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Sulfonyl 3-alkynyl pantetheinamides as mechanism-based crosslinkers of ACP dehydratase

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Abstract

The acyl carrier protein (ACP) plays a central function in acetate biosynthetic pathways, serving as a tether for substrates and growing intermediates. Activity and structural studies have highlighted the complexities of this role, and its protein-protein interactions have recently come under scrutiny as a regulator of catalysis. As existing methods to interrogate these interactions have fallen short, we have sought to develop new tools to aid their study. Here we describe the design, synthesis, and application of pantetheinamides capable of crosslinking ACPs with catalytic β -hydroxyacyl carrier protein dehydratase (DH) domains based upon a 3-alkynyl sulfone warhead. We demonstrate this process by application to the *Escherichia coli* fatty acid synthase and apply it to probe protein-protein interactions with non-cognate carrier proteins. Finally, we use solution phase protein NMR to demonstrate that sulfonyl-3-alkynyl pantetheinamide is fully sequestered by the ACP, indicating that the *crypto*-ACP closely mimics the natural DH substrate. This crosslinking technology offers immediate potential to lock these biosynthetic enzymes in their native binding states by providing access to mechanistically-crosslinked enzyme complexes, presenting a solution to ongoing structural challenges.

The acyl carrier protein (ACP) is a highly conserved, small (~9 kDa), acidic protein found in fatty acid synthase (FAS) and polyketide synthase (PKS) biosynthetic pathways.¹ The ACP serves as a central tether for all starting materials and intermediates in these pathways, mediating each chemical transformation in the elongation and elaboration of the metabolites. As the structure and activity of FAS and PKS enzymes continue to be refined, the role of the ACP has been drawn into further scrutiny.² Whether incorporated into a large megasynthase (type I systems) or as discrete enzymes functioning independently (type II systems), ACPs are highly flexible species that must interact with multiple catalytic enzyme partners through highly specific protein-protein interactions.³ Complicating this binding event is the ability of type II ACPs to sequester their elongating intermediates, shielding them from reactivity until favorable protein interactions are achieved.⁴

The need for new technologies to capture ACP dynamics and protein-protein interactions has become apparent in recent years.⁵ Due to inherent technical issues posed by the cloning and expression of large, multidomain synthases, ACP participation in these systems remains largely uncharacterized, and those systems that have been studied structurally show

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Supporting Information

Supporting figures; procedures for the syntheses of **4a** and **4b**; copies of ¹H and ¹³C-NMR spectra for all compounds; complete gel images; and full experimental details. This material is available free of charge via the Internet at <http://pubs.acs.org>.

disordered ACPs.⁶ To overcome the kinetic disadvantage of diffusion control, type II enzymes are thought to associate in dynamic complexes, the compositions of which can change during the course of a particular bio-synthetic pathway.⁷ In order to serve as a covalent tether ACP must be post-translationally modified with a 4'-phosphopantetheine (PPant) prosthetic arm. It is in this covalent, protein-bound form that these species react with the active site of catalytic partner proteins.⁸ The ACP must therefore functionally interact with all enzyme domains responsible for loading carbon units, condensing these units, modifying the condensation product, and releasing the final product from the synthase. Each step relies on specific protein-protein interactions to recognize and channel biosynthetic intermediates to the proper partner enzyme.

The binding affinity of ACP for its enzymatic partners is relatively weak, with $K_d \sim 1 - 10 \mu\text{M}$, such that it can reversibly interact with each enzyme in the biosynthetic pathway and move on to the next. Therefore, visualization of ACP in complex with partner enzymes for structural analysis has been met with only modest success. To overcome this obstacle, we have devised a general methodology for covalently crosslinking ACPs to partner domains by taking advantage of ACP's unique posttranslational modification (Scheme 1).⁸ Here we synthesize analogues of pantetheine, so called pantetheinamides, that contain mechanism-based inhibitors of FAS enzymes and append them to ACP. When these inhibitor-loaded *crypto*-ACPs interact with their enzymatic partners through protein-protein interactions (Scheme 1a), the synthetic PPant sidearm is allowed to enter the pocket of the catalytic protein (Scheme 1b), reacting with an active site residue and forming a covalent bond that traps both ACP and enzyme in a bound conformation (Scheme 1c). Initially applied to the study of ketosynthase domains,^{8b} formation of the crosslinked species is highly dependent on ACP-mediated protein-protein interactions and has been applied as a tool in elucidating ACP-partner protein interactions. Similar strategies have also been successfully applied to probe peptidyl carrier protein interactions within non-ribosomal peptide synthetase enzymes.⁹

