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A mechanism based protein crosslinker for acyl carrier protein dehydratases

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ABSTRACT

Recent advances in the structural study of fatty acid synthase (FAS) and polyketide synthase (PKS) biosynthetic enzymes have illuminated our understanding of modular enzymes of the acetate pathway. However, one significant and persistent challenge in such analyses is resolution of the acyl carrier protein (ACP), a small (~9 kDa) protein to which biosynthetic intermediates are tethered throughout the biosynthetic cycle. Here we report a chemoenzymatic crosslinking strategy in which the installation of a historical suicide substrate scaffold upon the 4′-phosphopantetheine (PPant) arm of the ACP is used to capture the active site of acyl carrier protein dehydratase (DH) domains in FAS. Through the synthesis of a small panel of related probes we identify structural features essential for ACP–DH crosslinking, and apply gel-based assays to demonstrate the stability as well as purification strategies for isolation of the chemoenzymatically modified ACP. Applying these carrier protein crosslinking techniques to the structural analysis of FAS and PKS complexes has the potential to provide snapshots of these biosynthetic assembly lines at work.

Recent advances in the structural study of fatty acid synthase (FAS) and polyketide synthase (PKS) biosynthetic enzymes have advanced our understanding of modular enzymes of the acetate pathway.[1–3] However, one persistent challenge in such analyses is resolution of the dynamic acyl carrier protein (ACP), a small (~9 kDa) protein to which biosynthetic intermediates are tethered throughout the biosynthetic cycle.[4] The ACP is inherently flexible, relying on specific protein–protein interactions to recognize and channel biosynthetic intermediates to the proper partner enzyme during each step of the iterative biosynthetic process. The transient nature of these interactions presents a challenge to traditional methods of structural analysis. To capture these interactions, we have pursued a strategy to install mechanism-based inhibitor moieties on the 4′-phosphopantetheine (PPant) arm of the ACP which react with the active site of partner proteins.[5–7] This process results in the selective crosslinking of the ACP with a single partner domain. Initially applied to the study of ketosynthase domains, formation of the resulting crosslinked species is highly dependent on ACP-mediated protein–protein interactions, and has been applied as a tool in structural analysis and investigations of ACP-partner protein interactions.

In this study we extend the repertoire of chemoenzymatic ACP-crosslinking to study the interactions of ACP and dehydratase (DH) domains in FAS. DH domains catalyze the dehydration of β-hydroxyacyl-ACP substrates to α,β-unsaturated acyl-ACPs during the reductive steps of fatty acid and polyketide biosynthesis (Fig. 1a).[8,9] In addition, DH domains play a key role in initiation of bacterial unsaturated fatty acid biosynthesis, isomerizing *trans*-2-decenoyl-ACP to *cis*-3-decenoyl-ACP. These reactions were first studied in the context of the *Escherichia coli* FAS, where they are catalyzed by the prototypical DH enzyme FabA.[10,11] These studies also yielded the discovery of the first mechanism-based inhibitor of a fatty acid biosynthetic enzyme, 3-decynoyl-*N*-acetyl-cystamine (NAC). This suicide substrate undergoes α-deprotonation in the FabA active site to form an electrophilic allene, which then modifies the active site histidine of FabA to irreversibly inactivate the enzyme.[12–14] Here we expand the use of this historical inhibitor scaffold to guide site-specific crosslinking of ACP and DH domains.

To this end, a small panel of pantetheine analogues was synthesized incorporating well-known inhibitor scaffolds of DH and other α-deprotonating enzymes (Fig. 1c, **1**–**8**). These pantetheine analogues can be transformed into CoA analogues and site-specifically incorporated into ACPs using the one-pot chemoenzymatic method depicted in Figure 1b.[15] In addition to the 3-decynoyl and 2,3-decadienoyl thioester inhibitors (**1** and **2**), we also examined 3-decynoyl-oxoesters and amides (**3** and **4**), a transition state analogue (**5**), 2-octynoyl thioesters and amides capable of forming reactive allenes upon γ-deprotonation (**6** and **7**), and a simple histidine reactive acyl-bromoacetamide affinity tag (**8**).[16–18] These pantetheine analogues were assayed for their relative abilities to modify the active site of FabA by testing their ability to block labeling by fluorescent probe **9**, a 3-decynoyl-NAC derivative which reacts with FabA in an active-site dependent manner (Fig. 2a). As expected, pre-incubation of FabA with the denaturing agent SDS or known

