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Revisiting the Modularity of Modular Polyketide Synthases

Chaitan Khosla^{*,§,±,¥}, Shiven Kapur[§], and David E. Cane[#]

[§] Department of Chemistry, Stanford University, Stanford CA 94305-5080

[±] Department of Chemical Engineering, Stanford University, Stanford CA 94305-5080

[¥] Department of Biochemistry, Stanford University, Stanford CA 94305-5080

[#] Department of Chemistry, Box H, Brown University, Providence RI 02912

Abstract

Modularity is a highly sought after feature in engineering design. A modular catalyst is a multi-component system whose parts can be predictably interchanged for functional flexibility and variety. Nearly two decades after the discovery of the first modular polyketide synthase (PKS), we critically assess PKS modularity in the face of a growing body of atomic structural and in vitro biochemical investigations. Both the architectural modularity and the functional modularity of this family of enzymatic assembly lines are reviewed, and the fundamental challenges that lie ahead for the rational exploitation of their full biosynthetic potential are discussed.

Modularity is a highly sought after feature in engineering design. Large-scale integrated circuits, automobile assembly lines and multipurpose chemical plants are just some examples of the power of modular engineering. (In contrast, notwithstanding its exquisite engineering elegance, a Swiss watch is not known for its modularity.) A modular system may be defined as a multi-component system that can be divided into smaller subsystems, which interact with each other and can be *predictably* interchanged for *functional* flexibility and variety. The two highlighted words merit particular attention as one considers the modularity of catalysts.

Guided by the above definition, the functional flexibility of a catalyst would either refer to its range of chemical transformations or the scope of its substrate tolerance. In the chemical catalysis literature, the term “modular” is frequently used, although it most often refers to the preparative modularity of a catalyst. (Unlike proteins, the synthesis of man-made catalysts is not necessarily governed by modular principles.) A few man-made catalysts with modular reactive or molecular recognition features are known. In such cases, modular reactivity is achieved by swapping the metal center of an organometallic catalyst [1]. Alternatively, modular substrate range results from systematically altering a particular feature of the ligand structure [2]. In practice, this kind of predictable functional modularity invariably encounters serious limitations due to the intimate interplay between the metal and ligand. The ability to fully decouple catalysis from recognition as is, for example, the case for the ultimate catalytic machine, the ribosome, remains a lofty but elusive goal for man-made catalysts.

Against this general backdrop, it is worth reassessing modular polyketide synthases (PKSs), a family of multifunctional catalysts that has received much attention owing to their ability to

*To whom correspondence should be addressed. E-mail: khosla@stanford.edu.

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synthesize a seemingly endless variety of complex natural products [3,4]. As implied in the above definition, it is the prospect of tapping into the functional modularity of these megasynthases (not merely their architectural modularity) that makes them attractive targets for engineering. Schemes 1 and 2 illustrate two different forms of functional modularity that one desires in a modular PKS. In both schemes E_1 , E_2 , E_3 and E_4 are sequentially acting catalysts, and B, C and D are intermediates in the polyketide biosynthetic pathway. In Scheme 1, E_2 is replaced with a different catalyst E_2' to alter a targeted functional group or stereocenter without affecting the rest of the natural product or the PKS turnover rate. Examples include replacing a ketoreductase (KR) domain of a PKS module with (i) a KR having different stereospecificity [5], or (ii) a tridomain comprised of a ketoreductase, dehydratase (DH) and enoylreductase [6], or (iii) an aminotransferase (AMT) for reductive amination of the β -ketoacyl intermediate (not yet demonstrated).

Scheme 2 illustrates a distinct modular principle, in which E_1 and E_2 are replaced by E_1' and E_2' , respectively, so as to accommodate the processing of an alternative functional group or stereocenter (introduced via the substrate A') without affecting the PKS turnover rate. Examples include regiospecific introduction of unnatural primer [7–9] or extender units [10, 11] into a polyketide backbone.

