Gating mechanism of elongating β-ketoacyl-ACP synthases

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Carbon-carbon bond forming reactions are essential transformations in natural product biosynthesis. During de novo fatty acid and polyketide biosynthesis, β-ketoacyl-acyl carrier protein (ACP) synthases (KS), catalyze this process via a decarboxylative Claisen-like condensation reaction. KSs must recognize multiple chemically distinct ACPs and choreograph a ping-pong mechanism, often in an iterative fashion. Here, we report crystal structures of substrate mimetic bearing ACPs in complex with the elongating KSs from *Escherichia coli*, FabF and FabB, in order to better understand the stereochemical features governing substrate discrimination by KSs. Complemented by molecular dynamics (MD) simulations and mutagenesis studies, these structures reveal conformational states accessed during KS catalysis. These data taken together support a gating mechanism that regulates acyl-ACP binding and substrate delivery to the KS active site. Two active site loops undergo large conformational excursions during this dynamic gating mechanism and are likely evolutionarily conserved features in elongating KSs.
Fatty acid synthases (FASs) and polyketide synthases (PKSs) iteratively condense and modify ketide units to produce a variety of natural compounds, ranging from fatty acids to complex bioactive molecules\(^\text{1-3}\). FASs and PKSs can exist as one or more polypeptide mega-synthases that contain distinct catalytic domains (type I) or as discrete enzymes, each possessing specific activities (type II)\(^\text{3-5}\). In these pathways, \(\beta\)-ketoacyl ACP synthases, alternatively ketosynthases (KSs), catalyze carbon–carbon formation via a precisely choreographed decarboxylative Claisen-like condensation to produce a \(\beta\)-ketoacyl species. In fatty acid biosynthesis (FAB, Fig. 1a), complete reduction at the \(\beta\)-position occurs before further chain extension, while in polyketide biosynthesis (PKB), the \(\beta\)-positions of pathway intermediates can be completely, partially, or not reduced to produce structurally and functionally complex natural products\(^\text{1,6}\). The small, 4-\(\alpha\)-helical acyl carrier protein (ACP) is used to shuttle substrates and intermediates to each catalytic domain or discrete enzyme in these pathways\(^\text{10,11}\). ACPs are initially translated to an inactive apo form and are subsequently post-translationally modified to an active holo form via attachment of a 4'-phosphopantetheine (PPant) arm at a conserved serine residue, providing a thiol moiety that ligates substrates and pathway intermediates to the ACP\(^\text{10}\). Protein–protein interactions (PPIs) between ACPs and their enzymatic partners (or domains) regulate the catalytic activities of FASs and PKSs and additionally provide a mechanism to ensure pathway orthogonality between primary (i.e., FAB) and secondary metabolism (i.e., PKB)\(^\text{3,5,6,12-17}\).

KS-catalyzed carbon–carbon bond formation is an essential reaction common to both FAB and PKB. The decarboxylative condensation reaction provides a thermodynamic driving force for the formation of complex natural products\(^\text{18,19}\). In addition, KS domains play an important role in chain length determination, ensuring product fidelity, and controlling pathway flux\(^\text{15,20,21}\). Therefore, efforts to engineer FASs and PKSs will depend on our ability to manipulate the specificities and activities of these domains/enzymes, as evidenced by the recent successes engineering the yeast FAS\(^\text{11-26}\).

KSs can be classified as elongating or initiating based on their role within a given pathway. Initiating KSs serve to catalyze the first condensation reaction of FAB or PKB pathways, converting holo-ACP to acyl-ACP, while all subsequent condensation reactions are catalyzed by elongating KSs\(^\text{14}\). Despite their distinct biosynthetic roles, both types of KS operate via a common ping-pong reaction mechanism, which can be disaggregated into two discrete steps\(^\text{11,27,28}\). In the first step, an acyl-ACP associates with a KS, and its acyl cargo is transferred from the PPant arm to a conserved, active site cysteine, producing a covalent acyl-KS adduct and holo-ACP. In the second step, malonyl-ACP replaces holo-ACP as the KS’s binding partner, subsequently undergoing a decarboxylative Claisen-like condensation with the acyl-KS adduct that produces \(\beta\)-ketoacyl-ACP (Supplementary Fig. 1). During the course of this ping-pong process, two distinct ACPs must sequentially interact with the KS, and thus must enforce exquisite temporal and spatial discrimination among the subtly different chemical states of these acyl-ACPs. The mechanisms used by KSs to coordinate these two half-reactions and how this coordination determines substrate specificity remains cryptic despite extensive biochemical and structural characterization.

The \(E.\ coli\) type II FAS has served as a model system for understanding ACP-mediated PPIs and FAB\(^\text{19,29-36}\). Here, we use this well-characterized system to better understand ACP-KS PPIs and KS substrate discrimination by structurally characterizing \(E.\ coli\) elongating KSs, FabB (KASI family KS), and FabF (KASII

