Flexing and Stretching in Nonribosomal Peptide Synthetases

Sabrina Pfennig[1,2] and Milton T. Stubbs[1,*]
[1]Institut für Biochemie und Biotechnologie
[2]DFG Graduiertenkolleg 1026 "Conformational Transitions in Macromolecular Interactions"
Martin-Luther-Universität Halle-Wittenberg, Kurt-Mothes-Straße 3, D-06120 Halle (Saale), Germany
*Correspondence: stubbs@biochemtech.uni-halle.de
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Re-engineering of nonribosomal peptide synthetase molecular assembly lines has been hampered by a lack of detailed knowledge concerning inter-domain substrate transfer. Recent structural studies of catalytically relevant domain-domain interactions provide valuable insights into this problem (Liu et al., 2011; Sundlov et al., 2012 [in this issue of *Chemistry & Biology*]).

An enormous variety of complex secondary metabolites are synthesized in vivo by large multi-domain enzymes, the nonribosomal peptide synthetases (NRPSs). In contrast to ribosomal peptide synthesis, in which the nascent peptide sequence is translated from a messenger RNA template via transfer RNA molecules, the NRPSs function directly as protein templates, with specific modules responsible for adding individual building blocks to the growing peptide chain. The composition of the resulting product is determined by the number and order of NRPS modules. A typical minimal module consists of an adenylation (A)-domain, a condensation (C)-domain, and a peptidyl carrier protein (PCP)-domain. The A-domains, responsible for substrate selection (and therefore ultimately product composition), activate substrate monomers by forming an adenylate intermediate. Activated moieties are transferred to the 4′-phosphopantheic acid (PPant) cofactor thiol group of *holo* PCP, which in turn can interact with upstream and downstream C-domains, responsible for peptide bond formation, so that the nascent peptide chain remains attached to a PCP thiol group. A dedicated thioesterase (TE) domain is often responsible for release of the final product from the ultimate PCP.

The biosynthesis of the *Escherichia coli* iron chelator enterobactin, accomplished by the two-module enterobactin synthetase (comprising the three proteins EntE, EntB, and EntF), nicely illustrates the typical sequence of reactions (Figure 1A). In the first step (1), 2,3-dihydroxybenzoic acid (DHB) is activated to the corresponding adenylate by the A-domain EntE. DHB is then transferred to the PPant side chain of the EntB PCP by thiol ester formation (2). In the second step, serine is activated (3) by the corresponding EntF A-domain and transferred to the following PCP domain (4). The condensation domain C then catalyzes peptide bond formation between the thiol-bound DHB and serine residues (5), resulting in PCP-bound DHB-Ser. This process is repeated to yield the linear depsipeptide $(DHB\text{-}Ser)_3$ bound to the EntF PCP thiol group. In the final stage (6), a TE domain catalyzes cyclisation and release of the trimeric macrolactone enterobactin $(DHB\text{-}Ser)_3$.

The antibiotic, antitumor, immunosuppressive, or antifungal properties of many nonribosomal peptides make them particularly interesting for pharmaceutical research. However, attempts to redesign NRPSs by module shuffling have met with limited success (for review, see Strieker et al., 2010). A comprehensive understanding of substrate recognition and transfer within the NRPS assembly line has therefore important implications for the design of modified or novel "natural" products. Detailed structural information is now available for a variety of NRPS components (reviewed recently by Strieker et al., 2010). The A-domain, composed of a large N-terminal and a small C-terminal domain connected via a short hinge, undergoes multiple reorganizations during catalysis (Gulick, 2009). Substrate, ATP, and Mg^{2+} are thought to bind to the large domain in an "open" conformation (c.f. Figure 1D), which leads to closure of the small domain onto the large domain ("adenylation" conformation; Figure 1B), catalysis of adenylate formation, and pyrophosphate release. Possibly as a result of electrostatic distribution changes (Yonus et al., 2008), the small domain rotates some 140° to facilitate substrate transfer to the *holo* PCP PPant thiol group ("thiolation" conformation; see Figure 1C). The four-helix bundle PCP-domains are surprisingly dynamic (Koglin et al., 2006), adopting alternative conformations in the *apo* and *holo* states. Less is known about the structural plasticity of the C-domains, whereas the TE-domains possess a conformationally variable lid domain (Bruner et al., 2002).

