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Active Site Labeling of Fatty Acid and Polyketide Acyl-Carrier Protein Transacylases

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Abstract

Metabolic engineering of fatty acids and polyketides remains challenging due to unresolved protein-protein interactions that are essential to synthase activity. While several chemical probes have been developed to capture and visualize protein interfaces in these systems, acyl carrier protein (ACP) transacylase (AT) domains remain elusive. Herein, we combine a mutational strategy with fluorescent probe design to expedite the study of AT domains from fatty acid and polyketide synthases. We describe the design and evaluation of inhibitor-inspired and substrate-mimetic reporters containing sulfonyl fluoride and β -lactone warheads. Moreover, specific active-site labeling occurs by optimizing pH, time, and probe concentration, and selective labeling is achieved in the presence of inhibitors of competing domains. These findings provide a panel of AT-targeting probes and set the stage for future combinatorial biosynthetic and drug discovery initiatives.

Graphical Abstract

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Conflicts of interest

There are no conflicts to declare.

Fatty acid synthases (FAS) and polyketide synthases (PKS) produce small molecules that provide cellular structure, facilitate signaling and protein trafficking, or aid in chemical defense and virulence.^{1, 2} Fatty acid and polyketide biosynthesis share a common foundation that is initiated by acyl-carrier protein (ACP) transacylase (AT) domains that select and load acyl-CoA substrates onto *holo*-ACPs *via* transient acylation of an active site residue (Fig. 1). In particular, malonyl-CoA ACP transacylases (MCATs) from FAS and PKS are related to the α/β -hydrolase superfamily, containing a large hydrolase core and a small ferredoxin-like subdomain.^{3–5} During fatty acid biosynthesis in *Escherichia coli*, the MCAT FabD loads malonate from malonyl-CoA onto an active site serine, followed by transesterification onto *holo*-AcpP.⁶ In contrast, during polyketide biosynthesis, AT enzymes select, load, and transfer a wider array of substrates.⁷

FAS is a valid therapeutic target,^{8, 9} yet AT domain inhibitors have not been explored as alternatives to combating drug resistance. Engineering PKS AT domains has produced novel candidates for drug screening.^{10, 11} Still, challenges remain, which are attributed to perturbed protein-protein interactions. While docking and structural studies have revealed insights into AT-ACP interactions,^{4, 12, 13} additional tools are necessary to develop general guidelines to engineer these interfaces

To begin investigating AT domains and AT-ACP interfaces, we combined probe design with protein mutational engineering. Crosslinkers have been used to capture and characterize protein-protein interactions.^{14, 15} Moreover, insertion of cysteines proximal to allosteric sites and subsequent thiol trapping spurred the discovery of compounds that bind and modulate protein activity.^{16, 17} Recently, this strategy enabled the structural elucidation of condensation domains from nonribosomal peptide synthetases.¹⁸ Herein, we demonstrate an approach to discover active site probes of FAS and PKS AT domains. We hypothesized that mutating the active site serine to cysteine within AT domains might enhance covalent targeting and report the design, synthesis, and evaluation of new fluorescent probes to interrogate these proteins.

To establish whether AT domain labeling was feasible, we incubated FabD with commercially-available affinity and fluorescent fluorophosphonates (Fig. S1a, ESI), which have previously been used to profile α/β serine hydrolases.¹⁹ These probes weakly labeled FabD, a FabD homolog from flavobacteria isolate (BBFL7), and PKS-derived ZmaA AT, but strongly labeled serine hydrolases (Fig. S1b–c). Concerned with fluorophosphonate stability and lack of FabD selectivity, we sought to develop additional probes.

Previously, our design of probes targeting FAS and PKS domains was inspired by known active site inhibitors.^{20, 21} We reasoned that a similar approach would prove fruitful for AT domains. Based on evidence that sulfonyl fluorides, such as phenylmethane sulfonyl fluoride and *para*-toluene sulfonyl fluoride interact with active site residues of FabD homologs,²² we hypothesized that fluorescent PTSF derivative **1** would irreversibly label AT domains (Fig. 2). Moreover, docking studies of thiolactomycin, a malonyl-CoA mimetic, unveiled critical hydrogen bonds with catalytic residues of FabD,²² inspiring the design of fluorescent β -lactones **2–4** as additional probes (Fig. 2). Notably, sulfonyl fluorides and β -lactones have garnered attention as targeted warheads in chemical biology.^{23, 24}

Following synthesis of **1–4** (Schemes S1–S3), we assessed the influence of pH on FabD labeling. At pH 5–6, FabD labeling with **1** was modest, with intensity stabilizing at pH 7 (Fig. 3a, Fig. S2a). By comparison, labeling with **2–4** was pH-independent (Fig. 3c–d, Fig. S2b–d). To evaluate non-specific labeling, we denatured FabD with 1% SDS at 95 °C prior to probe treatment. Under these conditions, non-specific labeling with all probes increased with pH. Interestingly, **1–2** labeled native FabD more intensely than denatured FabD across the pH spectrum, whereas labeling with **3–4** was specific only at pH 5. Overall, these findings suggests that selective active-site targeting can be achieved at lower pH. The high concentration of probe relative to FabD likely promotes binding at additional sites within the protein. Since the microenvironment within protein binding pockets can alter pK_a ,²⁵ it is possible that these off-target binding sites are more reactive at higher pH.