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**Fig. 1 FAB cycle and known AcpP-PP complexes.** a A schematic representation of the chemical transformations that occur during fatty acid biosynthesis, highlighting the importance of protein–protein interactions required at each step for substrate processing. The four quadrants at the center of the scheme show the structures of all FAS AcpP crosslinked complexes solved thus far. The top two structures are the crosslinked AcpP–KSs (AcpP–Fabf and AcpP–FabB) reported herein, while depicted below are the crosslinked AcpP–DHs (AcpP–FabZ and AcpP–FabA) solved and reported in previous publications\(^\text{14,33}\). Each domain is abbreviated as follows: MAT malonyl-ACP transacylase, KS ketosynthase, KR ketoreductase, DH dehydratase, ER enoylreductase, and AT acyl transferase. AcpP-DH structures depicted in bottom two quadrants are AcpP–FabZ (PDB: 6N3P), AcpP–FabA (PDB: 4KEH). b, c Cartoon representations of the crosslinked AcpP–FabB and AcpP–Fabf dimers. AcpP is colored cyan; the first KS monomer of AcpP–FabB and AcpP–Fabf is colored orange or light orange, respectively, while the second is colored white in both structures. d Electrostatic potentials (ESP) maps of the FabB and AcpP monomers. e ESP maps of the FabF and AcpP monomers. In all cases, the ESP is mapped onto the Connolly surfaces of the FabF and AcpP monomers using a blue to white to red color range, spanning from +15.0 kT e\(^{-1}\) to -15.0 kT e\(^{-1}\).
family KS), as substrate/intermediate complexes with ACP that approximate states formed during catalysis. In conjunction with molecular dynamics (MD) simulations and mutagenesis studies, these structures provide dynamical descriptions of two ACP-KS complexes, revealing conformational changes that regulate substrate processing. Specifically, we propose that the elongating KSs employ two conserved active site loops that open and close through a double drawbridge-like gating mechanism in order to regulate substrate processing. When open, the drawbridge expands the KS active site in order to accommodate PPant-AcpP substrates. The structural features underlying this gating machinery are conserved in related enzymes and similar conformational transitions will likely determine substrate selectivity and processing in other FAB and PKB pathways.

Fig. 2 KS pantetheineamide crosslinking probes and ACP one-pot method. a Pantetheineamide-derived α-bromo and chlorovinyl crosslinking probes used in this study. b An illustration of the one-pot chemoenzymatic loading of apo-ACP with pantetheineamide probe 1c to produce crypto-1c-ACP using the coenzyme A biosynthetic enzymes CoaA, CoaD, and CoaE and a promiscuous phosphopantetheinyl transferase (PPTase) Sfp.

Results
Crosslinking and crystallization of AcpP–KS complexes. We synthesized C12-α-bromo- (1a) and C16-α-bromopantetheineamide (1b) and loaded them onto the E. coli ACP (AcpP) using a one-pot chemoenzymatic method38 to produce crypto-1a-AcpP (C12AcpP) and crypto-1b-AcpP (C16AcpP) that bear a reactive warhead on their respective PPant arms39–40 (Fig. 2). These crypto-AcpPs were then reacted with FabB and FabF to produce uniformly crosslinked complexes, referred to herein as AcpP–KS, where “−” denotes a crosslinked complex (Supplementary Figs. 1 and 2). Using this method, we grew crystals of C12AcpP–FabF and C16AcpP–FabF and obtained x-ray crystallographic data sets with 2.35 Å and 2.30 Å resolution, respectively. The crystallographic asymmetric units (ASU) of these complexes were identical and contained one monomer of FabF crosslinked to a single AcpP. In addition, C12AcpP–FabF crystals diffracted to 1.55 Å and C16AcpP–FabF diffracted to 2.50 Å. The C12AcpP–FabB and C16AcpP–FabB ASUs both contain the functional FabB dimer crosslinked to two AcpP molecules (Fig. 2b, c). Additional analysis for the C12AcpP–FabF and C16AcpP–FabB structures can be found in Supplementary Notes 1 and 2.

The AcpP–KS interface. The AcpP–KS interacting surfaces are electrostatic (Fig. 2b–e) and composed largely of flanking charged and polar groups surrounding a central hydrophobic patch (Fig. 3). The AcpP and KS monomers provide negatively and positively charged residues, respectively, to the interface. Figure 3 provides a comparison of the AcpP–FabF and AcpP–FabB interfaces from this work. While a thorough analysis of the AcpP–FabB interface was recently performed by Milligan et al.34 herein we report an example of a KASII-type KS in complex with an ACP, AcpP–FabF. The AcpP–FabB and AcpP–FabB complexes can be broken into three regions. Region 1 electrostatic interactions include Lys65, Arg68, and Lys69 of FabF interacting with Glu13, Glu14, Asp35, and Asp38 of AcpP (Fig. 3a). In addition, Thr270 of FabF, which sits on a flexible loop that undergoes conformational rearrangements upon acyl-AcpP binding, interacts with Asp35 of AcpP. Region 2 interactions are formed between Arg127, Lys128, and Arg206 of FabF and Glu47, Glu48, Glu53, and Asp56 of AcpP (Fig. 3b). The central hydrophobic patch is comprised of interactions between Met44, Val40, and Leu37 of AcpP and Ile129, Ser130, Pro131, Phe132, Ala205, and Arg206 of FabF (Fig. 3c).

Fig. 3a, b). In FabF, individual ACP monomers, AcpP1 and AcpP2, bury surface areas of 67.12 Å² and 67.54 Å², respectively, while in FabB, AcpP1, and AcpP2 bury surface areas of 597.5 Å² and 607.4 Å², respectively. These small contact areas are consistent with the transient nature of molecular recognition between ACP and its binding partners14,33. In addition, the differences in contact areas of AcpP–FabF and AcpP–FabB interfaces are consistent with the differing orientations that the AcpPs adopt relative to their KS partners. It is also worth noting that when comparing the interfaces of these AcpP–KS complexes, AcpP’s helix I forms more extensive contacts with FabF than FabB, while FabB forms more hydrophobic interactions at region 3 (Fig. 3). While the differences at these interfaces are subtle, the manner in which AcpP engages with each of its cognate KSs may modulate the activities of FabF and FabB41,42. A time-resolved analysis of the FabF and FabB interfaces using MD simulation data is provided in Supplementary Note 3.

