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Resin-based investigation of acyl carrier protein interaction networks in *Escherichia coli*

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ABSTRACT

Protein–protein interactions play an integral role in metabolic regulation. Elucidation of these networks is complicated by the changing identity of the proteins themselves. Here we demonstrate a resin-based technique that leverages the unique tools for acyl carrier protein (ACP) modification with non-hydrolyzable linkages. ACPs from *Escherichia coli* and *Shewanella oneidensis MR-1* are bound to Affigel-15 with varying acyl groups attached and introduced to proteomic samples. Isolation of these binding partners is followed by MudPIT analysis to identify each interactome with the variable of ACP-tethered substrates. These techniques allow for investigation of protein interaction networks with the changing identity of a given protein target.

1. Introduction

In *Escherichia coli*, acyl carrier protein (ACP) plays an essential role of carrying growing biosynthetic substrates for a variety of metabolic processes. In addition to its primary role in fatty acid biosynthesis,[1] ACP has been shown to participate in a diversity of cellular processes including guanosine tetraphosphate (ppGpp) control through an interaction with SpoT,[2] and the recruitment of fatty acid precursors by association with membrane bound protein YchM.[3] The participation of ACP in these pathways adds to its central role in the biosynthesis of lipid A and phospholipids.[4] Given these functions, ACP has become an attractive target both as a cue to further understand cellular phenomena and their associated protein–protein interactions. Understanding these systems not only provides a vital next step in understanding bacterial metabolism, but also unveils new targets for antibacterial discovery.[5]

The mechanism employed to shuttle acyl-ACPs out of primary fatty acid biosynthesis and into auxiliary pathways is not well understood.[6] It has been shown that acyltransferase activity can be highly specific for an acyl-ACP carrying a particular number of carbons.[7] For example, LipB, the octanoyltransferase responsible for scavenging the acyl chain that is incorporated into lipoic acid is specific for octanoyl-ACP.[8] While enzymes such as LipB have been characterized, the mechanisms guiding their scavenging activity has yet to be defined.

Studies with the aim of discovering ACP partner enzymes have been largely conducted using Tandem Affinity Purification (TAP).[2,9] TAP tagging depends on the co-purification of the ACP with an interacting enzyme during expression. As such, there is no control over the form of the bait-ACP used to isolate its binding partners. Moreover, native ACP from *E. coli* lysate is purified predominantly in the active *holo* form, wherein the 4'-phosphopantheine has been post-translationally appended but not yet acylated. Based on the specificity of acyltransferase activity, it is possible that the prior studies missed a number of biologically important protein partners that selectively-bind to acylated ACPs. Using a chemoenzymatic strategy, we gain control over the form of the ACP used in affinity experiments and apply this tool to identify a class of yet undescribed acyl-ACP binding proteins.

2. Results

2.1. Preparation of ACP analogs

Two variable length acyl-ACPs, bearing 8-, and 12-carbon fatty acids, were prepared to probe for ACP binding partners that are dependent on acyl-chain length (Fig. 1). These chain lengths were

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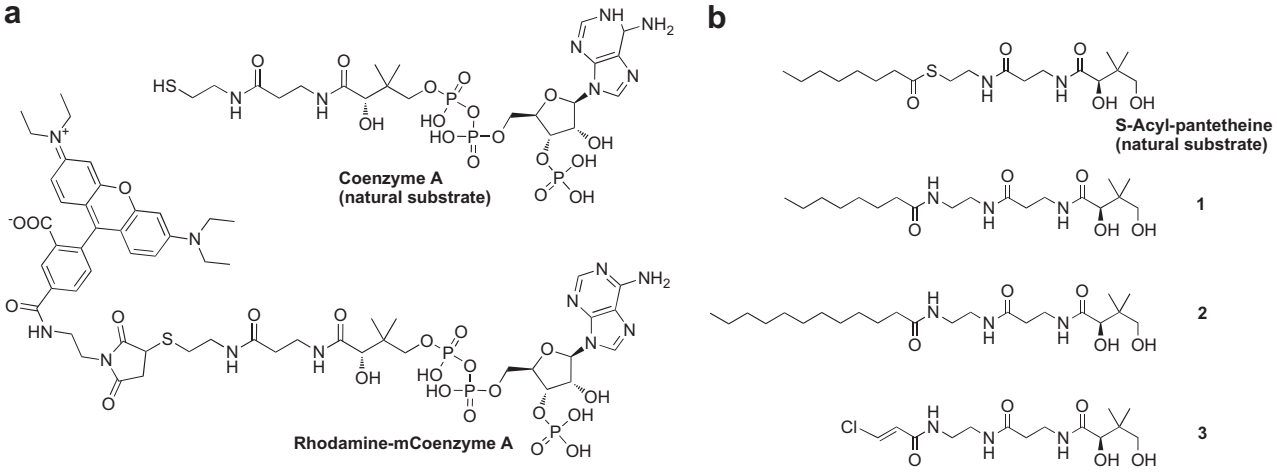

