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Manipulation of Carrier Proteins in Antibiotic Biosynthesis

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Summary

Engineering biosynthetic pathways into suitable host organisms has become an attractive venue for the design, evaluation, and production of small molecule therapeutics. Polyketide (PK) and nonribosomal peptide (NRP) synthases have been of particular interest due to their modular structure, yet routine cloning and expression of these enzymes remains challenging. Here we describe a method to covalently label carrier proteins from PK and NRP synthases using the enzymatic transfer of a modified coenzyme A analog by a 4′-phosphopantetheinyltransferase. Using this method, carrier proteins can be loaded with single fluorescent or affinity reporters, providing novel entry for protein visualization, Western blot identification, and affinity purification. Application of these methods provides an ideal tool to track and quantify metabolically engineered pathways. Such techniques are valuable to measure protein expression, solubility, activity, and native posttranslational modification events in heterologous systems.

Introduction

The biosynthesis of polyketide (PK) and nonribosomal peptide (NRP) natural products has been of great interest recently for potential new drug discovery and production [1–3]. Since elucidation of the modular nature of their biosynthetic machinery, PK and NRP synthases have been aggressively studied for directed engineering and combinatorial biosynthesis [4–6], while novel members of these molecular classes continue to be discovered in nature [7, 8]. Future adaptation of molecules within these classes depends, in part, upon the successful implementation of metabolic engineering or the directed manipulation of metabolite formation in heterologous hosts [9, 10]. While several examples of engineering PK and NRP synthases exist, routine manipulation and expression of complex synthases poses enormous challenges [11–15].

Due to difficulties in culturing and metabolite overproduction in natural producer strains such as actinomycetes, bacilli, and filamentous fungi, metabolic engineering of PK and NRP synthases has focused on the heterologous expression of biosynthetic clusters in host organisms more amenable to laboratory manipulation and industrial culturing, particularly *Streptomyces coelicolor* and more recently *E. coli* [15]. Modular PK/NRP biosynthetic enzymes pose difficulties in heterologous expression for several reasons. First, they are large enzymes, usually ranging in molecular weights between 300 and 800 kDa [1]. Their sheer size presents a major obstacle to their routine cloning and manipulation. Second, the majority of large megasynthase proteins heterologously expressed in *E. coli* either form insoluble aggregates or show no activity in soluble form. Current metabolic engineering efforts that examine alternative heterologous hosts and high-throughput combinatorial techniques face enormous challenges. Achieving proper expression, folding, and posttranslational modification of these biosynthetic proteins is a major obstacle to be overcome, and advanced techniques to rapidly verify expression and activity parameters are needed.

A common theme in the biosynthesis of PK and NRP molecules lies in the posttranslational modification of their synthases by 4′-phosphopantetheinyltransferases (PPTases) [16]. Specifically, carrier protein (CP) domains within each biosynthetic system are modified at a conserved serine residue with a 4′-phosphopantetheine moiety from coenzyme A (CoA). This modification, as illustrated by the conversion of the *apo*-carrier protein (*apo*-CP) to the phosphopantetheinylated *holo*-carrier protein (*holo*-CP) in Figure 1, is essential for activating the biosynthesis of PK and NRP metabolites in all known producing organisms. The development of suitablemetabolic engineering platforms must overcome issues related to abnormal protein folding, low solubility, and poor turnover in large modular synthases. Routine assays for 4′-phosphopantetheinylation might therefore provide an ideal entry to monitor the production of active synthases. The conventional methods to assay for 4′-phosphopantetheinylation rely on radiolabeled CoA and liquid scintillation [17]. Other techniques involve HPLC separation and analysis of purified proteins [18]. The implementation of these methods place major limitations on high-throughput operations, such as combinatorial biosynthesis, in which rapid and reliable assays for active biosynthetic proteins are required [10].

Sfp, the PPTase responsible for modifying surfactin synthase in *Bacillus subtilis*, demonstrates the most promiscuous activity of all known PPTases and is frequently used in metabolic engineering [17]. An interesting characteristic of Sfp lies in its ability to accept functionalized thioesters of CoA, such as acetyl-CoA and malonyl-CoA, two predominant intracellular metabolites. Walsh has used this ability to great benefit in the transfer of nonnatural NRP substrates and intermediates onto CP domains [19]. These aminoacyl-S-CoA derivatives have proven useful in elucidating the mechanisms of NRP synthases, particularly C domain specificity and procession. Since PPTase modification serves as a unifying marker of NRP and PK natural product biosynthesis as well as fatty acid biosynthesis, we reasoned that PPTases could also transfer derivatives of CoA bearing functionality considerably more complex than thioesters. Such a technique

