JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Structure and Substrate Sequestration in the Pyoluteorin Type II Peptidyl Carrier Protein PltL

Matt J. Jaremko, D. John Lee, Stanley J. Opella, and Michael D. Burkart*

Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0358, United States

Supporting Information

ABSTRACT: Type II nonribosomal peptide synthetases (NRPS) generate exotic amino acid derivatives that, combined with additional pathways, form many bioactive natural products. One family of type II NRPSs produce pyrrole moieties, which commonly arise from proline oxidation while tethered to a conserved, type II peptidyl carrier protein (PCP), as exemplified by PltL in the biosynthesis of pyoluteorin. We sought to understand the structural role of pyrrole PCPs in substrate and protein interactions through the study of pyrrole analogs tethered to PltL. Solution-phase NMR structural analysis revealed key interactions in residues of helix II and III with a bound pyrrole moiety. Conservation of these residues among PCPs in other pyrrole containing pathways suggests a conserved mechanism for formation, modification, and incorporation of pyrrole moieties. Further NOE analysis provided a unique pyrrole binding motif, offering accurate substrate positioning within the cleft between helices II and III. The overall structure resembles other PCPs but contains a unique conformation for helix III. This provides evidence of sequestration by the PCP of aromatic pyrrole substrates, illustrating the importance of substrate protection and regulation in type II NRPS systems.

Type II nonribosomal peptide synthetases (NRPSs) commonly combine with fatty acid and/or polyketide synthases (FAS/PKS) generating structurally diverse secondary metabolites with activities ranging from anti-infective to anticancer agents. The products of type II NRPSs are modified amino acids and are frequently aromatic. For example, proline, tyrosine, and salicylic acid are shuttled through type II NRPSs to form pyrrole, hydroxytyrosine, and methoxy-chlorosalicylic acid before incorporation into downstream pathways.[1] These moieties are electron-rich rings that can participate in hydrogen bonding and π-stacking interactions that can contribute to the bioactivity of their respective product. The pyrrole containing compounds prodigiosin and chlorizidine A exhibit promising antitumor activity,[2] for which a prodigiosin derivative recently entered stage II clinical trials (Figure 1).[3] Vancomycin (hydroxytyrosine) and chlorothricin (methoxy-chlorosalicylic acid) are known antibacterial and cholesterol reducing compounds, respectively.[4] All type II NRPS components in these compounds are generated while covalently attached to a peptidyl carrier protein (PCP).

As shown in Scheme 1, the PCP is responsible for shuttling peptide substrates to partner enzymes for type II NRPS catalyzed

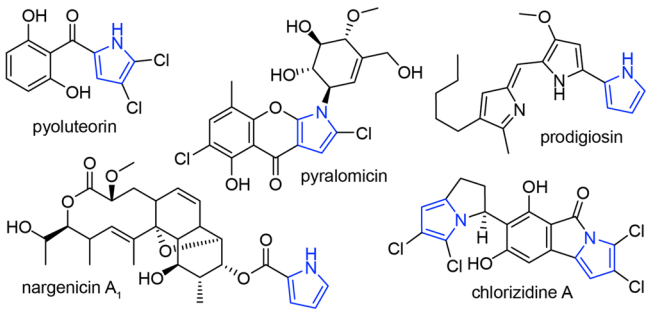

Figure 1. Structures of pyrrole containing natural products.

modifications. To prime the *apo*-PCP, a 4′-phosphopantetheine (PPant) arm is first coupled to a conserved serine by a 4′-phosphopantetheinyl transferase (PPTase), generating *holo*-PCP. In systems that incorporate a pyrrole, an adenlyation domain serves to load proline (Pro) onto the *holo*-PCP. The resulting prolyl-PCP is then recognized by a flavoprotein dehydrogenase, which catalyzes a four-electron oxidation of Pro to the corresponding pyrrolyl-PCP.[5] Further downstream processing can occur while tethered to the PCP, as illustrated by pyrrole halogenation in pyoluteorin biosynthesis (Scheme 1). In this system, the modified pyrrole is then transferred to a three-module type I PKS system that facilitates elongation and reduction, followed by cyclization to pyoluteorin.[6]