We then evaluated FabD labeling over 24 h at pH 7. Sulfonyl fluoride **1** was moderately reactive, and labeling plateaued at 12 h (Fig. 3e, Fig. S3a). On average, β -lactone **2** strongly labeled within 1 h, while **3–4** delivered comparable labeling after 2 h (Fig. 3f–h, Fig. S7b–c). All probes demonstrated increased non-specific labeling with time, as determined using denatured protein.

Since high concentrations of electrophilic probes often lead to non-specific, off-target labeling,^{26, 27} we determined whether specific, active site targeting of FabD could be achieved at lower concentrations. FabD labeling increased under both native and denaturing conditions as probe concentration increased; however, when FabD was treated with stoichiometric amounts **1–4**, non-specific labeling was minimal (Fig. 3i, Fig. S4).

To further demonstrate active site targeting, we generated FabD S92C, which altered the nucleophilicity of the key catalytic residue. Labeling of both wild type and mutant FabD with **1–2** did not differ substantially (Fig 3j, Fig. S5). In contrast, we observed a 3-fold enhancement in labeling of FabD S92C with **3**, and a 5-fold increase with **4**. Both FabD and FabD S92C were differentially labeled by lactones (**4**>**3**>**2**), highlighting the influence of stereochemistry and linker length on labeling proficiency. To confirm active site targeting, pretreating FabD S92C with *N*-ethylmaleimide (NEM) to block cysteines decreased labeling of all probes (Fig. S6). These data suggest labeling occurs in the active site, accompanied by some off-target labeling.

Next, using *E. coli* FAS enzymes downstream of FabD, we evaluated the selectivity of **1–4**. These proteins contain nucleophilic residues in their active sites. Probes **1–4** labeled ketosynthases FabF and FabH, ketoreductase FabG, and dehydratase FabA (Fig. S7). We further investigated whether **1** or **3** selectively labeled FabD in competition experiments. FabF and FabG were preferentially labeled over FabD by **1**, while FabF and FabA were preferred over FabD by **3**; however, FabD was preferred over FabG by **3** (Fig. 4a–b, Fig. S8). The selectivity of **3** for FabA was surprising, and we postulate that active site H70 was labeled.

To test if our probes target the active sites of FabF and FabA, we utilized covalent inhibitors and crosslinkers. In particular, cerulenin inhibits FabF,²⁸ and crosslinker sulfonyl-alkyne **5** irreversibly reacts with FabA (Fig. 4c).²⁹ We pretreated FabF with cerulenin, then followed

with FabD and **1–4**. Cerulenin pretreatment did not significantly perturb FabF or FabD labeling with **1** (Fig. 4d, Fig. S9). Interestingly, cerulenin pretreatment potentiates FabF labeling with **2**, **3**, or **4**, which suggests that an allosteric site is likely targeted. While cerulenin binding induces changes in the FabF active site,³⁰ the presence of a second molecule might cause additional unknown conformational perturbations that promote binding. When FabA was pretreated with **5**, followed by FabD and **1–4**, FabA labeling decreased by up to 4.5-fold, confirming active site targeting of FabA (Fig. 4e, Fig. S10). Additionally, **5** also increased FabD labeling up to 3-fold with **2** and **4**, while labeling with **1** and **3** was not substantially impacted by pretreatment with **5**. Overall, these data suggest that probe selectivity for FabD can be tempered by pretreatment with known active site modulators.

To further explore FabD selectivity, we examined the reactivity of **1–4** against other hydrolases. We surveyed cross-reactivity with TesA, a multi-functional SGNH hydrolase that cleaves long chain acyl-CoAs and acyl-ACPs.³¹ Sulfonyl fluoride **1** weakly labeled TesA, with no labeling observed by **2–4** (Fig. S11). In competition experiments between FabD and TesA, **1** preferentially labeled TesA. Intriguingly, when FabD and TesA were treated with **2–4**, neither protein was labeled. We attribute this to TesA-catalyzed probe inactivation, likely *via* nucleophilic attack at the β -lactone carbonyl by the active site serine of TesA, followed by hydrolysis. Next, we examined if **1–4** labeled serine hydrolases. Sulfonyl fluoride **1** was most promiscuous and labeled both thrombin and pig liver esterase (Fig. S12). In contrast, **3–4** did not label additional hydrolases (Fig. S13).