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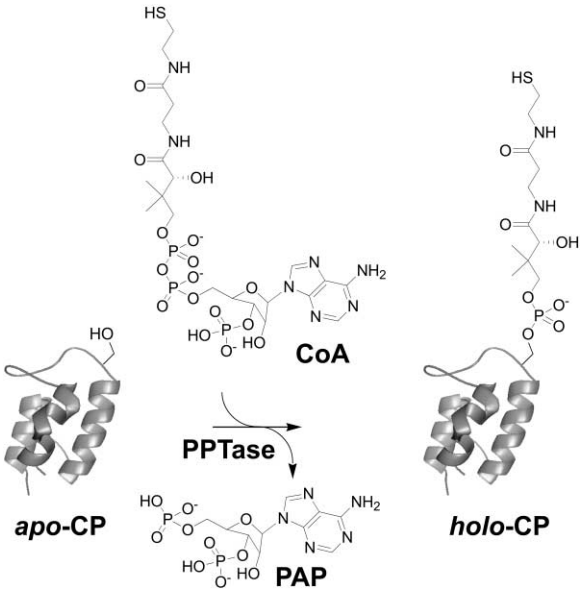

Figure 1. Posttranslational Modification of CP Domains in NRP and PK Synthases

PPTases transfer a 4′-phosphopantetheine residue from coenzyme A (CoA) to a conserved serine residue on each *apo*-CP domain, creating the *holo*-CP and 3′-phosphoadenosyl-5′-phosphate (PAP).

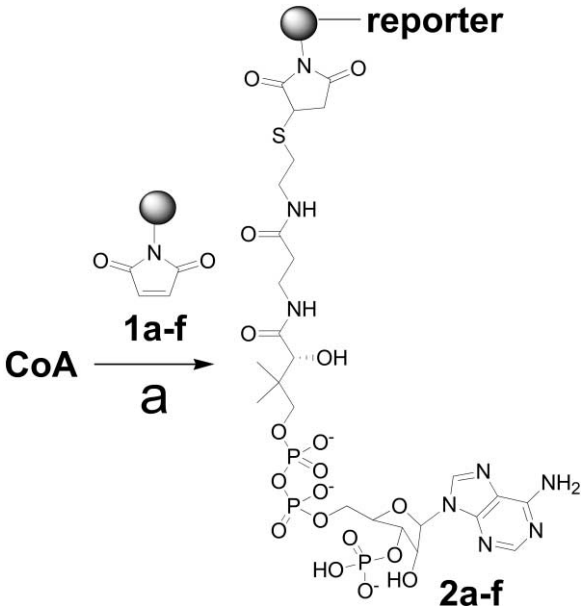

Figure 2. Synthesis of CoA Derivatives

Coenzyme A-reporter analogs (**2a-f**) are created through a Michael addition of the thiol in CoA across the olefin of reporter-linked maleimide (Figure 7) in one step. Excess maleimide reporter may be extracted with organic solvent or removed with thiol-teminating scavenger resin.

could lead to in vitro labeling of carrier proteins with a variety of reporters for the purpose of identification and isolation of PK and NRP biosynthetic machinery. Here we demonstrate the utility of chemo-enzymatic modification with CP domains from various PK and NRP synthases with synthetic CoA analogs and Sfp to visualize, isolate, and manipulate the biosynthetic proteins in vitro.

Results and Discussion

Synthesis of CoA-Reporter Analogs

Given our assumption that PPTases would accept substrates other than thioesters, we chose to create analogs of CoA that would require simple preparation and purification. To this end, maleimides were chosen for their specific reactivity with sulfhydryl groups. Michael attack of the thiol in CoA onto a maleimide-linked reporter molecule would result in selective and irreversible covalent attachment [20]. Unreacted maleimide reporter could then be removed by organic wash or with the use of a thiol-terminating scavenger resin. To investigate the feasibility of this approach (Figure 2), we synthesized several CoA derivatives (**2**) with the use of fluorescent-labeled and affinity reporter-labeled maleimides (Figure 7). Commercially available fluorescent maleimide **1a** (BODIPY FL *N*-(2-aminoethyl)maleimide) was first used to yield analog **2a**. Unreacted **1a** was extracted from the media using ethyl acetate. Thin layer chromatography was used to demonstrate completion of the reaction and successful extraction of unreacted maleimide. The same procedure was followed with Oregon green 488 maleimide **1b** and *N*-(7-dimethylamino-4-methylcoumarin-3-yl)maleimide **1c**. Affinity reporters were also synthesized. Biotin maleimides **1d** and **1e** were coupled to CoA in the same manner as the fluorescent dyes above, except thiol-terminating scavenger resin was used for extraction of the unreacted maleimides. α-mannosyl maleimide is not soluble in organic solvents; therefore, scavenger resin extraction is also used with this reporter.

