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Crosslinking Studies of Protein-Protein Interactions in Nonribosomal Peptide Biosynthesis

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SUMMARY

Selective protein-protein interactions between nonribosomal peptide synthetase (NRPS) proteins, governed by communication-mediating (COM) domains, are responsible for proper translocation of biosynthetic intermediates to produce the natural product. In this study, we developed a crosslinking assay, utilizing bioorthogonal probes compatible with carrier protein modification, for probing the protein interactions between COM domains of NRPS enzymes. Employing the Huisgen 1,3-dipolar cycloaddition of azides and alkynes, we examined crosslinking of cognate NRPS modules within the tyrocidine pathway and demonstrated the sensitivity of our panel of crosslinking probes toward the selective protein interactions of compatible COM domains. These studies indicate that copper-free crosslinking substrates uniquely offer a diagnostic probe for protein-protein interactions. Likewise, these crosslinking probes serve as ideal chemical tools for structural studies between NRPS modules where functional assays are lacking.

INTRODUCTION

Nonribosomal peptides (NRPs) constitute a prominent and diverse class of therapeutically beneficial natural products produced by bacteria and fungi (Sieber and Marahiel, 2005; Marahiel et al., 1997; Shen et al., 2002; Mootz and Marahiel, 1997). They include antibiotics such as tyrocidine and daptomycin, immunosuppressants like cyclosporine and rapamycin, and antitumor drugs like bleomycin. These metabolites are assembled by large multifunctional proteins known as nonribosomal peptide synthetases (NRPSs). NRPS enzymes are organized into sequential functional units, called modules, in which each module is responsible for recognition, activation, and incorporation of a single building block of the growing metabolite. The order and makeup of modules dictate the sequence and identity of amino acids in the resulting natural product peptide. Whether the modules are located on a single polypeptide (type I NRPSs), as seen in many fungal and bacterial systems, or on several discrete proteins (type II NRPSs), which are relatively rare, highly specific intermodular communication, mediated by protein-protein interactions, is required for proper biosynthesis of the natural product (Weissman and Muller, 2008).

Intramodular communication also plays a large role in NRP biosynthesis. Individual catalytic protein domains, each responsible for a specific role in the chain elongation process, must properly interact in order to ensure smooth processing of biosynthetic intermediates (Weissman and Muller, 2008). Central to these interactions are the peptidyl carrier protein (PCP) domains found in each module of the NRPS. They serve to covalently tether the growing NRP product during biosynthesis through a 4′-phosphopantetheine prosthetic group (Mercer and Burkart, 2007). PCP domains act as a scaffold, tethering the amino acid building blocks and growing peptidyl chain as they are modified and condensed by the other domains of the NRPS module. Due to the modular architecture of NRPS enzymes, endeavors toward biocombinatorial synthesis of novel therapeutic compounds by genetic reprogramming of their biosynthetic machinery have become a prime objective in this field of research (Eppelmann et al., 2002). In the past, several approaches, including exchange and deletion of modules (Schneider et al., 1998; Mootz et al., 2002) and translocation of domains (de Ferra et al., 1997), have been pursued for the generation of new therapeutic compounds using NRPS biochemistry. However, in many of these efforts efficient product formation has been hindered by the mismatch of native and new catalytic domains and poor substrate selectivity. An understanding of the intramodular protein-protein interaction between PCPs and other domains along with the intermodular communications between subunits within the synthetases could greatly aid the design of new biosynthetic routes for the production of novel medicinal compounds.

Past examination of protein interactions within NRPSs led to the identification of intermodular communication-mediating (COM) domains. Based on these studies (Hahn and Stachelhaus, 2004), a donor COM domain (COM^D) and an acceptor COM domain (COM^A) were defined as the short recognition regions (15–25 amino acids) located at the C terminus of the donating NRPS and N terminus of the accepting partner NRPS, respectively. In addition, COM domain exchange experiments demonstrated that the COM^D and COM^A domain of partner NRPSs formed a compatible set that is responsible for the selective interactions necessary for proper processing of NRP intermediates, whereas the COM^D and COM^A domain of nonpartner NRPSs form an incompatible set, preventing miscommunication between these enzymes. Due to the discovery and verification of the role of

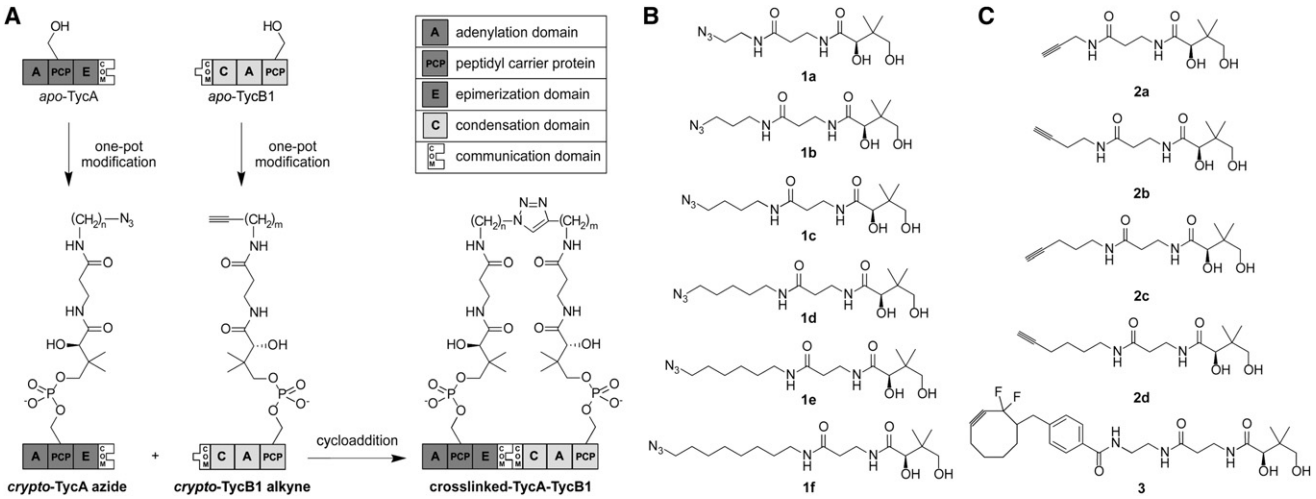

Figure 1. Overall Scheme of Crosslinking Assay and Panel of Bioorthogonal Pantetheine Crosslinking Probes

(A) Structural diagram of the crosslinking assay.

(B) Structure of azido pantetheine crosslinking probes.

(C) Structure of alkynyl pantetheine crosslinking probes.