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inhibitor scaffolds **1** or **2** each efficiently blocked labeling by **9** (Fig. 2a). 3-Decynoyl-oxoester **3** and 2-octynoyl thioester **6** showed decreased active site modification, blocking fluorescent labeling by **9** to a lesser degree, while pantetheine analogues **4**, **5**, **7**, and **8** showed no effect. This is consistent with previous studies on mechanism-based inhibition of FabA by 3-decynoic acid analogues, which found enzyme inactivation to be strongly dependent on the pK_a of the α-proton of the suicide substrate (thioester > oxoester ≫ acid/amide) and lead us to focus on the use of thioesters **1**, **2**, and **6** as ACP–DH crosslinking reagents.[12,13,19]

First, the ability of analogues **1**, **2**, and **6** to modify the *E. coli* FAS ACP (AcpP) was demonstrated. Using the CoA biosynthetic enzymes PanK, PPAT, and DPCK along with the permissive PPTase Sfp, we were able to observe modification of AcpP by **1**, **2**, and **6** by SDS–PAGE, in which a characteristic gel-shift to lower molecular weight was observed upon AcpP-incorporation of fatty acyl pantetheines **1**, **2**, or **6** (Fig. 2b).[20,21] Upon addition of FabA to AcpP modified by **1**, a faint band appearing at ~45 kDa corresponding to a putative AcpP–FabA complex was observed (Fig. 2b). While this band co-migrated with a persistent FabA disulfide, it could be clearly visualized using strongly reducing SDS–PAGE conditions. This putative AcpP–FabA complex was observed to be dependent upon the presence and amount of **1** added to the reaction mix (Fig. 2c). ACP, PanK, and Sfp were each also judged to be necessary components for this crosslinking to occur (Fig. S1). In addition, complex formation was highly sensitive to the integrity of the FabA active site, and was not observed in reactions in which FabA had been pre-denatured by boiling or inactivated by high concentrations of **9** (Fig. 2b). Pantetheine analogue **2** resulted in approximately equivalent results, while analogue **6** produced noticeably reduced crosslinking (Fig. S2).

For unambiguous identification of the crosslinked species we applied an orthogonal purification strategy to isolate inhibitor modified (termed *crypto*) AcpP from the reaction mixture.[22] The resulting *crypto*-ACPs were then incubated with FabA, allowing observation of a distinct gel-shift to higher molecular weight corresponding to formation of the ACP–DH complex. This shift was observed only in the presence of agents **1** or **6** (Fig. 2d). A similar result was seen when using a GFP-tagged AcpP, which facilitates visualization of the AcpP–FabA complex by removing it from the molecular weight region of the FabA disulfide (Fig. 2e). Both the GFP–AcpP and native AcpP–FabA complexes were excised, subjected to tryptic digest, and identified by MALDI-TOF/TOF and peptide mass fingerprinting (Fig. S3).

One consideration when applying Michael acceptors **2** and **6** to ACP–DH crosslinking of multidomain synthases is their potential reactivity with KS domains. Indeed, when *crypto*-AcpPs generated from **1**, **2**, and **6** were incubated with the *E. coli* KS enzyme FabB, each demonstrated ACP–KS crosslinking activity (Fig. S4). This could be avoided by pre-incubation of FabB with the KS-selective reagent cerulenin, a strategy which may prove useful for achieving ACP–DH specific crosslinking in multidomain synthases. The