In the present study we extend the repertoire of chemoenzymatic ACP crosslinking tools to study the interactions of ACP and dehydratase (DH) domains in FAS. β -Hydroxy-ACP DH enzymes are found in all FAS pathways and any PKS whose product contains olefinic or saturated methylene units. The enzyme FabA catalyzes two kinds of chemical reactions in the *Escherichia coli* fatty acid biosynthetic pathway: dehydration of (*R*)-3-hydroxydecanoyl-ACP to (*E*)-2-decenoyl-ACP and isomerization of (*E*)-2-decenoyl-ACP to (*Z*)-3-decenoyl-ACP.¹⁰ 3-Decenoyl-*N*-acetylcysteamine (**1**), shown Figure 1, first reported by Bloch to rapidly and irreversibly inactivate FabA, was the first instance of a "mechanism-based inhibitor".¹¹ Here the enzyme target catalyzes the formation of a covalent bond between the inhibitor and an active site residue, thus permanently inactivating the enzyme. In this work we expand the use of pantetheinamides by installing a sulfonyl 3-alkyne reactive functionality to facilitate specific cross-linking of ACP and DH domains.

Our studies originally began with the preparation of crosslinker **2** to expand the use of this historical inhibitor scaffold and guide specific crosslinking of ACP and DH domains.¹² When this pantetheine analogue was investigated, however, only low levels of crosslinking were observed. This observation was attributed to rapid release of the reactive functionality from *crypto*-ACP by nucleophilic cleavage of the thioester bond in **2** before formation of the ACP-DH complex or the decomposition of **2** itself. We therefore began investigating potential replacements of this war-head for increased stability.

To this end we recently reported the design, synthesis, and utility of a DH-specific probe **3** that contains a sulfonyl 3-alkyne reactive warhead.¹³ Replacement of the reactive thioester moiety of **1** with a non-hydrolyzable sulfone as in **3** not only eliminated the instability of

nucleophilic cleavage but also provided an α -proton with low enough acidity to avoid the nonenzymatic allene formation yet still facilitate DH-specific labeling. In addition, our previous studies suggested that mechanism-based inhibition of FabA by 3-decynoic acid analogues is dependent upon the pK_a values of the α -proton of the suicide substrate scaffold (thioester > oxoester >> acid/amide),¹³ which lead us to focus on the use of the sulfonyl 3-alkyne scaffold as second-generation ACP-DH crosslinking reagents. To this end, pantetheine analogues **4a** and **4b** were synthesized from the convergent assembly of commercially available 2-nonyn-1-ol, cysteamine for **4a** or 3-chloropropyl amine for **4b**, and a PMB-protected pantothenic acid (synthetic methods are provided in the Supporting Information.)

Our biochemical studies began by evaluating the ability of **4a** and **4b** to modify the *E. coli* FAS ACP (AcpP). Using the CoA biosynthetic enzymes CoaA, CoaD, and CoaE along with 4'-phosphopantetheinyl transferase (PPTase), Sfp, we were able to confirm loading of the analogues **4a** and **4b** onto *apo*-ACP using conformationally sensitive urea-PAGE analysis.¹⁴ A characteristic increase in electrophoretic mobility was observed upon AcpP-loading of fatty acid pantetheine **4a** and **4b** (*crypto*-ACPs) (Figure 2a). The conversion of *apo*-ACP to *crypto*-^{14b} was accomplished with quantitative yield at 37 °C for 1 h. Upon addition of FabA to the corresponding *crypto*-ACP modified by **4a** and **4b**, a clear band indicating irreversible covalent cross-linking between the ACP and the DH domain was observed after 12 h and 24 h at 37 °C. These products resulted in observed gel shifts of the FabA band from an observed mass ~20 kDa for the FabA to ~45 kDa for the AcpP-FabA crosslinked complex when analyzed by SDS-PAGE analysis. By observing the consumption of FabA at 12 h and 24 h (Figure 2b), it was clear that **4a** efficiently facilitated crosslinking between the *crypto*-AcpP and FabA, suggesting that the longer alkyl linker more closely mimicks the native substrate (*R*)-3-hydroxydecanolyl-ACP and correctly aligns a His residue with the α -proton of sulfonyl 3-alkyne in the FabA active site. A slight gel shift is seen in all bands within probe-containing lanes due to non-specific probe labeling during SDS-PAGE sample preparation. Subsequent screening allowed us to investigate pH dependence for the crosslink process (Figure 2c). While the maximum labeling of probe **3** was observed at pH 8.0 or 8.5,¹³ the crosslinking reaction to form **7** was unaffected by pH. As an ultimate test of crosslink efficiency, we conducted time-course studies using **4a** as ACP-DH crosslinking reagent at 37 °C at pH 7.0. As shown in Figure 2d, this study indicated that the improved stability of **4a** allowed successful crosslinking between the *crypt*-to-AcpP and FabA. Using pantetheinamide **4a**, we observed greatly improved crosslinking efficiency at yields greater than 90%, as estimated by band intensity after 36 h at pH 7.0.