Figure 1. Probes used. (a) Coenzyme A is linked to a rhodamine analog through a maleamide linkage and used as a fluorescent ACP probe. (b) *Crypto*-pantetheine probes including an octanoyl probe **1**, lauroyl probe **2** and *trans*-chloroacryloyl probe **3**. Each probe **1**–**3** is 'non-hydrolyzable' in media due to the presence of a stronger amide linkage, as compared to the thioester bond commonly occurring in natural acyl-CoAs.

selected due to their established roles in lipoic acid (8-carbon) and lipid A biosynthesis (12-carbon).[10] For further stability, non-hydrolyzable pantetheine amide probes **1** and **2** were used as mimics of the natural thioester linkages (Fig. 1). Using established protocols,[11] compounds **1** and **2** were synthesized, chemoenzymatically converted to their corresponding CoA analogs and appended to a His_6-tagged recombinant *E. coli* ACP to form the corresponding *crypto*-ACPs (Fig. 2a). Using an orthogonal purification strategy,[12] we were able to obtain pure His_6-tagged *crypto*-ACP by a single Ni-NTA chromatographic purification (Qiagen). Appendage of the acyl probe to *apo*-ACP was visualized using conformationally sensitive urea–PAGE analysis (Fig. 2b).[13]

Using activated thiol Sepharose 4B (GE Healthcare), we prepared samples of pure *apo*-ACP as a negative control and as a precursor to our ACP analogs. *Holo*-ACP, a positive control, was prepared in vitro. A standard procedure was also developed for preparation of the acylated *crypto*-ACPs. (see Experimental Procedures)

2.2. Demonstration of on-resin sustained ACP activity

A resin-based approach was used that applied purified *crypto*-ACPs (ACPs loaded with one of the pantetheine probes **1**–**3**) tethered to Affi-gel 15 resin (Bio-Rad). We selected Affi-gel 15 resin due to its previous use as ACP based affinity chromatography.[14] Affi-gel 15 is an agarose-based resin containing an activated *N*-hydroxysuccinimide ester that reacts readily with free amines such as those found on lysine residues. Upon incubation with resin, complete uptake of ACP was observed.

Sustained activity of resin bound protein was demonstrated by Rhodamine-mCoA labeling of *apo*-ACP or covalent crosslinking of *apo*-ACP and a GFP fusion with FabF, a β-ketoacyl-ACP synthase II (KASII). (Fig. 3) These labeling studies demonstrate continued interaction with both the phosphopantetheinyl transferase (PPTase) Sfp, which appends the pantetheine side arm to the ACP, as well as with a typical partner enzyme.[11,15]

2.3. Proteomic analysis of ACP affinity isolation

Crosslinking of the *Shewanella oneidensis MR-1* carrier protein with probe **3** was used in conjunction with multidimension protein identification technology (MudPIT). This was conducted as a preliminary study to validate our method by enriching a partner enzyme from soluble cell lysate. *Shewanella oneidensis MR-1* was used for its known production of polyunsaturated fatty acids by acyl carrier protein mediated machinery. Although this cluster was not enriched, *Shewanella* proved a viable organism to investigate carrier protein interaction in parallel to investigation in *E. coli*. The carrier protein is labeled with probe **3** while tethered to the resin, briefly washed with potassium phosphate buffer and

