

Acyl Carrier Protein Cyanylation Delivers a Ketoacyl Synthase–Carrier Protein Cross-Link

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S Supporting Information

ABSTRACT: Acyl carrier proteins (ACPs) are central hubs in polyketide and fatty acid biosynthetic pathways, but the fast motions of the ACP's phosphopantetheine (Ppant) arm make its conformational dynamics difficult to capture using traditional spectroscopic approaches. Here we report that converting the terminal thiol of *Escherichia coli* ACP's Ppant arm into a thiocyanate activates this site to form a selective cross-link with the active site cysteine of its partner ketoacyl synthase (FabF). The reaction releases a cyanide anion, which can be detected by infrared spectroscopy. This represents a practical and generalizable method for obtaining and visualizing ACP–protein complexes relevant to biocatalysis and will be valuable in future structural and engineering studies.

Polyketide synthases (PKSs) and fatty acid synthases (FASs) produce structurally diverse molecules with pharmacologically relevant properties. Acyl carrier proteins (ACPs) play a central role in these synthases by using their 18 Å long 4'-phosphopantetheine (Ppant) arm to shuttle building blocks and intermediates to appropriate catalytic partners that perform the chemistries programmed by the synthase (Figure 1A). The fast motions of ACPs allow the protein to protect and present its molecular cargo as needed but also make it difficult to study its structure and its interactions with other proteins.¹ Here we report a practical method for obtaining and visualizing ACP–protein interactions relevant to catalysis. We anticipate that this method will be valuable in future structural and engineering studies.

We recently reported that converting the ACP Ppant arm's thiol to a thiocyanate (ACP-SCN) turns the reactive end of the arm into a probe that reports on hydrogen bonding and local dynamics (Figure 1B).² The frequency of the thiocyanate band is sensitive to the local solvation environment: when the Ppant arm of ACP-SCN is solvent-exposed it absorbs at $\sim 2163\text{ cm}^{-1}$, but when the arm is buried, the probe band exhibits a red-shift of $5\text{--}10\text{ cm}^{-1}$.² Because the Ppant arm can enter the hydrophobic active site of catalytic partners,^{1,3} we hoped that this catalytically relevant change in the solvation environment could be tracked with the solvato-sensitive thiocyanate probe (Figure 1C).

To test this hypothesis, we identified a model ACP/partner system: the *Escherichia coli* fatty acid ACP, AcpP, and one of its

cognate ketoacyl synthases (KSs), FabF. During the fatty acid elongation cycle in *E. coli*, AcpP collaborates with FabF to catalyze a Claisen condensation reaction that extends the growing alkyl chain by two carbon units. While characterizing the AcpP–FabF interaction is paramount to understanding and manipulating FASs, the AcpP–FabF interaction is difficult to study because of its transient nature. To overcome this obstacle, Burkart and co-workers used solvatochromatic fluorescence and cross-linking studies to visualize the movement of the AcpP Ppant arm into the hydrophobic active site of FabF.^{4,5} That work confirmed that the AcpP Ppant arm experiences a change in solvation environment (from solvent-exposed to solvent-excluded) upon its interaction with FabF and validated the use of the AcpP/FabF pair to test our hypothesis.

We labeled AcpP with the thiocyanate probe to make the product AcpP-SCN and found that cyanylation did not perturb the protein's helical structure (Figure S1). AcpP-SCN exhibited a mode frequency of 2161 cm^{-1} , which on the basis of prior results from model molecules⁶ and proteins² suggests that the Ppant arm is mainly solvent-exposed in the absence of a catalytic partner (Figure 1D).

We predicted that the interaction between AcpP-SCN and FabF would induce a red shift of $6\text{--}7\text{ cm}^{-1}$ in the CN frequency of AcpP-SCN when the probe entered the solvent-excluded FabF active site. To our surprise, the introduction of FabF resulted in a decrease in intensity of the 2161 cm^{-1} band and the appearance of a new peak at 2120 cm^{-1} (Figure 1D). The 40 cm^{-1} shift in the CN stretching band indicated that the interaction of AcpP-SCN with FabF chemically transformed the CN-containing functional group.

