases

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Abstract

Assembly line polyketide synthases (PKSs) are remarkable biosynthetic machines with considerable potential for structure-based engineering. Several types of protein-protein interactions, both within and between PKS modules, play important roles in the catalytic cycle of a multimodular PKS. Additionally, vectorial biosynthesis is enabled by the energetic coupling of polyketide chain elongation to the channeling of intermediates between successive modules. A combination of high-resolution analysis of smaller PKS components and lower resolution characterization of intact modules and bimodules has yielded insights into the structure and organization of a prototypical assembly line PKS. This review discusses our understanding of key structure-function relationships in this family of megasynthases, along with a recap of key unanswered questions in the field.

Graphical Abstract

Introduction

Assembly line polyketide synthases (PKSs) are remarkable multifunctional catalytic machines responsible for the biosynthesis of a structurally diverse range of natural products[1] with a comparably broad spectrum of biological activities[1,2]. These modular enzymes are evolutionarily and mechanistically related to fatty acid synthases. Individual active sites operate sequentially on protein-bound reactive intermediates to yield a

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polyketide product; in most cases, a given enzyme within the protein assembly line catalyzes one and only one reaction in the overall biosynthetic pathway.

Perhaps the most notable feature of this family of PKSs is their immense size. For example, the 6-deoxyerythronolide B synthase (DEBS, a prototypical assembly line PKS[3]) is an $\alpha_2\beta_2\gamma_2$ homodimer with a molecular mass exceeding 2 MD[4]. The operational unit of an assembly line PKS is termed a “*module*” (Figure 1), which is comprised minimally of a ketosynthase (KS), an acyltransferase (AT), and an acyl-carrier protein (ACP) domain. The KS receives the growing polyketide chain from an upstream module via an ACP thioester-to-KS active site thiol acyl transfer reaction, hereafter referred to as “*chain translocation*”. It also catalyzes a decarboxylative Claisen condensation between this incoming polyketide chain and an ACP-bound malonyl (or substituted malonyl) extender unit, a reaction that is hereafter referred to as “*chain elongation*”. The AT transacylates, via a thioester exchange reaction, the extender unit from an appropriate acyl-CoA species onto the pantheineyl side chain of an ACP. Thus, the ACP serves as a tethering site that is occupied first by the extender unit and then the newly elongated polyketide chain. Importantly, each ACP domain in a homodimeric PKS shows preference for a subset of active sites in the homodimer. For example, an ACP domain can interact with either AT domain within the homodimeric module[5]; however, during chain elongation it only collaborates with the KS domain across the dimer interface, and during chain translocation it preferentially partners with the downstream KS domain from the same polypeptide chain[6]. Thus, in the truncated derivative of DEBS shown in Figure 1, the propionyl primer is predicted to hop from the KS1 domain shown explicitly to its ACP1 partner not shown. The diketide is then translocated to the KS2 domain also not shown. Upon further elongation to a triketide, it returns to the ACP2 domain shown in the Figure.

Components of a PKS Module

By now, prototypical structures of individual domains and multi-domain fragments covering an entire canonical PKS module have been solved at atomic resolution. In this section we provide a cursory overview of the salient structural features of these components, as these prototypes have been covered in-depth in numerous other review articles [3,7].

Starting at the interface between DEBS1 and DEBS2, corresponding to Modules 2 and 3, as illustrated for the truncated DEBS derivative shown in Figure 1, as well as for the interface between DEBS 2 and DEBS3, involving Modules 4 and 5, PKSs utilize docking domains to enhance the rate and specificity of intermodular chain translocation across distinct proteins. A representative NMR structure, comprising ~80 residues from the C-terminus of the donor DEBS Module 4 artificially fused to ~40 residues from the N-terminus of the acceptor DEBS Module 5, revealed coiled-coil interactions that stabilize individual homodimeric modules while also facilitating specific intermodular docking[8]. It should be noted that these interactions are relatively weak, with K_D parameters estimated in the range of 1–10 μ M[9,10], consistent with the transient lifetimes of intermodular ACP–KS complexes that has so far prevented their isolation and direct analysis.