Changes in the FabF active site accompany substrate binding. Electron density maps calculated using the 2.30 Å C16AcpP–FabF data set demonstrate adequate density for AcpP’s Ser36, the PPant moiety, and acyl cargo. The FabF active site cysteine residue (Cys163) forms a covalent bond with the α-carbon of the fatty acid substrate mimetic (1b), and the resulting stereocenter is found in the S configuration (Fig. 4a). The carbonyl group of the substrate coordinates to the FabF active site histidine residues, His303 and His340, in a manner similar to the published structure of AcpP–FabB34 (PDB: 5KOF) (Fig. 4b). Modeling and refinement of the crosslinker’s acyl chain required the remodeling of two active site loops. The first of which is the conserved GFGG loop (loop 1, residues 399–402) adjacent to the active site that contains the putative Phe400 gating residue42, and a second less conserved loop (loop 2, residues 265–275) that caps loop 1 (Fig. 5a). These loops undergo significant conformational changes, revealing a hydrophobic pocket that accommodates the acyl chain (Supplementary Fig. 4a–c and Supplementary Note 4). This pocket is distinct from the substrate binding site identified in the crystallized acyl-KS intermediate structures 2GFY43 and 1EK44 (Fig. 4c). Close analysis of the C16AcpP–FabB structure indicates that crystal contacts do not play a role in the conformational changes seen in these loops.

C16AcpP–FabB adopts an open conformation. A comparison of the apo-FabF (PDB: 2GFY)43, acyl-FabF (PDB: 2GFY)43,
and C16AcpP–FabF structures shows that Phe400 of loop 1 moves ~7 Å, as measured by changes in the Phe400 Cα position, into a pocket revealed by a conformational rearrangement of loop 2 (Fig. 5a). Gly399 and Gly402 of loop 1 act as pivot points as the loop transitions from a closed conformation to an open conformation (Fig. 5b, c). The open conformation is stabilized by a network of hydrogen-bonding interactions between the backbone amides of Gly399, Phe400, Gly401, and Gly402 and the side chains of Asn404 and Asp265 from loop 2 (Fig. 5b, c). Overlays of the apo-FabF and C16AcpP–FabF structures indicate that the C16-acyl chain of 1b would clash with Phe400 in the closed conformation of loop 1 (Fig. 5a). These results indicate that these loop rearrangements may act as a gate to provide access to the active site for acyl-ACP substrates. Interestingly, the recently deposited FabF (light orange) and FabB (bright orange) interfaces can be broken up into three regions, and the cartoon inset provides context regarding the viewing angle and the positional relationship of AcpP (cyan) to the overall AcpP–KS complex.

Fig. 3 AcpP–FabF and AcpP–FabB interface interactions. The FabF (light orange) and FabB (bright orange) interfaces can be broken up into three regions, and the cartoon inset provides context regarding the viewing angle and the positional relationship of AcpP (cyan) to the overall AcpP–KS complex. In addition, the white hole represents the active site to which the viewed AcpP is crosslinked as the majority of contacts are provided by the KS monomer not covalently crosslinked to AcpP. a A close-up view of the AcpP–FabF region 1 electrostatic interactions. b A close-up view of the AcpP–FabB region 2 electrostatic interactions. c A close-up view of the AcpP–FabF region 3 hydrophobic interactions. d A close-up view of the AcpP–FabB region 1 electrostatic interactions. e A close-up view of the AcpP–FabB region 2 electrostatic interactions. f A close-up view of the AcpP–FabB region 3 hydrophobic interactions.

C12AcpP–FabB trapped in closed acyl transfer conformation. Analysis of the 1.55 Å C12AcpP–FabB data (Supplementary Fig. 4d) demonstrates well-resolved electron density for the 4′-phosphopantetheinamide arm and the C12-acyl chain. In this structure, loops 1 and 2 are found in a catalytically competent closed conformation (Fig. 4d–f). Like the catalytic cysteine of FabF, Cys163 of FabB, reacts stereoselectively with the α carbons of 1a to produce the S stereoisomer (Fig. 4d). The carbonyl group of the substrate is coordinated to the oxyanion hole formed by the backbone amides of Cys163 and Phe400, and the probe’s acyl chain is positioned inside FabB’s fatty acid binding pocket (Supplementary Fig. 4d, e). Interestingly, the recently deposited AcpP–FabB structure (PDB: 5KOF)34 does not feature these interactions, and instead the carbonyl group forms hydrogen bonds with His298 and His333.
Comparison of C12AcP–FabB and dodecanoyl-FabB (C12-FabB, PDB: 1EK4) demonstrates that the active site and acyl chain geometries are nearly identical in the two structures (Fig. 4f). The similarity between the C12-FabB and C12AcP–FabB indicates that the latter approximates a catalytically relevant state for the transfer of substrate from acyl-AcpP to FabB’s active site cysteine. Both steps of the KS reaction form a tetrahedral intermediate that is stabilized by the oxyanion hole, which is only organized when loop 1 and the associated Phe392 residue (Phe400 in FabF), are in the gate-closed conformation.

**MSA analysis suggests conservation of loops 1 and 2.** Multiple sequence alignments (MSA) of type II FAS elongating KSs indicate that residues of loop 1 are highly conserved across the KASI (FabB) and KASII (FabF) families of KSs, while loop 2 is only conserved within, but not between, the two families (Supplementary Fig. 5). The consensus sequence for loop 1 is comprised of the conserved GFGG β-turn motif flanked by two conserved asparagine residues, Asn396 and Asn404 (FabF numbering). Interestingly, Asn404 interacts with a highly conserved Asp265 found at the start of loop 2 in both the open and closed states, providing a physical basis for the (proposed) coordinated movement of loops 1 and 2 (Fig. 5b, c). The conserved nature of loop 1 and Asp265 suggests that their proposed gating function is a general feature of type II FAS elongating KSs. Further analysis and comparison of FabF and FabB structure and function are provided in Supplementary Note 5.

