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Trapping the dynamic acyl carrier protein in fatty acid biosynthesis

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Abstract

Acyl carrier protein (ACP) transports the growing fatty acid chain between enzyme domains of fatty acid synthase (FAS) during biosynthesis.¹ Because FAS enzymes operate upon ACP-bound acyl groups, ACP must stabilize and transport the growing lipid chain.² The transient nature of ACP-enzyme interactions imposes a major obstacle to gaining high-resolution structural information about fatty acid biosynthesis, and a new strategy is required to properly study protein-protein interactions. In this work, we describe the application of a mechanism-based probe that allows site-selective covalent crosslinking of AcpP to FabA, the *E. coli* ACP and fatty acid 3-hydroxyacyl-ACP dehydratase. We report the 1.9 Å crystal structure of the crosslinked AcpP=FabA complex as a homo-dimer, in which AcpP exhibits two different conformations likely representing snapshots of ACP in action: the 4'-phosphopantetheine (PPant) group of AcpP first binds an arginine-rich groove of FabA, followed by an AcpP helical conformational change that locks the AcpP and FabA in place. Residues at the interface of AcpP and FabA are identified and

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Data Deposition

The atomic coordinates of AcpP=FabA have been deposited in the Protein Data Bank (accession code 4KEH).

Competing financial interests

The authors have none.

Supplementary Information

Supplementary information accompanies this paper.

Author Contributions

C.N., assisted by G.C., D.R.J. and J.B., determined the AcpP=FabA X-ray crystal structures. R.W.H., D.J.L., and B.O. conducted the protein NMR experiments under the supervision of S.J.O. F.I. and K.F. prepared the crosslinking probe. P.R.L.M. conducted molecular dynamics simulations under the supervision of J.A.M. C.N., G.C., B.W.H. and D.J.L. analyzed data and contributed to writing of the paper. S.-C.T. and M.D.B. directed the research, provided funding and wrote the final manuscript.

validated by solution NMR techniques, including chemical shift perturbations and RDC measurements. These not only support our interpretation of the crystal structures but also provide an animated view of ACP in action during fatty acid dehydration. Combined with molecular dynamics simulations, we show for the first time that FabA extrudes the sequestered acyl chain from the ACP binding pocket before dehydration by repositioning helix III. Extensive sequence conservation among carrier proteins suggests that the mechanistic insights gleaned from our studies will prove general for fatty acid, polyketide and non-ribosomal biosyntheses. Here the foundation is laid for defining the dynamic action of carrier protein activity in primary and secondary metabolism, providing insight into pathways that can play major roles in the treatment of cancer, obesity and infectious disease.

Acyl carrier protein (ACP) plays a central role in transporting starting materials and intermediates throughout the fatty acid biosynthetic pathway (Fig. 1A).^{3–5} In *Escherichia coli*, AcpP interacts with at least twelve enzymes involved in fatty acid biosynthesis, plus seven other enzymes from disparate biosynthetic pathways (Fig. 1A, Fig. S1).^{6–10} AcpP sequesters growing metabolites in an interior hydrophobic cavity that protects these intermediates from non-selective reactivity,¹¹ and selective protein-protein interactions are believed to be a prerequisite for the delivery of ACP-bound substrate to its catalytic partners.³ Given the importance of ACP-protein interactions in metabolism and cell regulatory processes, understanding this “switchblade mechanism” (Fig. 1C) is crucial,¹² though this has proven elusive due to the inherently transient nature of ACP-partner complexes.¹³

We recently deployed synthetic probes to study ACP activity and protein-protein interactions,¹⁴ including a sulfonyl-3-alkyne based probe (**1**, Fig. 1B) designed to capture ACP in functional association with 3-hydroxyacyl-ACP dehydratase with demonstrated specificity between *E. coli* AcpP and FabA (Fig. 1C–D).^{15,16} Probe **1** applied to AcpP and FabA creates a uniformly crosslinked species (AcpP=FabA) that forms reproducible crystals in tag-free form (Fig. S4). No crystals form without **1**, demonstrating the necessity of applying probes such as **1** to capture ACP in action.