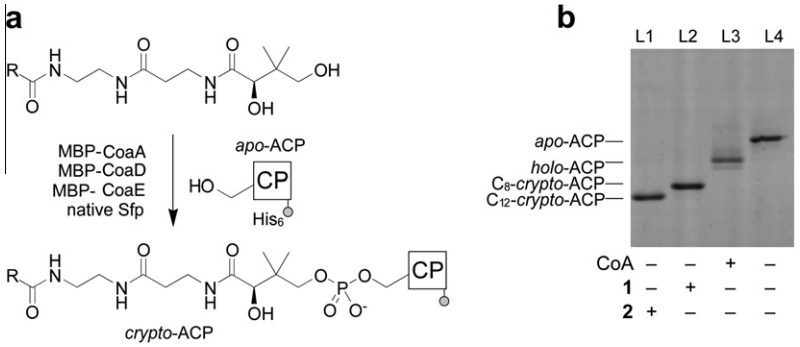

Figure 2. Preparation of non-hydrolyzable acyl-ACP analogs. (a) Probes **1** and **2** were appended enzymatically to *apo*-ACP and purified using an orthogonal purification strategy. After derivitization, the mixture was batch-bound to Ni-NTA resin. Low concentration imidazole washes removed CoA biosynthetic enzymes with maltose binding protein fusion tags and untagged Sfp. Pure, His_6 tagged ACP was then eluted by application of 300 mM imidazole. Side chain R is as in Figure 1. (b) Attachment by addition (+) of **1**, **2** or Coenzyme A to ACP was visualized by Urea–PAGE analysis. The (−) sign denotes that the compound was left out of the reaction mixture.