We hypothesized that the band at 2120 cm^{-1} was due to CN^- released from the SCN group. To test this theory, we repeated the titration experiment using AcpP- ^{13}C SCN, where the isotopic mass of ^{13}C would lead to a lower vibrational frequency of any bonds containing the carbon atom of the thiocyanate. Both the AcpP- ^{13}C SCN alone and AcpP- ^{13}C SCN treated with FabF displayed ^{13}C isotope frequency shifts of $\sim 50\text{ cm}^{-1}$ versus natural abundance (Figure S2), indicating that the new band at 2120 cm^{-1} in Figure 1D is due to a mode containing the thiocyanate carbon atom.⁷ Further support for

Received: March 9, 2017

Revised: April 13, 2017

Published: April 27, 2017

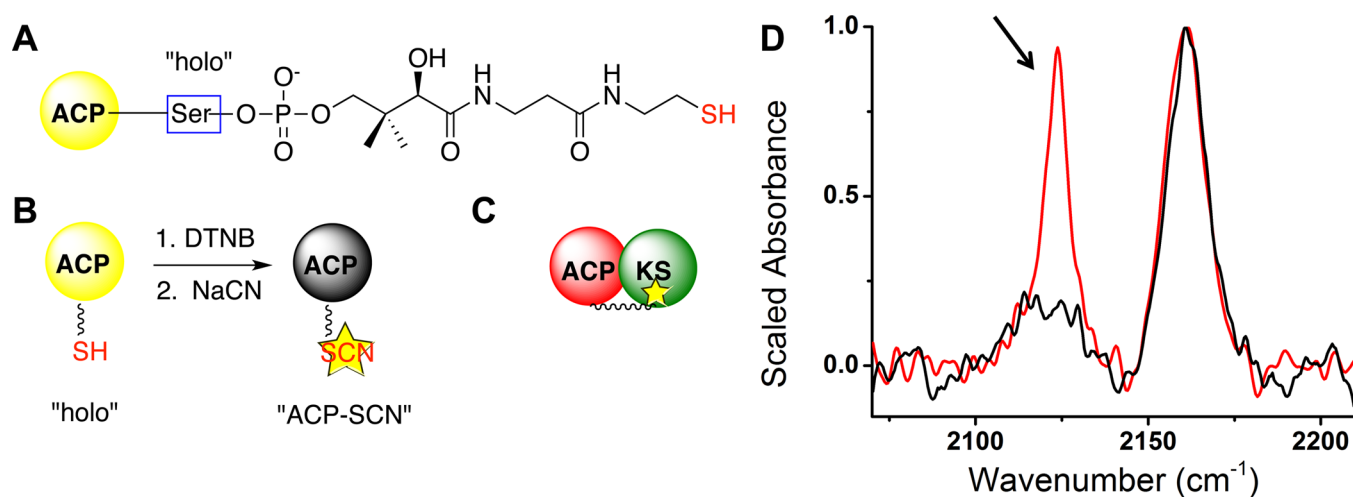


Figure 1. The thiol of the ACP Ppant arm (A) is converted into thiocyanate via a one-pot cyanylation reaction (B). We hypothesized that the labeled Ppant arm would gain access to the active site of cognate ketoacyl synthase (KS) enzymes (C) and that this change in solvation could be monitored by vibrational spectroscopy. Infrared analysis of cyanylated *E. coli* ACP, AcpP-SCN, titrated with its cognate KS (FabF) supports this hypothesis (D). The infrared CN stretching band of AcpP-SCN (black) absorbs at 2161 cm^{-1} , a frequency consistent with the label being water-exposed.² When AcpP-SCN was titrated with FabF, a new peak at 2120 cm^{-1} (arrow) was observed.

assignment of the 2120 cm^{-1} band to CN^- released from the SCN group includes the observation of this peak upon titration of FabF with NaCN in the absence of AcpP (data not shown).

We postulated that CN^- was released upon the formation of a cross-linked FabF–AcpP complex and therefore analyzed the reaction mixture using size-exclusion chromatography (SEC) (Figure 2A). For AcpP-SCN, we observed two peaks in the chromatogram (18 and 22 min), corresponding to the known equilibrium of the monomer and dimer AcpP states under nonreducing conditions.⁸ Titration of AcpP-SCN with FabF resulted in a decrease in the magnitudes of these peaks and the appearance of a complex larger than FabF alone eluting at 16 min, consistent with the formation of a FabF–AcpP adduct.

The most likely covalent link between AcpP and FabF occurs via a disulfide bond resulting from the FabF active site (Cys163) attacking AcpP-SCN's sulfur atom, releasing free CN^- , as shown in Figure 2A. To test this model, we titrated AcpP-SCN with FabF and analyzed the product via gel electrophoresis in the presence and absence of a disulfide reducing agent. We observed the covalent cross-linked product via gel electrophoresis under nonreducing conditions, but not reducing conditions (Figure 2B,C and Figure S3).

