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Minireview

Dissecting and Exploiting Nonribosomal Peptide Synthetases

Qing-Tao SHEN, Xiu-Lan CHEN, Cai-Yun SUN, and Yu-Zhong ZHANG*

State Key Laboratory of Microbial Technology, Shandong University, Ji'nan 250100, China

Abstract A large number of therapeutically useful cyclic and linear peptides of bacteria or fungal origin are synthesized via a template-directed, nucleic-acid-independent nonribosomal mechanism. This process is carried out by mega-enzymes called nonribosomal peptide synthetases (NRPSs). NRPSs contain repeated coordinated groups of active sites called modules, and each module is composed of several domains with different catalytic activities. The familiarity to these domains lays base for the future genetic engineering of NRPSs to generate entirely "unnature" products. The details about NRPSs domain structures and the exploitation of NRPSs are described in this review.

Key words nonribosomal peptide synthetase; domain; combinatorial biosynthesis

Over the past decade striking advances in microbial genetics have propelled a revolution in our ability to deduce, analyze and manipulate the biosynthesis of structurally complex and biologically important families of nature products, one most notable class was known as nonribosomal peptide (NRP). NRPs include many important pharmaceuticals, veterinary agents, and agrochemicals as a result of their unique composition and diverse structures. Some NRPs are illustrated in Table 1.

In addition to 20 proteinogenic amino acids, scientists have spotted several hundred different molecular ingredients in various nonribosomally manufactured peptides, most of which the ribosomal have never heard of [6]. These compounds include so-called right-handed amino acids, the rare twins of the standard left-handed model, as well as molecular relatives of amino acids such as acyl acids. Usually, they also contain loops, which are almost never found in standard proteins. These peculiar nonribosomal peptides are synthesized by mega-enzymes called nonribosomal peptide synthetases (NRPSs), the largest enzymes known in nature [6].

An explosion of discoveries and technological innovations helps us to understand how NRPS efficiently works, and rapidly enhances our capacity to "mute" the structure of nature products. In the near future, it will be possible to modify the structures of natural products in the same precision and convenience as their corresponding binding proteins can do [7]. Fully understanding the components of NRPS assembly line is required before these mega-synthetases can be effectively engineered to produce novel drugs.

Like modular PKSs, NRPSs are made up of a series of modules, and each is responsible for adding one particular unit to a growing peptide chain. Most NRPSs consist of four to ten modules, some—as the peptides they produce—can even reach up to 50 units in length. NRPS for an enzyme can be enormous. For example, the molecular weight of 11-module cyclosporin NRPS (1700 kD) [6] is far larger than that of myoglobin (20 kD), which is ribosomally produced to help store oxygen. Each module of NRPS is composed of domains including minimal domains and optional domains. Adenylation (A) domain, peptidyl carrier protein (PCP) domain, condensation (C) domain, and thioesterase (TE) domain are minimal and necessary for the whole process. While cyclization (Cy) domain, methyltransferase (MT) domain, and epimerization (Er) domain are optional ones. The order of biosynthetic modules from N- to C-terminus on each NRP and the number and type of catalytic domains within each module determine the order of structural and functional elements in the resulting natural products.

Although the immense sizes of NRPSs prevent resear-

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*Corresponding author: Tel, 86-531-8364326; Fax, 86-531-8364326; E-mail, zhangyz@sdu.edu.cn

Table 1 Several NRPs with different activities

NRP type	Cases
Antibiotic	Penicillin, bacitracin, tyrocidine, alamethicin [1], and trichoginA IV [2]
Antifungal drug	Echinocandin
Cytostatic agent	Bouvardins
Immunosuppressive compound	Cyclosporin
Sideropore	Mycobactin, Pychelin, Yersiniabactin [3–5]

chers from deducing their structure using the standard crystallization tricks, which is important to understand how an enzyme works, researchers have broken these enzymes apart and studied the structures of the subunits. The publication of the "TE" subunit structure in "*Structure*" marks the completion of sample structures for all subunits except the "C" domain [6]. Recent progress will help us understand the architecture of these mega-enzymes.