PPTases Can Selectively Transfer Fluorescent CoA Derivatives to Carrier Proteins

To investigate PPTase transfer of nonthioester CoA derivatives, Sfp was used for posttranslational modification of known, heterologously expressed CP domains (Figure 3). As a first experiment, we used VibB, a small protein from the *Vibrio cholera* vibriobactin biosynthetic machinery that consists of a modular NRP synthase system. VibB contains only one carrier protein domain (as represented by *apo*-CP in Figure 1) and as such is a perfect model system due to its small size and facile expression in *E. coli*. Cell lysate was collected from induced *E. coli* BL21 cells producing VibB from a pET24 expression vector. An aliquot of this lysate was incubated with **2a** and recombinant Sfp and analyzed by SDS-PAGE. When viewed under UV irradiation, recombinant VibB was visualized as a fluorescent band (Figure 4A). Coomassie staining of the gel confirmed the band to be fluorescently tagged VibB **3a** (32.6 kDa) (Figure 4B). Similarly, we tested the formation of other fluorescent reporters. Comparable labeling (i.e., conversion of *apo*-CP to **3b** or **c**) was obtained after repetition of this experiment with Oregon green 488 maleimide (Figure 7, **1b**) and *N*-(7-dimethylamino-4-methylcoumarin-3-yl)maleimide (**1c**) (data not shown). Further proof was obtained by sequence analysis. A gel identical to Figure 4A was

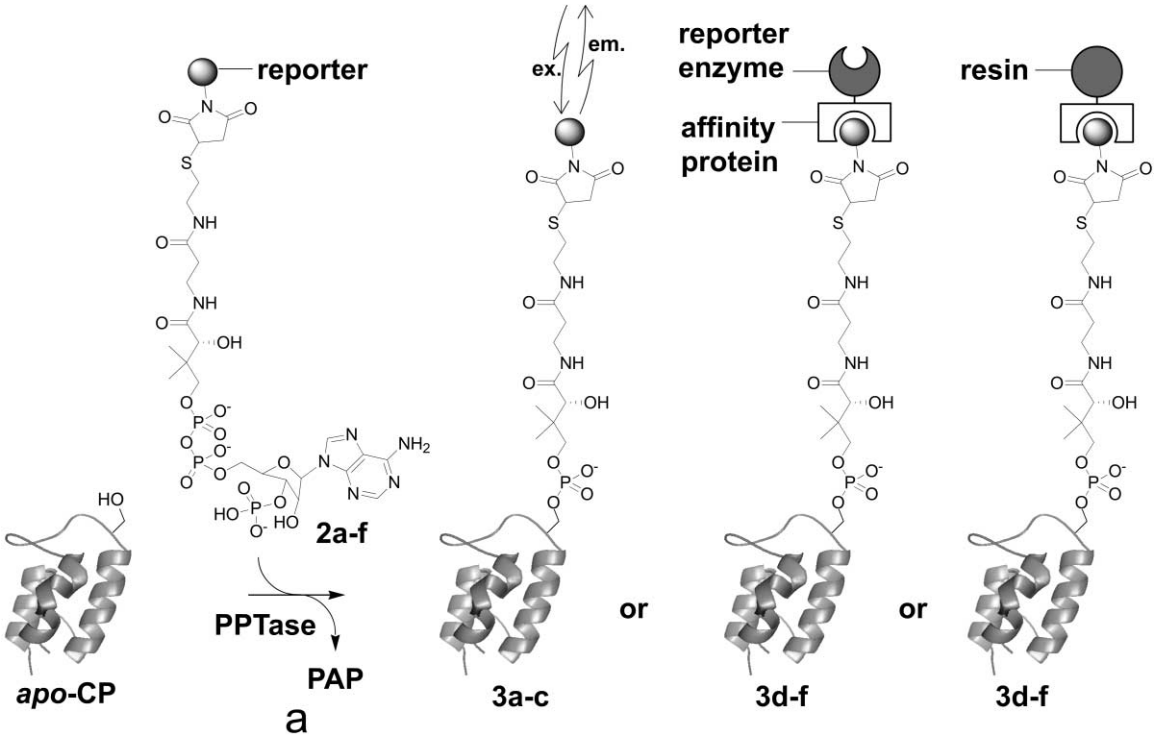

Figure 3. Functional Manipulation of PK and NRP Carrier Proteins

PPTase is used to catalyze the labeling of *apo*-CP at a conserved serine residue to yield 4′-phosphopantetheine-*S*-succinyl derivative **3a-f** and PAP. Fluorescent reporters may be used to visualize carrier protein domains at specific excitation and emission wavelengths with SDS-PAGE. Affinity reporters may be used with affinity proteins and reporter enzymes to visualize carrier protein domains via Western blot or for purification by affinity chromatography with resin bound affinity proteins.

electrophoretically transferred to a polyvinylidene fluoride membrane, and the fluorescent band corresponding to **3a** (VibB lane) was excised from the membrane. The resulting piece was subjected to N-terminal amino acid sequencing by Edman degradation [21]. The first 10 amino acids of the returned sequence, "maipkiasyp," mapped to the correct protein, VibB, when searched with BLAST against 1.4 million sequences in GenBank. All three fluorescent analogs **2a-c** could be used to label, visualize, isolate, and sequence VibB.

