Preparation of holo- and malonyl-[acyl-carrier-protein] in a manner suitable for analog development

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Abstract

The fatty acid biosynthetic pathway generates highly reduced carbon based molecules. For this reason fatty acid synthesis is a target of pathway engineering to produce novel specialty or commodity chemicals using renewable techniques to supplant molecules currently derived from petroleum. Malonyl-[acyl carrier protein] (malonyl-ACP) is a key metabolite in the fatty acid pathway and donates two carbon units to the growing fatty acid chain during each step of biosynthesis. Attempts to test engineered fatty acid biosynthesis enzymes in vitro will require malonyl-ACP or malonyl-ACP analogs. Malonyl-ACP is challenging to prepare due to the instability of the carboxylate leaving group and the multiple steps of post-translational modification required to activate ACP. Here we report the expression and purification of holo- and malonyl-ACP from *Escherichia coli* with high yields (>15 mg per L of expression). The malonyl-ACP is efficiently recognized by the *E. coli* keto-acylsynthase enzyme, FabH. A FabH assay using malonyl-ACP and a coumarin-based fluorescent reagent is described that provides a high throughput alternative to reported radioactive assays.

Introduction

The promise of biorenewable chemicals for the chemical industry extends beyond replacing the petroleum source of contemporary chemical precursors and reaches towards developing new molecules with alternative functionalization and structure [1]. To achieve this goal, enzymes, biosynthetic pathways, and microorganisms must be engineered to synthesize alternative substrates. One fundamental barrier to progress is preparing appropriate substrates, many of which are protein-bound intermediates and thus challenging synthetic targets.

Fatty acid biosynthesis (FAS) is one pathway targeted for engineering [2]. Prokaryotic fatty acid biosynthesis employs a dissociated multi-polypeptide system that allows for the targeting of individual enzymes, unlike eukaryotic FAS which uses a fused system [3]. The

products of the pathway are long chain hydrocarbons that have significant commercial value. The *Escherichia coli* FAS machinery can be manipulated into producing free fatty acids [4]. It has also been demonstrated that varying fatty acid chain length can be selected for in *E. coli* variants [5]. Alternatively, shorter compounds can be produced by truncating the pathway and will serve as novel starting points to prepare a wide range of compounds. It is widely believed that the pathways may be engineered for the production of custom molecules [6-8]

E. coli β -keto-acylsynthase III (FabH) catalyzes the Claisen-condensation of malonyl-ACP and acetyl-coenzyme A (acetyl-CoA) as the priming step of fatty acid biosynthesis (Figure 1) [9]. This reaction yields the four carbon acetoacetyl-ACP product which serves as the substrate for two NADH/NADPH-dependent reduction steps, generating butyryl-ACP that can be extended in a malonyl-ACP dependent reaction by the keto-acylsynthase I or II enzymes [10]. In vitro examination of this system requires high titers of malonyl-ACP.

Malonyl-ACP is challenging to prepare for multiple reasons, the first of which is that holo-ACP is required as a substrate (Fig 1) [11]. Holo-ACP is formed by the transfer of phosphopantetheine from CoA to serine 36 of apo-ACP, the unmodified polypeptide [12, 13]. This reaction can be performed on the benchtop or *in vivo* with overexpression of the holo-ACP synthase gene [11]. Malonyl-ACP is then formed by the transfer of the malonyl moiety from malonyl-CoA to holo-ACP and is catalyzed by the malonyl-CoA:ACP transacylase, FabD [14, 15]. Once prepared, malonyl-ACP spontaneously decarboxylates to form acetyl-ACP.

We developed a strategy to prepare holo-ACP by coexpressing apo-ACP and holo-ACP synthase in *E. coli* with a pETDuetTM (EMD Millipore) vector. This system allows for a simplified expression system that provides post-translational modification *in situ*. Because malonyl-ACP is labile, we developed a strategy to modify holo-ACP *in vitro*. The combination of improved expression techniques and a high-throughput fluorescence based assay will allow for facile modification of the ACP or malonyl-CoA donor to probe engineered enzymes *in vitro*.