Next, we extended our tandem chemical reporter and protein mutational efforts to PKS AT domains by testing whether CazM (from the chaetidoviridin PKS)³² and ZmaA AT (from the zwittermicin hybrid PKS)³³ could be labeled with TAMRA-fluorophosphonate (Fig. S1) or **1–4** (Fig. 2). TAMRA-fluorophosphonate selectively labeled CazM, which contains a native cysteine as the acyl-group acceptor (Fig. S14). Under denaturing conditions, both CazM and ZmaA were non-specifically labeled. By comparison, **1–3** labeled both CazM and ZmaA AT, but labeled CazM more intensely (Fig. S15).

We generated ZmaA S192C AT to explore the potential of enhancing labeling *via* mutagenesis. All probes labeled ZmaA S192C AT more strongly than wt (Figs. S14–S15), which suggests active site targeting and further validates the rationale of merging site-directed mutagenesis with probe design to improve site selectivity.

To further confirm selective active-site targeting of CazM and ZmaA S192C AT, we conducted time and concentration dependent studies. Sulfonyl fluoride **1** robustly labeled CazM within 4–8 h, and labeling was specific throughout the time course (Fig. 5a, Fig. S16a); whereas, ZmaA S192C never reached comparable labeling levels (Fig. 5b, Fig. S17a). Nonetheless, both PKS domains were stably tagged with lactones **2–4** within 1 h (Fig. 5a–b, Fig. S16–S17). Modest differences in initial CazM labeling intensity were apparent, depending on stereochemistry (**2**>**3**) and linker length (**4**>**3**). Initial labeling of ZmaA S192C with lactones demonstrated similar preferences (**2**~**4**>**3**).

CazM and ZmaA S192C showed distinct susceptibilities to non-specific background labeling. Minimal background labeling of CazM with **2–4** was observed within 4 h and increased with time (Fig. 5a, Fig. S16), while ZmaA S192C labeling was mostly specific up to 24 h (Fig. 5b, Fig. S17). Moreover, non-specific labeling of CazM was evident, even at sub-stoichiometric concentrations of **2–4** (Fig. 5c, Fig. S18). In contrast, non-specific labeling of ZmaA S192C occurred when probes were used in two-fold excess (Fig. 5d, Fig. S19). Overall, non-specific labeling of both PKS AT domains was most pronounced with **4**, suggesting that β -lactone protein binding positively correlates with hydrophobicity.

As a final demonstration of active site targeting, pretreatment of CazM and ZmaA S192C with NEM decreased labeling of the lactones, compared to the non-treated controls (Fig. 5e–f, Fig. S20). The differences in labeling between the NEM-treated and control samples were less apparent with **4** compared to **2–3**. This further indicates that lipophilicity may contribute to binding at locations distinct from the active site.

Conclusions

The promise of combining non-native starter and extender units to access novel and diverse metabolites with untapped biological potential make FAS and PKS AT domains particularly attractive engineering targets. However, the molecular basis of AT-ACP recognition in these pathways remains elusive, and the dearth of well-characterized AT domain inhibitors offers additional challenges to stabilizing these transient complexes. Herein, we merge site-directed mutagenesis with fluorescent probe design to accelerate the discovery of activity-based chemical reporters targeting FAS and PKS-derived AT domains. Of the salient features in this investigation, we generated a S192C mutant of ZmaA AT, unveiling robust active site targeting of this enzyme that would have otherwise remained cryptic. A similar strategy can be exploited to uncover chemical probes targeting the active sites of poorly understood domains in other carrier-protein mediated pathways. Remarkably, while FabD, CazM, and ZmaA are distant relatives of the α/β hydrolase superfamily,^{3, 32, 34} they were selectively labeled over several enzymes within this class. In studies with a competing FAS domain, selective FabD labeling occurred by inhibiting this domain, providing an outlet to circumvent probe promiscuity. Overall, while optimizing reaction conditions (pH, incubation time, and probe concentration) appear critical for specific active site targeting of these proteins, precisely tuning physicochemical parameters (*e.g.*, LogP) of the probes themselves may also further improve specificity. Ultimately, further optimization of the reporters developed herein will enable the discovery of novel AT domain inhibitors as potential therapeutics, inform the design of small molecules to probe the structural basis of AT-ACP recognition, and offer insights that can be leveraged to engineer ACP-containing pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

FAS and PKS AT domains. The AT domains initiates biosynthesis through transient acylation of an active site serine or cysteine by acyl-CoA. The acyl group is then transferred to the 4'-phosphopantetheine arm of holo-ACP. Downstream domains catalyze chain extension, modification, and off-loading of the product from the synthase. Palmitic acid and chaetidoviridin are representative fatty acids and polyketides, respectively.