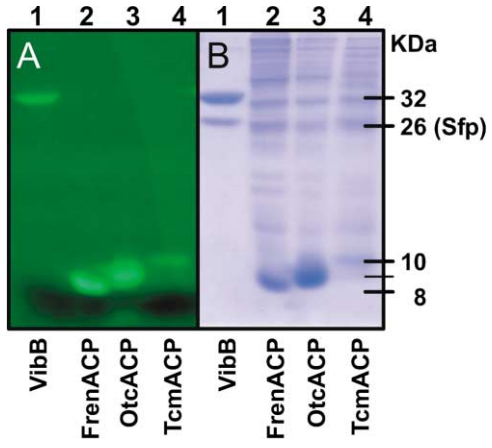

Figure 4. Functional Manipulation of Carrier Proteins

(A) Fluorescent visualization of carrier proteins from *E. coli* crude cell lysate after incubation with **3a** and Sfp. VibB (lane 1) is a small NRP carrier protein domain. FrenACP (lane 2), OtcACP (lane 3), and TcmACP (lane 4) are three type II PK carrier protein domains.
(B) Coomassie stain of the same gel.

Since Sfp has been shown to 4′-phosphopantetheinylate both modular and iterative NRP and PK synthases, we sought to demonstrate carrier protein labeling on other systems. Since iterative systems like type II PK carrier proteins comprise a major group of PK synthases [1], we chose ACPs from three different type II PK producer strains: frenolicin (*fren*) from *Streptomyces roseofulvus*, oxytetracycline (*otc*) from *S. rimosus*, and tetracenomycin (*tcm*) from *S. glaucescens*. These proteins were heterologously expressed in *E. coli* BL21 cells from pET22 vectors. Cell lysate from IPTG-induced cultures was treated with **2a** and recombinant Sfp and separated on SDS-PAGE. Each of these carrier proteins was labeled as **3a** and identified by comparing the uptake of fluorescence in Figure 4A versus Coomassie staining in Figure 4B.

Fluorescent Labeling of Carrier Protein Domains Can Be Used to Quantify Posttranslational Modification in Engineered Systems

For metabolically engineered systems, carrier proteins become active only after posttranslational modification. This modification can be conducted either by PPTases endogenous to the heterologous host or by the co-expression of a PPTase, often under low-level gene ex-

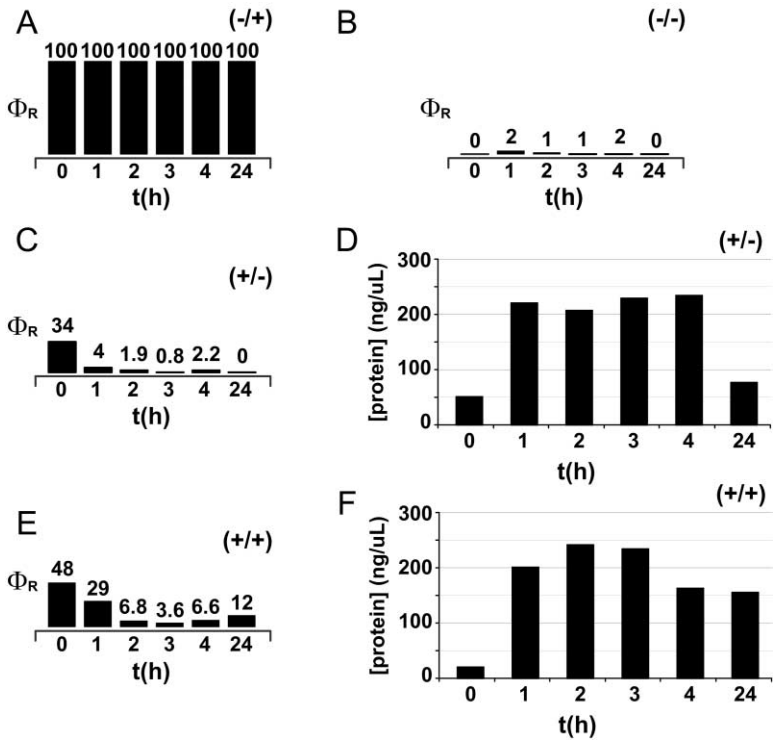

Figure 5. Relative Sfp Activity in Engineered Systems

The amount of TcmACP posttranslationally modified within the cell at various time points post-IPTG induction is reported as relative fluorescence (ϕ_r) as given by fluorescence intensity (ϕ_f)/protein concentration (ng/μl). Parenthetical + or − represent the presence or absence of (Sfp coexpression/exogenous Sfp added to cell lysate).

(A) (−/+) Culture without Sfp co-expression (−pREP4-Sfp) and incubated with exogenous Sfp and **2a** after cell lysis.

(B) (−/−) Culture without Sfp co-expression (−pREP4-Sfp) and incubated with only **2a** (no exogenous Sfp).