COM domains in NRP biosynthesis, exploitation of COM domains has become one of the most promising strategies to circumvent some of the problems previously encountered in NRP combinatorial biosynthesis. The development of systems to enable selective crosstalk between different NRPSs remains an important facet in future metabolic engineering of NRPS enzyme systems. To date, manipulation of COM domains has been pursued both in vitro (Hahn and Stachelhaus, 2006) and in vivo (Chiocchini et al., 2006); however, structural information concerning the protein-protein interactions between COM domains has yet to be generated. Based on secondary structure predictions (Hahn and Stachelhaus, 2006), COM domains are believed to possess α-helical structures similar to those of the docking domains found in polyketide synthases (PKSs) (Broadhurst et al., 2003). However, differences in the structural organization (Broadhurst et al., 2003; Sieber et al., 2002) and functional activity (Hahn and Stachelhaus, 2004; Tsuji et al., 2001) between COM and docking domains clearly indicate different modes of intermodular communication between NRPSs and PKSs. At present, it has been suggested, based on the crystal structure of the termination module of the surfactin biosynthetic cluster (Tanovic et al., 2008), that the COM^A domain consists of a more complex structural motif, the COM-hand motif, as a docking site for the helical COM^D domain as opposed to the four-helix bundle of docking domains found in PKSs. However, the proposed COM-hand motif was based on the interaction between the helical segment of the C-terminal *myc*-his_6 tag of the termination module SrfA-C and the condensation domain of a neighboring SrfA-C module, not that of its partner module SrfA-B.

Given the complexities of such studies, we recognized an opportunity to develop new tools for COM domain study. In particular, we saw great potential in development of new assays to evaluate protein-protein interactions between COM domains. We have previously developed specific assays for special module pairs, such as formation of diketopiperazine products in the analysis of tyrocidine biosynthesis (Stachelhaus et al., 1998), yet these are not generally applicable methods to study module interaction. More general assays are needed. We therefore sought to develop probes to examine the protein-protein interactions between partner COM domains in order to potentially validate the COM-hand motif and gain more in-depth structural insights into the role of the COM domain during peptide transfer. Here, we report the application of site-specific protein modification and azide-alkyne cycloaddition as tools for the examination of intermodular communication in NRP biosynthesis. A panel of pantetheine azides and alkynes was synthesized and loaded onto PCP domains of NRPS modules from the enterobactin, tyrocidine, and vibriobactin biosynthesis pathways by a one-pot chemoenzymatic synthesis (Worthington and Burkart, 2006). The modules containing the bioorthogonally tagged PCPs were incubated and then analyzed by SDS-PAGE and western blot to visualize crosslinking of NRPS modules (Figure 1A). Our results demonstrate this is a viable and specific method for the trapping of transient protein-protein interactions in NRP biosynthesis and confirm the importance of the COM domain in the mediation of intermodular communication of NRPS enzymes.

RESULTS

Design and Synthesis of Pantetheine Azides and Alkynes for Crosslinking

In this study, we chose to examine the enzymes responsible for the first condensation reaction in the tyrocidine pathway, TycA and TycB1. TycA performs the activation, loading, and epimerization of L-phenylalanine through the joint activities of an adenylation (A), a PCP, and an epimerization (E) domain. TycB1 is composed of a proline-activating A domain, a PCP domain, and a condensation domain (C). Because the two modules are located on discrete polypeptides, TycA interacts in *trans* with TycB1, meaning the two modules rely heavily on protein-protein interactions for the condensation reaction to occur. The condensation domain catalyzes formation of the first amide bond in tyrocidine

biosynthesis between the upstream TycA-bound D-Phe and the downstream L-Pro of TycB1 (Mittenhuber et al., 1989). In order for this condensation to occur, the PCP must first be posttranslationally modified with a 4′-phosphopantetheine cofactor at the side chain hydroxyl moiety of the invariant serine residue within the PCP. Then the adenylation domain activates and covalently attaches the aminoacyl substrates, D-Phe or L-Pro, to the cofactor through a thioester linkage of the acyl group with the thiol moiety of the 4′-phosphopantetheine prosthetic arm. This cofactor-PCP scaffold is what shuttles the aminoacyl substrates to the various domains, such as the C domain, for further transformations during peptide assembly. Due to the necessity of this prosthetic arm for condensation, we based our analogs on the cofactor-PCP scaffold. The incorporation of the azide and alkyne was based on a recent study (Krasinski et al., 2005), which employed the bioorthogonal [1,3]-dipolar cycloaddition reaction of azides and alkynes for the discovery of new acetylcholinesterase inhibitors. In this study, two site-specific ligands of acetylcholinesterase were decorated with alkyl azides and alkynes of varying chain lengths in order to find new triazole-linked bivalent inhibitors of acetylcholinesterase. Based on this approach, we designed and synthesized a panel of pantetheine azides and alkynes of varying lengths to investigate the protein interactions of COM domains involved in the biosynthesis of the NRP tyrocidine. In order to study the feasibility of using azide-alkyne cycloaddtion to study these interactions, a panel of pantetheine analogs **1–3** (Figure 1B) was synthesized (see Figure S1 available online). Utilizing the one-pot chemoenzymatic carrier protein modification method (Worthington and Burkart, 2006), these analogs were converted to their CoA analogs with CoA biosynthetic enzymes and then loaded onto the carrier proteins of the first two modules in the tyrocidine pathway via a phosphopantetheinyl transferase (PPTase). With this panel of bioorthogonally tagged TycA and TycB1 carrier proteins, designated *crypto*-PCP, we probed the ability of COM domain-mediated protein-protein interactions to catalyze cycloaddition between complementary *crypto*-PCPs, thus trapping this interaction. To test the specific nature of inter- and intramodular communication in this system, the carrier proteins EntB and VibB from the enterobactin and vibriobactin synthetases, as well as a TycA COM domain deletion mutant (TycAΔ23), were tested for crosslinking capabilities and specificity with alkyne-modified TycB1. In all cases, crosslinking of the proteins was analyzed by SDS-PAGE and western blot analysis. In order to confirm the identity of the crosslinked protein complexes, MALDI MS/MS was used.

Verification of PCP Modification with Pantetheine Analogs through Copper-Catalyzed Azide-Alkyne Cycloaddition

Conversion of all carrier proteins from *apo*- to *crypto*-PCP was accomplished using the one-pot chemoenzymatic carrier protein modification method (Worthington and Burkart, 2006). Each of the pantetheine analogs **1–3** was extended with the recombinant enzymes pantothenate kinase (PanK), phosphopantetheine adenylyltransferase (PPAT), and dephosphocoenzyme A kinase (DPCK) to produce the respective CoA analogs, followed by covalent transfer on to the TycA or TycB1 PCPs with the PPTase Sfp (Quadri et al., 1998). In order to first verify the successful loading of all bioorthogonal pantetheine analogs, the modified PCPs were then reacted with a corresponding coumarin alkyne **4** or coumarin azide **5** following the cycloaddition protocol (Alexander and Cravatt, 2005) and detected by UV visualization on SDS-PAGE gels (Figure 2). For the reaction of cyclooctyne **3** with **5**, copper sulfate, tris(2-carboxyethyl) phosphine (TCEP), and tris-(benzyl triazolylmethyl)amine ligand (TBTA) were omitted from the reaction and all other variables were kept constant. As seen in Figure 2, all PCPs displayed fluorescence, confirming the covalent modification of these carrier proteins in vitro with the panel of pantetheine analogs **1–3**. To verify complete posttranslational modification of TycA and TycB1 with analogs **1–3**, BODIPY-CoA and additional Sfp were added to the one-pot reactions of TycA and TycB1. It was found that addition of BODIPY-CoA and fresh Sfp did not result in fluorescent labeling of TycA and TycB1, an indication that TycA and TycB1 underwent complete loading by **1–3** and no *apo*-PCP domains remained (see Figure S2).

