

CBI Student Sabbatical Proposal:
Characterization of docking domains from the curacin
A biosynthetic pathway

Jonathan R. Whicher, Chemical Biology

Advisor: Dr. Janet L. Smith, Biological Chemistry

Sabbatical Host: Dr. David Sherman

Location: University of Michigan, Life Sciences Institute

Duration: 9 weeks

Time Frame: February 1, 2011 – March 31, 2011

Summary:

Docking domains are structural domains located at the N- and C-terminus of modules in polyketide synthases (PKSs) that promote interaction and facilitate intermediate transfer between modules. The purpose of my sabbatical is to biochemically characterize the docking domains from the curacin A biosynthetic pathway, which appear to have different structures than previously characterized docking domains. This will be accomplished through binding experiments, which are designed to determine the specificity and affinity of the curacin A docking domain interaction, and biochemical assays, which will probe intermodular intermediate transfer mediated by curacin A docking domains.

Introduction. Polyketides are natural products that are produced and secreted by microbes to provide the producing organism with a competitive advantage in its environment. Due to this inherent biological activity, many polyketides or polyketide analogues have been investigated for and are currently used as therapies for human diseases [1]. Thus, the study of polyketide biosynthesis has become an important area of research with the ultimate goal of engineering biosynthetic pathways to produce novel therapeutics [2-5]. Polyketides are synthesized from acyl-CoA precursors by large protein complexes called polyketide synthases (PKS) [6]. The large protein complexes of type I PKSs are organized into modules, each of which catalyzes a specific elongation and up to several specific modifications of a certain intermediate in the biosynthesis of the natural product [7, 8].

Modules in PKS pathways can be covalently linked or on separate polypeptides. Modular association and intermediate transfer between modules on separate polypeptides is mediated by docking domains, which are structured domains at the C-terminus (attached to acyl carrier protein domains (ACPs)) of the upstream module and the N-terminus (attached to the ketosynthase domains (KSs)) of the downstream module [9-16]. There is increasing interest in docking domains for PKS engineering due to their ability to mediate interaction and intermediate transfer between non-interacting modules [9, 17-21]. To date, most structural and biochemical studies of PKS docking domains have focused on actinobacterial PKSs, specifically pikromycin synthase (Pik) and 6-deoxyerythronolide B synthase (DEBS) [9-17, 22, 23]. I conducted sequence alignments, secondary structure predictions, and bioinformatic analyses that indicate the docking domains from cyanobacterial PKSs, such as the curacin A (Cur) pathway [24], have

different structures from actinobacterial docking domains. These analyses agree with a prior bioinformatic analysis which indicated that cyanobacterial docking domains cluster into a class distinct from actinobacterial docking domains [25]. The characterization of cyanobacterial docking domains is essential for engineering PKS pathways to take advantage of the incredible chemical diversity of cyanobacterial polyketides. In addition, further characterization of PKS docking domains, especially docking domains that appear to have different structures to those previously characterized, will provide valuable insights into structural elements that allow for docking of PKS modules. These insights could facilitate the design of docking domains that can mediate intermediate transfer more efficiently and can be used in PKS engineering.

Our docking domains will be characterized with a combination of binding and structural experiments similar to those used for the characterization of Pik and DEBs docking domains [14]. In addition, an *in vitro* assay will assess the ability of cyanobacterial docking domains from the curacin A pathway to mediate intermediate transfer between actinobacterial modules. For this assay, curacin docking domains will be substituted onto the well characterized system consisting of module 5 (Pik AIII) and module 6 (PikAIV) of the Pik pathway and product formation will be used as a readout for the ability of docking domains to mediate intermediate transfer [21, 23, 26, 27].

The structural studies will be carried out in my current lab, Dr. Janet Smith's lab, which specializes in protein crystallography. A sabbatical in the Sherman laboratory will provide an opportunity to quickly develop expertise for the binding experiments and PikAIII/PikAIV *in vitro* assay. Furthermore, it will provide an opportunity to perform the proposed experiments under the guidance of scientists who completed similar

experiments. The Sherman laboratory successfully determined the binding constants of docking domains from actinobacterial PKSs [14], has experience with biolayer interferometry, developed the PikAIII/PikAIV assay, synthesized the substrates for the assay, and determined the role of native docking domains on intermediate transfer between PikAIII and PikAIV [21, 23, 26, 27].