The major component of a PKS module is the KS and AT domains, which together comprise 50–90% of the mass of each module at just under 200 kD for each subunit of the homodimeric protein. Two highly similar high-resolution structures of KS-AT fragments from DEBS Module 3 and Module 5 have been reported[11,12]. In addition to revealing an extensive protein-protein interface between the two KS subunits that presumably is a major contributor to the stability of an intact homodimeric module, the KS-AT didomain assumes an extended conformation with the C₂ axis of symmetry running through the N-terminal docking domain and the KS, and each AT domain extending outwards, with the AT-KS•KS-AT complex in a near linear array with a bend of 19–25°[11,12]. The KS and AT domains are separated by a linking domain with a ferredoxin-like protein fold. The overall conformation of the two KS-AT fragments of DEBS modules is identical to their counterparts from the vertebrate fatty acid synthase[13], and also overlaps well with structures of *trans*-AT PKS modules, which lack an integrated AT domain but retain a subdomain analogous in structure to the KS-AT linker[14].

The C-terminus of the KS-AT interface most often connects to a ketoreductase (KR) domain. In most cases, this KR catalyzes reduction of the β-ketothioester product of chain elongation with exclusive control of the α- and β-carbon stereochemistry. In some cases, such as DEBS Modules 2, 5, and 6, the KR domain catalyzes the direct reduction of the (2*R*)-2-methyl-3-ketoacyl-ACP intermediate generated by the linked KS domain. In others, such as DEBS Module 1, the KR domain catalyzes an epimerization at the α-carbon to the corresponding (2*S*)-2-methyl-3-ketoacyl intermediate prior to β-ketoreduction. Occasionally, KR catalytic domains can be redox-inactive but epimerase-active, as in the case for the KR domain of DEBS Module 3. The crystal structure of the KR domain from DEBS Module 1 has been solved[15]. This 51kD monomeric protein is not expected to make contact with the two-fold axis of symmetry of a PKS module and includes a larger structural subdomain and a smaller catalytic subdomain[15]. Additional structures of KR domains from other PKS modules have validated these architectural features, while also highlighting key differences in the active sites of KR domains with distinct reduction stereospecificity, and both with and without intrinsic epimerase activity.[16–20].

At the C-terminus of a canonical PKS module is the 10-kD ACP domain. This small but critical domain is post-translationally modified with a phosphopantetheine (Ppant) group that tethers the growing polyketide chain as it is shuttled between the KS, KR, and other optional tailoring domains, and ultimately to either a downstream KS or a terminal thioesterase (TE) domain. The solution NMR structure of the ACP domain of DEBS Module 2 has been solved[21]; like all other proteins of this family, ACP2 is a 3-helix bundle.

The TE domain, which is responsible for the formation of a macrolactone in its natural context, forms a 66 kD homodimer with an axis of symmetry that is shared with the core module. The remarkable substrate channel of the DEBS TE domain passes through the entire length of the protein, presumably allowing it to accept the acyclic substrate from one side and release the 14-membered macrocyclic product on the opposite side of the active site[22]. The structures of TE domains from other PKSs have also been solved, and appear to have similar features[23,24].

Recently, a crosslinked ACP-AT complex from the vicenistatin synthase has been crystallized. Its structure provides the first insight into the binding interface between these two domains[25]. The vicenistatin AT is atypical in that it transacylates a dipeptidyl substrate between two distinct ACP domains, but the findings nonetheless have broader implications for understanding assembly line PKSs. Using 1,2-bis(maleimido)ethane as a bifunctional crosslinking agent and a non-catalytic Ser→Cys mutant of the AT, Miyanaga and co-workers were able to prepare a covalent AT-ACP adduct that yielded crystals diffracting to 2.34 Å. Most of the residues responsible for ACP binding to the AT were located on helix II of the 3-helix domain, an observation that is entirely consistent with the results of mutagenic analysis of the AT-catalyzed reaction[26].

Architecture of full PKS modules

To date, there are not yet any atomic resolution structures of intact PKS modules. Our current understanding of assembly line PKS structure is therefore based on combining high-resolution structural data for individual domains or didomains derived from PKS modules, as summarized above, with lower resolution models of intact modules derived from: (i) single-particle electron cryo-microscopy (cryo-EM), (ii) small angle X-ray scattering (SAXS), and/or (iii) homology to the porcine fatty acid synthase that has been characterized at ~3.2 Å resolution[13].