The AcpP=FabA crystals diffracted to 1.9 Å (Table S2), and we solved the AcpP=FabA crystal structure by molecular replacement using *apo*-FabA dimer (PDB: 1MKA)¹⁷ and two *butyryl*-AcpP (PDB: 2K94) as search templates (Fig. S5). Final refinement revealed the structure of an AcpP2=FabA2 complex (Fig. 2A), consistent with protein sizing data in solution. The dimeric FabA forms a “double hotdog” topology,¹⁸ with two anti-parallel “hotdog” helices surrounded by a combined 14-stranded β sheet (Fig. 2A).^{17,19} The two AcpP monomers adopt a four-helix bundle fold³ and dock helices II-III with the β5-6 loop of FabA (Fig. 2D). The contact area is small (539 Å² and 503 Å² for the first and second AcpP=FabA), consistent with the transient nature of AcpP-partner interactions. AcpP has a negatively charged cleft between helices II and III, which interacts with a positively-charged arginine-rich patch on FabA (the “Positive Patch”, Fig. 2C).³ The AcpP=FabA interfaces are mainly electrostatic but also include conserved, hydrophobic residues (Fig. 2D, detailed in SI). The high sequence conservation of negatively charged residues on helices II and III at the AcpP=FabA interface (Fig. S2–3) is consistent with previous reports of ACP-partner

complex structures (Fig. 4D),^{7,20–23} strongly supporting the presence of the Positive Patch in ACP partner proteins.

Only the position of R137 differs between the two FabA protomers, but in the ACP structures many residues of helix III move extensively (Fig. 2D and SI), resulting in different topology near the contact interface between helices II and III (Fig. 2C and SI). Thus the first AcpP=FabA interaction likely represents a snapshot when AcpP completes its docking with FabA, resulting in less disorder of AcpP1. Accordingly, the second AcpP=FabA interaction would represent a snapshot of AcpP in transition, where extensive movement of helix III is necessary in order for AcpP2 to bind or dissociate from FabA.

The natural FAS substrates contain both PPant and acyl chain moieties (Fig. 1B), and the application of probe **1** shows how both bind to FabA (Fig. S6–7). Probe **1** covalently connects the active site S36 of AcpP and H70 of FabA and binds in a highly conserved tunnel of FabA (detailed in SI). Unlike acyl-AcpP structures that contain a hydrophobic interior pocket to sequester the acyl chain,¹³ the AcpP in the AcpP=FabA complex structure contains no interior pocket (Fig. 2E) and closely resembles *apo*-AcpP (Table S4),²⁴ because five conserved hydrophobic residues between helices II-III move inward and collapse the interior pocket (Fig. 2F). This drastic change reflects a dynamic AcpP moving from the sequestered-substrate state to the open switchblade state to position the substrate within partner enzyme FabA (Fig. 1C–D).

Further characterization of the dynamic interactions between AcpP and FabA was achieved through comparisons with two-dimensional ¹H/¹⁵N HSQC spectra of *holo*-AcpP and *octanoyl*-AcpP. Many resonances displayed chemical shift perturbations (CSPs) in residues that define the hydrophobic pocket of AcpP (Fig. S11). Titrating unlabeled *apo*-FabA into each sample allowed us to observe CSPs resulting from dynamic, non-covalent association with FabA in solution (Fig. 3A–B). In the *holo*-AcpP-FabA titration experiment, we observed significant CSPs in residues spanning helices II and III and the adjacent loops (Fig. S12). In the *octanoyl*-AcpP-FabA titration experiment, additional CSPs were observed in residues lining the hydrophobic pocket of AcpP (Fig. 3C), which we attribute to the translocation of the bound acyl chain out of the AcpP pocket into the FabA active site to complete the “switchblade” process.

We then acquired a two-dimensional HSQC spectrum of the AcpP=FabA complex in 1:2 stoichiometry, with one AcpP crosslinked to each FabA homodimer. When overlaying the HSQC spectra of the AcpP=FabA complex with the *holo*- and *octanoyl*-AcpP titration data (Fig. 3A–B) obtained with experiments incorporating TROSY,²⁵ we observed a striking correlation between the CSP shifts in the two AcpP species and the HSQC spectrum of the complex; the chemical shift of each residue migrates toward the observed chemical shift in the complex as the concentration of FabA increases. The similarities between CSPs of the transient binding event and our crosslinked complex (Fig. 3C–F) indicate that the binding conformation in the crosslinked complex is truly indicative of the natively-bound conformation.