Adenylation (A) Domain

The structure of A domain, responsible for core task of recruiting new amino acids for the growing protein, has been determined with most details. In 1997, Mohamed MA and his colleagues at Philipps-University of Marburg in Germany reported the crystal structure of a 556-residue A domain from *Bacillus brevis*, the source of antibiotic gramicidin; this subunit recognizes phenylalanine [8]. They found that this subunit carries the same chemistry as the ribosomal pathway when activating its substrate as aminoacyl adenylate, but shares no sequence homology with tRNA synthetase I or II [9]. Instead, it shows sequence homology with acyl-CoA ligases and firefly luciferases, and it has nearly an identical fold to *Photinus pyralis* luciferase [10] except the difference in relative rotations of two subdomains. The fold of luciferase is nearly identical to that of PheA despite their relatively low sequence identity of 16%, whereas the A domain of the NRPS shares a sequence identity of 30%–60% [11], indicates that this fold can be general for A domain [12]. PheA consists of two major subdomains, a smaller C-terminal subdomain (B) of about 100 residues and a larger 400-residue N-terminal subdomain (A). Most residues important for substrate recognition are provided by subdomain A except Lys^{517} which is provided by a loop protruding from subdomain B.And this Lys^{517} uses its positively charged ammonia group to coordinate the carboxyl group of the phenylalanine and the ribose phosphate region of AMP, and brings them into the right conformation for the reaction [12]. Several amino acids within subdomain A, between core motifs A4 and A5 [13], form a pocket that could accommodate the sidechain of the substrate. By using the PheA crystal structure and extensive sequence alignments of 160 kinds of "A" domains [13], ten residues that formed the binding pocket where the substrate was to be adenylated were found to be crucial for substrate recognition.

A major role of these residues is to distinguish the sidechains of possible substrates. Within these residues, residues at positions 235 and 517 are nearly invariant, whereas those at positions 278 and 299 are highly variable even in phylogenetic clusters of the same selectivity (Fig. 1). The latter are therefore thought to parallel the wobble position in ribosomal codons [12,13].

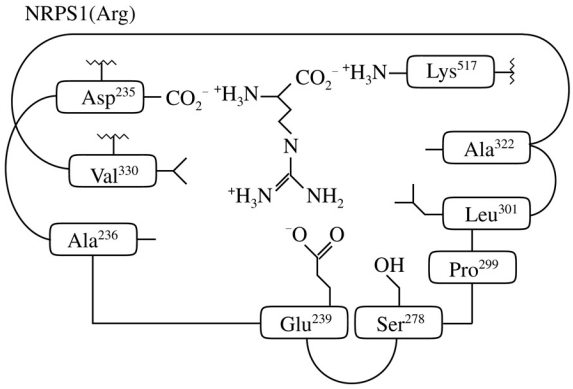

Fig. 1 A two dimensional representation of the specificity pocket from the arginine activity domains of NRPSs [14]

Peptidyl Carrier Protein (PCP) Domain

The second domain, for which the structure is now known, is the PCP domain. This domain is responsible for the fixation of activated substrate before it is passed on to different reaction centers. Before peptide biosynthesis can begin, PCP domain of the polypeptide synthetase must be

"primed" or post-translationally modified from the inactive apo form to active holo one by the covalent attachment of a 4′-phosphopantetheine (Ppant) cofactor to a particular serine residue. This modification is catalyzed by amember of the phosphopantetheinyl transferase (PPTase) enzyme family [15]. The -SH of the Ppant prosthetic group acts as a nucleophile of acyl-CoAs for polyketide synthetases or aminoacyl-AMPs for polypeptide synthetases [16]. The trimodular aminoadipoyl-cysteinyl-valine (ACV) synthetase of penicillin biosynthesis has one PCP domain for each amino acid-activating module, whereas the undecapeptide immunosuppressive drug cyclosporin A is assembled on a mega-synthetase containing 11 PCP domains [7]. The PCP domain of the third module of the *B. brevis* tyrocidine synthetase III (TycC) (TycC3-PCP) was characterized and shown to be functional *in vitro*. The solution structure of this domain has been solved using nuclear magnetic resonance (NMR) spectroscopy [17]. All three carrier proteins (FAS ACP, PKS ACP, and PCP) comprise about 80 residues and are composed of a distorted anti-parallel four-helix bundle with a long loop between the first two helices. The invariant serine residue, where the cofactor binds, is located at the interface between the loop and the second helix; the cofactor shows no interactions with the protein and protrudes into the solvent [12,17].