On the basis of the precise stereochemical, mechanistic, and structural studies of FabA inactivation by **1**,¹⁰ we propose that **4a** and **4b** loaded onto ACP act via the mechanism shown Scheme 2. When the DH and *crypto*-ACP interact (**5**), the reactive 3-decynoyl unit is positioned precisely in the FabA active site via protein-protein interactions, such that active site residue His₇₀ of FabA can deprotonate the acidic α -sulfone proton, generating electrophilic allene **6**. This, in turn, reacts with the His₇₀ residue to form AcpP-FabA crosslinked products **7** and **8**.

Next, we tested the ability of the ACP-DH crosslinking reaction to visualize protein-protein interactions between native and non-native ACP•DH pairs.⁸ In addition to the FAS carrier protein AcpP, the type II PKS carrier proteins FrenACP (from *Streptomyces roseofulvus*), ActACP (from *Streptomyces colicolor*), and OtcACP (from *Streptomyces rimosus*), as well as the NRPS carrier proteins VibB (from *Vibrio cholerae*) and EntB (from *E. coli*) were loaded with **4a** and incubated with FabA.⁸ Crosslinking with FabA was seen only with FrenACP, ActACP, and OtcACP, while VibB and EntB remained uncrosslinked (Figure 2e–2f). Clear preference for *E. coli* AcpP could be seen by comparison of intensity in the

crosslinked bands. This result reflects the sequence and activity-based homology between type II FAS ACPs and type II PKS ACPs.¹⁵ Indeed, DH-specific fluorescent probes based on the this sulfonyl alkyne scaffold labeled DH enzymes from recombinant type I and type II FAS and PKS systems.¹³ A lack of cross-linking between FabA and NRPS ACPs indicates a distinct specificity in protein-protein interactions that are not satisfied with these pairs. These experiments indicate that, as seen with ACP-KS crosslinking,⁷ the formation of ACP•DH complexes is driven by the native the protein-protein interactivity within the ACP-DH pairing.

Finally, we wished to understand the relevance of studying pantetheinamide probe **4a** as a true surrogate of ACP-DH interactions. The suitability of this probe to mimic sequestration of the natural substrate within the hydrophobic cavity of *E. coli* AcpP was determined by solution phase protein NMR. We linked pantetheinamide **4a** to ¹⁵N-labeled AcpP, purified the *crypto*-AcpP, and collected the HSQC spectrum. A comparison with the *holo*-AcpP spectrum under identical conditions is shown in the HSQC overlay and corresponding chemical shift perturbation plot in Figure 3. In particular, major perturbations of residues Asp₃₅ and Thr₃₉ and Glu₄₇ within helix II; and Ala₅₉ and Glu₆₀ in helix III are indicative of substrate sequestration, as previously demonstrated for AcpP and other ACPs within type II FAS and PKS pathways.^{2d, 4, 16} Of particular interest are the large perturbations seen at positions Thr₆₃ and Tyr₇₁ of helix IV, which display interactions with the sulfonyl-3-alkynyl moiety. These results demonstrate that association with FabA allows for the release of probe from the ACP binding pocket and into the active site of the enzyme, and indicate that comparison of ACP in free and crosslinked states could shed light on the molecular details of this “switchblade” mechanism.