(C) (+/−) Culture with Sfp co-expression (+pREP4-Sfp) and incubated with only **2a** (no exogenous Sfp).

(D) Recombinant protein concentration (carrier protein) in C (+/−) as a function of time postinduction.

(E) (+/+) Culture with Sfp co-expression (+pREP4-Sfp) and incubated with exogenous Sfp and **2a**.

(F) Recombinant protein concentration (carrier protein) in (E) (+/+) as a function of time postinduction.

pression [11, 12]. The fluorescent CP domain labeling technique provides a robust and useful means to compare the in vivo activity of native and differentially expressed heterologous PPTases. By fluorescently tagging unmodified CP domains in cell lysate, purifying the protein, and spectrophotometrically comparing fluorescently tagged protein versus total protein, one can quantify the amount of in vivo posttranslationally modified protein. In this manner, different promoters may be compared and optimized. We demonstrated this technique with a common co-expression system, whereby the CP domain was expressed in a pET vector (with a T7 promoter) and the PPTase was expressed in a pREP4 vector (with a *lacI* promoter). We chose a small CP domain, TcmACP in a pET22 vector, both with and without co-expressed Sfp in a pREP4 vector.

To determine the relative activity of co-expressed Sfp, we induced a set of cultures of BL21(DE3) *E. coli* transformed with *tcm* ACP, a subset cotransformed with *sfp*. The cells were harvested at several postinduction time points. The cell lysates were treated with an excess of **2a**, and a subset was treated with additional recombinant Sfp to compare in vitro activity of co-expressed PPTase. The Tcm ACP in each sample was purified by nickel chromatography with EDTA elution. Purified protein **3a** was then analyzed for relative fluorescent intensity as a function of total protein concentration, and these results were tabulated to reveal amount of in vitro labeling in the engineered system (Figure 5). Here carrier proteins unmodified in vivo were fluorescently tagged in the cell lysate. This experiment indicates that Sfp insufficiently tags Tcm ACP when expressed at a low level prior to induction by IPTG. Here, the *lac* promoter allows basal levels of expression ("leaky" expression) that results in nearly 50% unmodified Tcm ACP. However, protein concentration at this time point (time = 0) is 5- to 10-fold lower than at maximal production levels. After induction, 4′-phosphopantetheinylation of the CP follows a time-dependent lag, reaching a maximum at 3 hr postinduction with just 4% unmodified protein. We conclude that this system is sufficient for production of modified CP domains under high expression.

This study offers a simple means to evaluate transcriptional regulation as it applies to posttranslational modification of biosynthetic enzymes. Both promoter level and gene copy number remain critical issues for metabolic engineering efforts [22], and posttranslational modification must be carefully optimized. Selective use of promoters to control these events will be essential to the production of active enzyme and downstream products.

Carrier Protein Western Blot

While fluorescent techniques could be used to identify proteins by direct visualization with very low expression (25 μg/l), where the Coomassie-stained gel indicated little to no protein present, we also examined more sensitive reporter systems. It was found that Sfp would also accept biotinylated derivatives **2d-e**, therein allowing protein identification by Western blotting [23]. To this end, we prepared **2d** and **2e** from *N*-biotinoyl-*N*′-(6-maleimidohexanoyl)hydrazide (**1d**) and biotinyl-3-maleimidopropionamidyl-3,6-dioxaoctanediamine (**1e**), respectively. Aliquots containing **2d** were incubated with Sfp and cell lysate from *apo*-VibB-producing *E. coli*. Following SDS-PAGE, the gel was electrotransferred to nitrocellulose and incubated sequentially with streptavidin-linked alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT)

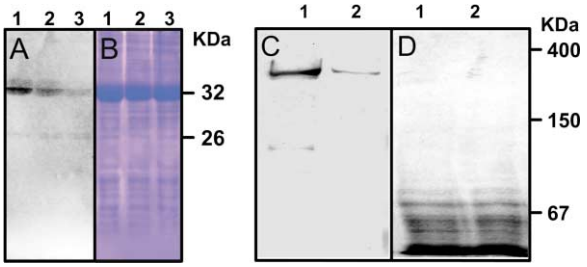

Figure 6. Western Blot

(A) Following SDS-PAGE separation of cell lysate (Figure 4A), analysis using reporter **2e** (lane 1) or **2d** (lane 2). Lane 3 represents CoA spiked with 10% **2d** used in the PPTase labeling reaction.
(B) Coomassie stain of the same gel.
(C) Western blot visualization of DEBS1, DEBS2, and DEBS3 from crude cell lysate of *S. erythreaea* after incubation with Sfp and **3e**. Lane 2 is a 2-fold dilution of sample volume from lane 1.
(D) Coomassie stain of the gel in (C). Here, DEBS proteins are not visible by Coomassie.