Crosslinking Analysis of Cognate and Noncognate NRPS proteins via Copper-Catalyzed Azide-Alkyne Cycloaddition

As an initial test of the crosslinking capabilities of cognate and noncognate *crypto*-PCPs loaded with compounds **1a–f** and **2a–d**, we utilized traditional Cu-catalyzed azide-alkyne cycloaddition. It was thought that this would provide a good positive control to our in situ and strain-promoted crosslinking approaches, as the Cu-catalyzed variant of the Hüisgen 1,3-dipolar cycloaddition reaction is known to proceed at high velocities and may not require protein-protein interaction for catalysis. Accordingly, pantetheine analogs **1a–f** and **2a–d** were independently loaded onto TycA and TycB1 via the one-pot method. The azide-loaded TycA modules were then reacted with the corresponding alkyne-loaded TycB1 modules through the addition of $CuSO_4$, TCEP, and TBTA. After a 1 hr incubation period, crosslinking reactions were subjected to SDS-PAGE (strongly reducing) for analysis of crosslinking by gel shift. The same assay was performed between alkyne-loaded TycA modules and azide-loaded TycB1 modules. These results (see Figure S4) showed partial crosslinking of TycA and TycB1 in all cases. Figure 3A shows the crosslinking of TycA-**1c** with TycB1-**2a** as an example of a negative (lane 1) and positive (lane 2) result for the crosslinking reaction between TycA and TycB1 in Cu-catalyzed conditions. In lanes 1 and 2, two bands at 120 kDa, corresponding to TycA and TycB1, can be seen; and a new third band in lane 2 at 240 kDa, corresponding to a gel shift of the crosslinked TycA-TycB1 complex, is observed. A similar shift, along with unreacted TycA and TycB1 starting material, was observed for all crosslinking reactions (Figure S4). To definitively identify the high molecular weight (240 kDa) band, it was excised and subjected to proteolytic digest. MALDI MS/MS analyses resulted in the identification of peptides corresponding to 23% sequence coverage of TycA and 9% of TycB1 (see Figure S5).

We performed the same crosslinking assay between TycB1 and noncognate PCPs, EntB, and VibB from the enterobactin and vibriobactin biosynthesis. EntB and VibB were loaded with azido pantetheine analogs **1a**, **1e**, and **1f** and TycB1 was loaded with pantetheine alkyne **2a**. As seen in Figures 3B and 3C, bands are seen at 30 kDa (corresponding to VibB/EntB) and 120 kDa (TycB1), along with a new band formed at 150 kDa corresponding to the crosslinked EntB-TycB1 and VibB-TycB1 complexes.