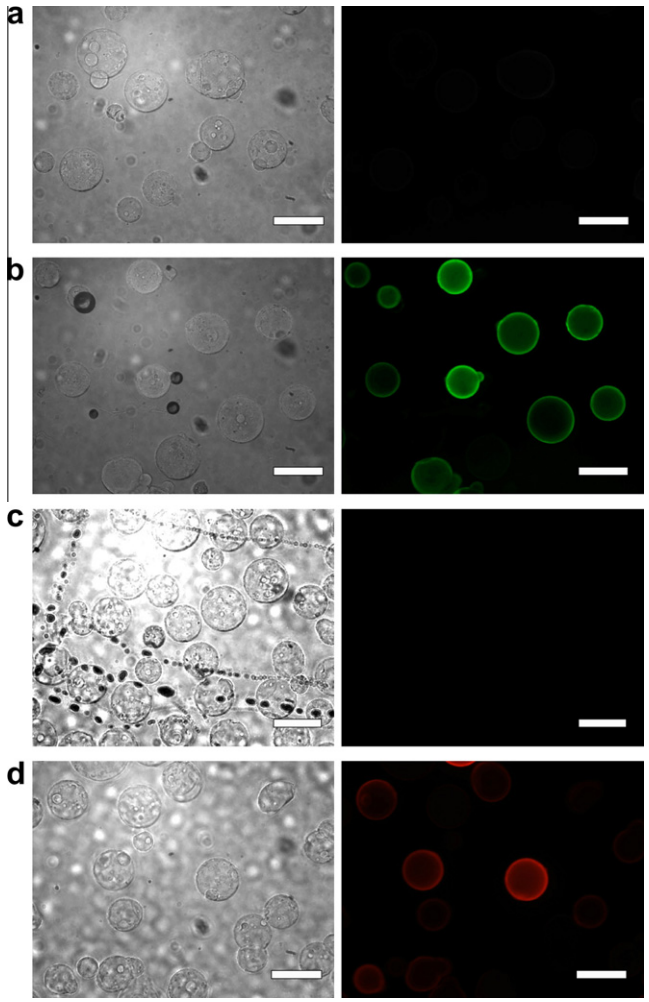

Figure 3. Visualization of resin-bound ACP using fluorescence microscopy. Images denote white light (left) and fluorescence (right) from the same sample. Images (a) and (b) show *crypto*-ACP beads incubated with MBP-CoaA, MBP-CoaD, MBP-CoaE, native Sfp and KASII-GFP. Crosslinking probe chloroacrylate pantetheine **3** is also introduced in (b) resulting in a covalent adduct between ACP and KASII-GFP and a green fluorescence observed by excitation at 475 ± 30 nm. Compound **3** is omitted in negative control (a). Images (c) and (d) show *crypto*-ACP beads incubated with Rhodamine-mCoA. Sfp is also introduced in (d) resulting in fluorescent labeling and a red fluorescence observed by excitation at 570 ± 35 nm. Sfp is omitted in negative control (c). The white bar denotes 300 μm.

incubated with soluble cell lysate from the native organism with inversion at 37 °C for 12 h. The samples were stringently washed, reduced, alkylated and finally tryptically digested for LC–MS/MS analysis. LC–MS/MS analysis showed specific enrichment of the KASII enzyme, the primary target of this probe, which is a result of a covalent crosslinking. (Table 1) This experiment also shows moderate enrichment of the dehydratase domain that, as evidenced of the high level of counts in the negative control, is primarily due to protein–protein interaction with ACP. Prior to this experiment, *apo*- and *holo*-ACP forms were not separated.

Moderate enrichment of partner enzymes in the negative control, in which resin bound *apo*- and *holo*-ACP is not incubated with the pantetheine analog but also incubated with cell lysate overnight, prompted our study based on protein–protein and substrate–protein interactions alone.

MudPIT analysis of acyl-ACP partners in *E. coli*, was performed by incubation of the soluble cell lysate from *E. coli* with purified *apo*-, *holo*- and acyl *crypto*-ACP bound to resin. (Table 2) The resin is allowed to incubate overnight at 4 °C with inversion and samples

Table 1
Spectral counts of ACP-resin after incubation with *Shewanella oneidensis MR-1* cell lysate

Proteins[a]	Negative control[b]	Positive control[c]	FC
3-oxoacyl-[acyl-carrier-protein] synthase II (NP_718355.1)	82	801	9.8
30S ribosomal protein S12 (NP_715866.1)	7	37	5.3
30S ribosomal protein S6 (NP_719461.1)	9	47	5.2
Ribosomal subunit interface protein (NP_718956.1)	5	16	3.2
Holo-ACP synthase (NP_716972.1)	5	15	3.0
(3*R*)-hydroxymyristoyl-ACP dehydratase (NP_717251.1)	213	568	2.7
Acyl carrier protein (NP_718356.1)	1431	1721	1.2

[a] Included proteins show >2 fold change enrichment and ⩾15 spectral counts in the positive control. ACP is included to show presence in both samples.
[b] Negative control is included in which the resin is incubated with phosphate buffer only.
[c] Positive control sample is ACP preloaded with **3** which has been omitted in the negative control.

are prepared as described above for MS analysis with the exception that washes are performed in 50 mM Tris buffer pH 8.0 only.

3. Discussion

Results are analyzed by spectral counting; a semi-quantitative measure of relative protein abundance.[16] Our technique is validated by the enrichment of previously annotated *holo*-ACP partner enzymes (Table 2). Of the five proteins we identified as hits in our *holo*-ACP set, three have been previously described as partner enzymes by the aforementioned TAP-tagging experiments or by other means.[17] Dihydrodipicolinate synthase (DHPS), one of the enzymes we identified has not been previously shown to interact with *holo*-ACP. DHPS has been shown to be a key step in the synthesis of *s*-lysine and *meso* diaminopimelate, key components of bacterial cell walls.[18] Therefore, this finding suggests that ACP may control levels of lysine formation and, indirectly, cell wall formation by acting as a regulator of DHPS.