(Figure 6A). Here, carrier protein could be detected at a limit of 100 pg/lane (or 5 ng/ml). As expected, we did encounter some conditions that yielded a high background level due to the labeling of native *E. coli* proteins. To counter this effect, we found that diluting derivative **2d** with CoA lowered the background and increased the selection of carrier proteins within *E. coli* cell lysate (Figure 6A, lane 3). This experiment qualitatively illustrates that Sfp accepts both CoA and its analogs **2** with comparable efficiency. Additionally, we examined the effects of tether length. A second botinylated derivative **2e** demonstrated roughly double the efficiency for streptavidin association by Western blot, suggesting a steric requirement for streptavidin binding.

This technique also imparts modest utility in identifying CP domains from the lysate of native cultures. While natural product producer strains express PPTases sufficient for posttranslational modification of their native carrier proteins, a small percentage of unmodified sites remain after cell lysis. These unmodified CP domains may still be used for in vitro reporter tagging with Sfp for protein visualization. Western blotting of PK synthase enzymes has been demonstrated using the 6-deoxyerythronolide B synthase (DEBS) system from *Saccharopolyspora erythraea* and polyclonal antibodies raised against the recombinant proteins [24]. We chose to use the DEBS system for our first native CP-tagging experiments. A type I modular PK synthase, DEBS represents a class of synthases in which cloning, expression, and purification difficulties are particularly acute. We found that the DEBS proteins from native culture could be identified by our CP-labeling Western blot techniques following incubation of cell lysate with Sfp and **2e** (Figure 6B). Here, DEBS1, DEBS2, and DEBS3, with molecular weights of 365.1, 374.5, and 331.5 kDa, respectively, ran as one band and were readily visualized in amounts below the detection limit of Coomassie visualization. A faint band seen at 150 kDa was a native biotin-labeled protein. Unfortunately, tagging efficiency remained only modest, and Western blot visualization proved to be acutely sensitive to the media for culture growth, the timing of cell harvesting, and the conditions of cell lysate

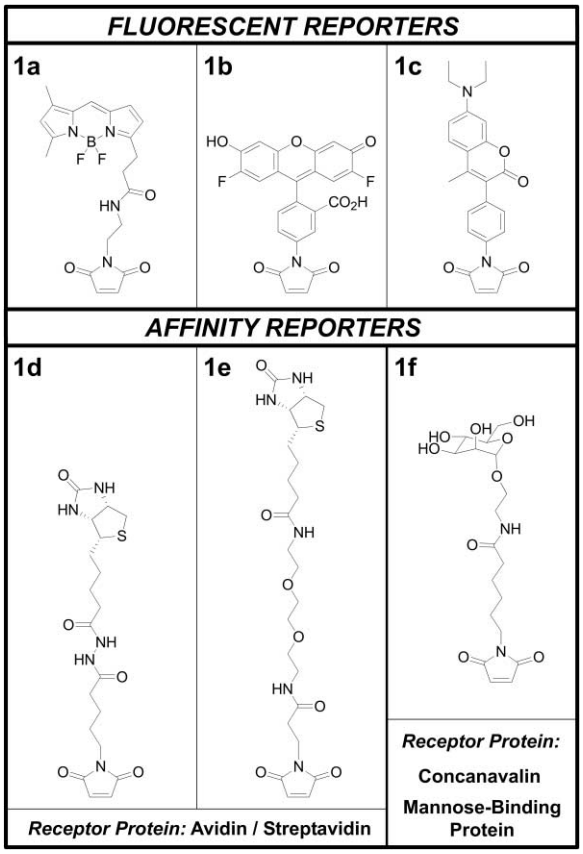

Figure 7. Maleimide Reporters Used in Coenzyme A Analog Preparation

preparation. Clearly, natural PPTases in producer organisms effectively modify the majority of available CP domains. We are currently investigating methods to revert or inhibit 4′-phosphopantetheinylation to alleviate this issue.

Affinity Chromatography

We reasoned that the above labeling methods could be transferred to affinity purification techniques in order to isolate synthases with carrier protein domains [25]. Cell lysate with *apo*-VibB was incubated with Sfp and **2d**, and the mixture was run over a small column loaded with streptavidin-linked agarose resin. Following washing, the resin was boiled to release biotin bound protein. A sample was subjected to SDS-PAGE and a Western blot against streptavidin-phosphatase conjugate. Both the Coomassie-stained gel and the Western blot indicated that biotin-tagged VibB **3f** was successfully purified with biotin affinity chromatography.