From the crystal structure we identified acidic residues E41, E47, E53 and E60 of AcpP that interact with FabA (Fig. 2D and S6), and correspondingly observed large CSPs in these residues between helices II and III (Fig. 3C–E). Additionally we found large CSPs in the hydrophobic helix II residues observed in the binding interface of the crystal structure, such as L37, V40, and M44 (Fig. 2D, 3, and S6). Similarly, the crystal structure observation is consistent with CSP plots that compare *octanoyl*-AcpP with crosslinked AcpP=FabA (Fig. 3C–E, Table S8): the strong CSPs for A59, E60, E41 and E47 correspond with side-chain interactions within the Positive Patch of FabA. Large CSPs in S36, L37, and D38 correspond with a change in the PPant position as it extends into the FabA. T63 undergoes significant rotation when comparing the *octanoyl* and the AcpP=FabA complex. Ultimately, these CSP observations both complement and corroborate binding observations found in the crystal structure.

To study the detailed dynamics of AcpP and its interaction with FabA, we measured Residual Dipolar Couplings (RDCs)²⁶ from weakly aligned samples of *octanoyl*-AcpP and AcpP in the AcpP=FabA complex (Fig. 4A–B). The empirical RDCs were combined with accelerated molecular dynamics (AMD) simulations²⁷ to provide structural-dynamic information in the μ s regime (detailed in SI).²⁸ Within this framework, we identified the optimal acceleration parameters, and hence optimal conformational space sampling criteria for the correlation of experimental and theoretical RDCs (Fig. 4A), and calculated the averaged NH order parameters at both fast (ns) and slow RDC-optimized (μ s) timescales (Fig. 4B). On fast-time scales (ns), no substantial differences in the order parameters between *octanoyl*-AcpP and AcpP=FabA are observed. By contrast, in the slow (μ s) time regime, substantial differences in the structural-dynamic behavior of *octanoyl*-AcpP and AcpP=FabA are identified, indicating that AcpP is dramatically stabilized in the presence of FabA, especially in the N-terminal region of helix II and the helix II-III loop. There is a striking correlation between the AcpP=FabA binding interface observed in the crystal structure (Fig. 2), the NMR titration data (Fig. 3), and molecular dynamics simulations (Fig. 4B–C), all highlighting key dynamic residues.

These results provide a window into the dynamic properties of AcpP, which sequesters elongated substrates in its interior cavity with motion at the helix II-III loop on a μ s timescale. A likely order of events is that the Positive Patch of FabA first interacts with PPant attached to S36 of AcpP. Once in proximity, residues R132 and K161 of FabA form salt bridges with E41 and E47 on helix II of AcpP, anchoring the complex; while R136 and R137 serve to pry away helix III of AcpP through interactions with A59 and E60, thus disrupting shielding of the sequestered substrate. V40 and L37 on AcpP form hydrophobic interactions with L138 and V134 on FabA. All of these binding events serve to stabilize AcpP in an open conformation, allowing the sequestered substrate to release from AcpP and insert into the pocket of FabA as the AcpP hydrophobic cavity collapses. Together, these results provide an unprecedented verification of the switchblade mechanism (Fig. 1C). We surmise that the identity of the sequestered substrate can affect the positioning of helix II and helix III of AcpP, thereby modulating successful binding and switchblade events for selective catalysis.

The application of crosslinking probes to gain structural insights now lays a foundation for defining the dynamic events associated with the mechanism of action of ACP. The approach can be applied to other carrier protein partners from primary and secondary metabolism such as FAS, polyketide synthase and non-ribosomal peptide synthetase,^{3,29,30} as well as other carrier protein-dependent pathways that play major roles in the treatment of cancer, obesity and infectious disease (Table S9).

Methods Summary

All proteins used were overproduced in *E. coli* BL21(DE3) (Novagen) and purified by Ni-affinity followed by FPLC chromatography. The AcpP=FabA complex was generated as previously reported and crystallized at room temperature by sitting drop vapor diffusion at 30 mg/mL in 10 mM sodium phosphate (pH 8.0), 350 mM sodium acetate, 1 M LiCl and 35 % PEG3350. Data were collected on beamline 12-2 at the Stanford Synchrotron Radiation Lightsource (SSRL) and beamline 8.2.2 at the Advanced Light Source (ALS) and processed with HKL2000. The AcpP=FabA crystallographic phases were determined by molecular replacement using FabA as the search template. Protein NMR data were collected at the UCSD Biomolecular NMR facility. Details of the molecular dynamics simulations are included in Supplementary Discussion. Detailed experimental procedures are described in the Supplementary Methods.