Given that PCP attachment is essential for product formation, protein−protein interactions between the PCP and partner enzymes are presumed to be critical, although the structural details of these binding events remain unknown. In pyoluteorin biosynthesis, Pro is loaded onto the PCP PltL by PltF and then oxidized to pyrrole by PltE (Scheme 1). The homologous adenylation and dehydrogenase enzymes in undecylprodigiosin biosynthesis cannot catalyze pyrrole formation with PltL,[5] illustrating the importance of PCP identity, substrate demonstration, and protein−protein interactions.

Substrate sequestration in carrier proteins has been recognized as an important phenomenon in type II fatty acid and polyketide synthases.[7] Type II carrier proteins, as opposed to type I, are stand-alone enzymes that must recognize up to five partner proteins in a particular order. When not interacting with a partner, the carrier protein has been shown to protect the extending substrate from reactive compounds in the cytosol. Here the substrate localizes in a hydrophobic cleft between helix II and III

Received: April 30, 2015
Published: September 4, 2015

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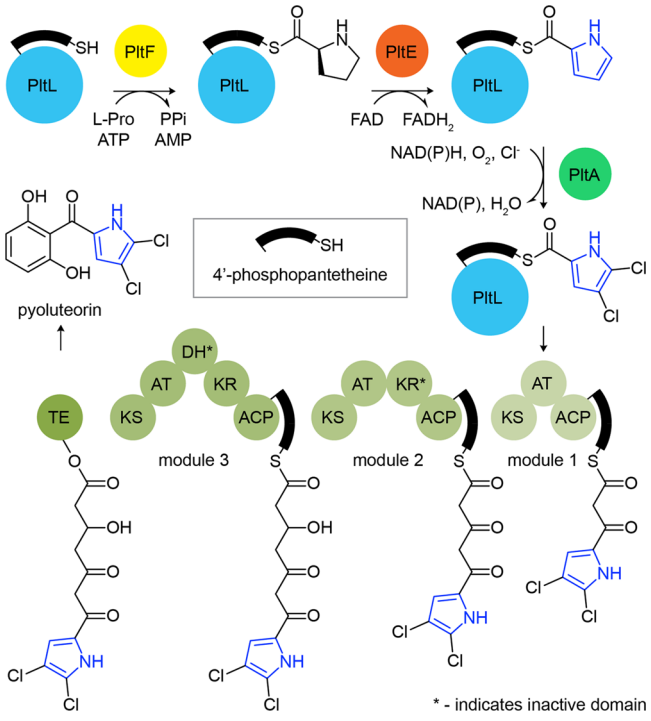
DOI: 10.1021/jacs.5b04525
J. Am. Chem. Soc. 2015, 137, 11546−11549

Scheme 1. Biosynthesis of Pyoluteorin



of the acyl carrier protein (ACP). NMR structural studies of acylated *E. coli* fatty acid ACP reveals sequestration of chain lengths greater than C4,[7d,e] which shields the growing metabolite from nucleophiles other than the cognate partner protein.[8] Similar interactions are also seen in type II PKS, where helix II and III of actinorhodin ACP were shown to interact with cyclic and linear intermediate analogs.[7f,g] While PCPs have been shown to interact with partner enzymes at the helix II/III interface,[9] no studies have demonstrated PCP substrate sequestration. The overall helical structure of PCPs is similar in helix II and III; however, amino acid distributions are notably diverse, creating significant electrostatic differences.[10] These surfaces may be key to protection of various intermediates to discriminate for reactivity with proper partner enzymes. Structural studies of carrier proteins can provide insight into the regulation of substrate modification. Currently, there are no structures of a peptidyl-bound PCP, and there are only two structures of stand-alone type II PCPs, BlmI and A3404, for which functions are unknown.[10,11]