The PCP structure provides no evidence for the interaction of the loaded substrate with something like a binding pocket. It seems that PCP domains have no substrate selectivity; instead, these domains can be fused to noncognate domains when recombinant NRPS systems are constructed. Functional domain fusions between the A and PCP domains [18], or PCP and C domains [19] have been described.

Condensation (C) Domain

Successive transpeptidation of thioesterified substrates attached to the individual modules allows the peptidyl chain to grow unidirectionally from the amino- to carboxyl-terminal under the control of C domain. The C domain (about 450 residues) is the site of peptide bond formation and chain translocation which catalyzes the peptide-bond formation between two adjacent modules: an upstream peptidyl-S-PCP donor is attacked by a downstream aminoacyl-S-PCP acceptor nucleophile. C domain contains 7 conserved amino acids core motifs, with "HHXXXDG" being most highly conserved [20]. An active site organized by an arginine-aspartate salt bridge, a key histidine acting as a general base, and an asparagine instead of a serine stabilizing the proposed tetrahedral intermediate by hydrogen bonding is important for C domain [21]. C domains catalyze crucial elongation reactions and are the focus of unanswered questions in the NRPS field. The question about how C domain is responsible for the substrates recognition is solved by using many different approaches such as mutational analysis, the making of chimeric proteins or fusion proteins [22,23], the use of aminoacyle-S-CoA substrates [24], and the use of aminoacyl-*N*-acetylcysteamine thioesters [25]. David used aminoacyl-SNACs as small-molecule substrate analogues to mimic both the upstream (donor) substrate, aminoacyl- or peptidyl-S-PCP domain, and the downstream (acceptor) substrate, aminoacyl-S-PCP domain, and found that in general C domains would specifically recognize the sidechain and *L*- versus *D*-configuration of both upstream and downstream substrates [25], while some others reported that C domains have a high substrate selectivity for the coming aminoacyl-S-PCP nucleophile acceptor but are less specific for the incoming peptidyl-S-PCP electrophile donor [24] just as that the ribosomal synthetases transfer peptidyl chain from P site to A site by recognizing the right aminoacyl tRNA in A site.

Although the importance of the C domain in the elongation reaction has been demonstrated by deletion and mutational experiments [26], the catalytic mechanism of peptide-bond formation is unknown and no structural information is currently available for this central domain.

Thioesterase (TE) Domain

Once the elongating chain reaches the final carrier protein domain, the resulted peptide is generally released from the Ppant group that carries the acyl chain during peptide biosynthesis by a reaction catalyzed by a 30–35 kD C-terminal TE domain. The peptide is initially transferred to the conserved active site serine nucleophile located within the GXSXG signature motif located about 100 amino acids from the N-terminus of the TE domain [27]. Deacylation of the resulted peptide-Sery-TE intermediate is proceeded through hydrolysis to release a linear peptide (e.g., the TE of ACV synthetase) or through intramolecular capture by an -OH or -NH_2 group in the peptidyl chain to release a cyclic lactone or lactam (e.g. enterobactin or gramicidin S). Recent advances have shown that the excised TE domain of EntF is both a cyclotrimerizing lactone synthesis and an elongation catalyst for ester bond formation between covalently tethered DHB-Ser moieties

[28]. Further studies [29,30] have shown that this TE domain catalyzed cyclization is quite nonspecific and therefore could be used to prepare a wide range of macrocylic peptide compounds, which may have clinically relevant biologically activity. An additional role for the external TEs is to perform a housekeeping function by hydrolyzing mis-acylated monomers or acyl chains stalled on carrier protein domain [31]. The dimer of TE domains is observed in an asymmetric unit, one is "C" ("closed") conformation and the other is "O"("open") conformation. The catalytic residues (Ser^{80}, His^{207}, and Asp^{107}) are important for the β-hydroxy-acyl-heptapeptidyl-O-Ser TE acyl enzyme intermediate (Fig. 2) [32].