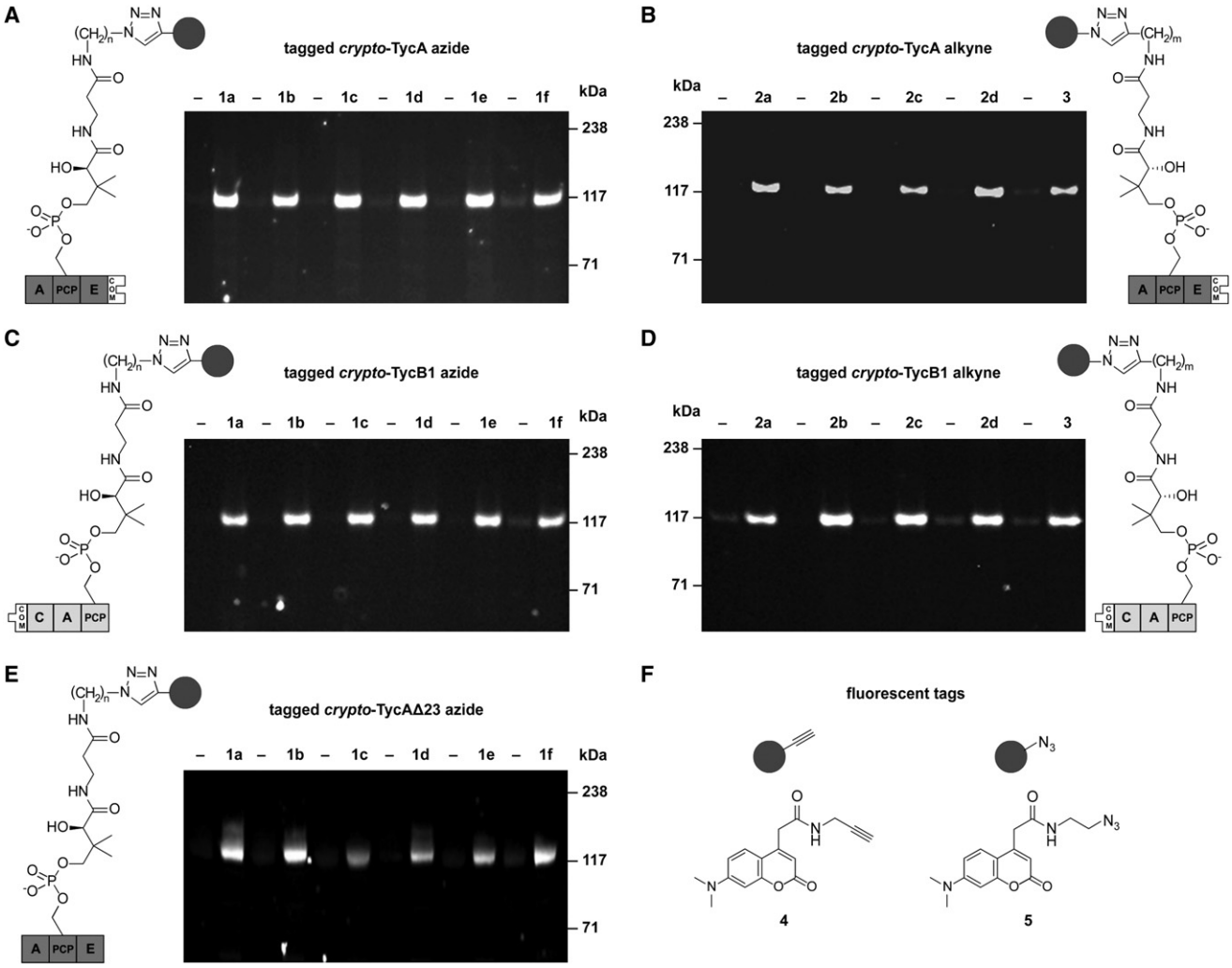

Figure 2. Verification of PCP Modification

Conversion of the carrier proteins of TycA, TycAΔ23, and TycB1 from *apo*- to *crypto*-PCP with the panel of crosslinking probes **1–3** was accomplished using the one-pot carrier protein modification conditions: 2.0 μM of carrier protein TycA, TycAΔ23, or TycB1, 0.4 mM of a pantetheine analog **1–3**, 3.3 μM PanK, 3.3 μM PPAT, 3.3 μM DPCK, and 10 μM *Bacillus subtilis* Sfp. Verification of the modification was established by tagging the *crypto*-PCP with the corresponding coumarin alkyne **4** or coumarin azide **5** using the following conditions for the cycloaddition protocol: addition of 50 μM coumarin alkyne **4** or azide **5**, 1 mM $CuSO_4$, 1 mM TCEP, and 0.1 mM TBTA ligand. For the reaction of cyclooctyne **3** with **5**, $CuSO_4$, TCEP, and TBTA were omitted from the reaction and all other variables were kept constant. Negative controls (–) consisted of one-pot reactions without Sfp. The fluorescently tagged *crypto*-PCPs were detected by UV visualization on SDS-PAGE gels.

(A) SDS-PAGE gel of fluorescently tagged *crypto* TycA azides **1a–f**.

(B) SDS-PAGE gel of fluorescently tagged *crypto* TycA alkynes **2–3**.

(C) SDS-PAGE gel of fluorescently tagged *crypto* TycB1 azides **1a–f**.

(D) SDS-PAGE gel of fluorescently tagged *crypto* TycB1 alkynes **2–3**.

(E) SDS-PAGE gel of fluorescently tagged *crypto* TycAΔ23 azides **1a–f**.

(F) Structures of fluorescent coumarin alkyne **4** and azide **5**.

Crosslinking Analysis of Cognate NRPS Proteins TycA and TycB1 via In Situ Copper-Free Azide-Alkyne Cycloaddition

Having established the loading and reactivity of PCP-tethered bioorthogonal crosslinking probes, we probed the ability of TycA-TycB1 protein-protein interactions to catalyze in situ crosslinking reactions (i.e., without Cu catalysis) in the C domain active site. The one-pot carrier protein modification protocol was again applied to modify TycA and TycB1 with analogs **1a–f** and **2a–d** (cyclooctyne **3** was analyzed separately, as detailed below). After loading, TycA and TycB1 were incubated for a longer period of time, 24 hr, to facilitate the slower reaction rate (~4-fold slower than Cu-catalyzed triazole formation) associated with in situ triazole formation in previous studies (Mock et al., 1983, 1989). In addition, the Universal His Western Blot Kit 2.0 (Clontech) was utilized for analysis for its lower limit of detection. Western blot analysis of all in situ crosslinking reactions produced a band at 240 kDa in all lanes, including the negative controls (See

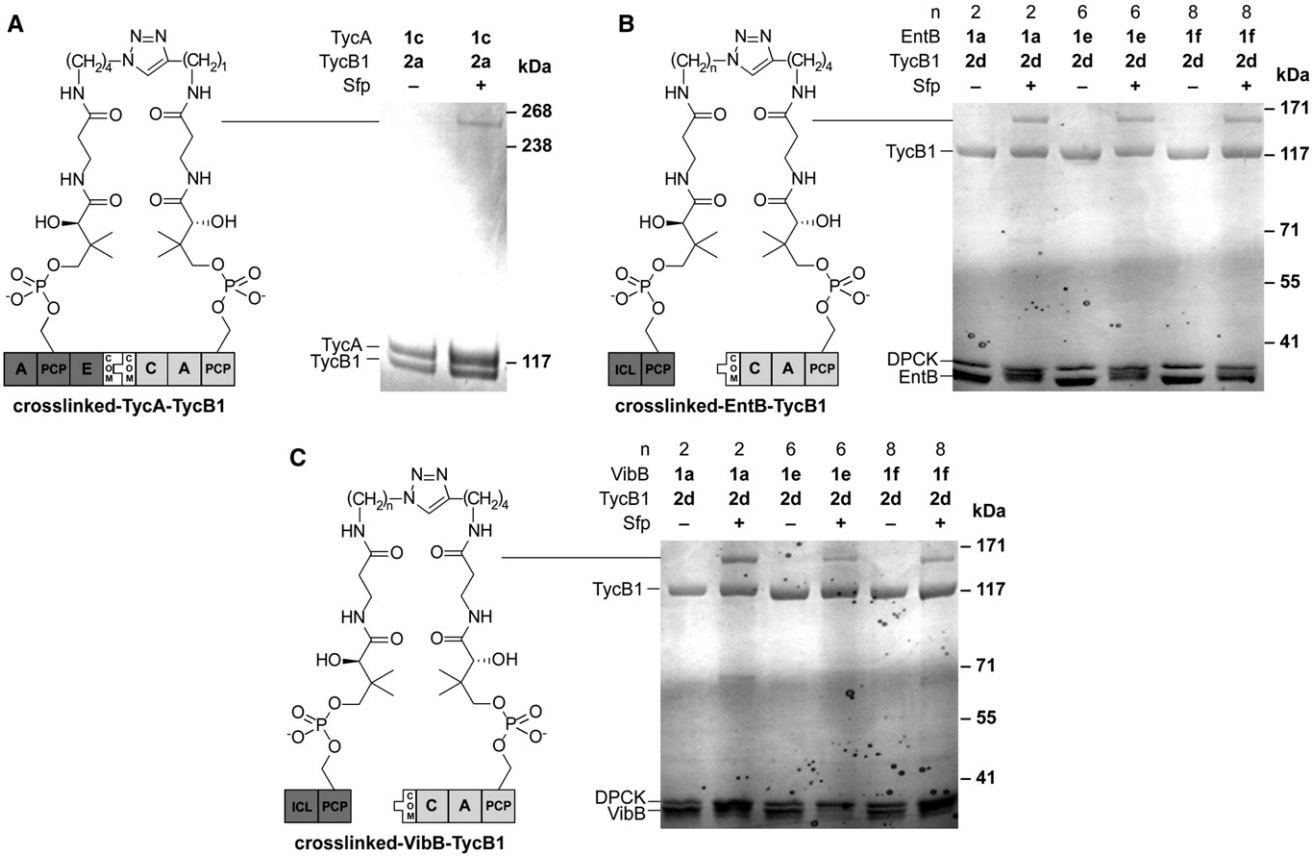

Figure 3. Copper-catalyzed Crosslinking of NRPS Proteins

NRPS proteins TycA, TycB1, VibB, and EntB were crosslinked by first converting the carrier proteins of TycA, TycB1, VibB, and EntB from *apo*- to *crypto*-PCPs with crosslinking probes through the one-pot carrier protein modification conditions: 2.0 μM of carrier protein EntB, VibB, TycA, or TycB1, 0.4 mM of a pantetheine analog **1a**, **1c**, **1e**, **1f**, **2a**, or **2d**, 3.3 μM PanK, 3.3 μM PPAT, 3.3 μM DPCK, and 10 μM *B. subtilis* Sfp. Then copper-catalyzed cycloaddition of the *crypto*-PCP azides and alkynes were performed under the following conditions: 25 μl of the one-pot carrier protein modification reactions EntB, VibB, or TycA with a pantetheine azide **1a**, **1c**, **1e**, or **1f**, 25 μl of the one-pot carrier protein modification reactions TycB1 with corresponding pantetheine alkyne **2a** or **2d**, 1 mM $CuSO_4$, 1 mM TCEP, and 0.1 mM TBTA ligand. Negative controls (–) consisted of one-pot reactions without Sfp.