Here we show that pyrrole PCPs actively sequester their pyrrole cargo. Bioinformatic analysis of PltL and other pyrrole PCPs revealed a secluded PCP subfamily with several conserved residues. Using chemoenzymatic techniques developed in our laboratory,[12] we attached PPant-Pro and PPant-pyrrole mimetics to PltL to study interactions between the PCP and substrate. NMR analysis revealed direct interactions of the pyrrole with residues in helix II and III not observed in other PCPs.[13] Through NOE techniques, the pyrrole acyl substrate was localized in a 3D solution NMR structure of PltL. The results shed light on how type II NRPS PCPs regulate peptidyl substrates and for the first time reveal direct interactions between the substrate and PCP.

Due to the unique nature of pyrrole biosynthesis, we first hypothesized that pyrrole PCPs could encompass a subfamily of PCPs. Sequence homology, generated with MUSCLE,[14] was used to compare PCPs that tether pyrrole, Pro, and other amino acids as final products (Figure S1). The comparison highlights conserved residues and polarity in pyrrole PCPs, particularly at the helix II N-terminus and in helix III. A phylogenetic tree generated with ClustalW[15] further illustrates the seclusion of pyrrole PCPs (Figure S2). By comparison, PCPs that load Pro as a final product are dispersed among PCPs that load other amino acids, emphasizing the unique conservations in pyrrole PCPs. Some of the conserved residues play an important part in substrate stabilization and possibly protein−protein recognition.

To analyze the interaction between PltL and peptidyl substrates, NMR experiments were considered. However, the natural thioester between the pantetheine and substrate is susceptible to hydrolysis in aqueous solution, and this instability is further aggravated under conditions for NMR studies.[7a,16] Therefore, we prepared pantetheine mimetics with Pro and pyrrole for attachment onto PltL employing an amide linkage in lieu of the thioester (SI A.1, A.2). These Pro and pyrrole probes were then loaded onto PltL via a one-pot chemo-enzymatic strategy as previously described.[12] Posttranslational modification was verified by urea PAGE and LCMS (Figures S3, S4). To locate which PltL residues are perturbed by addition of the Pro and pyrrole moieties, $^{1}H-^{15}N$ HSQC experiments were conducted on ^{15}N-*holo*-PltL, ^{15}N-prolyl-PltL, and ^{15}N-pyrrolyl-PltL, and chemical shift perturbations (CSPs) were calculated (Figure 2). Analysis revealed several key residues that were altered with the