In summary, we have demonstrated new tools for the study of protein-protein interactions between ACP and DH in FAS and PKS based on the development of a pantetheinamide with a sulfonyl alkyne scaffold. The synthesis of this probe is straightforward, offering easy access to synthetic modulation. The probe itself is stable toward one-pot chemoenzymatic methodology, sequesters within the hydrophobic cleft of *crypto*-AcpP, and forms the designed AcpP-FabA crosslinked product in high yield. This scaffold could offer immediate development of optimized pantetheine analogues for investigating protein-protein interactions for type I and type II PKS biosynthetic pathways. This sulfonyl 3-alkynyl pantetheinamide provides a general approach for elucidating ACP interactions with other DH-like enzymes, such as the product template (PT) domain in fungal nonreducing PKSs¹⁷ and the thioester hydrolase (TH) domain in fungal type I iterative PKSs.¹⁸

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Structure of DH probes and crosslinking agents. First-generation pantetheine crosslinker **2** was designed as a directed mimic of 3-decynoyl-*N*-acetylcysteamine (**1**).¹² We later evaluated a DH-specific reactive probe **3** that contains a sulfonyl 3-alkyne reactive warhead engineered to avoid hydrolysis or nonenzymatic inactivation.¹³ Second-generation crosslinkers **4a** and **4b**, based on probe **3**, provide the mechanism-based activities of **1** and **2** with improved hydrolytic stability.

Figure 2.

Evaluation of ACP-DH crosslinking reagents **4a** and **4b**. (a) Pantetheinamides **4a** and **4b** were appended enzymatically to *apo*-AcpP using one-pot chemoenzymatic method. Conversion of *E. coli apo*-AcpP to its corresponding *crypto*-ACP when treated with 500 μ M **4a** or 500 μ M **4b** was visualized by Urea-PAGE analysis. a = *apo*, h = *holo*, c = *crypto*. (b) SDS-PAGE depicting the crosslinking of AcpP and FabA when treated with 500 μ M **4a** or 500 μ M **4b** at 37 $^{\circ}$ C for 12 h or 24 h. (c) SDS-PAGE gel denoting pH dependence of the crosslinking reaction for 12 h at 37 $^{\circ}$ C. (d) Time-course studies depicting the crosslinking of AcpP to FabA with 500 μ M **4a**. The crosslinking reaction by **4a** loaded onto ACP accomplished with high yield (> 90 %) after 36 h at 37 $^{\circ}$ C. (e) Effect of carrier protein identity on FabA crosslinking. Pantetheinamide **4a** was used to modify FAS and PKS carrier proteins AcpP (FAS), Fren (PKS), Act (PKS), Otc (PKS); (f) and NRPS carrier proteins VibB, and EntB, indicating the clear preferential interaction of FabA with AcpP from FAS over the other ACPs from PKS and peptidyl carrier protein from NRPS. The CPs (30 μ M) were modified by 500 μ M **4a** using one-pot chemoenzymatic method. All one-pot chemoenzymatic conditions included treatment with 0.26 μ M CoaA, 0.56 μ M CoaD, 0.44 μ M CoaE, and 0.31 μ M Sfp for 1 h at 37 $^{\circ}$ C. *crypto*-ACPs were incubated with 15 μ M FabA of *E. coli* FAS for 12 h at 37 $^{\circ}$ C.

Figure 3.

Solution phase NMR of *crypto*-ACP demonstrating probe sequestration. (a) ^1H , ^{15}N -HSQC spectra of purified *crypto*-ACP comprised of ^{15}N -enriched *E. coli apo*-AcpP^{16b} modified chemoenzymatically with **4a** (blue) and *holo*-AcpP (green). Significant shifts are annotated by residue number. (b) Structure of AcpP highlighting residues that show the largest chemical shift perturbations between *holo*- and *crypto*-forms. (c) Chemical shift perturbation plot of *crypto*-AcpP versus *holo*-AcpP.

Scheme 1.

General representation of the crosslinking strategy with a FAS dehydratase (DH) and its ACP loaded with a cross-linking pantetheine probe. (a) Protein-protein interaction. (b) Switchblade mechanism. (c) Mechanism-based inactivation and crosslinking.

Scheme 2.

Schematic representation of the crosslinking of ACP and dehydratase (DH) domains using probe 4a.