Fig. 2.

Inhibitors, substrates, and probes for AT domains. Sulfonyl fluoride **1** is a mimetic of phenylmethane sulfonyl fluoride or *p*-toluene sulfonyl fluoride. β-Lactones **2–4** and thiolactomycin are malonyl-CoA mimetics; the carbons that mimic the malonate core are highlighted. The functional units in each probe are depicted by color with reactive warhead (red), linker (black), and fluorescent reporter (blue)

Fig. 3.

In vitro active site labeling of *E. coli* FabD with **1–4**. (a–d) SDS-PAGE analysis depicting the fluorescence (top) and total protein (bottom) from pH-dependent labeling of 10 μM FabD with (a) 50 μM **1**, (b) 50 μM **2**, (c) 50 μM **3**, or (d) 50 μM **4** in PBS for 12 h at 37 °C. (e–h) SDS-PAGE analysis depicting the fluorescence (top) and total protein (bottom) from time-dependent labeling of 10 μM FabD with (e) 50 μM **1**, (f) 50 μM **2**, (g) 50 μM **3**, or (h) 50 μM **4** in PBS pH 7 at 37 °C. (i) SDS-PAGE analysis depicting the fluorescence (top) and total protein (bottom) from concentration-dependent labeling of 5 μM FabD with 5–25 μM **1–4** in PBS pH 7 for 24 h at 37 °C. (j) SDS-PAGE analysis depicting the fluorescence (top) and total protein (bottom) comparing labeling of 5 μM FabD or 5 μM FabD S92C with 25 μM **1** or 5 μM **2–4** in PBS pH 7 for 8 h at 37 °C. The graph (right) depicts semi-quantitation of fluorescent band intensities relative to total protein. Data is reported as the mean ± SEM from 3 experiments. Specific active-site labeling of FabD was assessed in the absence of SDS (–). Non-specific labeling was evaluated by denaturing FabD with 1% SDS for 5 min at 95 °C, followed by probe addition (+). Full-scale gel images are provided in Supporting Information Fig. S2–S5

Fig. 4.

Evaluation and modulation of labeling specificity. (a–b) SDS-PAGE analysis depicting fluorescence (top) and total protein (bottom) of competitive labeling between 5 μ M FabD and 5 μ M FabF, 5 μ M FabG, or 5 μ M FabA with (a) 25 μ M **1** or (b) 25 μ M **3** in PBS pH 7 for 4 h at 37 $^{\circ}$ C. (c) Structures of cerulenin and sulfonylethyne **5**, covalent inhibitors of FabF and FabA, respectively. (d) Modulation of FabD versus FabF labeling specificity by pretreating 5 μ M FabF with 1 mM cerulenin in PBS pH 7 for 1 h at 37 $^{\circ}$ C, followed by the addition of 5 μ M FabD and 10 μ M **1-4** in PBS pH 7 for 8 h at 37 $^{\circ}$ C. (e) Modulation of

FabD versus FabA labeling specificity by pretreating 10 μ M FabA with 1 mM **5** in PBS pH 7 for 18 h at 37 °C, followed by the addition of 10 μ M FabD and 50 μ M **1–4** in PBS pH 7 for 4 h at 37 °C. For d–e, top row depicts fluorescence, middle row depicts total protein, and bottom graphs depicts semi-quantitation of fluorescent bandintensities relative to total protein. Full-scale gel images are provided in Supporting Information Fig. S8–S10

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Fig. 5.

In vitro active site labeling of PKS AT domains. (a–b) SDS-PAGE analysis depicting the fluorescence of time- dependent labeling of (a) 5 μM CazM or (b) 5 μM ZmaA S192C with 5 μM **1–4** in PBS pH 7 at 37 °C. (c, d) SDS-PAGE analyses depicting the fluorescence (left) and total protein (right) of concentration-dependent labeling of (c) 5 μM CazM or (d) 5 μM ZmaA S12C with 2.5–10 μM **1–4** in PBS pH 7 at 37 °C for 24 h. (e,f) SDS-PAGE analysis depicting the fluorescence (top) and total protein (middle) from incubation of (e) 5 μM CazM or (f) 5 μM ZmaA S192C with 1 mM NEM in PBS, pH 7 for 1 h at 37 °C, followed by the addition of 5 μM **2–4** in PBS, pH 7 for 1 h at 37 °C. The graphs (bottom) depict semi-quantitation of fluorescence intensities normalized to total protein, reported as mean ± SEM from 3 experiments. Full images are provided in Supporting Information Figs. S16–S20.