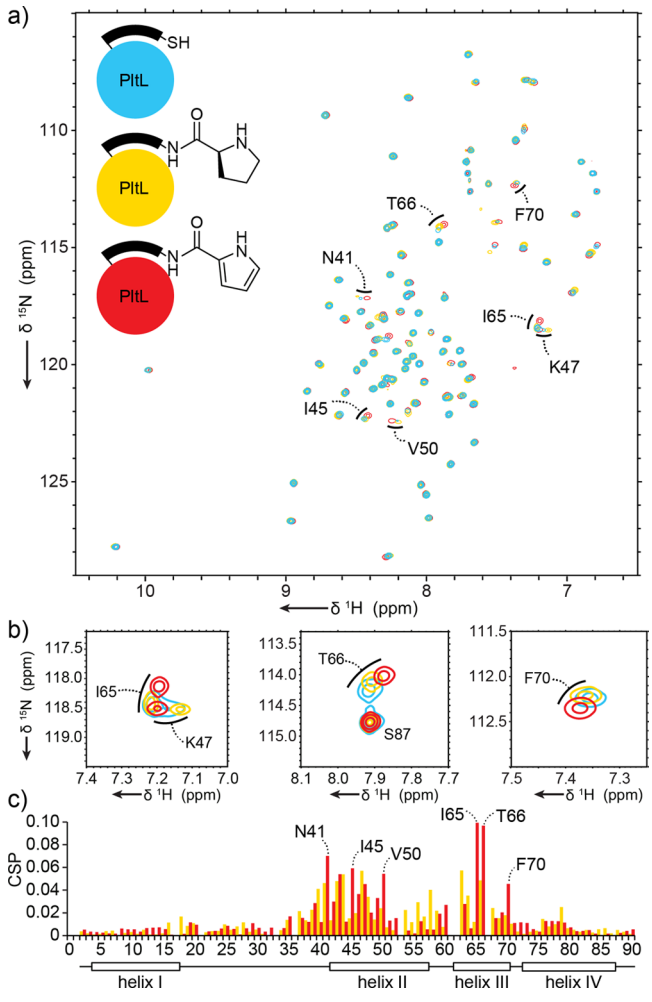


Figure 2. (a) HSQC overlay of ^{15}N-*holo*-PltL, ^{15}N-prolyl-N-PltL, ^{15}N-pyrrolyl-N-PltL. (b) Highlights of ^{15}N-HSQC data, illustrating perturbations of residues I65, T66, and F70. (c) CSP plots of ^{15}N-prolyl-PltL and ^{15}N-pyrrolyl-PltL relative to ^{15}N-*holo*-PltL.

DOI: 10.1021/jacs.5b04525
J. Am. Chem. Soc. 2015, 137, 11546−11549

attachment of the Pro and pyrrole moieties. Residues in helix II and proximal to Ser42 (covalently attached to PPant) showed significant movement, specifically Asn41, Ile45, Lys47, and Val50. However, the largest movements were seen in helix III, including residues Ile65, Thr66, and Phe70 (Figure 2). The side chains of Asn41, Lys47, and Val50 do not protrude toward the helix II/III cleft and may indicate general movement of helix II, although other residues, as mentioned below, have direct contact with the substrates. The CSP results of PltL coincide with other carrier protein−substrate interactions, where the majority of the interactions occur at helix II and III. Overall, the perturbations were larger for pyrrolyl-PltL compared to prolyl-PltL. This may indicate further sequestration or stabilization of the more hydrophobic pyrrole moiety compared to Pro.

In conjunction with the PltL perturbations, movement was also observed in protons of the pyrrole (Figure 3). Due to the unique chemical shifts of pyrrole protons, a ^{1}H NMR experiment could be used to observe the protons in PltL-bound pyrrole. Three isolated peaks of the pyrrole shift upfield when pyrrole-*N*-pantetheine is tethered to PltL, compared to the probe alone, or in solution but unattached to PltL (Figure 3). This indicates that the pyrrole is located in a more electron-rich environment, in this case, the hydrophobic cleft between helix II and III. PltL was also shown to interact with the solvatochromatic compound 4-DMN (Figure S15).[17] The NMR and fluorescent data suggest that the pyrrole is solvent protected. A ^{1}H−^{1}H NOESY experiment was then performed on pyrrolyl-PltL to observe any specific proton interactions between the pyrrole and PltL residues. The pyrrole proton NOESY strips contained several peaks corresponding to Leu35 Hδ*, Ile45 Hδ1*, Ile65 Hγ2*/δ1*, and Thr66 Hα, all of which are located in the cleft of helix II and III (Figure S16). Correspondingly, pyrrole proton shifts can also be visualized within NOE strips of hydrophobic residues of the PltL cleft (Figure S17).

To further visualize the interactions between PltL and peptidyl substrates, the solution NMR structures of both *holo*-PltL (PDB ID: 2N5H) and pyrrolyl-PltL (PDB ID: 2N5I) were determined with Cyana 3.97 (Figures 4a−b, S18).[18] The overall structure of PltL consists of four bundled helices as seen with all carrier protein structures (Figure S19).[9−11] PltL has both positive and negative electrostatic surfaces similar to other PCPs (Figure S20), but different from FAS and PKS ACPs, which have primarily negative surfaces.[10] Unlike the multiple states observed for the type I excised PCP TycC3,[19] a single state was observed for both PltL structures similar to that seen in other PCPs.[10,13,20] Further similarities with PCPs include a linker region and helical turn between helix I and II and a Pro situated on the N-terminus of helix III.[10,13] However, PltL contains Gly67 that interrupts the center of helix III, consequently positioning neighboring residues closer to helix II (Figure 4c). For example, Ile65 and Thr66 are only in position to interact with peptidyl substrates due to the helical interruption. Several other pyrrole carrier proteins contain Gly and/or Pro in the middle of helix III, likely interrupting these