Due to denaturation involved in the recovery from streptavidin/biotin affinity purification, we also examined the nondenaturing conditions given by the affinity between carbohydrate-tagged proteins (i.e., α-mannosylated proteins) and lectin-linked agarose resins (i.e., concanavalin A). Here, we coupled maleimide **1f** to CoA to yield α-mannoside **2f** [26]. Incubating **2f** with cell lysate of *E. coli* producing recombinant VibB and exogenous Sfp, we produced **3f** (VibB) with α-mannosyl

groups. An aliquot of this mixture was bound to concanavalin A-linked agarose and washed on a small column. Bound protein was eluted off the agarose with a gradient of glucose, and the purified protein was identified with SDS-PAGE to yield a single band that was identified by Western blotting against concanavalin A-peroxidase conjugate (data not shown). This protocol therefore produced pure, nondenatured α-mannosylated VibB **3f**. In the purified form, VibB is not catalytically active, as the 4′-phosphopantetheinyl thiol remains covalently bound to the reporter. However, other domains associated with the CP domain (for example, condensation, adenylation, and thioesterase domains in NRP synthases) retain activity, and functional studies on these domains remains viable. We conclude that this technique can be used with a variety of affinity methods and will further allow functional characterization of other active domains within a purified synthase. We are currently investigating methods by which to reconstitute activity from labeled carrier protein domains.

Significance

Here we have shown a robust system for specifically labeling carrier protein domains within PK and NRP synthases. This technique provides novel access to the fluorescent labeling, Western blotting, and affinity purification of carrier proteins. We suggest that these tools provide a powerful means to screen, quantify, and isolate these enzymes. Given the size and complexity of multidomain biosynthetic systems, techniques are needed to quantify expression, solubility, folding, activity, and posttranslational modification of these proteins in heterologous expression systems. These techniques can serve as powerful diagnostic tools in metabolic engineering and combinatorial biosynthesis programs and may also be applicable in the search for natural product biosynthetic machinery in novel producer strains.

Experimental Procedures

Coenzyme A Analog Preparation

Six different analogs were prepared using the maleimides in Figure 7. Fluorescent maleimides **1a-c** (Molecular Probes, Seattle, WA), **1d** (Sigma-Aldrich, Milwaukee, WI), and **1e** (Quanta Biodesign, Powell, OH) were obtained. α-mannoside **1f** was prepared according to Ahmed et al. [25]. An aliquot of maleimide **1** (4.8 μl of 25 mg/ml solution of **1a** in DMSO, 13.5 μl of a 10 mg/ml solution of **1b** in DMSO, 8.7 μl of a 10 mg/ml solution of **1c** in DMSO, 5.2 μl of a 25 mg/ml solution of **1d** in DMSO, 6.0 μl of a 25 mg/ml solution of **1e** in DMSO, and 4.0 μl of a 25 mg/ml solution of **1f** in DMSO) was added to coenzyme A disodium salt (300 μg, 0.37 μmol) in 1.9 ml MES acetate and 100 mM $Mg(OAc)_2$ at pH 6.0 containing 300 μl DMSO. The resulting solution was vortexed briefly, cooled for 30 min at 0°C, and warmed at room temperature for 10 min. CoA-maleimide formation was followed by thin layer chromatography (butanol/HOAc/water, 5:2:4). Extraction of the completed reaction with ethyl acetate (3 × 10 ml) was effective in removing excess **1a** and **1c**; the other maleimides were removed using scavenger resins *N*-linked 3-thiopropanoic acid PL-PEGA (Polymer Laboratories, Amherst, MA) or PS-thiophenol (Argonaut, Forester City, CA). This procedure provided stock solutions containing 100–125 μM **2a-f** as determined by fluorescence analysis.

Carrier Protein Labeling Procedure

One liter of *E. coli* BL21 (DE3) cells induced to express recombinant VibB, FrenACP, OtcACP, and TcmACP, each in pET22b vectors (Novagen, Madison, WI), were pelleted, resuspended, and lysed by sonication in 30 ml 0.1 M Tris-Cl (pH 8.0) with 1% glycerol in the presence of 500 μl of a 10 mM protease inhibitor cocktail containing bestatin, pepstatin A, E-64, and phosphoramidon (Sigma-Aldrich) and sonicated by pulsing for 5 min on ice. Alternatively, a lysozyme digestion was used in which the pellet was resuspended in lysis buffer A (20 mM Na_2HPO_4 [pH 7.8], 500 mM NaCl, 1 mg/ml lysozyme) and cooled on ice, and lysis buffer B (5% Triton X 100, 20 U/ml DNase I, 20 U/ml RNase) to 20% volume was then added. A 40 μl aliquot of a 100 μM solution of **2a** (Figure 2) was added 200 μl of cell lysate containing overexpressed protein and 1 μl of a 34 mg/ml solution of purified Sfp, and the reaction was incubated at room temperature for 30 min in darkness. When required (Figure 5), recombinant His-tagged carrier proteins were purified by nickel chromatography using Ni-NTA His-Bind Resin (Novagen) according to manufacturer prodecure and dialyzed against 0.1 M Tris-HCl (pH 8.4) with 1% glycerol. Proteins were precipitated with 10% trichloroacetic acid, pelleted, and washed, and the pellet was resuspended in 1:1 mixture of 1.0 M Tris-HCl (pH 6.8) and 2× SDS-PAGE sample buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 0.02% bromophenol blue). The samples were in boiled for 5 min and separated using SDS-PAGE electrophoresis on a 12% Tris-Glycine. Tagged proteins were visualized by trans-illumination ($\lambda = 365$ nm) and the resulting images captured with CCD camera using a 475 nm cutoff filter. Protein concentration was determined using the Bradford method with bovine serum albumin (Sigma-Aldrich) as a standard.