The large number of conformational states available to NRPS domains make them demanding targets for structure determination. Whereas the *apo* PCP:TE interaction could be elucidated using NMR (Frueh et al., 2008), the sheer size of NRPS domains poses significant experimental challenges. Crystallization of the entire termination module SrfA-C (consisting of C-, A-, PCP-, and TE-domains) of the surfactin NRPS revealed that A- and C-domains form a contiguous structural platform, with the (*apo*) PCP domain in a position compatible with an interaction with the C-domain (Tanovic et al., 2008; Figure 1D).

A two-pronged approach has been used to trap the PCP:A-domain interaction (Sundlov et al., 2012 [in this issue of *Chemistry & Biology*]). In addition to fusing the stand-alone aryl acid activating domain EntE to the PCP-domain of EntB with a four-residue linker, a mechanism-based vinylsulfonamide inhibitor was utilized, resulting in formation of a covalent DHB-adenylate-thioether that

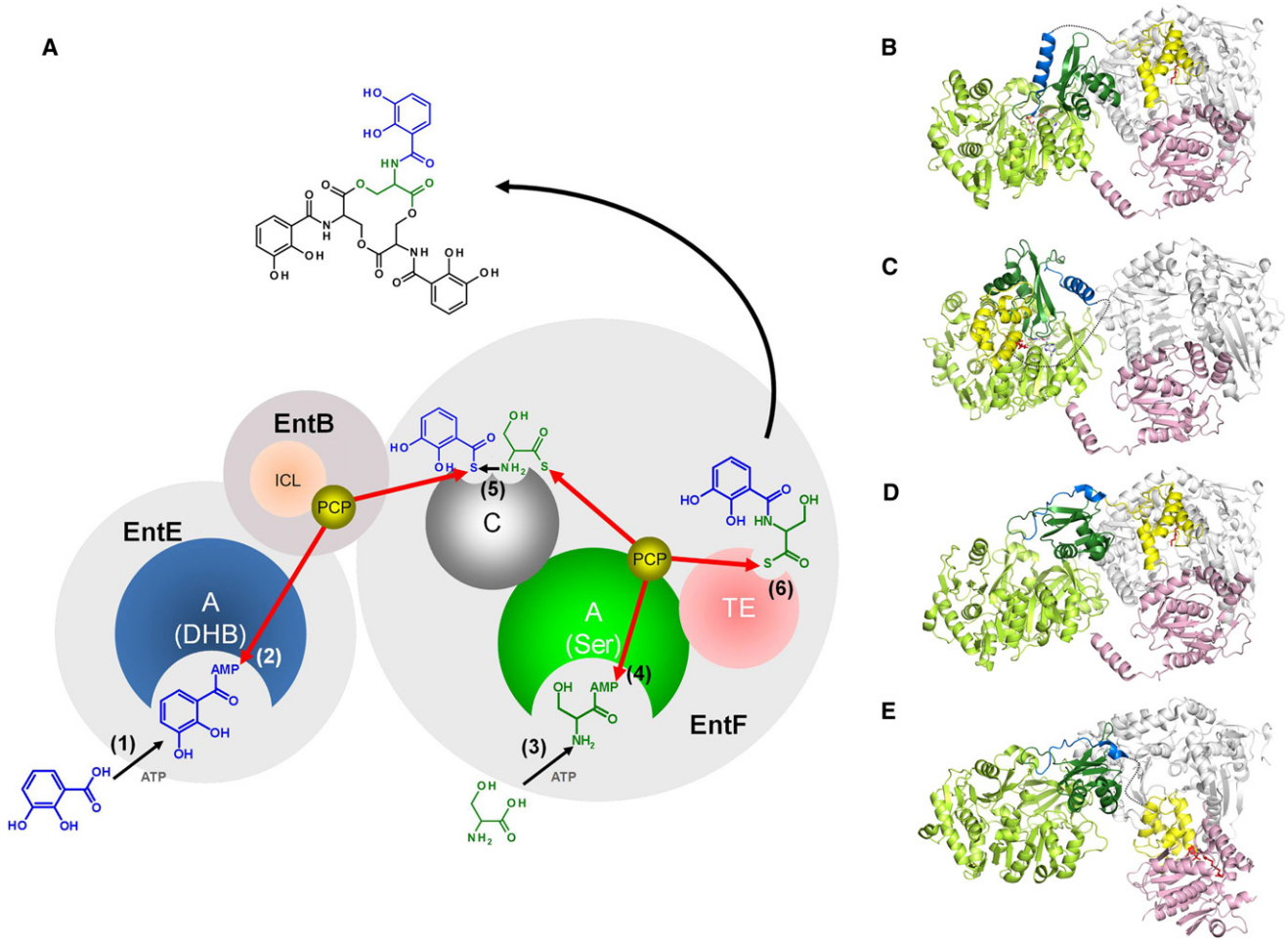

Figure 1. The NRPS Molecular Assembly Line
(A) Biosynthesis of the catechol siderophore enterobactin.
(B) A-domain adenylation conformation, based on DhbE (May et al., 2002) superimposed on the Srf A-C termination module (Tanovic et al., 2008).
(C) Thiolation conformation for transfer from the A-domain to the PCP PPant group (Sundlov et al., 2012).
(D) Putative PCP:C-domain interaction observed in the Srf A-C module (Tanovic et al., 2008); A-domain in "open" conformation.
(E) Domain organization for transfer to TE domain (Liu et al., 2011). A-domain, large subdomain light green; small subdomain, dark green; C-terminal helix, blue; PCP domain, yellow; PPant cofactor, red; C-domain, white; TE-domain, pink.