To determine if these proposed gating elements are conserved in condensing enzymes outside of type II FAS, we aligned *E. coli* FabF with representative KSs from type II PKS, type I FAS, and type I PKS (Fig. 6a). Loop 1 of FAS and PKS KSs is generally conserved, although there is modest sequence variation. Notably, the Phe400 FabF gating residue is conserved in type I FAS and type II PKS but is replaced by valine or isoleucine in the KS domains of the type I PKS deoxyerythronolide B synthase (DEBS). Modification of the gating residue may modulate substrate specificity, as the KS domains from DEBS accept and extend substrates with β-substituents and utilize bulkier alpha-branched extender units, namely methylmalonyl-CoA.

Loop 2 of these KSs is significantly less conserved than loop 1. Only Asp265 can be considered highly conserved, as it is present in all KSs except the KS monomer (actKS) of the heterodimeric type II PKS KS/chain length factor (CLF) complex from actinorhodin biosynthesis. In contrast to FAS KSs, these enzymes iteratively extend a growing polyketide within the KS binding pocket only unbinding the polyketide once multiple extensions using malonyl-ACP have been catalyzed to produce a fully extended poly-β-keto-ACP. The size of the polyketide produced by actKS is determined by CLF, which accommodates...
the growing polyketide chain, mediating substrate dissociation when the polyketide intermediate can no longer be accommodated within its binding pocket\(^\text{[49]}\). Therefore, given that iterative type II KSs do not accept acyl-ACPs and instead only process polyene PKSs, such as Iga1\(^\text{[50,51]}\) and ApeO\(^\text{[52]}\), that require substrate offloading in-between condensation steps\(^\text{[52]}\), the same substitution is not observed, instead these enzymes possess the conserved Asp265 found in type II FAS KSs.

**Mutagenesis studies of FabF gating mutants.** To determine if the proposed gating function of loops 1 and 2 have any effect on KS activity, we identified a panel of FabF mutations that we hypothesized would attenuate FabF’s ability to accept and/or process acyl-ACP substrates. These mutations were designed to alter FabF’s proposed gating function by modulating loop 1’s flexibility (flex modulation), destabilizing (disfavoring) the gate-open conformation (destabilization), or occluding the pocket occupied by Phe400 in the gate-open conformation (pocket blockade). To test this hypothesis, we adapted a crosslinking assay...
that utilizes α-bromo and chloroacrylate mechanistic crosslinkers with different fatty acid chain lengths as substrate mimetics\(^{29}\) (Fig. 2a). We hypothesized that our panel of FabF gating mutants would generally retain wild-type (wt) activity toward smaller C2 and C3 chain length crosslinkers, 1c and 2a, but would crosslink less efficiently with longer C8 and C12 chain length crosslinkers, 2b and 1a, which would require transitions between gate conformations to access the KS active site.

Before assaying these gating mutants, we first evaluated the crosslinking activity of a gate removal mutant, F400A. The results show that both F400A and wt FabF crosslink with generally equal efficiency with short- and long-chain acyl-AcpP substrates (Fig. 6). Interestingly, the F400A mutant underwent crosslinking with the C8Cl (2b) with higher relative efficiency to wt (133%) both in our single time point assay and at a faster rate in our time-course crosslinking studies (Supplementary Figs. 6–11). These results suggest that mutating Phe400, a key gating residue\(^{27}\), to alanine enhances the accessibility of probe to the KS active site cysteine\(^{27}\). In addition, we prepared two FabF interface mutants, K65E and K65A/K69A, to demonstrate that crosslinking can also report on PPIs. Both of these mutants showed reduced crosslinking towards all probes, but crosslinking
efficiency was most significantly reduced with long-chain crosslinkers (Fig. 6). These results suggest that well-coordinated PPIs are important for chain-flipping \( ^5 \) substrates into the KS active site.

To prevent loop 1 transitions from the closed to open conformation, we mutated FabF’s Gly310 to a phenylalanine or methionine residue to introduce a bulky amino acid at a position that would occlude the pocket occupied by Phe400 in the open conformation (pocket-blockage, Supplementary Fig. 12). Interestingly, G310F and G310M crosslink with 73 and 70% efficiency with C2aBr (1c) but only 33 and 30% efficiency with C12aBr (1a), respectively. Differential crosslinking activity between short and long substrates was more pronounced for the chloroacrylate crosslinkers as G310F and G310M crosslinked with 85 and 82% efficiency with C3Cl (2a) but only 5 and 1% efficiency with C8Cl (2b), respectively (Fig. 6). These results indicate that blocking the open conformation reduces FabF’s ability to accept longer acyl-ACP substrates but not that of smaller substrates that more closely resemble malonyl-AcpP.

Similar patterns emerge when examining the crosslinking efficiencies of our D265A and D265N mutants of loop 2. These mutants are hypothesized to disfavor the open conformation by replacing the ion-dipole interactions involving the conserved Asp265 from loop 2 with weaker interactions. These mutations should therefore disrupt the conserved D265 hydrogen-bonding network between loops 1 and 2 that stabilizes the open conformation. D265A and D265N mutants crosslinked with 72 and 77% efficiency with C2aBr (1c) and 51 and 64% efficiency with C12aBr (1a), respectively. Furthermore, D265A and D265N crosslinked with 63 and 73% efficiency with C3Cl (2a) but only 2 and 1% efficiency with C8Cl (2b), respectively.

In contrast, loop flexibility-modulated mutants, G399A and G402A, did not demonstrate pronounced differences in cross-linking relative to wt FabF in our assay. The G399A mutant reacted with all crosslinkers with similar efficiency, as wt FabF, while crosslinking efficiencies of the G402A mutant only showed differential crosslinking activity when comparing C3Cl (2a) (87%) and C8Cl (2b) (24%) (Fig. 6). G399A also crosslinked similarly to wt FabF in our time-course crosslinking assay (Fig. S5). While it was expected that the G399A mutant would show a difference in crosslinking efficiency, it is important to note that such a substitution is observed in nature, as the KSs from type II polyene synthases, Igal1\(^ {50,51} \) and ApeO\(^ {32} \), both have alanine at this position (Fig. 5a). In addition, the dihedral angles of G399A in both the closed and open conformation are not in the Ramachandran forbidden zone (Fig. 5b, c) and can therefore be reasonably accessed by the G399A mutant.