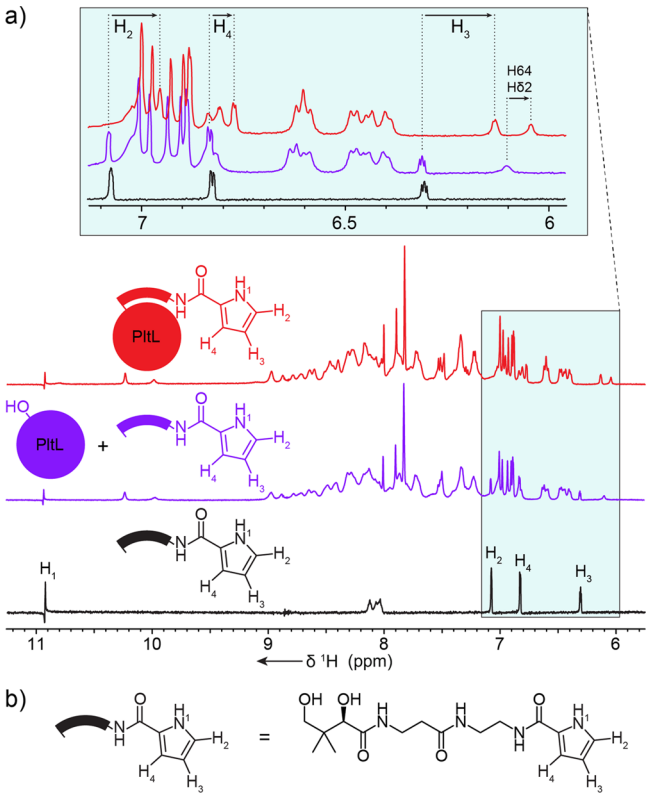


Figure 3. Pyrrole NMR shift analyses. (a) ^{1}H NMR experiment of pyrrolyl-*N*-pantetheine probe isolated (red), with *apo*-PltL (blue), and covalently attached to PltL (green). The enlarged spectra reveal perturbations of pyrrole protons, suggesting pyrrole-PltL interactions. (b) Structure of pyrrolyl-*N*-pantetheine probe.