mimics approach of the PCP-PPant arm. As anticipated, crystals of this locked chimeric protein reveal the EntE-derived A-domain to be in the thiolation conformation, with extensive contacts between PCP helices I to III and both A-domain subdomains (Figure 1C). Mapping the contact region to the A-domain BasE of acinetobactin biosynthesis allowed generation of mutant variants able to load substrate to EntB, confirming the potential for engineering A-domain:PCP contacts.

No major structural rearrangements of the PCP domain are observed; surprisingly, however, the C-terminal helix of the EntE small subdomain is dissociated from the remainder of the A-domain. Moreover, the chimeric protein crystallizes as dimers so that the A-domain of one monomer interacts with the PCP-domain of the second, i.e., acts in *trans*. Despite designing the linker based on that in EntF, it is obvious that a *cis* interaction would not be possible based on the present structure. It seems likely that a *cis*-transfer would require "melting" of the A-domain C-terminal helix, which is indeed apparent in the corresponding SrfA-C termination module A-domain (Tanovic et al. (2008); see Figure 1D).

In an alternative approach, Liu et al. (2011) have utilized an α-chloroacetyl-amino CoA analog to obtain a locked EntF-derived PCP-TE didomain. The catalytically relevant interaction involves PCP helices III and IV and residues of the TE α/β core and helical lid. Only minor domain rearrangements from the NMR structure (Frueh et al., 2008) are observed, suggesting that the PCP-TE domains adopt a near-transfer orientation in the absence of other domains. In contrast, superposition with the structure of SrfA-C (Figure 1E) hints that the PCP-TE domains must reorient with respect to the A- and C-domain platform, indicating yet another degree of flexibility.