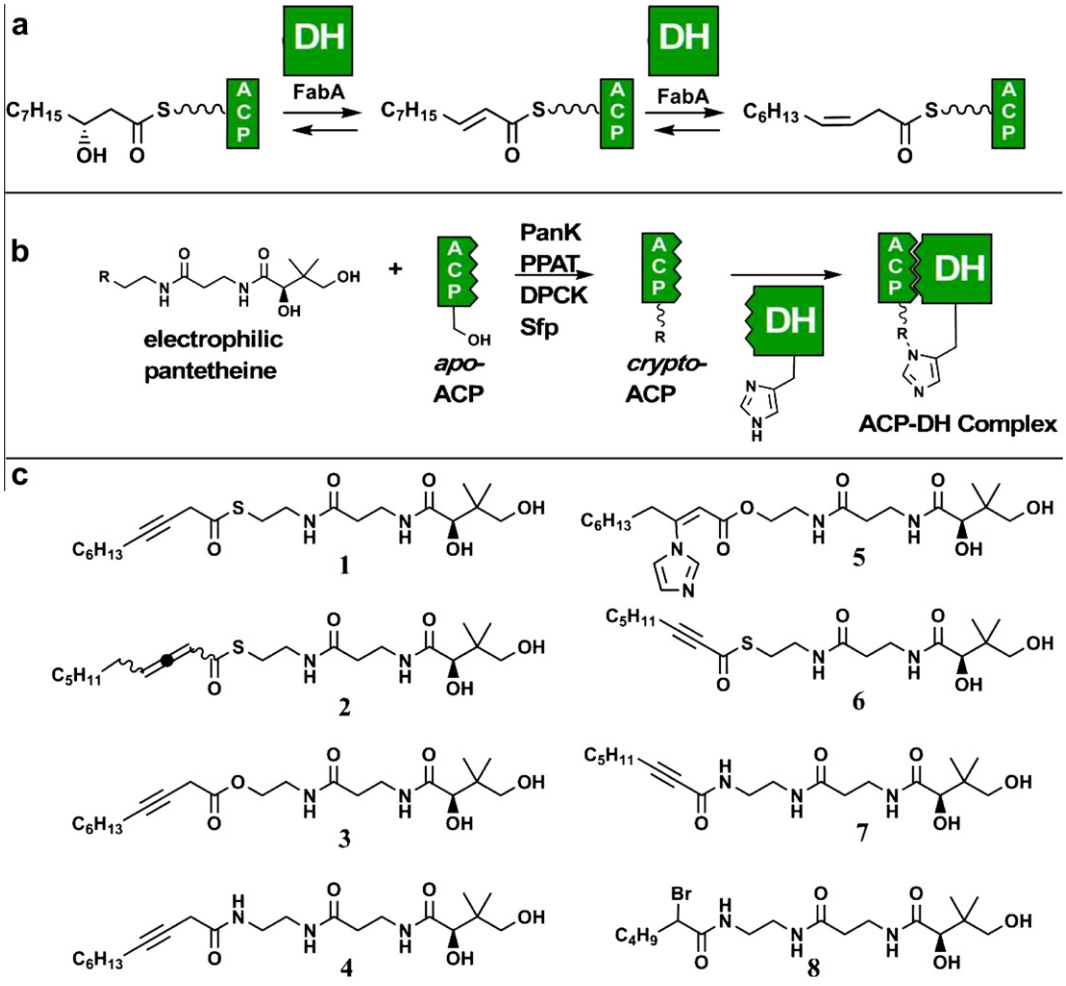

Figure 1. General strategy for site-specific mechanism-based crosslinking of ACP and DH domains. (a) Dehydration and isomerization reactions catalyzed by the *E. coli* FAS DH enzyme FabA. (b) Reactive ACPs can be generated through the CoA biosynthetic enzymes (PanK, PPAT, and DPCK) and the PPTase Sfp. Upon addition of an ACP-partner protein, the native protein–protein interactions result in a transient interaction, which is captured by the electrophile. (c) Structures of electrophilic pantetheine analogues **1**–**8**.