Materials and Methods

Materials- All materials were purchased from Sigma-Aldrich unless noted otherwise. All buffers were made with double distilled water.

Expression and purification of holo-ACP- Holo-ACP was expressed in *E. coli* BL21* DE3 cells harboring a pETDuetTM-vector (EMD-Millipore) containing the apo-ACP and holo-ACP synthase genes under control of individual T7 promoters. Apo-ACP was cloned into *Bam*HI and *Eco*RI of the first multiple cloning site (MCS) while holo-ACP synthase was inserted into the *Nde*I and *Xho*I restriction sites in the second MCS of the pETDuetTM vector. The holo-ACP expression cassette is designed to include a 6xHis tag at the N-terminus and the holo-ACP synthase cassette includes no modifications. *E. coli* cells were transformed with the protein expression vector and grown to mid log phase (OD₆₀₀=0.5) at 37 °C in a shaking incubator (Thermo Scientific MaxQ 4000) and induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). At the moment of induction, temperature was reduced to 18 °C. After 16-18 hours cells were pelleted and resuspended in 25 mL lysis buffer (20 mM Tris(hydroxymethyl)aminomethane (TRIS), 500 mM sodium chloride, 1 mM 2-mercaptoethanol pH=8.1). The cell suspension was then passed through an Emulsiflex C5 homogenizer (Avestin) five times at 15000 psi. Cell debris was removed from the lysate by centrifugation at 14000 rpm for 1 h in a Fisher Scientific Sorvall Legend XTR centrifuge operating at 4 °C. The clarified lysate (CL) was applied to a 5 mL nickel-NTA column (Qiagen) pre-equilibrated with lysis buffer. The flow through (FT) was collected and set aside on ice. The column was washed with 25 mL of the base buffer 20 mM TRIS, 500 mM sodium chloride, 1 mM 2-mercaptoethanol, pH 8.1 (W1), then washed again with 25 mL of the base buffer plus 30 mM imidazole (W2), followed by 25 mL of the base buffer plus 50 mM imidazole (W3). Protein was eluted in three 5 mL fractions with a buffer consisting of the base buffer plus 100 mM imidazole, followed by three 5 mL fractions with the base buffer plus

250 mM imidazole. Fractions were analyzed by SDS-PAGE by loading 2.5 μ L of each elution and wash fraction onto a 20% SDS PAGE gel.

Fractions containing holo-ACP were pooled and diluted to a final salt concentration of 50 mM and loaded onto a 2.6 cm diameter column containing 150 mL Q-Sepharose Fast Flow resin (GE healthcare). A two buffer system was employed to wash and run the column. Buffer A consisted of 25 mM 3-morpholinopropane-1-sulfonic acid (MOPS), 1 mM 2-mercaptoethanol, pH 7.1. Buffer B was buffer A plus 1M potassium chloride, pH 7.1. All buffers were made with a filter sterilized stock of MOPS buffer. The Q-column was loaded at a flow rate of 2 mL/min and then washed at the same flow rate with 50 mL of Buffer A, followed by elution with a 200 mL linear gradient from 200-600 mM potassium chloride by adjusting the ratio of A/B. The column was then washed with 25 mL Buffer B, then 200 mL Buffer A. Fractions (8 mL) were collected and analyzed by SDS-PAGE.

Determining the amount of free thiol on holo-ACP- Purified holo-ACP (~10 mg) was concentrated to 500 μ L using a 3 kDa MWCO centrifugal filter unit (EMD Millipore) and then passed over a 5 mL G25 column equilibrated with 20 mM TRIS, 100 mM sodium chloride, pH 7.5 to remove reductant. Fractions containing ACP were pooled and the volume reduced to 1 mL.