Dutta et al. used cryo-EM to study the architecture of intact Module 5 of the pikromycin synthase (PikAIII), a 328 kDa homodimer that is closely related to DEBS Module 5[27**]. The electron densities of PikAIII (7.3–9.5 Å resolution) were modeled using the crystal structures of the homologous domains previously reported for DEBS. The resulting architecture (Figure 2a) revealed a remarkable difference between PikAIII and both structurally characterized KS-AT didomains from DEBS. Notably, the module forms an arch-shaped dimer with the KS as the capstone. Each AT domain is bent downward, with the KR domains forming the legs of the arch. As a result, the C-terminal ACP domains reside within the reaction chamber constructed by the arch. The relative orientation of the N-terminal coiled coil and the KS domains of PikAIII is analogous to that observed in the KS-AT didomain of DEBS Module 5, but the AT domains are rotated 120° relative to their position along the C₂ axes of symmetry in the porcine fatty acid synthase, both KS-AT fragments of DEBS, as well as a KS-AT fragment of the mycocerosic acid synthase, all previously solved at atomic resolution[28**]. Moreover, in the cryo-EM structure of PikAIII, the AT forms an extensive interface with the KS of the opposite dimer.

An alternative model of a complete PKS module (Figure 2b) has been proposed based on small angle X-ray scattering (SAXS) analysis of the Module 3+TE protein shown in Figure 1, as well as a homodimeric subunit (DEBS3) of DEBS harboring Modules 5 and 6 along with the TE[29*]. The models of these complete PKS modules show their KS-AT regions in the same extended, only slightly bent linear conformation that has been observed in the high-resolution X-ray structures of the DEBS and the mycocerosic acid synthase KS-AT fragments, as well as the vertebrate fatty acid synthase. Additionally, the bimodular DEBS3 was inferred to assume a tree-shaped conformation, with Modules 5 and 6 arranged co-linearly but with minimal intermodular interactions. Considerable flexibility was observed

via SAXS analysis, presumably reflecting the multidomain architecture of these extraordinarily large proteins.

Very recently, Herbst et al. reported high resolution hybrid crystal structures of the KS-AT condensing region and the DH- Ψ KR-ER-KR modifying region of the *Mycobacterium smegmatis* mycocerosic acid synthase (MAS), a fully reducing iterative PKS that shows closer sequence resemblance to PKSs than to fatty acid synthases[28]. The condensing and modifying regions of MAS are connected by a thin strand of two short peptide loops, indicative of considerable flexibility between these two architectural units. This iterative PKS structure further informs our understanding of the structure of assembly line PKSs.

The *trans*-AT assembly line PKSs, which lack dedicated AT domains, have been considerably less extensively characterized. These systems use shared AT domains that operate *in trans* to acylate all ACP domains of the PKS with the same extender unit, most often malonyl-CoA. Davison, et al. characterized Module 5 of the virginiamycin synthase (VirA5) using SAXS, and uncovered some surprising structural features[30]. This module has a KS domain and two discrete ACP domains with flexible inter-domain linkers and a C-terminal docking domain that allows for interaction with the next module downstream. Such dual-ACP modules are prevalent in about a third of *trans*-AT PKSs, especially where intermediates undergo β -methylation[31]. It is thought that the first ACP participates in the chain elongation reaction and transfers the chain to its partner ACP, which affords greater interaction with modifying enzymes by virtue of being located in a more flexible region of the module. The overall structure of VirA5 depicts a dimerized KS domain forming the base, followed by an interdomain linker with homology to the KS-AT linker regions found in *cis*-AT systems. Indeed, the molecular envelope encompassing the KS region of VirA5 agrees with seven AT-less crystal structures reported by Lohman et al.[14], all of which contain this compact KS-AT interdomain linker that is hypothesized to serve as the site for *trans*-AT docking on PKS modules of this sub-family; such a proposal is consistent with the observation that *trans*-ATs have broader ACP specificity than their *cis*-AT counterparts[32]. Abutting this linker is the proximal ACP, followed by an elongated and flexible 28-residue linker region and the second ACP. Unlike the N-terminal docking domain, the C-terminal docking domain does not self-dimerize, suggesting that the latter region only associates with the corresponding N-terminal docking domain of the downstream module.

Structural models for KS-ACP interactions

A distinctive pattern of KS-ACP interactions is the hallmark that distinguishes assembly line PKSs from other homologous polyketide synthases as well as vertebrate fatty acid synthases. The latter families of multi-enzyme assemblies catalyze chain growth via a committed partnership between a KS and an ACP domain from chain initiation to its ultimate release. In contrast, as summarized in Figure 3, the catalytic cycle of an assembly line PKS module requires that each KS catalyze chain translocation in partnership with one ACP domain, followed by chain elongation by intramodular interaction with a distinct ACP. Thus, translocation and elongation may be viewed as two KS-catalyzed half-reactions that collectively comprise a ping-pong turnover mechanism. Failure to discriminate between two

very similar ACP partners in either half-reaction would lead to retrograde movement of the growing polyketide chain, clearly a forbidden process on assembly lines such as DEBS.