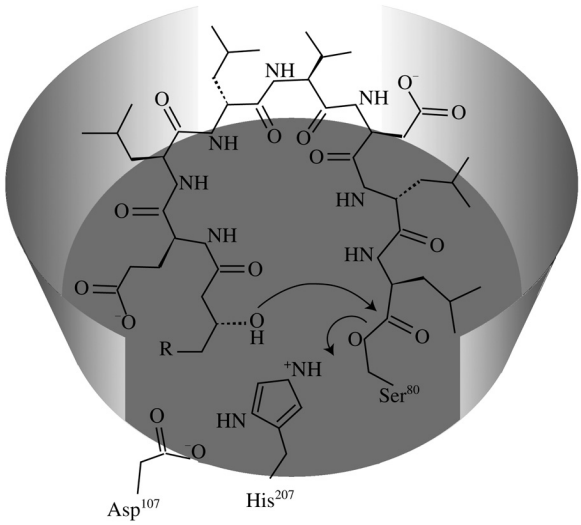

Fig. 2 The β-hydroxy-acyl-heptapeptidyl-O-Ser TE acyl enzyme intermediate is schematized in the hydrophobic active site bowl

Methyltransferase (MT) Domain

A variety of NRPs contain N-methyl amino acids, and some hybrid PK/NRP molecules contain C-methyl groups introduced by dedicated C-methyltransferases (C-MTs). The strategy appears to have MT domains, whether N-MT or C-MT specific, embedded within specific modules. In the case of cyclosporin synthetase (CssA) [33], seven of the eleven amino acids (MeBMT[1], Abu[2], Scr[3], MeLeu[4], *L*-Vol[5], MeLeu[6], *L*-Ala[7], *D*-Ala[8], MeLeu[9], MeLeu[10], MeVal[11]) are N-methylated, exerting a strong effect on conformer preference and perhaps on cyclization efficiency of the linear undecapeptidyl-S-enzyme intermediate. The typical domain order in these seven modules of CssA synthetases is C-A-MT-PCP, supporting the concept that each aminoacyl-S-PCP thioester intermediate in these modules can then be N-methylated via the cosubstrate S-adenosyl methioine (SAM); in two cases (enniatin synthetase and PF 1022A synthetase) N-methylation has been determined to occur on the amino acid monomer prior to peptide bond formation [34,35]. Modification of the upstream amino acid (i.e. acylation of threonine), however, was required for condensation with methylated amino acid [36]. C-methyltransferases are much less common in the NRPS, PKS, and NRPS/PKS assembly lines but two of them appear to be embedded in the 9-domain 350 kD HMWP1 subunit of the synthetase that makes the virulence-conferring sideropore yersiniabactin (Ybt) from the plaque bacterium *Yersinia pesis* [5]. *O*-Methyltransferase activities are also proposed for both NRPS and PKS systems. The saframycin Mx1 NRPS biosynthesis cluster from *Myxococus xanthus* possesses a standalone, 220 amino acid O-methyltransferase located immediately downstream of the two large NRPS genes [37].

Cyclization (Cy) Domain

The most dramatic chemical modifications performed on elongation peptide chains by some NRPS assembly lines are the heterocyclization of cysteine or serine/threonine residue to thiazoline or oxazoline five-membered heterocycle respectively, which alter the residue connectivity patterns and provide both metal-chelating and intercalating groups to the modified peptides. The domains that are responsible for catalytic activities, the Cy domains, are variants of the 50 kD peptide bond-forming Condensation domains [38]. The presumption has been that Cy domain first catalyzes peptide bond condensation and then carries out cyclization of the thiol sidechain of Cys or the hydroxyl sidechain of Ser/Thr onto the just formed peptide bond to form thiohemiaminal/hemiaminal intermediate, which are then dehydrated to yield the C=N bond in the thiazoline and oxazoline rings [5].

Linkers

Intermodular linkers may play a crucial role in the communication between PKS and NRPS modules. Khosla and coworkers [39] have hypothesized that chain transfer between modules depends upon the presence of evolutionarily optimized linkers providing connectivity between adjacent modules. The linker regions are typically short

stretches of hydrophilic residues that have the potential to mediate α-helical coiled-coil interactions that would establish protein-protein contacts between modules [40]. The sequence analysis done by Shen and coworkers [41] showed no apparent intermodular linker within NRPS/NRPS modules or PKS/PKS modules. However, they did identify interpolypeptide linkers (hydrophilic regions consisting of mainly basic or acidic residues such as Asp, Glu, and Arg) between PKS and NRPS modules within PKS/-NRPS hybrid systems. This finding helps to develop a new strategy of combinatorial biosynthesis capable of producing a variety of diverse "unnatural" products [41].