**MD simulations support a KS gating mechanism.** Using these AcpP–KS crystal structures as initial coordinates, we performed MD simulations of C10, C12, and C16-AcpP-KS complexes, where “a” denotes a noncovalent complex (Supplementary Figs. 1 and 13), to probe the dynamic properties of the gating processes in acyl-AcpP-KS complexes. Models of the ACP-bound acyl-AcpP-KSs were constructed by manually replacing the crosslinkers in the crystal structures with the natural saturated substrates encountered by the KSs (Online Methods). In addition, simulations of apo-FabB and apo-FabF were performed using previously reported crystal structures (PDB: 2VB9\(^ {34} \) and 2GFW\(^ {45} \), respectively) as initial coordinates. Lastly, in an attempt to computationally visualize the transition between the open and closed conformations of loops 1 and 2, we performed additional MD simulations of apo-FabF and apo-FabB using KS structures prepared by removing the AcpP monomers from the AcpP–KS complexes reported herein. We distinguish these structures from the crystallographic apo-FabB and apo-FabF structures by referring to them as apo-FabB\(^ * \) and apo-FabF\(^ * \). Because these apo-structures feature their loops in conformations that may not be stable in the absence of an associated acyl-ACP, we hypothesized that they may more readily undergo conformational transitions. Each of the ten systems described above was subjected to three independent 500 ns MD simulations. Replicates were initiated using different velocities and a total of 15 μs of MD data were collected. General analysis for all simulated systems is provided in Supplementary Figs. 14–27 and additional discussion regarding substrate interactions is provided in Supplementary Notes 6 and 7.

From this data, no transition between the closed and open loop conformations was sampled during the course of any simulation, suggesting that the coordinated movement of loops 1 and 2 between their open and closed states is a relatively slow process, likely occurring on timescales of tens or hundreds of μs\(^ {35} \). Nonetheless, analysis of the per residue backbone and side chain root mean square fluctuations (RMSFs) of the loop residues, shown in Fig. 7d–i and Supplementary Figs. 28–30, suggests the following: Firstly, while the loops of apo-FabB and apo-FabF have similar RMSFs, the loops of apo-FabF\(^ * \) and the acyl-AcpP-FabF complexes show greater fluctuations than do those of apo-FabB\(^ * \) and the acyl-AcpP-FabB complexes, respectively. Of the structures shown in Fig. 7d–i, apo-FabF\(^ * \) is the most significant. RMSF analysis shows that loop 2 of apo-FabF\(^ * \) fluctuates more in this structure than any other simulated structures. Despite this dynamism, apo-FabF\(^ * \)’s loop 1 remains fairly rigid. It is possible that the transition of loop 2 from its open to closed conformation first requires the motion of loop 1 toward the active site. In fact, this movement of loop 1 would restore the oxanion hole absent in the C16AcpP–FabB structure (Fig. 3). Therefore, the large RMSFs observed for apo-FabF\(^ * \)’s loop 2 reveal a so-called frustrated loop unable to assume its preferred open conformation in the absence of acyl-AcpP (Fig. 7). These findings, along with published studies of PKS ACPs\(^ {16} \), suggest that ACPs may not only be responsible for substrate delivery but also may induce loop motions that promote catalysis through allostery.

Identification of this frustrated loop suggests that the motions of loops 1 and 2 are correlated. To evaluate this hypothesis, we determined the average correlations of the motions of residues of loops 1 and 2 sampled during the course of the MD simulations of apo-KS, apo-KS\(^ * \), and acyl-AcpP-KS structures. Both FabB and FabF appear to form the same general network of interloop interactions to choreograph loop motion (Fig. 8). Nonetheless, the co-relatedness of the loops are distinct in the open (Fig. 8c, e) and closed (Fig. 8a–d) states, with a greater correlation found in the closed conformation. Furthermore, the average correlation of loop 1 and 2 residues is slightly greater in simulations of acyl-AcpP-FabF complexes than simulations of either apo-FabF or apo-FabF\(^ * \). On the basis of this analysis, it is evident that loops 1 and 2 move in concert with one another and that the GFGG hinge motif is found to be most correlated to the highly conserved Asp265 residue of loop 2. Interestingly, these motifs are more highly correlated in FabF than in FabB, although higher positive average correlations between Asp265 and intra-loop 2 residues Pro271, Ser272, and Gly273 are observed in simulations of apo-FabF and its acyl-AcpP complexes.

The residues of loop 1 and 2 of FabB and FabF, regardless of enzyme state, that are most strongly correlated are residues of the GFGG motif of loop 1 and Asp265 of loop 2. In fact, the closed and open conformations of these loops can be clearly distinguished by differences in these intra- and interloop interactions. That we do not observe changes in these networks of interactions within any KS simulation indicates the stability of the open and closed states. According to simulation data, the
open conformation (apo-FabF* and alyl-AcpP-FabF) is characterized by hydrogen-bonding interactions involving the GFGG motif and Asp265 and Asn404. Namely, Gly399 forms a hydrogen bond with Asn404 while the negatively charged Asp265 residue simultaneously coordinates Asn404 and the backbone amides of all residues of the GFGG motif. In the open conformation of FabF, average distances of 5.09 Å, 3.91 Å, 3.66 Å, and 4.12 Å, respectively, are computationally observed between the center of mass of the carboxylate moiety of Asp265 and the backbone amide nitrogens of Gly399, Phe400, Gly401, and Gly402 (Fig. 9). Given the highly conserved nature of Asp265 and its substitution with a less potent hydrogen-bond acceptor (e.g., Asn265 in actKS) in iterative type II KSs, it is reasonable to postulate that the coordination of the motion of loop 1 and loop 2 is mediated by Asp265.