(A) SDS-PAGE gel-shift analysis of copper-catalyzed crosslinking assays between *crypto* TycA azide **1c** with *crypto* TycB1 alkyne **2a**.

(B) SDS-PAGE gel-shift analysis of copper-catalyzed crosslinking assays between *crypto* EntB azides **1a**, **1e**, or **1f** and *crypto* TycB1 alkyne **2d**.

(C) SDS-PAGE gel-shift analysis of copper-catalyzed crosslinking assays between *crypto* VibB azides **1a**, **1e**, or **1f** and *crypto* TycB1 alkyne **2d**.

Figure S6). Due to the presence of this band in the negative control, we attributed this band to being a false positive. To verify this result, we used the same western blot methodology to analyze reactions in which TycA-**1c** and TycB1-**2a** had been crosslinked using Cu-catalyzed cycloaddition conditions (See Figure S7). The absence of a band in the negative control for the Cu-catalyzed cycloaddition of TycA-**1c** and TycB1-**2a** and the presence of a band observed in the lane for which Sfp was added indicates the ability to visualize the high molecular weight TycA-TycB1 complex with high sensitivity. Thus, it was concluded that TycA and TycB1 were most likely not capable of catalyzing in situ triazole formation when loaded with analogs **1–2**.

Crosslinking Analysis of Protein-Protein Interactions in Cognate and Noncognate NRPS via Strain-Promoted Copper-Free Azide-Alkyne Cycloaddition

The results of our in situ NRPS-mediated crosslinking reactions yielded inconclusive results, indicating the highly transient protein-protein interactions of NRPS may be incapable of providing the necessary transition-state stabilization required for triazole formation. In the acetylcholinesterase study, triazole formation was catalyzed in situ due to both entropic effects caused by enforced proximity and proper alignment of the reactants and enthalpic stabilization of the triazole-like transition state caused by engagement in hydrogen bonding and stacking interactions with amino acid residues within the active site. Unlike the AChE inhibitor study, our studies probe the highly transient and noncovalent interactions of two very large protein modules that have been decorated with bioorthogonal tags. This led us to examine methods to enhance the rate of azide-alkyne cycloaddition for the capture of these short-lived interactions. Foremost among current methods for the acceleration of biologically compatible azide-alkyne [3+2] cycloadditions is the use of alkynes activated by ring strain (Agard et al., 2004; Baskin et al., 2007). Thus, we synthesized cyclooctyne pantetheine **3**, which incorporates both a ring-strained cyclooctyne as well as difluoro

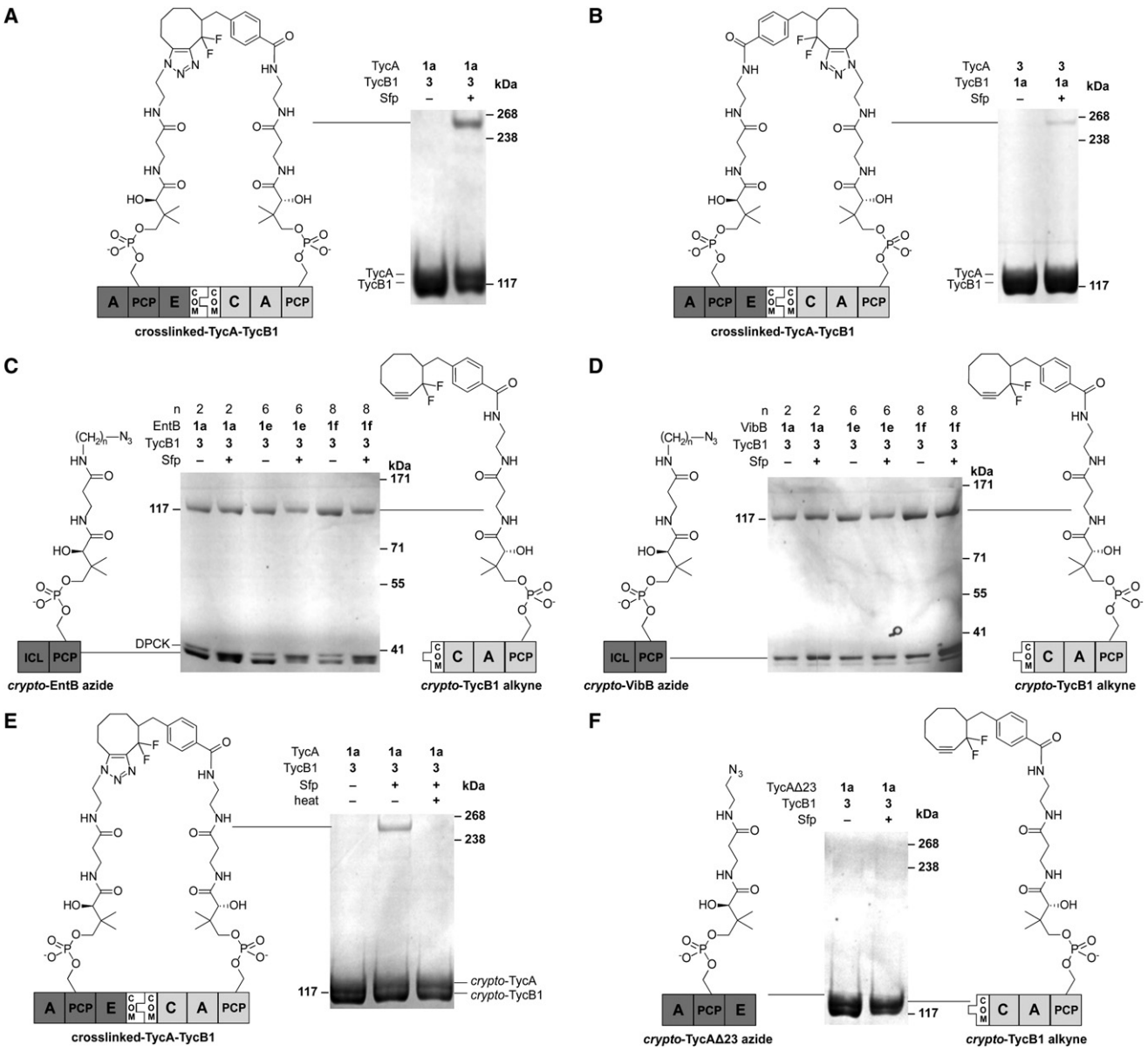

Figure 4. Strain-Promoted Copper-Free Azide-Alkyne Cycloaddition of NRPS Proteins