Expression Time Course Studies

Cultures of BL21(DE3) with TcmACP and (+/−)Sfp were grown in 100 ml of LB medium supplemented with the corresponding antibiotics. Gene expression was induced at $OD_{590} = 0.6$ with 1 mM ITPG. At the indicated time points, 15 ml aliquots were removed from the culture, cooled, and pelleted. Pellets were lysed and spun, and 250 μl of lysate was added to 100 μl of a 100 μM solution of **2a** (Figure 3). The reaction initiated with (+/−)1 μl (30 mg/ml) purified Sfp or 1 μl water. Reactions were incubated in the dark at room temperature for 30 min, and the proteins were purified by nickel chromatography with EDTA elution. 150 μl of the eluates were analyzed for fluorescent intensity (excitation, $\lambda = 492$ nm; emission, $\lambda = 535$ nm).

Western Blotting

Following SDS-PAGE separation of cell lysate using reporter **2e** or **2f**, the gel was electrophoretically transferred to nitrocellulose. Blots were incubated with 5% milk in TBST for 30 min at room temperature with shaking. The blots were then assayed with 10 ml of 5% milk in TBST solution containing either 10 μl of 25 mg/ml concanavalin A-peroxidase (Sigma-Aldrich) or 10 μl of 25 mg/ml streptavidin-alkaline phosphatase conjugate (Pierce Chemical Co., Rockford, IL). Following incubation at room temperature for 1 hr, the blot was washed 3× for 10 min with 20 ml of TBST at room temperature and incubated in 2 ml of either peroxidase substrate solution (Sigma-Aldrich) containing 0.6 mg/ml 3,3-diaminobenzidine tetrahydrochloride in 50 mM Tris (pH 7.6) and 5 μl 30% hydrogen peroxide or alkaline-phosphatase substrate solution containing 0.15 mg/ml BCIP, 0.30 mg/ml NBT, 100 mM Tris (pH 9.0), 5 mM $MgCl_2$ (pH 9.5) (Sigma-Aldrich).

For DEBS Western, *Saccharopolyspora erythraea* was grown according to Caffrey et al. [24], in minimal medium (0.2 M sucrose, 20 mM succinic acid, 20 mM K_2SO_4 [pH 6.6], 5 mM Mg_2SO_4, 100 mM KNO_3, 2 ml/l trace element solution). 100 ml 1 liter of culture was inoculated with a 100 ml 3-day growth and allowed to grow for 4 days. Cells were centrifuged and resuspended in 50 ml resuspension buffer: 50 mM Tris-Cl (pH 7.5), 50% (v/v) glycerol, 2 mM DTT, 0.4 mM PMSF, 100 μg/ml DNase, 20 μg/ml RNase, and 1 μl/ml bacterial protease inhibitor coctail (Sigma-Aldrich). The suspension was sonicated 10× 30 s and ultracentrifuged 2 hr at 40,000 × g, and the supernatant was labeled with Sfp and **3e**. The reaction product was separated by a 3%–8% Tris-acetate SDS-PAGE. The resulting gel was blotted onto nitrocellulose and developed as above with streptavidin-alkaline phosphatase conjugate and BCIP/NBT.

Affinity Chromatography

Following cell lysis, 200 μl supernatant was combined with 40 μl of either **2d-e** or **2f** and 1 μl of 11 mg/ml purified Sfp and allowed to react for 30 min at room temperature in the dark. For **2d-e**, 20 μl of agarose-immobilized streptavidin (4 mg/ml streptavidin on 4% beaded agarose, Sigma-Aldrich) was added, and the samples were incubated at 4°C for 1 hr with constant vigorous shaking. After centrifugation, the supernatant was decanted and the samples were washed 3× with a solution containing 100 mM Tris-HCl (pH 8.4) and 1% SDS in water. After washing, the samples were boiled in 50 μl 1× SDS sample buffer for 10 min and centrifuged, and the supernatant run on a 12% Tris-Glycine SDS-PAGE gel and analyzed by Western blot. For **2f**, 20 μl of agarose-immobilized concanavalin A (4 mg/ml Jack bean concanavalin on 4% beaded agarose, Sigma-Aldrich) was added with binding buffer (1.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1 mM $MnSO_4$, 10 mM KCl, 10 mM Tris [pH 6.7]) and incubated at 4°C for 12 hr. The beads were washed with binding buffer with 1% Triton X-100, and labeled carrier proteins were eluted with binding buffer with 20 mM glycine, 60 mM NaCl, 1% Triton X-100, and a gradient of 0–500 mM glucose. The elutate was run on a 12% Tris-Glycine SDS-PAGE gel and analyzed by Western blot.

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