It should be noted that in both of the above schemes, wild-type E_3 and E_4 have adequately broad substrate tolerance so as to process C' and D' with chemical and kinetic fidelity. Hence, modularity is not required of these PKS components. Below we review our current knowledge of the architectural modularity of a modular PKS, followed by a critical assessment of its functional modularity as exemplified in Schemes 1 and 2.

ARCHITECTURAL MODULARITY

The depiction of modular PKS-catalyzed biosynthetic pathways through schemes as in Figure 1 has become common practice. The derivation of such schemes rests upon analysis of the primary sequence of the modular PKS, which reliably identifies individual catalytic centers, generally known as domains, within the megasynthase. The organization of these catalytic domains into modules is governed by the principle that, insofar as possible, active sites clustered along the covalent polypeptide backbone are responsible for catalyzing successive reactions in the polyketide biosynthetic pathway and vice versa. While this approach reliably maps individual enzymes from the “assembly line” onto corresponding transformations in the biosynthetic pathway, its oversimplification precludes any description of limitations to the architectural (or functional) modularity of the PKS. There are a significant number of examples, for instance, of apparently competent catalytic KR or DH domains that clearly are functionally silent, based on the structure of the resulting polyketide natural product. Unfortunately, there are as yet no clear guidelines for identifying such catalytically silent domains absent knowledge of product structure.

Within the past few years, several high-resolution structures of prototypical components of the 6-deoxyerythronolide B synthase (DEBS) have been solved [12•,13•,14•,15•,16]. Together, these structures have facilitated the assembly of an atomic structure model of a typical PKS module (Figure 2). Not surprisingly, this model bears a close resemblance to the experimentally determined atomic structure model of the mammalian fatty acid synthase [17]. At the onset, it must be recognized that such models present a snapshot of the PKS (or fatty acid synthase) as opposed to a motion picture of the megasynthase during its catalytic cycle. It is conceivable that conformational dynamics, both within and between domains, are critical to PKS function [18,19,20•,21•,22•]. Notwithstanding this caveat, available atomic models provide a relatively clear picture of the architectural modularity of a PKS, in conjunction with other experimental information. In particular, using an engineering analogy, three architecturally

relevant questions can now be addressed. First, can the assembly line be deconstructed into structurally intact subsystems? Second, to what extent are sub-systems from heterologous sources architecturally compatible with each other? And finally, how universal are the connectors linking subsystems that we wish to interchange in order to achieve functional flexibility?

It has been known for some time now that individual PKS modules retain structural and functional integrity as stand-alone, yet still multifunctional proteins [23,24]. More recently, it has also become clear that the ketosynthase (KS), acyltransferase (AT), acyl carrier protein (ACP), KR and DH domains of a PKS module all retain structural and catalytic integrity as stand-alone proteins so long as the domain boundaries have been judiciously selected [16,25, 26,27••]. (Note that “catalytic integrity” here refers to the intrinsic reactivity of individual active sites. Domain disconnection markedly reduces the turnover number of the module (Figure 3), which is of course a property of the full system.) Therefore, assuming that an intact ER domain can also be disconnected into a stand-alone protein, the domains and modules of greatest interest to a PKS engineer can be regarded as architecturally autonomous subsystems.

In silico analysis of the architecture of a typical PKS module (e.g. Figure 2) also suggests that the KS, AT and KR domains do not directly contact each other. Direct interactions involving these domains are limited to contacts with inter-domain linkers and, of course, with the mobile ACP domain. We speculate that heterologous ACP domains are architecturally compatible with KS, AT and KR domains. Our assumption is based on the fact that ACP-KS, ACP-AT and ACP-KR interactions are weak (but functionally important; vide infra), as well as on the extensively documented structural and functional integrity of a hybrid module in which the ACP of DEBS module 5 has been replaced by its counterpart from module 6 [24,28]. If so, then the question of whether heterologous combinations of catalytically active domains are architecturally compatible is less important than whether the individual domains are compatible with their scaffolding linkers.