Preliminary work:

My current work in the laboratory focuses on designing, cloning, expressing, and purifying the curacin docking domain constructs that are necessary for crystallization, binding experiments, and biochemical assays. For the binding studies, three Cur docking domain constructs will be made: 1) Full-length ACPs with C-terminal docking domains (flACPdd) from modules CurG-CurL, 2) Full-length CurG ACP with the C-terminal docking domain containing a 9 residue C-terminal truncation (CurGACPdd Δ 9), and 3) full length ketosynthase-acyltransferase (KS-AT) with N-terminal docking domains (KSATdd) from modules CurH-CurM. All of the flACPdd constructs and the CurGACPdd Δ 9 construct were designed, PCR amplified, and ligated into an expression vector. Furthermore, 4 of the flACPdd (CurG flACPdd, CurH flACPdd, CurI flACPdd, and CurK flACPdd) and CurGACPdd Δ 9 were expressed and purified. In addition, all of the KSATdd constructs were designed and PCR amplified and 2 of the 6 (CurHKSATdd and CurJKSATdd) were ligated into an expression vector, expressed and purified. Prior to the start of my sabbatical in the laboratory of Dr. David Sherman I hope to have purified each docking domain constructs necessary to complete the binding studies.

The PikAIII/PikAIV assay is designed to determine the ability of curacin A docking domains to mediate intermodular intermediate transfer between actinobacterial

modules [21, 23, 27]. Currently, I am developing a method to efficiently substitute docking domains onto PikAIII and PikAIV utilizing restriction enzyme sites. I hope to have PikAIII fused to the CurG ACPdd and PikAIV fused to CurH KSdd cloned and purified prior to my sabbatical.

Proposed Studies:

Goal 1. Characterization of the binding interaction between curacin A docking domains.

Equilibrium dissociation constants (K_d) for all combinations of flACPdd and KSATdd pairs will be obtained to assess the affinity and specificity of the curacin docking domain interactions. These K_d values will be compared to those of actinobacterial docking domains [14] to determine if the structural variability between cyanobacterial and actinobacterial docking domains alters the affinity of the binding interaction. In addition, K_d values between CurG flACPdd Δ 9 and CurH KSATdd will be determined. This will indicate if the final 9 residues of the ACPdd are necessary for the binding interaction as shown for actinobacterial docking domains. If the 9 residue C-terminal truncation on the ACPdd does not affect binding, then further truncations will be made to elucidate the residues necessary for binding.

To complete the binding experiments I will use the Octet Red system, which employs biolayer interferometry (BLI) to measure protein-protein interactions [28]. BLI detects the interference pattern of white light reflected from a protein layer bound to a biosensor tip and an internal reference layer in the biosensor tip. Protein binding to the tip causes the interference pattern to shift thus allowing the measure of protein-protein interactions. This method is optimal for the binding experiments that I proposed because

it uses small quantities of protein, lacks the complications of surface plasmon resonance that arise from microfluidics, and it does not require protein labels. Members of the Sherman laboratory are currently using the octet Red to determine the affinity of docking domain interactions. Therefore, a sabbatical in the Sherman laboratory will be a great opportunity to gain expertise in the Octet Red and complete the proposed binding experiments under the guidance of Sherman lab members currently performing similar experiments.

Goal 2: Determine if cyanobacterial docking domains can mediate intermediate transfer in actinobacterial PKS pathway

The Sherman laboratory developed the PikAIII/PikAIV transfer assay that is ideal for probing the ability of the curacin docking domains to mediate intermediate transfer. PikAIII and PikAIV are consecutive monomodules in the actinobacterial Pik pathway, whose interaction is mediated by docking domains [23]. In addition, PikAIV contains a thioesterase domain (TE), which cleaves intermediates transferred from PikAIII to allow for their quantitation. Therefore, product formation can be used as a readout for effectiveness of intermediate transfer. The docking domain pair between CurG and CurH will be substituted onto the PikAIII/PikAIV system and steady state kinetic parameters will be determined. These values will be compared to the steady state kinetic parameters for the PikAIII/PikAIV system with the natural docking domains, determined previously [27]. This comparison will indicate how the structural differences between actinobacterial and cyanobacterial docking domains affect intermediate transfer efficiency between modules and will provide insights into the structural features that are necessary to mediate intermediate transfer. Furthermore, this analysis will indicate if

cyanobacterial docking domains can mediate intermediate transfer between actinobacterial modules, which is important for engineering PKS pathways with modules from both actinobacterial and cyanobacterial PKSs.

The PikAIII/PikAIV assay is complicated because it involves the action of 6 enzymes from in 2 multi-domain proteins. Furthermore, cloning foreign docking domains into the plasmids containing the multi-domain proteins is a difficult task. The Sherman laboratory has expertise in PikAIII/PikAIV transfer assay [21, 23, 27] and fusing foreign docking domains to multi-domain proteins. Therefore, a sabbatical in the Sherman laboratory will provide an opportunity to quickly learn the cloning techniques to make the docking domain swaps and fusions and the experimental techniques to perform the PikAIII/PikAIV assays. Furthermore, I will be able to complete these experiments under the guidance of the Sherman lab members, which will be essential for troubleshooting experimental problems that may arise. Such an experience is essential for successful completion of my thesis project and can only occur while working in the laboratory of Dr. David Sherman during a sabbatical.