The carrier proteins of TycA, TycB1, VibB, and EntB were converted from *apo*- to *crypto*-PCPs with crosslinking probes through the one-pot carrier protein modification conditions: 2.0 μM of carrier protein EntB, VibB, TycA, or TycB1, 0.4 mM of a pantetheine analog **1a**, **1e**, **1f**, or **3**, 3.3 μM PanK, 3.3 μM PPAT, 3.3 μM DPCK, and 10 μM *B. subtilis* Sfp. Then strain-promoted copper-free cycloaddition of the *crypto*-PCP azides and alkynes were performed under the following conditions: addition of 25 μl of the one-pot carrier protein modification reactions EntB, VibB, or TycA with a pantetheine analog **1a**, **1e**, **1f**, or **3** and 25 μl of the one-pot carrier protein modification reactions TycB1 with corresponding pantetheine analogs **1a** or **3**. Negative controls (–) consisted of one-pot reactions without Sfp.

(A) SDS-PAGE gel-shift analysis of strain-promoted copper-free crosslinking assay between *crypto* TycA azide **1a** with *crypto* TycB1 alkyne **3**.

(B) SDS-PAGE gel-shift analysis of strain-promoted copper-free click crosslinking assay between *crypto* TycA alkyne **3** with *crypto* TycB1 azide **1a**.

(C) SDS-PAGE gel-shift analysis of strain-promoted copper-free crosslinking assays between *crypto* EntB azides **1a**, **1e**, or **1f** and *crypto* TycB1 alkyne **3**.

(D) SDS-PAGE gel-shift analysis of strain-promoted copper-free crosslinking assays between *crypto* VibB azides **1a**, **1e**, or **1f** and *crypto* TycB1 alkyne **3**.

(E) SDS-PAGE gel-shift analysis of strain-promoted copper-free crosslinking assay between predenatured *crypto* TycA azide **1a** with *crypto* TycB1 alkyne **3**.

(F) SDS-PAGE gel-shift analysis of strain-promoted copper-free crosslinking assay between *crypto* TycAΔ23 azide **1a** with *crypto* TycB1 alkyne **3**.

substituents at the propargylic position to further increase the reaction rate (Codelli et al., 2008). Once again, application of one-pot chemoenzymatic modification of TycB1 with **3** yielded the *crypto*-PCP, which was examined for crosslinking activity with cognate azide-loaded TycA modules. After a 1 hr incubation period, crosslinking reactions were subjected to SDS-PAGE (strongly reducing) for analysis of crosslinking by gel shift. The same assay was performed between *crypto* TycA alkyne **3** and *crypto* TycB1 azides. These results (see Figure S8) showed partial crosslinking of TycA and TycB1 in all cases. Figure 4A shows the

crosslinking of TycA-**1a** with TycB1-**3** as an example of a negative (lane 1) and positive (lane 2) result for the crosslinking reaction between TycA and TycB1 in Cu-free conditions. Figure 4B shows the crosslinking of these modules with the opposite configuration of pantetheine analogs. Based on these results, gel-shift analysis demonstrated that cyclooctyne **3** not only crosslinked the cognate partner modules TycA and TycB1 but did so with a greater efficiency than that observed by application of either Cu-catalyzed or in situ click chemistry with analogs **1a–f** and **2a–d**, as observed by the relative intensities of the crosslinked bands at 240 kDa (Figures 3A and 4A). However, the intensity of the bands for the Cu-catalyzed crosslinking experiments may have been less due to protein precipitation that commonly occurs when using Cu-catalyzed azide-alkyne cycloaddition. In addition, it was observed by densitometry comparison that crosslinking was more efficient when cyclooctyne **3** was loaded on to TycB1 and the companion azide **1a** was loaded on TycA (Figure 4A), as opposed to the opposite configuration (Figure 4B).

After establishing the crosslinking ability of cyclooctyne **3** with TycA and TycB1, we investigated the specificity of this interaction by testing the ability of TycB1-**3** (which promoted the most efficient crosslinking in the TycA-TycB1 system) to crosslink the noncognate carrier proteins EntB and VibB loaded with pantetheine azides **1a**, **1e**, and **1f**. After carrier protein loading, the reactions were combined and incubated overnight followed by analysis by SDS-PAGE (Figures 4C and 4D). In contrast to when Cu-catalyzed azide-alkyne cycloaddition was used, no bands were visible in the region at 150 kDa corresponding to EntB-TycB1 and VibB-TycB1 complexes.

To further validate the necessity of protein-protein interactions in guiding the intermodular crosslinking of TycA and TycB1 via strain-promoted copper-free azide-alkyne cycloaddition, *crypto* TycA-**1a** was predenatured by boiling prior to crosslinking with *crypto* TycB1-**3**. No TycA-TycB1 complex was observed (Figure 4E, lane 3). As a final test, we examined the COM domains in greater detail. A deletion mutant of TycA, lacking the last 23 C-terminal amino acid residues corresponding to the COM domain, was overexpressed and purified. Previous studies had shown that this mutant (TycAΔ23) retains A domain and PCP activity, but is incapable of performing the condensation of its natural substrate with that of TycB1 (Hahn and Stachelhaus, 2004). After verification of carrier protein loading by pantetheine azides **1a–f** through Cu-catalyzed cycloaddition with a fluorescent alkyne and SDS-PAGE analysis (Figure 2E), the ability of *crypto* TycAΔ23-**1a–f** to crosslink TycB1-**3** was tested. Incubation under identical conditions to those used with wild-type TycA azides demonstrated no similar crosslinking (see Figure S8). Figure 4F shows the crosslinking of TycAΔ23-**1a** with TycB1-**3** as an example of a negative (lane 1) and positive (lane 2) result for the crosslinking reaction between TycAΔ23 and TycB1 in Cu-free conditions.

