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A nonradioactive high-throughput assay for screening and characterization of adenylation domains for nonribosomal peptide combinatorial biosynthesis

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ABSTRACT

Adenylation domains are critical enzymes that dictate the identity of the amino acid building blocks to be incorporated during nonribosomal peptide (NRP) biosynthesis. NRPs display a wide range of biological activities and are some of the most important drugs currently used in clinics. Traditionally, activity of adenylation domains has been measured by radioactive ATP-[^{32}P]pyrophosphate (PP_i) exchange assays. To identify adenylation domains for future combinatorial production of novel NRPs as potential drugs, we report a convenient high-throughput nonradioactive method to measure activity of these enzymes. In our assay, malachite green is used to measure orthophosphate (P_i) concentrations after degradation by inorganic pyrophosphatase of the PP_i released during aminoacyl-AMP formation by action of the adenylation domains. The assay is quantitative, accurate, and robust, and it can be performed in 96- and 384-well plate formats. The performance of our assay was tested by using NcpB-A_4, one of the seven adenylation domains involved in nostocyclopeptide biosynthesis. The kinetics of pyrophosphate release monitored by this method are much slower than those measured by a traditional ATP-[^{32}P]PP_i exchange assay. This observation indicates that the formation of the adenylated amino acid and its release are the rate-limiting steps during the catalytic turnover.

With the emergence of antibiotic resistance, there is a constantly increasing need for the discovery and development of novel small biologically active compounds. Natural products are major sources of drug leads in the modern pharmaceutical industry [1,2]. Some of the most therapeutically useful and best understood natural products are of nonribosomal peptide (NRP)[1] origin [3,4]. NRPs are structurally very diverse and display a broad spectrum of biological activities [5], including phytotoxins [6,7], siderophores [8,9], biosurfactants [10], antifungal [11–13], antiviral [14], antibacterial [15,16], immunosuppressant [17], and antitumor [18–20] agents. These secondary metabolites are biosynthesized on large multienzyme complexes, the nonribosomal peptide synthetases (NRPSs), which are composed of repeating elongation modules that contain three core catalytic domains: the adenylation (A), thiolation (T), and condensation (C) domains [21,22]. An A domain, one of the three core components of the repeating modules (-C-A-T-)$_n$, is responsible for the loading of the correct amino acid/amino acid-like component onto its downstream adjacent partner T domain. The structural diversity of NRPs is derived primarily from the building block-activating A domains in each NRPS module. More than 300 different precursors have been identified in NPRs [23]. The role of A domains is two-fold: (i) selection and activation of the amino acid substrate (Fig. 1A) followed by (ii) transfer of the activated amino acyl-AMP to the downstream T domain (Fig. 1B). Condensation of the upstream amino acid or growing peptide chain to the amino acid covalently tethered through thioester linkage to the downstream T domain is then achieved by action of the C domain (Fig. 1C).

The modular nature of NRPS provides an attractive opportunity for the development of combinatorial biosynthetic approaches to manipulate genes of NRP biosynthetic pathways to produce hybrid compounds with novel biological properties [24,25]. With A domains being primary determinants of substrate selectivity during NRP formation, it is easy to envision the combinatorial production

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[1] *Abbreviations used:* NRP, nonribosomal peptide; NRPS, nonribosomal peptide synthetase; A, adenylation; T, thiolation; C, condensation; PP_i, pyrophosphate (P_2O_7); P_i, orthophosphate (PO_4); FPLC, fast protein liquid chromatography; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; CTAB, hexadecyltrimethylammonium bromide; PCR, polymerase chain reaction; LB, Luria–Bertani; IPTG, isopropyl-β-D-thiogalactopyranoside; Ni-NTA, nickel–nitrilotriacetate; PAGE, polyacrylamide gel electrophoresis; RT, room temperature; DTT, dithiothreitol; TCEP, tris-(2-carboxyethylphosphine); ACVS, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase; Ybt, yersiniabactin.