Discussion

KSs catalyze multi-step reactions that require the association and dissociation of two distinct acyl-AcpPs and involve the intermedici of an acyl-enzyme adduct. Enzymes that catalyze such complex chemical transformations often implement gating mechanisms to control substrate access, exert mechanistic selectivity, and/or control reaction order. Zhang et al. previously showed that Phe400 of Str. pneumoniae FabF (SpFabF) acts as a KS gating residue. The F400A mutant of SpFabF has reduced catalytic activity relative to wt for its native reaction, but efficiently produces tricarboxylic acid lactone, a common shunt product of condensing enzymes that results from the mispriming and extension of malonyl-ACP. These results suggest a compromised gating system that no longer controls reaction order. The proposed gating role of Phe400 is also supported with previously published structural data. In structures of apo-FabB and apo-FabF, Phe400 initially blocks access to the active site cysteine but after transacylation rotates away to form the malonyl-ACP binding site for the pong half-reaction.

Results reported herein not only confirm and build upon the role of Phe400 as a KS gating residue but also provide additional context for its mechanism and regulation. With this information, we propose that elongating KSs use a double drawbridge-like gating mechanism to direct substrate binding and control the timing and sequence of the transacylation and condensation half-reactions. From this study we propose the model shown in Fig. 10. Here, ACP binds to apo-KS, upon which ACP chain-flips its cargo into the KS active site as KS loops 1 and 2 move in a coordinated manner to assume an open conformation, forming a transient, hydrophobic delivery/extraction pocket that temporarily accommodates the substrate (Fig. 10b). Loops 1 and 2 then return to their closed conformations, directing the substrate’s acyl chain into the acyl binding pocket while simultaneously restoring the oxanion hole that facilitates transacylation (Fig. 10c). Upon transacylation, holo-FabF dissociates and Phe400 rotates to form the malonyl-ACP binding pocket (Fig. 10d).

The proposed gating mechanism coordinated by loops 1 and 2 is in agreement with our mutagenesis studies, MD simulations, and MSA analysis. FabF pocket-blockage mutants, G310M and G310F, and loop 2 destabilization mutants, D265A and D265N, show reduced crosslinking with long-chain acyl-AcpP substrates as compared with short-chain substrates. These results, taken together, indicate that blocking transitions to the gate-open configuration or disfavoring the gate-open configuration by disruption of the the interloop interaction network involving impair KS activity with acyl-AcpPs. Interrogation of these trapped catalytic states using long time-scale MD simulations demonstrates that the open and closed conformations of FabF are stable and coordinated by a conserved network of hydrogen-bonding interactions. Furthermore, this network of interactions serves to correlate the motions of loops 1 and 2 and is generally conserved in elongating KSs in type I PKS, type I FAS, and type II PKS.

Given our gating model, we now propose a general mechanism used by these synthases to regulate substrate processing...
Fig. 8 Average cross correlations of the motions of key loop 1 and 2 residues sampled computationally. Cross correlations were determined by computing a motion vector for each residue of loop 1 and loop 2 from its prior position to its current over the course of each simulation. In this manner, motion vectors for each pair of residues of loop 1 and 2 are calculated for each frame of simulation data. Heat maps generated from the cross-correlation analysis of the motions of loop 1 and 2 residues of 

- a apo-FabF
- b apo-FabB
- c apo-FabF*
- d apo-FabB*
- e acyl-AcpP-FabF
- f acyl-AcpP-FabB

Residues are sampled using molecular dynamics (MD) simulations. a The heat map has been divided into four quadrants; quadrants II and III illustrates cross correlations of the motions of residues within loops 1 and 2, respectively. Inter-loop cross correlations are shown in quadrants I and IV. Note that each heatmap is symmetric about the diagonal that bisects quadrants II and III.
and catalysis. Transition to the open-conformation disrupts the oxyanion hole, which suggests that (trans)acylation of the active site cysteine occurs after the gate adopts the closed conformation. Consequently, the gate could act as a sensor that only reorganizes the oxyanion hole when the correct substrate is bound. Therefore, the gate and F400 may prohibit FAS KSs from reacting with fatty acyl intermediates that are not appropriate condensation substrates (i.e., β-hydroxy-acyl-ACPs).

A careful consideration of loops 1 and 2 and their potential specific functions reveals some interesting insights as well. Ramachandran analyses of the GFGG motif’s fluctuations over the course of MD simulations illustrate the rigidity of loop 1 in both the closed and open conformations. The φ and ψ angles of the GFGG residues sampled computationally are narrowly distributed with hinge residues, Gly399 and Gly402, possessing distinct backbone dihedral angles in the open and closed conformations (Fig. 5b, c). This is in contrast to Ramachandran analysis of the hinge residues in loop 2, which shows that although the φ and ψ angles of Pro272 and Pro273 are distinct in the open and closed states in our crosslinked structures, they sample similar φ, ψ angle distributions during simulations (Fig. 5e, f and Supplementary Figs. 31 and 32). These results, along with the increased sequence variation in different condensing enzyme families, suggests loop 2 may modulate loop 1 gating events, potentially conferring different activities or substrate specificities to distinct KSs. This is an appealing hypothesis, as loop 2 sits on top of loop 1, effectively capping it in when in the closed conformation. Furthermore, the dynamism of loop 2 in our apo-FabF* simulations indicates that acyl-AcpP substrates stabilize the gate-open conformation. In fact, AcpP binding or the chain flipping of acyl cargo into the KS active site may serve to trigger gating events by stabilizing loop 2’s open position. Movement of loop 2 would thereby allow loop 1 to assume the gate-open conformation, raising the drawbridge and allowing substrate access to the active site. While more work is needed to test this hypothesis, a recent study by Robbins et al. demonstrated that mutation of S315A from loop 2 of DEBS KS1 increased the formation of propionate, which results from the decarboxylation of the methylmalonyl-ACP substrate. These findings suggest an important role for loop 2 residues in DEBS and that the altered