An aliquot of the ACP sample (50 μ L) was added to a 950 μ L solution containing 100 mM TRIS and 200 μ M 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), pH 7.5. After a 30 min incubation at RT, absorbency at 412 nm was measured using a quartz cuvette and a Cary® 50 UV-Vis spectrophotometer (Varian). A similar reaction with 50 μ L of the G25 equilibration buffer added to the 950 μ L reaction mix listed above served as a blank for the A_{412} measurements. Each reaction was performed in triplicate. Similar assays were performed on control reactions that included the loading of only buffer containing reductant on the G25 column. These results

indicated that reductant and ACP eluted in resolved, non-overlapping peaks and DTT did not contaminate the ACP fractions.

Another aliquot was analyzed by ^1H NMR. The NMR sample (600 μL) consisted of 229.87 μL ACP, 5% D_2O , 200 μM DSS, 25 mM MOPS, 100 mM sodium chloride, 1 mM dithiothreitol, pH 7.0. Signals from five resolved methyl peaks upfield of the bulk protein signal were observed using an 18.8T Bruker instrument equipped with an AvanceIII console and a 5 mm cryoprobe. The area under each peak was integrated and compared to the internal DSS standard to determine protein concentration.

Expression and Purification of FabD and FabH: The genes encoding FabH and FabD were inserted into the pET29a and pET28b expression vectors (EMD Millipore) respectively. The constructs were unmodified and contained no tags. The expression was conducted as described above for holo-ACP. The FabH clarified lysate was applied to the 150 mL Q-Sepharose column and washed with 50 mL of a 100 mM sodium phosphate, 1 mM 2-mercaptoethanol, pH 7.0 buffer, followed by a linear gradient from 0-500 mM potassium chloride using same buffer, supplemented with 1 M potassium chloride. The column was then washed with 25 mL of the 1M potassium chloride buffer followed by 200 mL of the phosphate buffer with no potassium chloride. Fractions containing FabH as determined by SDS-PAGE were concentrated to ~5 mL using a 10kDa-cutoff Amicon filter (EMD) and applied to a 600 mm Superdex 200 column with a diameter of 26 mm (GE Healthcare). The S200 Column was run at a flow rate of 0.5 mL/min. Buffer used was 100 mM sodium phosphate and 1 mM dithiothreitol. The fractions containing FabH were concentrated and stored in 50% glycerol. The procedure for purifying FabD was the same as FabH, but the Q-column buffers consisted of 25 mM MOPS in place of sodium phosphate. The S200 buffer for FabD contained 25 mM MOPS, 100 mM sodium chloride, and 1 mM dithiothreitol.

Malonyl-ACP Preparation: Malonyl-ACP was prepared in a reaction containing 90 μ M holo-ACP, 2.25 mM malonyl-CoA, 6 μ M FabD, 25 mM MOPS, 100 mM sodium chloride, and 1 mM dithiothreitol, pH 7.1 in a final volume of 4 ml and incubated for 16-20 hours at 0 °C. Complete conversion to malonyl-ACP was verified by MALDI-TOF MS using a Voyager-DE Pro MALDI-TOF instrument (Applied Biosystems). The spotting conditions were as follows: protein was diluted to ~10 μ g/ μ L in DI water, this was then mixed 1 μ L:1 μ L in alpha-Cyano-4-hydroxycinnamic acid (ACH), and one μ L was spotted on a JBI 100 well MALDI plate. Following enzymatic conversion, malonyl-ACP was adsorbed onto a 1 ml nickel-NTA column (Qiagen). Reaction components were removed by extensively washing the column with a buffer containing 25 mM MOPS, 500 mM sodium chloride, 1 mM dithiothreitol, pH 7.1. Malonyl-ACP was eluted with the same buffer containing 25 mM MOPS, 500 mM sodium chloride, 1 mM dithiothreitol, and 30, 50 or 250 mM imidazole. Fractions containing malonyl-ACP were concentrated and exchanged into a buffer containing 25 mM MOPS, 100 mM sodium chloride, pH 7.1 using a 3 kDa-cutoff centrifugal filter device as described above. Protein was then diluted with glycerol to a final concentration of 10-25% (v/v) and 500 μ M malonyl-ACP and stored at -80 °C.