By now it is well recognized that multiple protein-protein interaction mechanisms play a role in the orderly transition of the growing polyketide chain between successive KS and ACP domains, the prototypical example being that of docking domains (Figure 1) [10,33]. Because this mode of association is inherently modular, it has been extensively engineered to facilitate chain translocation between heterologous PKS modules[9*,34,35].

In addition to these docking domains, KS-ACP interactions also play a key role in both polyketide chain translocation[36] and chain elongation[37]. While the ACP surfaces involved in both interactions have been relatively well characterized through mutagenic analysis[38,39], the complementary interface(s) on the KS domain have not yet been mapped. The crystal structures of KS domains from assembly line PKSs revealed an active site tunnel near the dimerization interface, whereas the cryo-EM structure of the PikAIII module identified a second tunnel on the opposite side of the KS from the N-terminal docking domains.

To visualize the ACP binding sites on the KS surface, we computationally generated KS-ACP docking models for the crystallographically characterized KS domain of DEBS Module 5. Our models were constrained by experimental data identifying ACP residues that play key roles in chain translocation from ACP4 to KS5 (Figure 4A) or in chain elongation between ACP5 and KS5 (Figure 4B). Analogous models between DEBS ACP4/5 and KS5 were also generated with the module conformation that has been proposed based on cryo-EM analysis of PikAIII (Figures 4C&D). These models reveal notable differences.

In the models shown in Figures 4A and 4B, both ACP4 and ACP5 dock at the canonical entry point to the KS active site that has been identified in all PKSs and vertebrate fatty acid synthases to date. The major difference between the proposed mode of interaction during chain translocation (Figure 4A) and elongation (Figure 4B) lies in the relative orientation of the ACP on the KS-AT fragment surface. Namely, the C-terminus of Helix 1 makes extensive contact with the KS during chain translocation, whereas during chain elongation Helix 1 is rotated up and away from the homodimer interface. In contrast, the corresponding models based on cryo-EM analysis (Figures 4C and 4D) predict that the ACP domains dock on opposite sides of the KS domain. In both models for chain translocation (Figures 4A and 4C), the distance between the C-terminus of ACP4 and the N-terminal docking domain of Module 5 can be bridged, at least in theory, by the C-terminal docking domain of Module 4, although this distance is considerably larger in the X-ray structure derived model than the cryo-EM derived model (86 Å versus 37 Å). Which of these alternative modes of KS-ACP interaction represent the catalytically relevant chain translocation and chain elongation states remains to be established. This problem represents an exciting frontier for the leading edge of structural biology methods, and has been elevated to special significance given recent studies demonstrating that domain-domain interactions are considerably more important to the fidelity of assembly line polyketide biosynthesis than are enzyme-substrate interactions[40].

Energetics of assembly line PKS turnover

Whereas protein-protein and protein-substrate interactions can, in principle, account for the specificity of assembly line biosynthesis (i.e., which enzyme preferentially acts upon which substrate), the actual channeling of intermediates along the preferred pathway requires tight coupling between chain elongation (the only exergonic reaction in the catalytic cycle of each module) and the switch in ACP specificity of the KS from its elongation partner to its translocation partner. This general principle underlying vectorial processes in biology was elegantly articulated by William P. Jencks several decades ago[41]. A turnstile mechanism has recently been proposed for this switch[42*]. While its structural basis remains unknown, the designation “turnstile” is apt because it precludes more than one polyketide chain from residing on each monomeric unit of a module, even under maximum turnover conditions. As shown in Figure 3, this gating is apparently achieved through an elongation-coupled closure of the turnstile to the upstream ACP partner; the turnstile reopens upon translocation of the newly elongated polyketide chain to the next stage of the assembly line.

A variety of mechanisms can be hypothesized for the operation of this remarkable turnstile. The simplest model involves a conformational change in the module following chain elongation. Indeed, cryo-EM analysis has revealed such a change[43*], although it remains to be established whether the observed conformational switch is responsible for orderly transition of the growing polyketide chain through the module. Alternatively, a covalent mechanism could be postulated in which the elongation-associated carboxyl leaving group is transiently captured by an appropriate active site residue, such as Lys for example, resulting in an electrostatically induced decrease in nucleophilicity of the active site Cys residue in the KS domain. This too represents an exciting mechanistic challenge at the interface between computational and experimental structural biology.