Considerably less attention has been focused thus far on understanding the universality of the connectors that link catalytically active domains. Indeed, the very existence of these linkers is essentially ignored in commonly used cartoon representations of modular PKSs such as that in Figure 1. As seen in Figure 2, however, these linkers comprise a significant fraction of the total protein in a PKS module and have well defined architectural features of their own. Some linkers, such as the N-terminal coiled coil linker of module 5, do not engage in significant protein-protein interactions with the remainder of the module and not only can be expressed as stand-alone proteins [29••] but also can be interchanged without loss of function [24,30]. These connectors appear to be truly universal. In contrast, connectors such as the KS-to-AT linker or the AT-to-KR linker may be universal or context dependent. On one hand, they engage in extensive interactions with adjacent catalytic domains; on the other, a majority of such non-covalent interactions involve residues that are highly conserved across different modules [13••]. This aspect of the architectural modularity of a PKS module remains to be explored. If the recombination of catalytic domains and linkers from heterologous sources results in perturbed protein-protein interactions, then the PKS turnover rate will likely suffer even if individual active sites are well folded and retain their intrinsic catalytic properties. A vivid example is illustrated in the third and fourth entries in Figure 3, which summarize the adverse consequence of deleting the AT-to-KR linker (shown in red) on the rate of chain elongation catalyzed by the KS and ACP domains [27••]. Experiments suggest that this impairment is not due to changes in the structure or reactivity of individual domains. Rather, it highlights the potentially critical role of the AT-to-KR linker in facilitating important protein-protein interactions between the ACP and the paired KS and AT domains.

In summary, whereas the complete modules of a multimodular PKS can be accurately viewed as architecturally modular sub-systems connected by universal linkers, the architectural modularity of individual modules remains to be fully explored. To do so, it will be essential to understand at an atomic level the structural implications of recombining heterologous catalytic domains and their scaffolding linkers at an atomic level.

FUNCTIONAL MODULARITY

The sub-systems of a digital signaling device may be architecturally modular, and the connectors that interface these sub-systems may also be universal. However, if the replacement of a sub-system with a functionally equivalent unit results in impedance mismatching, signal transfer across the modified interface is incomplete and some of the signal is reflected back. By analogy, architectural modularity of a modular PKS is a necessary but of itself insufficient condition for the design of kinetically competent hybrid PKSs. Developing methods for accurately predicting the scope and limitations to the functional modularity of PKS domains and modules, as illustrated in Schemes 1 and 2, is arguably the most far-reaching goal for this field of research.

Thus far, the predominant method for exploring functional modularity has been through the construction and *in vivo* (qualitative) product analysis of domain- or module-swapped PKSs. Notwithstanding the conceptual simplicity and power of this approach, it has limited ability to differentiate between kinetically robust versus attenuated hybrid PKSs and even lesser capacity to dissect the nature of the kinetic bottleneck in an unproductive hybrid PKS. More recently, it has been possible to analyze biochemically the properties of hybrid modules and multimodular PKSs [31,32•], which in turn has provided useful insights into the nature of the problems and how they may be surmounted. Perhaps not surprisingly, many of the early chimeric PKS modules were kinetically impaired due to the selection, largely based on analysis of multiple alignments of primary sequence, of what have turned out to be suboptimal fusion junctions between heterologous domains and linkers (for example, see [31]). With the emergence of high-resolution structural data, it is now possible to exploit the architectural modularity of PKSs based on direct structural knowledge of domain junctions in the design of structurally robust chimeric modules and multi-modules. Therefore, we will highlight the major outstanding challenges for understanding and predictably exploiting the functional modularity of PKSs through three illustrative examples below.