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**Fig. 9 Analysis of the fluctuations of inter-loop hydrogen bonding networks.** Histograms showing the distances between the hydrogen-bond donating Asp265 and highly conserved (hydrogen-bonding accepting) residues, Gly399, Phe400, Gly401, Gly402, and Asn404 sampled computationally. Histograms of these distances sampled in molecular dynamics (MD) simulations of **a** apo-FabF, **b** apo-FabB, **c** apo-FabF*, **d** apo-FabB*, **e** acyl-AcpP·FabF, and **f** acyl-AcpP·FabB, respectively.
S315A mutant activity may be indicative of an impaired gating system. The importance of KS-catalyzed carbon–carbon bond formation in biosynthetic pathways cannot be overstated. These enzymes perform complex reactions and employ poorly understood biochemical regulatory mechanisms to ensure substrate specificity and product fidelity. Here, the combination of chemical, structural, and computational biology together support a gating mechanism used by elongating KSs to recognize and process acyl-ACP substrates. These results provide broadly applicable insights into KS activity, function, and substrate selectivity as well as further our understanding of the E. coli AcpP interactor.

**Methods**

**Ketosynthase production, purification, and tag cleavage.** The N-terminal His-tag FabF and FabB recombinant proteins were expressed in *E. coli* BL21 and cultured in Terrific Broth (MilliporeSigma). Cells were grown in the presence of 50 mg·L\(^{-1}\) kanamycin, induced with 0.5 mM Isopropyl \(\beta\)-d-1-thiogalactopyranoside (IPTG) at OD\(_{600}\) = 0.8, and incubated at 37 °C for 4 h. The cells were spun down by centrifugation at 500 \(\times\) g for 30 min and the collected pellets were resuspended in lysis buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, pH 8.0) along with 0.5 mg·mL\(^{-1}\) lysozyme ( Worthington Biochemical Corp). The pelleted cells were lysed by sonication (4 s pulses for 5 min). Due to the high stability of AcpP, irrelevant proteins were precipitated by dialyzing in same volume of isopropanol into the lystate at the speed of 0.1 mL·min\(^{-1}\). The resulting mixture was spun down by centrifugation at 11,200 \(\times\) g for 1 h and the supernatant was injected directly into FPLC. AcpP was purified by HiTrap Q HP ion exchange chromatography column in 50 mM Tris buffer with a gradient of NaCl from 0 M to 1 M and the native AcpP eluted around 0.3 M NaCl. The eluted protein was collected and concentrated using Amicon Ultra Centrifuge Filters (Millipore) with 3 kDa molecular weight cut off.

**Holo-AcpP apofication and apo-AcpP purification.** The purified protein was resulted in a mixture of holo-AcpP and apo-AcpP. The phosphopantetheine moiety on holo-AcpP was removed by acyl carrier protein hydrolase (AcpH) in lysis buffer for 16 h\(^{28}\). Final reaction concentrations: 5 mg·mL\(^{-1}\) AcpP mixture, 0.01 mg·mL\(^{-1}\) AcpH, 30 mM Tris (pH 7.4), 10% glycerol, 0.25 mM DTT. The resulting pure apo-AcpP, was analyzed by conformationally sensitive urea PAGE (2.5 M urea), and further purified by FPLC using HiLoad 16/600 Superdex 75 PG (GE Biosciences) size exclusion column to remove AcpH. The eluted protein was collected and concentrated using Amicon Ultra Centrifuge Filters (Millipore Sigma) with 3 kDa molecular weight cut off.

**Apo-AcpP loading and crypto-AcpP purification.** All crosslinkers were loaded onto apo-AcpP using a one-pot chemoenzymatic method\(^{28}\). This method utilizes three CoA biosynthetic enzymes (CoaA, CoaD, CoaE) to form CoA analogues and a phosphopantetheinyl transferase (PPTase, Stp) to load them onto apo-AcpP, resulting in crypto-AcpP. Final reaction concentrations: 1 mg·mL\(^{-1}\) apo-AcpP, 0.04 mg·mL\(^{-1}\) Stp, 0.01 mg·mL\(^{-1}\) CoaA, 0.01 mg·mL\(^{-1}\) CoaD, 0.01 mg·mL\(^{-1}\) CoaE, 50 mM potassium phosphate pH 7.2, 12.5 mM MgCl\(_2\), 1 mM DTT, 0.2 mM pantetheinamide probe (1a,1c or 2a,2b) (Fig. 2), and 8 mM ATP. The stock solutions of crosslinkers 1a–1c, 2a, and 2c were prepared by dissolving them in DMSO to a final concentration of 30 mM. Reactions were incubated at 37°C for 16 h and then purified by HiLoad Superdex 75 (GE Biosciences) size exclusion column. The eluted protein was collected and concentrated using Amicon Ultra Centrifuge Filters (Millipore Sigma) with 3 kDa molecular weight cut off up to a concentration at 1–3 mg·mL\(^{-1}\).
Crosslinking reaction and crosslinked complex purification. The crosslinking reactions were carried out by mixing crypt-o-AcpP with its partner protein (FabF or FabB) in 5:1 ratio at 37 °C for 16 h. Reactions were examined by 12% SDS PAGE and purified by HiLoad 16/600 Superdex 200 PG (GE Biosciences) size exclusion column using minimal buffer (20 mM Tris, 50 mM NaCl, pH 7.4). The resulting protein complex was greater than 95% in purity, determined by SDS PAGE, and was concentrated to 8–10 mg·mL⁻¹ using Amicon Ultra Centrifuge Filters (MilliporeSigma) with 30 kDa molecular weight cut off. The concentrated crosslinked complex was immediately used for protein crystallization or flash-frozen and stored in −80 °C freezer for later use.