DISCUSSION

We recently reported the synthesis and application of mechanism-based crosslinking probes that selectively crosslinked acyl carrier proteins from *Escherichia coli* type II fatty acid biosynthesis with their cognate ketosynthase domains (Worthington et al., 2006). This involved the chemoenzymatic synthesis of electrophile-incorporating CoA analogs, which were transferred to an *apo*-carrier protein by use of the promiscuous PPTase Sfp (Quadri et al., 1998). Addition of ketosynthase, which utilizes a nucleophilic cysteine for catalysis, resulted in covalent crosslinking of the two enzymes, trapping this normally transient interaction. While this initial study utilized individual domains, we sought to apply a similar approach to the investigation of the protein-protein interactions that govern selective communication between partner modules in NRP biosynthesis. This led to the synthesis of a small panel of crosslinking probes incorporating bioorthogonal azides and alkynes, designed to be loaded onto PCPs and used to immobilize intermodular interactions through chemoselective Hüisgen 1,3-dipolar cycloaddition reaction (Figure 1A). Since its development, the copper-(I)-catalyzed Hüisgen 1,3-dipolar cycloaddition of azides and alkynes has been shown to be useful for tagging of various biomolecules (Wang et al., 2003), activity-based protein profiling (Speers et al., 2003), and combinatorial chemistry for the generation and/or optimization of lead compounds (Lewis et al., 2002). Our approach was inspired by the target-guided synthesis of acetycholinesterase inhibitors (Lewis et al., 2002), which utilized conserved features of acetycholinesterase itself to catalyze the [3+2] cycloaddition reaction of azide and alkyne inhibitors binding to proximal sites of the enzyme. Similarly, we envisioned that decoration of two partner NRPS modules with the proper azido and alkynyl pantetheine analogs might promote a copper-free [3+2] cycloaddition due to the tight but transient intermodular protein interactions between their complementary COM domains.

Previous studies employing active-site promoted, copper-free azide-alkyne cycloaddition have been mainly applied to azide and alkyne inhibitors that bind a single enzyme at adjacent sites (Mocharla et al., 2004; Whiting et al., 2006). Because the transient nature of NRPS intermodular communication may not be conducive to such in situ approaches, we also explored the use of the ring strain-promoted [3+2] cycloaddition in addition to terminal azides and alkynes in trapping these protein-protein interactions. Cyclooctynes have recently been reported as promising new tools for bioconjugation (Agard et al., 2004; Baskin et al., 2007; Ning et al., 2008) due to their increased rate of triazole formation with azides. In addition, copper toxicity to the cell is eliminated for in vivo experiments (Baskin et al., 2007; Laughlin et al., 2008). The additional installation of electron-withdrawing substituents, such as fluoro groups observed at the propargylic position of **3**, are postulated to further decrease the azide-alkyne HOMO-LUMO gap and increase the rate of cycloaddition accordingly (Baskin et al., 2007). It was our belief that this increased rate might prove useful in compensating for the potentially transient nature of the protein-protein interactions in NRPS biosynthesis.

In this study, a panel of pantetheine azides and alkynes were examined for their utility in studying protein-protein interactions in NRPS biosynthesis. Due to their bioorthogonal reactivity, azide and alkyne functionalities are prime candidates for investigating biological macromolecules under physiological conditions. Application of this method to the first two modules of the tyrocidine synthetase, TycA and TycB1, resulted in the successful modification of the *apo*-PCP domains of these modules with pantetheine analogs **1–3** through a one-pot carrier protein modification protocol. Likewise, it was shown that after one-pot loading of

TycA and TycB1 with analogs **1–2**, each of the *crypto*-PCP domains produced were able to undergo Cu-catalyzed [3+2] cycloaddition reaction with corresponding azide or alkyne fluorescent reporters, regardless of the identity of the NRPS module loaded or specific structural features of the bioorthogonal pantetheine analog (Figure 2). Cyclooctyne **3** was able to undergo [3+2] cycloaddition reaction with the azide fluorescent reporter without the use of copper. After incubation under the reaction conditions specified, we demonstrated full modification of *apo*-PCP to *crypto*-PCP by addition of BODIPY-CoA and fresh Sfp, which did not result in fluorescent labeling of TycA or TycB1 (Figure S2). These experiments demonstrated the practicality of our experimental approach by elimination of two major concerns, the compatibility and efficiency of analogs **1–3** with carrier protein modification and azide-alkyne cycloadditon.

After verification of carrier protein modification, crosslinking assays were performed using Cu-catalyzed azide-alkyne cycloaddition (Figure 3). Individual loading of TycA and TycB1 with analogs **1–2**, followed by coincubation under Cu-catalyzed [3+2] cycloaddition conditions produced all possible combinations of crosslinked complexes between TycA and TycB1 (see Figure S4), suggesting that this protocol is insensitive to the protein-protein interactions of two modules mediated by the COM domains. In addition, these results indicated that crosslink formation was insensitive to changes in the azide-alkyne linker or the final position of the triazole formed. A final piece of evidence as to the negligible role of protein-protein interactions in guiding the Cu-catalyzed crosslinking of these domains was provided by the demonstration that alkyne-loaded TycB1 was capable of undergoing a similar cycloaddition with noncognate azide-loaded PCPs EntB and VibB. This experiment was notable, however, in that it demonstrated that small azide and alkyne moieties on these large, 120 kDa megaproteins were capable of locating each other even in these large contexts and reacting at an observable rate. Although nothing could be ascertained about the role of the COM domain from these experiments, they served as a positive control for following in situ azide-alkyne cycloaddition experiments in which copper and other reagents were omitted from the TycA-TycB1 reaction mixture. Interestingly, identical loading of TycA and TycB1 with bioorthogonal pantetheines **1–2** and incubation under all possible combinations in the absence of Cu showed no conclusive formation of the TycA-TycB1 complex. This indicates the inability of short-lived NRPS protein-protein interactions to mediate triazole formation at an appreciable rate.

The finding that Cu-catalyzed azide-alkyne crosslinking occurred at such a high rate as to negate the influence of protein-protein interactions, while NRPS intermodular communication was apparently too fleeting a phenomenon to promote the azide-alkyne activation necessary for in situ crosslinking, led us to explore the use of more subtle methods for alkyne activation, namely through the use of ring strain employed by cyclooctyne **3**. It was found upon incubation of *crypto* TycB1-**3** with *crypto* TycA-**1a–f**, copper-free azide-alkyne cycloaddition of the two modules occurred. The extent of TycA-TycB1 crosslinking was the same for TycA loaded with **1a–f**, suggesting again that probe length and triazole positioning were not major factors in catalysis of the cycloaddition. Intriguingly, the degree of TycA-TycB1 complex formed by use of cyclooctyne **3** was measurably greater than that observed when using Cu-catalyzed cycloaddition of the two proteins. This may be either due to increased promotion of complex formation by intact protein-protein interactions or, alternately, simply an artifact of the protein precipitation commonly encountered when using Cu-catalyzed azide-alkyne cycloaddition. This crosslinking was also found to be folding dependent, as boiling of TycA after loading with azide **1a** prior to incubation with *crypto* TycB1-**3** prevented crosslinking. We further validated the discrimination ability of **3** to capture only native protein-protein interactions of NRPS enzyme by assaying for *crypto* TycB1 alkyne **3** crosslinking with azide-loaded, noncognate NRPS modules EntB and VibB. In contrast to the use of Cu-catalyzed azide-alkyne cycloaddition, no crosslinked complexes were observed, suggesting that proper protein-protein interaction is required for crosslinking with **3** (Figures 4C and 4D). The specific role of the COM domain in promotion of these interactions was probed by examining bioorthogonal crosslinking of TycA-TycB1 with a COM domain-deficient mutant. Due to the absence of the last 23 C-terminal amino acids of TycA, TycAΔ23 cannot transfer its natural substrate (D-Phe) to the acceptor amino acid L-Pro on the next module (TycB1) in order for peptide formation to occur. As expected, crosslinking of TycAΔ23 loaded with pantetheine azides **1a–f** to TycB1-**3** did not occur due to a lack of the proper protein interactions, normally promoted by COM^{D}_{TycA}-COM^{A}_{TycB1}, between the two NRPS modules (see Figure S8). In this sense, the effect of COM domain mutation on the crosslinking ability of the TycA-N_3/TycB1-**3** pair mirrors the effect seen on peptide bond formation (Hahn and Stachelhaus, 2004).