As shown in Figure 1, module 3 of DEBS lacks a functional KR domain. Thus, introducing KR activity into this module without compromising its overall turnover rate constitutes a good test of the functional modularity outlined in Scheme 1. Chen et al. attempted to do so by fusing the KS-AT didomain of module 3 to a heterologous KR-ACP didomain at a junction that maintains the module's architectural integrity [32•]. To do so would require effective collaboration between a KS and an ACP derived from different modules. Therefore, they first evaluated the specificity of the KS domain of module 3 for ACP domains from modules 1, 2, 5 and 6 (all of which bear a functional KR). Surprisingly, a 1000-fold range in reaction rate was observed in the catalysis of C-C bond formation (Figure 4). Because ACP5 had the closest k_{cat} and k_{cat}/K_M to those of ACP3, a chimeric module was designed in which KS-AT from module 3 was fused to KR-ACP from module 5. Not only did the resulting hybrid module have a k_{cat} 70% that of the wild-type module, but it also catalyzed reduction of the corresponding β -ketoacyl biosynthetic intermediate. While this example illustrates the potential for functional domain interchange, it could be argued that it falls short of the above definition of modularity because the selection of the KR-ACP didomain from module 5 required trial-and-error evaluation of several ACP domains harboring functional KR neighbors. Predictable interchange of subsystems in this case would require the identification of a common "epitope" on all ACP domains of PKS modules that dictates KS-ACP specificity so as to enable selection

of the appropriate KR-ACP didomain based on *in silico* sequence or structure analysis. Some progress towards the identification of such an epitope has been made in related (Type II) PKSs and fatty acid synthases [33,34]; however, a general solution to this problem has not yet been proposed in the context of modular PKSs. Importantly, it must be recognized that this epitope does more than simply facilitate specific KS-ACP recognition but profoundly influences the transition state of the C-C bond forming reaction, as exemplified by the dramatically different values of k_{cat} for C-C bond formation in the presence of alternative ACPs [32••].

Another aspect of the functional modularity of a modular PKS that is still incompletely understood is the flexibility for regiospecific introduction of an unnatural extender unit. Because extender unit acyl transfer onto the ACP is not typically rate-limiting within the catalytic cycle of a given module, the challenge is two-fold. On one hand the need to maintain the architectural integrity of the module is paramount as one interchanges (or engineers) AT domains (see, for example, Figure 3). On the other hand, downstream active sites may also require exchange (or engineering) in order to accommodate the unnatural extender unit (Scheme 2). The architectural modularity of a PKS module is discussed above. We simply note that at least two alternatives to AT domain interchange have been proposed that minimize architectural perturbations in the PKS module. In one approach, the substrate binding pocket of the AT domain is mutated to alter its specificity [11,35]. In another, the AT domain is inactivated and complemented with a co-expressed acyl transferase with the desired specificity [36]. In contrast, no satisfactory approaches have yet emerged for exploiting PKS modularity in order to process the unnatural extender unit through downstream active sites without an appreciable kinetic penalty. For example, a two- to four-fold reduction in C-C bond formation rates was observed in the case of two DEBS KS domains when the natural nucleophilic substrate, methylmalonyl-ACP, was replaced by the unnatural analog malonyl-ACP [32••]. Similarly, a 6-fold reduction in kinetic specificity constant was observed in chain transfer between modules, when the incoming polyketide chain harbored an unnatural α -carbon substituent [37]. Developing predictable strategies for alleviating these kinetic penalties will require a deeper understanding of the structural basis for KS domains to discriminate between subtly different substrates, something that is beyond the scope of available structural models.

The challenges that lie ahead for understanding and exploiting the functional modularity of modular PKSs are perhaps best exemplified by the especially fascinating (but vexing) example of DEBS module 2. Table 1 shows the specificity of this module for accepting and elongating seven diketides and triketides [38••]. Remarkably, whereas certain substrates are transferred onto the KS active site with extremely high efficiency, the KS is still incapable of catalyzing their elongation. (A similar feature has also been observed with module 3 of the pikromycin synthase [39].) Thus it appears that the very same KS can exhibit entirely different specificity for the two successive chemical reactions that it catalyzes, self-acylation and decarboxylative condensation. How does one take this Janus-like behavior into account as one exchanges KS domains or even entire modules? Clearly it is not possible to divide the same active site into subsystems that can be separately interchanged to alleviate kinetic bottlenecks in chain transfer versus chain elongation. Presumably, the trick will lie in predicting which heterologous KS domains have the most desirable combination of recognition features for a given situation, although it remains to be seen how this will be accomplished.