Quaternary Structure of NRPS

By dissecting NRPS into several modules, the structures of each module and relative domains have been delineated into details, however, information on advanced structure of NRPS is rare though it is very important not only for understanding reactions on or between enzymes but also for engineering enzymes by swapping modules or domains. PKSs are known to function as homodimeric enzyme complexes just as fatty acids synthases of eukaryotes are composed of two identical subunits. Biophysical and biochemical methods are applied to NRPS subunits from the gramicidin S (GrsA-ATE), tyrocidine ($TycB_1$-CAT and $TycB_{2-3}$-AT.CATE), and enterobactin (EntF-CATTe) biosynthetic systems, all these three NRPS systems investigated are functionally active as monomer and further experimental proof is needed [42]. However, by using a deletion of the Cy1 domain and separate inactivating mutations in the Cy2, A, PCP, and C2 domains in the six-domain (Cy1-Cy2-A-C1-PCP-C2) enzyme VibF from the vibriobactin synthetase assembly line, Hillson *et al.* [43] reported an NRPS subunit that can be homodimerized and that there is a continuum of functional oligomerization state between monomer and dimer in nonribosomal peptide synthetases.

Combinatorial Manipulation of Diverse Structure of Non-Ribosomal Peptides

The understanding of individual domains, individual modules and quaternary structure of NRPS is much helpful for genetic engineering to expand vastly the molecular diversity of these pharmacologically important metabolites. There are three degrees of freedom in NRP synthesis: (I) the length of the NRP chain, which is determined by the number of modules; (II) the choice of primer and extender units, each controlled by both gatekeeper A domain and C domain; (III) modification of amino acid residues, with the help of Er, Cy, and MT domains. Accordingly, some strategies have been developed such as gain or loss-of-function, precursors-directed biosynthesis, domain replacement, module swapping. The presence of methyltransferase domains in modules 2, 3, 4, 5, 7, 8 and 10 of the cyclosporin synthetase results in *N*-methylation of the corresponding amide bonds, so, the number of methyl residues enlarges end-products though the actual synthetic potential is usually limited to a certain extent by the intrinsic chemistry of each module [7,44]. In 2003, Du *et al.* [45] demonstrated that BlmIV-A(1) activates Cys and catalyzes both in *cis*-aminoacylation of BlmIV-PCP(1) (for NRPS-1) and in *trans*-aminoacylation of BlmIII-PCP(0) (for NRPS-0), which underscores the flexibility and versatility of NRPSs in both structure and mechanism for natural product biosynthesis. Before the full potential of combinatorial biosynthesis can be realized, there are fundamental issues that must be addressed. One serious limitation to the manipulation arises from the possibility that downstream modules in a NRPS may not accept or process efficiently the anomalous product of an engineered upstream module. In *cis*-fusions were more active than in trans ones in protein-protein recognition between three tyrocidine synthetases TycA, TycB, and TycC [46]. Another is about substrate channeling. Specific intrapolypeptide channeling has been shown to be mediated by the covalent attachment of the two modules by intrapolypeptide linkers [39].

Besides the genetic engineering of homologous recombination in the native producing organism, a major alternative to the use of homologous recombination has been to use appropriate vectors to transfer the entire NRPS to a heterologous host or hosts, in which the background genetic methodology is already higherly developed. In 2002, the genetically engineered strain *Bacillus subtilis* KE30 has been reported as an excellent surrogate host for the heterologous expression of an entire nonribosomal peptide synthetase (NRPS) gene cluster. They developed four *Escherichia coli/B. subtilis* shuttle expression vectors, and overproducing hybrid NRPS proteins in *B. subtilis* KE30 was obtained [47].

Future Directions of NRPS Combinatorial Biosynthesis

Proteins built ribosomally are subjected to certain res-

trictions, so researchers are harnessing a non-ribosomal system that might produce new drugs in future. Four directions are mentioned by Cane *et al*. [7] in 1998: to biosynthesize new natural products; to increase the size and diversity of combinatorial biosynthetic libraries; to advance technology needed for the rapid and convenient development of new pathways; to incorporate unusual precursors into NRPSs to expand the biosynthetic libraries dramatically. Though progress has been achieved in the past years, there are still many challenges before genetic engineering. Recombinant synthesis of longer and more complex peptides will still be restricted to alteration of existing structures by manipulations of NRPS gene clusters located on chromosomes or artificial chromosomes. Besides targeted replacements of domains and modules, reprogramming of NRPS by altering the substrate specificities of A-domains is a promising tool for the near future to get novel peptides [48].

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Edited by
Wei-Hong JIANG