To determine if the disulfide cross-link occurred at Cys163, we titrated AcpP-SCN with FabF preincubated with cerulenin, an inhibitor of the KS active site cysteine.⁹ Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis showed that the inhibited FabF does not form a cross-link with AcpP-SCN (Figure S4). The 2120 cm^{-1} infrared (IR) band was not observed, indicating that the release of CN^- requires that AcpP's Ppant arm enters the FabF active site (Figure S4). The attachment point between AcpP and FabF was further confirmed by tandem proteolysis mass spectrometry: digestion of the cross-linked product with trypsin and chymotrypsin yields a product consistent with a FabF peptide fragment tethered to an AcpP peptide fragment via a disulfide bond between the FabF Cys163 active site and AcpP Ppant arm (Figures S5–S10).

Reactions of cysteine sulfonyl thiocyanates with thiols are known to afford asymmetric disulfides.¹⁰ While the resultant disulfides are typically stable only in protic solvents under very

acidic conditions, it appears that this reaction can take place in the active site of FabF and that the local environment facilitates the formation of the FabF–AcpP disulfide bond. Because unlabeled *holo*-AcpP does not readily form a cross-link with FabF *in vitro* (Figure S11), we speculate that the cyanylation of AcpP mimics a nascent substrate and induces a conformational change that alters the orientation of AcpP with respect to KS¹¹ and/or facilitates cross-linking via an $\text{S}_{\text{N}}2$ -type reaction by providing a robust leaving group. Once the arm is “activated”, a stable Ppant–Cys cross-link can be formed, as observed previously *in vivo*¹² and in engineered nonribosomal peptide synthetase systems.¹³

The simple installation of a thiocyanate probe on the AcpP Ppant arm provides a reactive and spectroscopically unique handle that facilitates the creation of a mechanistically relevant FabF–AcpP cross-linked product. IR spectroscopy using relatively conventional commercial instrumentation can be used to monitor formation of the cross-linked product via release of CN^- . To extend the potential impact of these findings, we investigated whether other ACPs could be cross-linked to FabF via this cyanylation approach and if the appearance of an IR peak at 2120 cm^{-1} could serve as a general readout for productive FabF–ACP interactions. Previous studies showed that FabF productively interacts with the polyketide ACP from frenolicin biosynthesis (ACP_{fren}), but not the polyketide ACP from actinorhodin biosynthesis (ACP_{act}).¹⁴ Thus, if FabF–ACP cross-links are selectively formed when FabF is titrated with a functional cyanylated ACP, we expect to see evidence of a covalent cross-link and release of CN^- for ACP_{fren} but not ACP_{act} . Indeed, titration of ACP_{fren} -SCN with FabF led to a cross-linked product (Figure S12) also marked by CN^- in the IR spectrum (Figure S13). Titration of ACP_{act} -SCN with FabF afforded neither a cross-link (Figure S12) nor the release of CN^- (Figure S13). Similarly, no cross-link or 2120 cm^{-1} peak was observed upon titration of ACP-SCN with a bovine serum albumin (BSA) protein control (data not shown). These data suggest that the cross-linking reaction between ACP-SCN and FabF is selective for functional interactions and can potentially be used to monitor interactions of ACP with other cognate catalytic partners.