The two identified protein partners enriched by C8-ACP (Table 2), have not been previously annotated experimentally as specific for octanoyl-ACP. However, our top hit, LpxD, is a well-characterized acyltransferase from the lipid A biosynthetic pathway. In previous studies, LpxD has shown relaxed substrate specificity.[19] From our result of preferential binding to an acyl-ACP with only eight carbons, we propose even greater substrate promiscuity than reported.

The dataset enriched by C12-ACP also contained proteins that have not been annotated as ACP partners. However, previous research suggests possible interaction networks. For example, hypoxanthine phosphoribosyltransferase (Hpt), an enzyme involved in purine salvage, has been shown to interact with ppGpp, the signaling molecule shown to be under ACP-dependent control.[20] In parallel to the previously elucidated relationship between ACP and ppGpp[2], we propose that acyl-ACPs may play a allosteric regulatory role in purine uptake. Control over purine levels, precursors to both ppGpp and coenzyme A, suggest a number of possible regulatory roles that ACP plays by interaction with Hpt.

In summary, we have designed a resin-based approach to aid in the investigation of ACP–protein interactions. This method has important applications for studying the interactome of a specific protein within the variable of changing protein identity. We have used this tool to demonstrate probe specificity as well as to enrich for post-translationally modified carrier protein partners. Our initial crosslinking studies and fluorescent labeling experiments have demonstrated on-resin activity of ACP, as well as a novel technique

Table 2
Enzymes enriched from soluble *E. coli* lysate using selected ACP resins

	Average				FC	
Proteins found after enrichment with holo-ACP resin[a]	*Apo-*	*Holo-*	—	—	*Holo-/Apo-*	—
3-oxoacyl-[acyl-carrier-protein] synthase 1	6.0	136.7	—	—	22.8	—
3-oxoacyl-[acyl-carrier-protein] synthase 2	16.3	109.3	—	—	7.1	—
Dihydrodipicolinate synthase	9.0	30.3	—	—	3.4	—
Bifunctional protein glmU	7.3	16.7	—	—	2.1	—
Ribonucleoside-diphosphate reductase 1 subunit alpha	48.7	101.0	—	—	2.1	—
Proteins found after enrichment with C8-crypto-ACP resin	*Apo-*	*Holo-*	*C8-*	—	*C8-/Apo-*	*C8-/Holo-*
UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase LpxD*	6.3	7.7	72.3	—	11.4	9.4
Ribosomal RNA large subunit methyltransferase I	8.7	2.3	19.7	—	2.3	8.4
Proteins found after enrichment with C12-crypto-ACP resin	*Apo-*	*Holo-*	*C12-*	—	*C12-/Apo-*	*C12-/Holo-*
Glutamyl-tRNA synthetase	8.0	8.7	22.0	—	2.8	2.6
Hypoxanthine phosphoribosyltransferase	20.3	24.0	68.0	—	2.6	2.2
Protein	*Apo-*	*Holo-*	*C8-*	*C12-*	—	—
Acyl carrier protein	80.0	79.3	21.3	84.7	—	—

[a] Proteins denoted as enriched have ⩾15 average spectral counts in *holo-* or acyl crypto-ACP of interest. ACP is included to show presence in all samples. *Holo* ACP hits have >2 fold change enrichment over *apo-* control. Acyl crypto ACP hits have >2 fold change enrichment over *apo-* and *holo-* controls. All hits have a *T* test value of <0.05. All experiments were performed in triplicate.
* *T* test against *Holo*-ACP = 0.05.

to covalently enrich fatty acid biosynthetic enzymes using resin bound ACP. We have also demonstrated the ability to load carrier proteins with a variety of lipid chain lengths to enrich substrate specific partner enzymes. Spectral counts are semi-quantitative and show a substantial amount of variability in this experiment so confirmation of all proposed protein partners is a necessary validation step to be performed in the future. To further these studies, acyl-ACP partner enzyme enrichments will be validated by reverse pull down experiments and the role that ACP plays in these pathways with be more closely analyzed.

4. Experimental

4.1. Preparation of ACP affinity resin

Coupling of ACP to Affi-gel 15 resin was conducted in 0.1 M MOPS pH 7.5 and the resin capped with 0.1 M glycine ethyl ester according to manufacturer's instructions.