Relevance to Thesis Research:

My thesis research at the University of Michigan focuses on the structural biology of PKS pathways. In particular, my current project is aimed at understanding the molecular basis for docking domain interactions. For this project I proposed to solve the structures of docking domains from the curacin A biosynthetic pathway, which appear to have different structures than previously characterized docking domains. In addition to the structure I believe the two experiments I proposed for my sabbatical, binding studies and biochemical assays, will be essential to fully characterize and understand the

interactions between curacin A docking domains. Therefore, the projects that I am proposing to complete during my sabbatical are not only directly related to my thesis project but are essential for successful completion of it. Currently, I am nearly finished all the steps of cloning and protein purification for the proposed binding experiments. The best way to quickly develop expertise in biolayer interferometry and the PikAIII/PikAIV based docking domain assays necessary to perform these experiments is to spend time in the Sherman lab during a sabbatical.

A sabbatical in the laboratory of Dr. David Sherman is a great opportunity for me to gain expertise in and perform the binding and assay experiments that I proposed. For this sabbatical, I will physically move to the Sherman laboratory, immerse myself in the lab to complete my proposed experiments under the guidance of lab members experienced in performing similar experiments, and not participate in Smith lab activities. Such an experience will allow me to gain access to the full array of equipment and personnel in the Sherman lab to facilitate the completion of the proposed experiments. The proposed binding experiments and complex enzyme assays were developed following admission to the CBI training program as a sabbatical project to be performed in the laboratory of Dr. David Sherman. As a result, my proposed sabbatical was formulated to fulfill the requirements stated in the student handbook that it should be “preferably in a research collaborators laboratory” and a “self-contained project that is relevant to the student’s thesis work”. I believe my proposed sabbatical follows these guidelines because I am working in a collaborator’s lab, the Sherman lab, and the experiments that I proposed, which are aimed to biochemically characterize the interactions of curacin docking domains, will complement the structural characterization

of curacin docking domains that will be completed in the Janet Smith lab. Thus, my proposal is a sabbatical in the sense that I will be working only in the Sherman laboratory for its duration and I will be performing binding and assay experiments on polyketide synthase pathways, which require a new set of skills and techniques that are different from those needed for structural biology experiments.

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life sciences institute
SEPTEMBER 29, 2010

Prof. Vincent Pecoraro
Department of Chemistry
University of Michigan
930 North University Avenue
3823 Chemistry Building
Ann Arbor, MI 48109

Dear Prof. Pecoraro,

I am writing this letter in regards to the sabbatical experience that Jonathan Whicher is proposing to conduct in my laboratory between the dates of February 1, 2011 and March 31, 2011. As a member of Jonathan's thesis committee and a collaborator on his thesis project, I believe a sabbatical in my laboratory will provide him with a key opportunity to develop new skills and perform fundamental experiments for his thesis project. My laboratory has the expertise and all necessary materials, including active polyketide synthase modules and synthetic substrates to enable successful completion of the docking domain interaction studies and biochemical assays that Jonathan has proposed. In addition, the techniques acquired during Jonathan's sabbatical will be subsequently applied in Prof. Smith's laboratory to complete additional biochemical analyses in pursuit of his thesis research.

I enthusiastically agree to have Jonathan Whicher pursue his sabbatical in my laboratory with essential mentoring support toward the aims outlined in his sabbatical proposal.

With best wishes.

Sincerely,

A handwritten signature in black ink that reads 'David H. Sherman'.

David H. Sherman, Ph.D.
Hans W. Vahlteich Professor of Medicinal Chemistry (College of Pharmacy)
Professor of Microbiology & Immunology (Medical School)
Professor of Chemistry (College of Literature, Science & Arts)
Director, Center for Chemical Genomics



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Janet L. Smith, PhD

Martha L. Ludwig Professor of Protein Structure and Function
Department of Biological Chemistry

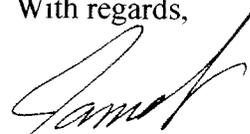
September 16, 2009

Dr. Vincent Pecoraro
Department of Chemistry
University of Michigan
930 North University Avenue
3823 Chemistry Building
Ann Arbor, MI 48109

Dear Vince,

I'm writing to support of the sabbatical plan that Jonathan Whicher has developed to spend time in David Sherman's laboratory from February 1, 2011 to March 31, 2011. Jon's project involves both the structural biology and the biochemistry of polyketide synthase (PKS) pathways. The structural biology experiments of his thesis project will be completed in my laboratory. The Sherman laboratory has developed several convenient PKS assays, which Jon will apply to his project along with binding studies. Therefore, a sabbatical in the Sherman laboratory will be a great opportunity for Jon to perform the experiments set forth his sabbatical proposal and to learn techniques that will benefit other aspects of his thesis project. Thus, I strongly support Jon's sabbatical plan.

With regards,



Janet L. Smith