Construction of ketosynthase mutants. Ketosynthase mutants were generated using the site directed mutagenesis method developed by Liu and Nisamish25. All constructs were verified via Sanger sequencing (Eton Bioscience Inc.). Primers used for mutagenesis can be found in Supplementary Fig. 33.

Crosslinking assay. Each reaction was set up to contain 40 μM of crypto-AcpP, 20 μM of FabF, and buffer (25 mM Tris, 150 mM NaCl, 10% glycerol, 0.5 mM TCEP, pH 7.4). To stop the reaction at the designated time point, 2.5 μL of the reaction solution was mixed with 10 μL of 3X SDS dye. Samples were analyzed by 12% SDS-PAGE and the intensity of protein bands was measured by ImageJ60. The crosslinking efficiency of mutants was normalized to the average wild type crosslinking efficiency to obtain a normalized relative crosslinking efficiency. The plotted data represent the average normalized crosslinking efficiency of each mutant and the error bars are represented as standard deviation (±SD).

General synthetic methods. General synthetic methods are provided in Supplementary Note 8 and NMR characterization of compounds and intermediates is provided in Supplementary Figs. 40–43.

Crystallization, structure determination, and refinement. The crystals of the crosslinked complexes were grown by vapor diffusion at 6 °C. In detail, 1 μL of crosslinked complex (8–10 mg·mL⁻¹) was mixed with 1 μL of corresponding crystallization condition and the mixture was placed inverted over 500 μL of the well solution (hanging-drop method). The AcpP–FabF complexes crystallized in 26–30% PEG 8 K, 0.1 M sodium cotateylate pH 6.5, and 0.3 M NaOAc, yielding numerous orange rods amongst a heavy precipitate. Seeding trials were performed to increase size and quality of the samples. The C12AcpP–FabF complexes produced crystals in two conditions: 20% PEG 8 K, 0.2 M Mg(OAc)₂, 0.1 M sodium cotateylate pH 6.5 as well as 30% PEG 8 K, 0.2 M (NH₄)₂SO₄, 0.1 M sodium cotateylate, pH 6.5. Both conditions yielded square plates and required 1–2 weeks for complete growth of crystals. The C16AcpP–FabF complexes produced crystals in 18–24% PEG 8 K, 0.1 M sodium cotateylate, and 0.3 M NaOAc, pH 6.5.

All data were collected at the Advanced Light Source synchrotron at Berkeley. Data were indexed using iMosFLM61. The crystals were processed using the program mosfilm61 and scaled using the program SCALEX62,63. The crosslinked complexes were grown by vapor diffusion at 6 °C. In detail, 1 μL of crosslinked complex (8–10 mg·mL⁻¹) was mixed with 1 μL of corresponding crystallization condition and the mixture was placed inverted over 500 μL of the well solution (hanging-drop method). The AcpP–FabF complexes crystallized in 26–30% PEG 8 K, 0.1 M sodium cotateylate pH 6.5, and 0.3 M NaOAc, yielding numerous orange rods amongst a heavy precipitate. Seeding trials were performed to increase size and quality of the samples. The C12AcpP–FabF complexes produced crystals in two conditions: 20% PEG 8 K, 0.2 M Mg(OAc)₂, 0.1 M sodium cotateylate pH 6.5 as well as 30% PEG 8 K, 0.2 M (NH₄)₂SO₄, 0.1 M sodium cotateylate, pH 6.5. Both conditions yielded square plates and required 1–2 weeks for complete growth of crystals. The C16AcpP–FabF complexes produced crystals in 18–24% PEG 8 K, 0.1 M sodium cotateylate, and 0.3 M NaOAc, pH 6.5.

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Preparation of apo-KS structures for MD simulations. Simulations of apo-FabB and apo-AcpF were performed using two of the crystallographic structures derived from previously reported 1.55 Å apo-FabB (PDB: 2V9B64) and 2.40 Å apo-AcpP (PDB: 2GFW55) and a second set generated by modifying the crosslinked structures of E. coli AcpP–FabB and AcpP–FabF reported herein by deleting coordinates of this moiety were performed using Avogadro 1.1.166 [http://avogadро.org/], Gaussview 5.0.9, and Pymol v.1.8.6, and Pymol v.2.2.3. Schrodinger was used to add poorly resolved C- and N-terminal residues omitted in the dimeric AcpP–FabB and AcpP–FabF complexes. The protonation state of titratable residues in the apo-KSs and acyl-AcpP–KS dido-
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Author contributions
J.J.L, J.A.M, M.D.B., and J.P.N. designed and supervised the project. T.D.D. synthesized probe molecules. J.T.M, W.E.K., T.G.B., and A.C. purified protein, optimized cross-linking, carried out crystallization, and obtained crystals. J.T.M. collected x-ray diffraction data and refined and analyzed crystallographic models. J.T.M designed and produced gating mutants. W.E.K. and A.C. performed crosslinking assays. A.P. performed MD simulations. A.P., J.T.M., and J.A.M analyzed simulation data. J.T.M., A.P., J.J.L, J.A.M, M.D.B., and J.P.N. wrote the manuscript. All authors edited the manuscript. W.E.K., T.D.D., A.C., and T.G.B. contributed equally to this work.

Competing interests
The authors declare no competing interests.

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