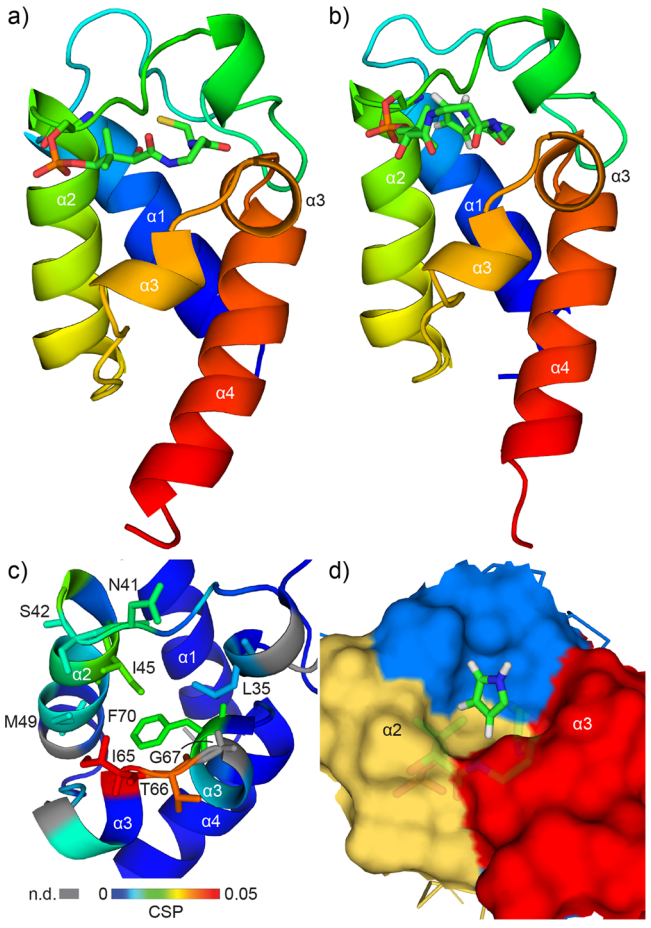


Figure 4. Solution NMR structures of PltL species. (a) *holo*-PltL. (b) pyrrolyl-PltL. (c) Expanded view of pyrrolyl-PltL with colors corresponding to CSP values from Figure 2. Key residues are labeled. CSPs not determined are shown in gray. (d) Expanded view of surface-filled pyrrolyl-PltL structure illustrating pyrrole sequestration between the helix II/III cleft.

DOI: 10.1021/jacs.5b04525
J. Am. Chem. Soc. 2015, 137, 11546−11549

helices (Figure S1), while other PCPs do not contain an interrupter (Figure S19).

Spectroscopic observation of the PPant arm of *holo*-PltL was facilitated by ^{15}N and ^{13}C isotope labeling of the tethered molecule (Figures S8, S10). This was achieved by cotransformation of PltL and Sfp constructs for *in vivo* production of *holo*-PltL (SI B.3). NOEs from the PPant to the protein were observed on both ends, but only intra-NOEs were observed in the middle of the PPant arm. These NOEs provided constraints between the thiol end and residues in both helix II and III, yielding a loop conformation with the thiol positioned between helix II and III.

The solution NMR structure of pyrrolyl-PltL was determined containing the solvent protected pyrrole localized between helix II and III (Figures 4d, S21). The structure of pyrrolyl-PltL differs slightly with the *holo*-PltL, where the hydrophobic cleft between helix II and III expands to accommodate the pyrrole. The PPant in *holo*-PltL has more constraints than in pyrrolyl-PltL, possibly due to the pyrrole displacing the PPant further into solution. The position of the pyrrole protects the reactive 4 and 5 positions of the aromatic ring, which are eventually chlorinated (Scheme 1).[21] In this position, the pyrrole is inaccessible to the halogenase PltA and, therefore, the interaction between PltL and PltA must induce a conformational change in PltL to display the pyrrole to PltA. These structural data highlight substrate sequestration by PltL in the biosynthesis of pyrrole and point to a similar role for other PCPs in type II NRPS pathways.

In this report, the structure of the first pyrrole carrier protein was determined, and the protein's interaction with substrate mimetics is revealed. This work illustrates the features of PCPs that are responsible for binding and protection of substrate intermediates and suggests the existence of similar PCP-dependent regulation in other type II NRPS biosynthetic pathways. For example, the methoxy-chlorosalicylic acid in chlorothricin is generated while attached to the type II PCP ChlB2,[1a] which has high sequence identity to PltL (36%) and therefore may have similar sequestration events. These studies also provide important information toward understanding the protein interfaces that facilitate specificity within pyrrole biogenesis. Future work with type II NRPS enzymes will provide a more complete understanding of protein−protein recognition and offer the prospect of enhanced bioactive compounds via synthetic biology.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b04525.

NMR structures were deposited to the PDB under IDs 2N5H (*holo*PltL) and 2N5I (pyrrolyl-PltL) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*mburkart@ucsd.edu

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Funding was provided from NIH GM095970 and NIH/NCI T32 CA009523. We thank J. Beld for the solvachromatic probe, C. T. Walsh for the plasmid constructs, Drs. X. Huang and A. Mrse for NMR assistance, and Dr. Y. Su for MS services.

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DOI: 10.1021/jacs.5b04525
J. Am. Chem. Soc. 2015, 137, 11546−11549