Concluding Remarks

Assembly line PKSs present phenomenal opportunities and challenges at the interface between structural biology and biosynthetic engineering. Although advances in whole-genome sequencing have led to an explosive pace of discovery of these remarkable catalytic machines, a majority of these megasynthases are “orphans” in that their products are still unknown[44]. Extensive analysis of a few prototypical assembly lines has led to considerable support for the theory that protein-protein interactions play critical roles in vectorial biosynthesis, more so than just enzyme-substrate recognition. At the same time, the exceptionally large size and inherent flexibility have so far precluded high-resolution structural analysis of intact PKSs or their trapped catalytically relevant states. Furthermore, although PKS structural data continues to accumulate, its co-deployment with computational approaches to develop experimentally testable mechanistic hypothesis has not yet begun in earnest. Because such advances will be essential for this field of research to realize its full potential, the time is ripe for structural biologists of all persuasions to pay close attention to assembly line PKSs.

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Highlights

- High and Low-resolution analysis yields insight into modular architecture of PKSs
- Energetic coupling of chain elongation controls channeling of intermediates
- Protein-protein interactions are critical for the fidelity of PKS turnover
- Detailed structural information could greatly enhance rational engineering

Figure 1. A truncated three-module derivative of the 6-deoxyerythronolide B synthase (DEBS)
 The first two PKS modules are shown in a distinct color, whereas the domains of Module 3 are colored for ease of comparison with the cryo-EM derived model of PikAIII (Figure 2A) and SAXS-derived model of DEBS Module 3 (Figure 2B). LDD = loading didomain; KS = ketosynthase; AT = acyltransferase; ACP = acyl carrier protein; KR = ketoreductase; KR⁰ = redox-inactive, epimerase-active ketoreductase; TE = thioesterase. Although the above assembly line PKS operates as a homodimer, for ease of visualizing the biosynthetic pathway, only one set of active sites are shown. Covalently linked domains are shown touching each other. The grey tabs refer to peptide docking domains that facilitate chain translocation between modules located in distinct polypeptide chains, such as Modules 2 and 3.

Figure 2. A.) Cryo-EM structure of PikAIII and B.) SAXS-derived model of DEBS Module 3
Equivalent domains are shown in the same color for ease of comparison between the two models and also with Figure 1.

Figure 3. The catalytic cycle of DEBS Module 2 (as depicted in Figure 1)

Step I: translocation of the diketide from the ACP domain of Module 1 onto the active site cysteine of the KS domain of Module 2. Step II: AT-catalyzed attachment of a methylmalonyl extender unit onto the Ppant arm of the ACP. Step III: Elongation of the KS-bound diketide chain by the ACP-bound methylmalonyl extender unit, followed by reduction of the ACP-bound (as depicted in Figure 1). Step I: translocation of the diketide from the ACP domain of Module 1 onto the active site cysteine of the KS domain of Module 2. Step II: AT-catalyzed attachment of a methylmalonyl extender unit onto the Ppant arm of the ACP. Step III: Elongation of the KS-bound diketide chain by the ACP-bound methylmalonyl extender unit, followed by reduction of the ACP-bound (as depicted in Figure 1). Step IV: translocation of the elongated diketide from the KS domain of Module 2 back to the ACP domain of Module 3.

Figure 4. KS-ACP docking models

Docking models are shown for Module 5 of DEBS. Panels A and C feature ACP4 (orange)-KS5 (green) interaction during chain translocation, whereas Panels B and D show ACP5 (yellow)-KS5 (green) interaction during chain elongation. Helix numbers are included for clarity. The models in panels A and B are based on the X-ray structure of the KS-AT fragment of DEBS Module 5 (PDB code: 2HG4), whereas those in panels C and D were derived by fitting individual domains into the cryo-EM maps (EMD-5651 and EMD-5653) of PikAIII.(Footnote)1

(Footnote)¹ Homology models of ACP4 and ACP5 were generated using SWISS-MODEL[45] and the solution NMR structure of the ACP domain of DEBS Module 2 (PDB code: 2JU1). All docking models were generated using ClusPro server[46]. A Ppant group was added to both ACP domains *in silico*. A distance restraint of 10–20 Å between active site residue of KS (C199) and the Ppant attachment site of ACP (S34) was introduced into each docking simulation. Additional experimental constraints used in this calculation are based on extensive mutagenesis data[38,39]. Top 20 models were superimposed and manually analyzed. Models that have lowest energy were selected as representatives. Rigid body fitting was performed using Chimera[47]. Real-space refinement with secondary structure restraints and group B-factor refinement was performed using PHENIX[48]. Figures were generated using PyMOL software (*The PyMOL Molecular Graphics System, Version 1.7 Schrödinger, LLC*).