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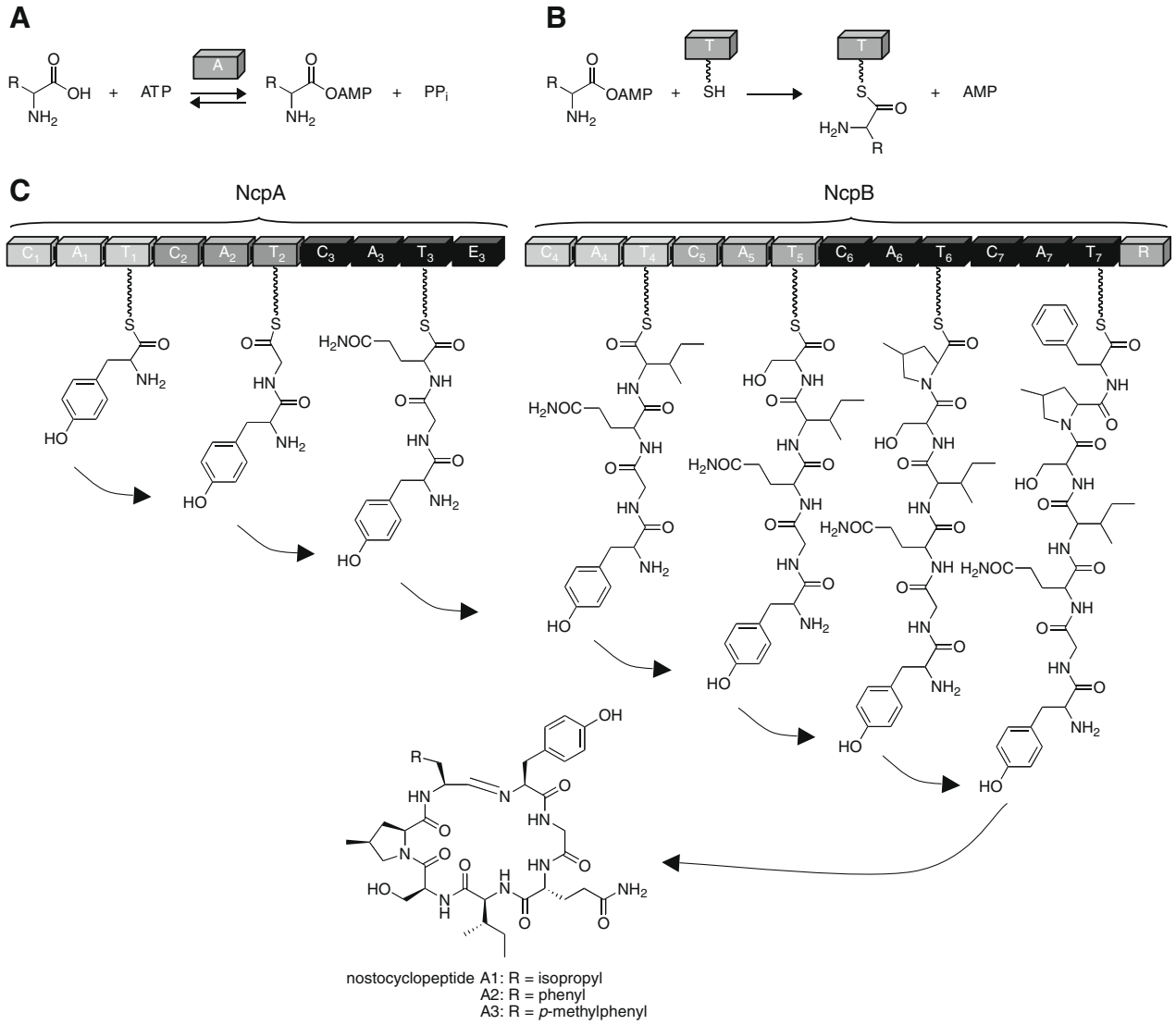

Fig. 1. (A) Conversion of amino acid to amino acyl-AMP by action of A domain. (B) Covalent attachment of amino acyl to T domain by thioester formation on the phosphopantetheinyl arm of the holo T domain. (C) Nostocyclopeptide biosynthesis with NcpB-A_4 as our model A domain.

of new drugs by engineering A domains with relaxed or altered substrate specificity. Adenylation domains generally display very high substrate specificity. However, there are A domains that activate multiple amino acid substrates [26]. CytC1 from the cytotrienin-producing strain of a *Streptomyces* sp. [27] and SgcC1 from *Streptomyces globisporus* producing the C-1027 enediyne antitumor antibiotic [28] are examples of A domains that display such broad substrate specificity. The identity of most of the proteinogenic and nonproteinogenic amino acid building blocks to be incorporated into the growing natural product can be predicted by the A domain substrate specificity-conferring code established by Stachelhaus and coworkers [29] and recently further refined by Rausch and coworkers [30]. The signature sequences appear to be degenerate given that there is more than one code for the activation of a unique amino acid by an A domain based on eight key residues found in the active site of the enzyme. However, examples of codes for which the predicted substrate specificity does not correspond to the actual activated amino acid have been reported [31]. Li and Jensen recently demonstrated the direct activation of D-Ala during fusaricidin biosynthesis by the Fus-A6 domain of *Paenibacillus polymyxa* PKB1 for which no substrate could be predicted [32]. It is now becoming evident that, in addition to the eight key residues deciphered by Stachelhaus and coworkers [29], there are other residues involved in dictating the identity of the substrate(s) to be activated by a given A domain. These additional residues have not been identified or characterized systematically.

The successful identification of novel A domains for combinatorial biosynthesis is critically dependent on the number of constructs that can be tested. For this reason, it is of particular interest to develop a rapid, reliable, sensitive, and quantitative high-throughput assay for A domain activity. A traditional method for analyzing A domains for aminoacyl-AMP formation is the radioactive ATP-[^{32}P]pyrophosphate (PP_i) exchange assay (Fig. 2, steps 1 and 2) [33]. As reported recently, this assay can be performed in a 96-well format [34]. However, most facilities are not equipped to handle radioactive materials in a high-throughput fashion. Thus, we sought to develop a simple nonradioactive high-throughput assay for identification of active A domains. Here we report an assay in 96- or 384-well plates that combines the use of commercially available and inexpensive inorganic pyrophosphatase to convert the PP_i produced during aminoacyl-AMP formation to orthophosphate (P_i) with a molybdate/malachite green reagent [35] to mea-