4.2. Synthesis of lauroyl pantetheine probe (2)

A two-step procedure was used to prepare probes **1** and **2**. (Fig. 4.) The following section provides experimental conditions for the preparation of probe **2**. Synthesis of probe **1** was previously described and comparable methods were used to prepare probe **2**.[12]

Lauroyl chloride (107 mg, 0.492 mmol) was added drop wise to a solution of PMP-pantetheinamine[21] (93 mg, 0.246 mmol) in dry pyridine (97 mg, 1.23 mmol) and CH_2Cl_2 (50 mL). The reaction was allowed to stir for 2 h. The contents were removed by rotary evaporation. Flash chromatography with a gradient of hexanes to 15% MeOH in EtOAc to yield **4** as a white solid (69 mg, 43%).

An aliquot of 1 M HCl (1 mL) was added drop wise to PMP-protected lauroyl pantetheine **4** (65 mg, 0.116 mmol) in THF at rt. After 1 h at rt, the reaction was quenched by the addition of a satd. NaH_2CO_3 and extracted with EtOAc (3 × 40 mL), washed with brine, and dried with Na_2SO_4. Flash chromatography (EtOAc to 15% MeOH in EtOAc) afforded product **2** as a white solid (40 mg, 0.090 mmol, 78%) ^{1}H-NMR (400 MHz, CD_3OD) ∂ ppm 3.88 (1H, s), 3.86 (1H, m), 3.42 (dd, *J* = 20.0. 12.0 Hz, 2H), 3.26 (br s, 3H), 2.40 (t, *J* = 8.0 Hz, 2H), 2.18 (t, *J* = 8.0 Hz, 2H), 1.59 (2H, m), 1.37 (1H, s), 1.29 (br s,18H), 0.92 (s, 3H), 0.89 (s, 3H), 0.88 (s, 3H);^{13}C NMR (120 Mhz, CD_3OD) ∂ ppm 175.48, 174.87, 172.94, 76.17, 69.17, 39.18, 38.94, 38.74, 36.00, 35.43, 35.22, 31.89, 29.55, 29.45, 29.28, 29.17, 25.76, 22.55, 20.17, 19.74, 13.26 MS (ESI) 444.00 [M+H], 466.27 [M+Na].

4.3. Preparation of ACP analogs

To prepare *holo-ACP*, apo-ACP (3 mg) was added Sfp (45 μg) to 20 μM CoA in potassium phosphate buffer (PPB) pH 7.4 containing 12.5 mM $MgCl_2$ and 1 mM DTT at 37 °C for 12 h. To prepare acyl-ACPs, apo-ACP (3 mg) was added with native Sfp (45 μg), MBP-CoaA (100 μg), MBP-CoaD (100 μg), MBP-CoaE (100 μg), and 20 μM of **1**, or **2** in PBB pH 7.4 containing 12.5 μM $MgCl_2$ and 8 mM ATP (10 μL of a 200 mM stock) at 37 °C for 12 h.

4.4. Rhodamine mCoA labeling

To 100 μL of 50:50 *apo*/*holo*-ACP resin was added Sfp (4.5 μg) and 20 μM Rhodamine-mCoA in 25 mM PPB pH 7.4 containing 12.5 mM $MgCl_2$. Sfp was omitted in the negative control.

4.5. Crosslinking of KASII-GFP

To 50 μL of 50:50 *apo*/*holo*-ACP resin was added native Sfp (4.5 μg), MBP-CoaA (10 μg), MBP-CoaD (10 μg), MBP-CoaE (10 μg), 20 μM trans-chloroacryloyl pantetheine **3** and KASII-GFP (25 μg) in PBB pH 7.4, 12.5 mM $MgCl_2$, and 8 mM ATP. Compound **3** was omitted in the negative control. All reactions were incubated