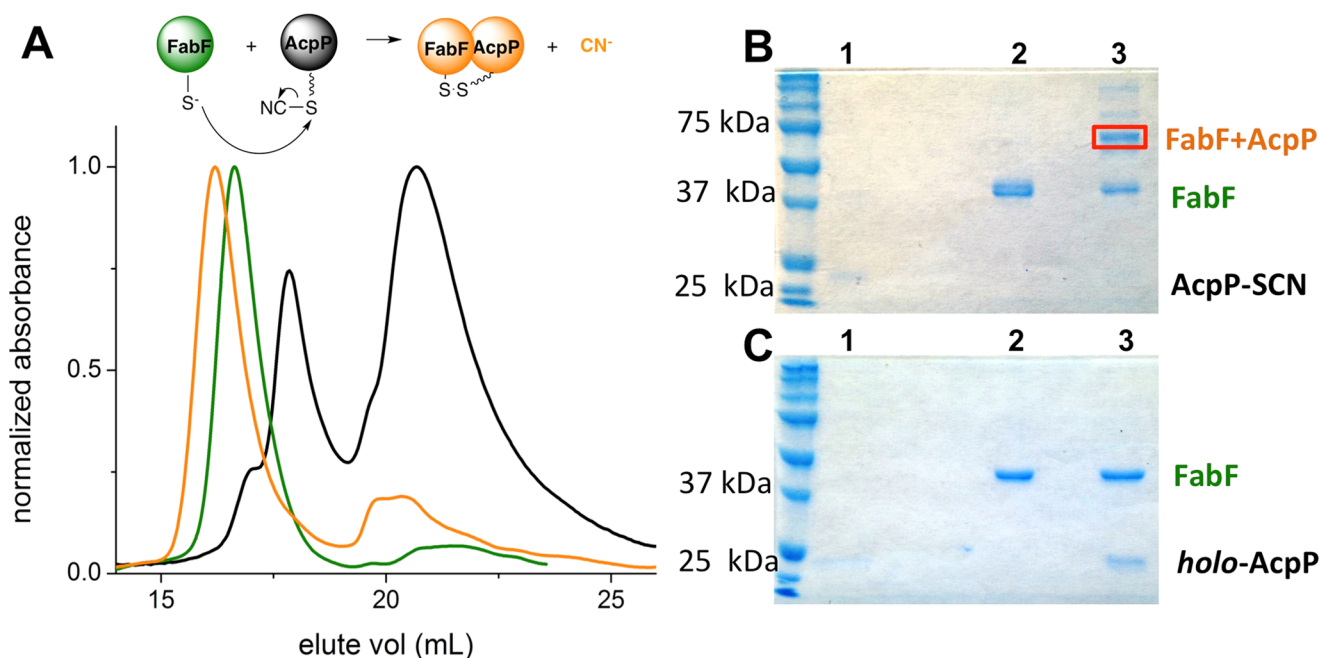


Figure 2. Size-exclusion chromatography (280 nm) shows that when AcpP-SCN (black) is titrated with FabF (green) a higher-molecular weight complex is formed (orange) (A). AcpP partly forms a dimeric complex under nonreducing conditions,⁸ giving rise to two peaks in the chromatogram. The complex eluting at 16 mL was collected and analyzed via nonreducing (B) and reducing (C) sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels (lane 1, AcpP; lane 2, FabF; lane 3, complex at 16 mL). The disappearance of the high-molecular weight complex (red box), and the increase in intensity of the AcpP and FabF bands observed under reducing conditions, support the idea that AcpP and FabF are linked via a disulfide bond.

The thiocyanate-facilitated cross-linking of ACPs to FabF is a practical method for obtaining protein complexes relevant to biocatalysis. Previous efforts to visualize AcpP–protein interactions required isotopic labeling, multistep synthesis of pantetheine-based probes, and/or chemoenzymatic loading of synthetic probes onto AcpP.^{15,16} While these approaches target interactions of ACP with diverse catalytic partners, thiocyanation with the IR method offers an alternative in which the simple bio-orthogonal modification of AcpP activates the protein for covalent linkage to FabF, and possibly other enzymes containing an active site cysteine.

Future work will focus on using this method to obtain crystal structures of FabF–ACP complexes and to identify residues that guide functional interactions. The ability to visualize functional FabF–ACP interactions *in situ* via the presence or absence of a peak in the IR spectrum at 2120 cm⁻¹ will also be explored as a low-barrier tool for screening functional unnatural biosynthetic partners. Given that cyanylation of ACPs and IR spectroscopy are amenable to high-throughput approaches, these methods could allow rapid progress toward building hybrid synthases capable of creating new chemical diversity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b00219.

Methods and additional figures (PDF)

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Funding

We gratefully acknowledge the Research Corporation for Scientific Advancement Cottrell College Science Award (L.K.C.), National Institutes of Health Grant R15GM120704 (L.K.C.), National Science Foundation (NSF) CAREER Grant CHE-1652424 (L.K.C.), NSF CAREER Grant CHE-1150727 (C.H.L.), a Henry Dreyfus Teacher-Scholar Award (C.H.L.), summer research fellowships from Haverford College (K.J.S.T., G.A.T., and C.P.F.), and the B. A. Rudolph Foundation (K.J.S.T.). The content is solely the responsibility of the authors and does not necessarily represent the views of the NIH or NSF.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Hsin-Yao Tang at the Wistar Institute Proteomics and Metabolomics Facility for analysis of proteomics data and Prof. Chaitan Khosla for generously providing FabF and AcpP expression plasmids.

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