These studies demonstrate the importance of domain arrangement and conformational plasticity in the production

of nonribosomal peptides. The strategies described by Liu et al. (2011) and Sundlov et al. (2012) promise to lead to a better understanding of the workings of these complex and fascinating molecular machines and serve as a first step in the development of tailor-made NRPSs.

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Exploiting Effectors of Rac GTPase

Hakryul Jo[1,*] and Hongbo R. Luo[2,*]
[1]Environmental Health Sciences, Yale University School of Public Health, 7th Floor, 1 Church Street, New Haven, CT 06510, USA
[2]Department of Pathology and Laboratory medicine, Harvard Medical School and Children's Hospital Boston, Enders Research Building, Room 811 Boston, MA 02115, USA
*Correspondence: hakryul.jo@yale.edu (H.J.), hongbo.luo@childrens.harvard.edu (H.R.L.)
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Targeting a specific arm of signaling pathways is of great interest. In this issue of *Chemistry & Biology*, Bosco et al. exploit the interactive interface between Rac GTPase and its effector p67^{phox} to specifically inhibit reactive oxygen species production without perturbing other Rac-mediated cellular processes.

The coordinated assembly of multiprotein complexes is essential for the transmission of multiple downstream signaling pathways. Chemical tools that interfere with the assembly of such complexes are highly desirable to tease out the intracellular signaling networks. Upon stimulation, NADPH oxidase, a key cellular enzyme responsible for generating the reactive oxygen species (ROS), is assembled into an active complex at the membrane. ROS is the central weapon of phagocytes to combat the invading microorganisms. It also plays important regulatory functions during neutrophil chemotaxis (Hattori et al., 2010). Inefficient generation of ROS due to genetic mutations of the components of NADPH oxidase leads to chronic granulomatous disease, which is typically characterized by the inability to fight infection and aberrant inflammation. Beyond their well-characterized traditionally roles in innate immunity and inflammation, more recently, ROS and NADPH oxidase are actively being scrutinized as the key mediators of a multitude of pathological conditions caused by oxidative stresses, including neurological diseases, cardiovascular pathologies, and cancer (Bedard and Krause, 2007; Kleinschnitz et al., 2010; Williams and Griendling, 2007). Accordingly, specific pharmacological inhibitors of ROS production by NADPH oxidase are being sought after for the therapeutic benefits of various human pathologies contributed by the oxidative stresses.

Targeting the assembly of active NADPH oxidase complex is an efficient way to inhibit ROS production. The central components of active NADPH oxidase consist of two membrane-bound subunits, gp91^{phox} (or Nox2) and p22^{phox}, four cytosolic proteins, p47^{phox}, p67^{phox}, p40^{phox}, and small GTPase Rac. In response to external stimuli, the cytosolic components are translocated to the membrane and interact with the membrane-bound components to assemble an active NADPH complex, which then transfer electrons from NADPH to oxygen to generate the reactive superoxide anion (Figure 1). A series of protein-protein interactions are critical for an efficient assembly of fully active NADPH oxidase complex. For example, the C-terminal part of cytosolic subunit p67^{phox} interacts with p47^{phox}, while the N-terminal half of p67^{phox} is capable of binding to Rac. The GTP-bound activated Rac recruits p67^{phox} to the membrane, hence facilitating the assembly of NADPH oxidase complex and its activation. As demonstrated by a small molecule inhibitor (Gao et al., 2004), targeting Rac activity is a legitimate strategy to interfere with the assembly of NADPH complex and ROS production. However, given the pleiotropic regulatory functions of Rac in a wide range of cellular functions, the "global" inhibition of Rac activity inevitably comes with the risk of unintended side effects, concomitant with inhibition of ROS generation. On the other hand, the "pathway-selective" inhibition of downstream of Rac signaling may specifically abrogate ROS production without affecting other Rac-dependent cellular processes. Because the specific interactions between