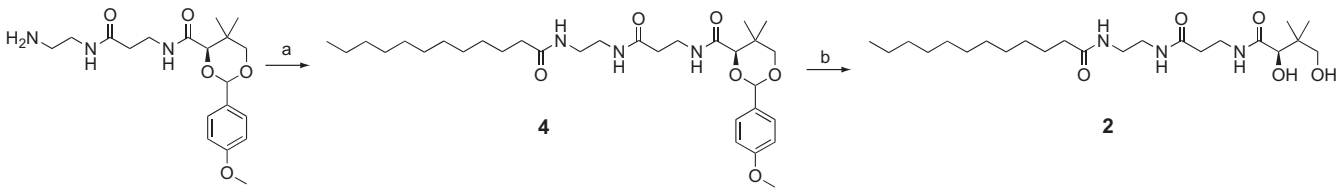

Figure 4. Synthetic route to lauroyl pantetheine (**2**) (a) Lauroyl chloride (1.1 equiv), pyridine (1 equiv), DCM, 2 h. (b) 1 M HCl/THF, 1 h.

at 37 °C for 1 h. Background fluorescence was removed by washing with 1 M NaCl (3 × 200 μL).

4.6. Culturing conditions

E. coli K12 or *Shewanella oneidensis MR-1* was streaked on LB-agar and incubated overnight at 37 °C. A single colony was picked and used to inoculate a 5 mL liquid LB starter culture and rotated overnight at 37 °C. 2 mL of this starter culture was then used to inoculate 1 L of autoclaved LB media. This culture was allowed to grow until stationary phase (OD_{600} ~1.0). At this point the cells were harvested and centrifuged (8000*g*, 20 min, 4 °C) and cell pellets were frozen overnight.

4.7. Lysate preparation

Cells were resuspended in lysis buffer (25 mM potassium phosphate, pH 7.4, 100 mM NaCl) and cell lysis performed by two passes through a French pressure cell. Soluble cell extract were cleared by centrifugation (20,000*g*, 30 min, 4 °C) and quantified to 1 mg/mL.

4.8. MS sample preparation and analysis

Affi-gel resin (50 μL) was used per sample (100 μL of 1:1 suspension in 0.1 M MOPS buffer pH 7.5) Protein pull-down experiments were conducted by incubating beads with 5 mL lysate (with a total protein concentration of 1 mg/mL overnight). After constant inversion 12 h at 4 °C, the lysate was removed and the resin was washed with a solution of 6 M urea, 0.1% SDS and 50 mM Tris pH 8.0 (3 × 10 mL). Lysate used in non-covalent enrichments were washed with 50 mM Tris pH 8.0 (3 × 10 mL) only. The supernatant was removed and resin was resuspended in 8 M urea in 50 mM Tris pH 8.0 (200 μL) All samples were reduced by treatment with 10 mM TCEP, alkylated by treatment with 12 mM iodoacetamide. At this point, the solution was diluted with 50 mM Tris pH 8.0 to a final concentration of 2 M Urea (400 μL). This was followed by the addition of trypsin (1 μg) and 2 mM $CaCl_2$. Samples were allowed to digest 12 h at 37 °C. At this point, digestion of samples was halted by the addition of formic acid to a final concentration of 5% and frozen at −80 °C until analysis. Tryptic peptides enriched by ACP loaded with probes as well as *apo* and *holo* ACPs, were loaded onto a biphasic strong cation exchange/reverse phase capillary column (Agilent) and analyzed by 2D-LC separation in combination with tandem MS. Peptides were eluted in a five-step MudPIT experiment and data were collected in an ion trap mass spectrometer (ThermoFisher LTQ) set in a data-dependent acquisition mode with dynamic exclusion turned on (60 s). Spray voltage was set to 2.75 kV and the flow rate through the column was 0.25 L/min.

4.9. Filtering of MudPIT results

MudPIT filtering conditions were set to annotate proteins as enriched if they have >15 spectral counts when averaged over three samples, are present in all 3 samples, have >3 fold change over the average counts in the apo-control, and <5 counts in the negative control. The identification of partner enzymes specific for C8 or C12 ACP were defined as having >15 spectral counts and >2 fold enrichment over both apo and holo ACP samples and a *T* test value $\leqslant 0.05$.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.10.053.

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