Supplementary Material

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Acknowledgments

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Figure 1. *E. coli* AcpP and crosslinking strategy

a, AcpP is a small, acidic protein comprised of four α -helices that interacts with at least 19 catalytic enzymes, 12 of which belong to FAS (10 shown here). The apolar interior of helix II ($\alpha 2$) and helix III ($\alpha 3$) form a hydrophobic cavity that sequesters the growing metabolite attached to the PPant arm. **b**, (top) A native substrate of FabA and (middle) modified AcpP with targeted sulfonfyl-3-alkynyl crosslinking probe, derived from (bottom) the crosslinking pantetheinamide analog **1**. **c**, Proposed mechanism of FabA. Protein-protein interactions between AcpP and FabA induce release of the sequestered substrate from AcpP into the active site of FabA, where dehydration is catalyzed. **d**, Crosslinking strategy to form AcpP=FabA with mechanism-based crosslinking probe **1**.

Figure 2. Structure of crosslinked AcpP=FabA

a, X-ray crystal structure of AcpP=FabA at 1.9 Å. **b**, The molecular surface mapped with calculated vacuum electrostatic potential of AcpP=FabA. Blue shading indicates electro-positive and red shading indicates electro-negative protein surfaces. **c**, Rotating **b** 90° at the interfaces between each AcpP=FabA to visualize electrostatic pairing. **d**, Expanded view of both interfaces in AcpP=FabA, indicating salt bridges and hydrophobic interactions between helix II (α2) and helix III (α3) of AcpP and the Positive Patch of FabA. **e**, Comparison between hydrophobic cleft of AcpP with (top) sequestered substrate (from PDB: 2FAE, with long interior hydrophobic cavity outlined with dashed line) and (bottom) AcpP1 in AcpP=FabA (reduced interior cavity). **f**, The interior cavity of 2FAE labeled with the hydrophobic residues. The contraction of these hydrophobic residues collapses the interior cavity in AcpP=FabA.

Figure 3. NMR studies

a, HSQC spectra of ^{15}N -octanoyl-AcpP in the absence of FabA (green), and with increasing (yellow to red) concentrations of FabA. Chemical shift perturbations (CSPs) are observed in AcpP residues that interact with FabA or the bound acyl chain. In magenta is the overlaid HSQC of ^{15}N -AcpP=FabA. **b**, Expanded views of select residues. **c**, CSPs were measured for each ^{15}N -octanoyl-AcpP residue in the absence and presence of 1 molar equivalent of FabA and plotted by residue number. **d**, AcpP residues from c where CSPs larger than 0.065 ppm are indicated in red. **e**, CSPs measured between ^{15}N -octanoyl-AcpP and the ^{15}N -AcpP=FabA were measured and plotted by residue number. **f**, AcpP residues from e where CSPs larger than 0.25 ppm are indicated in red. In NMR convention, protein residue number precedes residue letter; the converse applies with crystallography.

Figure 4. Molecular dynamics and protein-protein interactions

a, Experimental RDC data correlated with theoretical RDCs. (left) ^{15}N -*octanoyl*-AcpP (black) with Pf1 bacteriophage and (red) 5% neutral charge compressed polyacrylamide gel, and (right, magenta) crosslinked AcpP=FabA with Pf1 bacteriophage. **b**, Order parameter calculations of (left) *octanoyl*-AcpP and (right) AcpP=FabA. Nanosecond (blue) timescale compared to microsecond [RDC-optimized] (dotted) timescale routines. **c**, Sausage plot of order parameter differences on the microsecond timescale between *octanoyl*-AcpP and AcpP=FabA. Color and thickness depict relative disorder, where red represents maximal difference of 0.5. (Detailed in SI.) **d**, Residues of the Positive Patch mediating protein-protein interactions in known structures. (blue) FabA, (cyan) COL (PDB: 4DXE), (purple) stearyl ACP desaturase (PDB: 2XZ0), (orange) ACPS (PDB: 1F80), (magenta), ACP-P450 (PDB: 3EJB), (yellow) ACP-STAS (PDB: 3NY7), and (green) ACP-BioH (PDB: 4ETW).