CONCLUSION

In summary, the foundational knowledge for the assessment and rational exploitation of the functional modularity of modular PKSs is only just beginning to emerge, even for a prototypical PKS such as DEBS. Given the embarrassment of riches that awaits exploitation within nature's vast repertoire of modular PKSs, one clearly cannot wait for a case-by-case dissection of individual PKS structures and properties. The challenge therefore lies in the accurate

identification of those features of a PKS that are functionally modular, and in the development of predictive algorithms that exploit this modularity. The old serenity prayer, “give us grace to accept the things that cannot be changed, courage to change the things that can be changed, and the wisdom to distinguish one from the other” is perhaps more applicable to modular PKSs than any other family of biochemical machines discovered in recent times!

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Figure 1. Modular organization of 6-deoxyerythronolide B synthase (DEBS)

Chain elongation occurs minimally through the combined action of the ketosynthase (KS), acyl transferase (AT), and acyl carrier protein (ACP) domains. The final oxidation state of the α -carbon is controlled by the specific combination of ketoreductase (KR), dehydratase (DH) and enoylreductase (ER) domains present in a given module. Once processed, the polyketide chain is either passed to the KS domain of the downstream module or cyclized and released by the thioesterase (TE) domain at the C-terminus of the polyketide synthase. The loading didomain (LDD) is responsible for the selection and subsequent loading of the appropriate priming unit. KR $^\circ$, inactive ketoreductase domain.

Figure 2. Composite atomic model of DEBS module 5

Orange, N-terminal coiled coil linker that facilitates docking between modules 4 and 5; blue, ketosynthase (KS) domain; green, acyltransferase (AT) domain; yellow, KS-AT linker; red, AT-KR linker; cyan, ketoreductase (KR) domain; magenta, acyl carrier protein (ACP) domain. (Only a single ACP domain is shown for clarity.) The sequences (colored by domain/linker of origin) of junctions at which the KS, AT, KR and ACP domains can be deconstructed into stand-alone proteins, or alternatively recombined, are indicated with black arrows. The latter half of the AT-KR linker (red, residues 902–911) and the KR-ACP linker (black, residues 1360–1377) are not shown, as there is no reliable data based on which they can be modeled. The relative orientation of the ACP and KS domains is predicted by *in silico* docking analysis, whereas the relative orientation of the KR domains and the remainder of the module is based on the X-ray structure of homologous porcine fatty acid synthase [17].

Reaction/Catalyst	Relative k_{cat}
>6200
1000
1
<0.03

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Figure 3. Relative turnover rates of alternatively deconstructed versions of DEBS module 3
 Domains and linker regions are colored as in Figure 2. The relative turnover number of the intact module (first entry) is compared with that of a derivative in which: (i) a stand-alone ACP is used (second entry); (ii) the KS and AT are discrete proteins (third entry); or (iii) an analog of (ii) lacking the post-AT linker (fourth entry). In the third and fourth entries, only the KS is shown as a dimer.

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Figure 4. Steady-state kinetic analysis of chain elongation by the KS domain of DEBS module 3 in the presence of five different ACP domains from DEBS

A 1000-fold variation in specificity is observed, with both k_{cat} and k_{cat}/K_M values decreasing as the natural ACP domain (ACP3) is replaced by the ACP domains from modules 1, 2, 5 and 6 of DEBS. Data taken from [32••].

Scheme 1.

Scheme 2.

Table 1

Relative rates of chain transfer to the KS domain of DEBS module 2, and chain elongation by this module of alternative diketide and triketide analogs.

Substrate	Relative rate of chain transfer	Relative rate of chain elongation
1	1
0.21	0.01
0.16	0
7.6	1.5
0.48	0.2
1.9	0
9.8	0