Very interesting among these findings was the observation that TycA-TycB1 crosslinking was noticeably more efficient for the TycA-**1a**/TycB1-**3** pair than when the NRPS modules were loaded in the opposite configuration. This could be due to the possible deleterious interactions of cyclooctyne **3**, which contains a racemic center at the homobenzylic position, with the epimerization domain of TycA. Most tantalizing is the hypothesis that the observed crosslinking preference of the TycA-**1a**/TycB1-**3** pair is due to formation of the triazole bond within the TycB1 condensation domain substrate channel (Keating et al., 2002). It is known that the triazole formed in the 1,3-dipolar cycloaddition of azides with alkynes mimics the location of the atoms and the electronic properties of a peptide bond (Bock et al., 2006). However, this last possibility seems at odds with the lack of selectivity for any single TycA azide (**1a–f**) loaded, as well as with current knowledge of the substrate selectivity of the TycB1 condensation pocket for donor and acceptor substrates (Belshaw et al., 1999). In future studies we plan to probe the underlying cause of this crosslinking specificity by developing bioorthogonal crosslinkers that more closely resemble the substrates of the natural TycA-TycB1 condensation reaction (Lundquist and Pelletier, 2001).

SIGNIFICANCE

We have developed bioorthogonal crosslinking probes that are compatible with carrier protein modification by Sfp and sensitive to the selective protein interactions between NRPSs governed by COM domains. It was discovered that the intermodular interaction of NRPS modules TycA and TycB1 was too transient to catalyze in situ [3+2] cycloaddition

of PCP-loaded azides and alkynes, regardless of the specific structural characteristics of the bioorthogonal pantetheine analogs used. However, a ring strain-activated cyclooctyne, 3, showed highly efficient, protein-protein interaction-dependent crosslinking of cognate NRPS modules. Based on these results, we aim to apply these tools toward structural studies of the interactions that occur during selective communication between NRPS modules. In particular, with these probes covalently attached to the PCP, we hope to observe the specific interdomain dynamics and conformational states of the PCP and condensation domain implicated in the promotion of NRPS-mediated peptide-bond formation. Similar to the manner in which the use of active site-directed inhibitors in combination with crystallographic analysis has proven a valuable method for gaining insight into the mechanism employed by single enzymes, so might our method to capture transient protein-protein interactions prove key to unraveling the cryptic mechanisms employed by multifunctional biosynthetic assembly lines.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Proteins

Recombinant proteins PheATE (TycA), ProCAT (TycB1), and pTrcHis-TOPO-TycAΔ23 (TycAΔ23) were expressed and purified as previously described (Stachelhaus et al., 1998). Recombinant proteins EntB and VibB were expressed and purified as previously described (La Clair et al., 2004).

Verification of PCP Modification with Pantetheine Analogs by Copper-Catalyzed Azide-Alkyne Cycloaddition

The one-pot reaction for carrier protein modification of TycA and TycB1 was carried out with pantetheine analogs **1–3** and then verified by copper-catalyzed azide-alkyne cycloaddition with a fluorescent tag utilizing the following procedure. One-pot reaction mixtures contained the following (final volume of 50 μl): 50 mM potassium phosphate buffer (pH 7.0), 8 mM ATP, 12.5 mM $MgCl_2$, 3.3 μM PanK, 3.3 μM PPAT, 3.3 μM DPCK, 10 μM *B. subtilis* Sfp, and 2.0 μM of carrier protein enzyme TycA/TycB1. The reaction was initiated by addition of 0.4 mM pantetheine analog and incubated at 37°C for 1 hr. Then the corresponding 50 μM coumarin azide/alkyne, 1 mM $CuSO_4$, 1 mM TCEP, and 0.1 mM TBTA ligand were added and allowed to react at room temperature for 1 hr. Reactions were stopped with the addition of 10 mM dithiothreitol (DTT) and 20 μl of 2× loading buffer. Samples were run on 4%–12% SDS-PAGE gels and visualized with UV.

To verify 100% conversion of carrier protein modification of TycA and TycB1, the same one-pot reactions were performed as mentioned above and then 200 μM BODIPY-CoA and 10 μM Sfp were added and incubated at 37°C for 1 hr. Reactions were stopped with the addition of 10 mM DTT and 20 μl of 2× loading buffer. Samples were run on 4%–12% SDS-PAGE gels and visualized with UV.

Copper-Catalyzed and Copper-Free Crosslinking Assays between TycA/EntB/VibB and TycB1 with Pantetheine Analogs

Copper-catalyzed crosslinking assays were performed utilizing the following procedure. After a 1 hr incubation at 37°C of one-pot reactions for carrier protein modification of EntB, VibB, TycA, and TycB1 with pantetheine analogs **1–3**, 25 μl of the azido/alkynyl pantetheine-tagged TycA/EntB/VibB one-pot reactions were reacted with 25 μl of the corresponding azido/alkynyl pantetheine-tagged TycB1 one-pot reactions with the addition of 1 mM $CuSO_4$, 1 mM TCEP, and 0.1 mM TBTA ligand for 1 hr at room temperature. Reactions were stopped with the addition of 10 mM DTT and 20 μl of 2× loading buffer. Samples were run on 3%–8% SDS-PAGE gels and stained with Coomassie blue in order to detect crosslinked products.

Copper-free crosslinking assays were performed utilizing the following procedure. After a 1 hr incubation at 37°C of one-pot reactions for carrier protein modification of EntB, VibB, TycA, and TycB1 with pantetheine analogs **1–3**, 25 μl of the azido/alkynyl pantetheine-tagged TycA/EntB/VibB one-pot reactions were reacted with 25 μl of the corresponding azido/alkynyl pantetheine-tagged TycB1 one-pot reactions for 24 hr. Reactions were stopped with the addition of 10 mM DTT and 20 μl of 2× loading buffer. Samples were run on 3%–8% SDS-PAGE gels and were either stained with Coomassie blue in order to detect crosslinked products or blotted onto PVDF membrane and developed utilizing the Universal His Western Blot Kit 2.0.

SUPPLEMENTAL DATA

Supplemental Data include eight figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00075-1.

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