FabH Activity Assays: A fluorometric assay was adapted [16] to monitor the appearance of CoA via a reaction of a fluorescent reagent 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM) with the newly formed CoA thiol. The FabH assay was conducted with 50 μ M acetyl-CoA, 50 μ M malonyl-ACP (or malonyl-CoA), 100 mM phosphate buffer, pH 7.0, at 30 °C in a volume of 100 μ L. FabH concentrations ranged from 0.5-5 μ M for the reactions with malonyl-CoA and 1-25 nM for the reactions with malonyl-ACP. Six 10 μ L aliquots were removed from the reaction at various times and quenched with 190 μ L of 10 μ M CPM in 100% DMSO solution. Fluorescence was measured with excitation at 384 nm and emission at 470 nm using a Cary Eclipse fluorimeter. The fluorimeter was calibrated by measuring CoA at concentrations ranging from 0-5 μ M in the DMSO-based quench solution.

Size exclusion chromatography of holo-ACP- Fractions containing holo-ACP from the Q-column were concentrated to 2-5 mL using a 3 kDa molecular weight cutoff centrifugal filter device (EMD Millipore) and loaded onto a 475 mm Superdex 75 (S75; GE Healthcare) column with a diameter of 26 mm. The column was washed with 25 mM MOPS, 100 mM sodium chloride, and 1 mM dithiothreitol. The column was run with a flow rate of 0.5 mL / min using the same buffer and 3.5 ml fractions were collected. SDS PAGE was used to analyze the fractions. Fractions containing holo-ACP were concentrated to <1 mL and diluted with glycerol to a final concentration of 25% (v/v) and stored at -80 °C.

Results and Discussion

Expression and identification of a stable complex

We developed a method that permits simultaneous expression of the ACP polypeptide and holo-ACP synthase enzyme in *E. coli* to facilitate recovery of holo-ACP. The ability to prepare ACP with a high percentage in the holo form eliminates the need to separate holo-ACP and apo-ACP for downstream analysis. Furthermore, this coexpression method saves an additional step of CoA-dependent apo-ACP conversion in vitro.

ACP from the *E. coli* cell lysate was purified using an immobilized metal ion affinity column as shown in Figure 2. ACP eluted in the same fractions as the coexpressed holo-ACP-synthase. MALDI-TOF MS analysis revealed that a high percentage of the ACP pool was modified with a phosphopantetheine moiety.

The ACP and holo-ACP synthase proteins were not separated by a S200 size exclusion column. Although a very small amount of ACP eluted as a monomer from a gel-filtration column, the predominate pool of ACP eluted much earlier and at the same time as the holo-ACP synthase as shown in Figure 3. ACP and holo-ACP synthase appeared to form a multimer that was stable and eluted in a single, sharp peak at a volume of 200 mL that corresponds to a molecular weight of 95.1 kDa (Figure 3) [17, 18]. This is a surprise because the masses of holo-

ACP and holo-ACP synthase are 10.6 kDa and 13.9 kDa, respectively, though the *E. coli* holo-ACP synthase purifies as a homodimer [19]. It appears, based on the SDS-PAGE analysis that ACP and holo-ACP synthase elute with 1:1 stoichiometry. The molecular weights, stoichiometry, and dimer model for one partner suggest formation of a 4:4 heterooctamer with a theoretical molecular mass of 97.4 kDa. This predicted mass is only 2.5% greater than the observed mass of the ACP:holo-ACP synthase complex. Because estimates of molecular weight using gel filtration chromatography are sensitive to the shape of the analyte, it is additionally possible that the complex may be either a 1:1 heterodimer, 2:2 heterotetramer or 3:3 heterohexamer.

The formation of a discrete multimeric complex is likely important for holo-ACP synthase function. It is important to note that the abundance of apo, holo, and acetyl-ACP in the complex is similar to the abundance of these forms in the free ACP that eluted much later from the column (data not shown). This is not surprising as *E. coli* holo-ACP synthase can utilize both CoA and acetyl-CoA with comparable efficiencies [19].

Separation of ACP and holo-ACP synthase

Initial attempts to disrupt the ACP-holo-ACP synthase complex with a size exclusion chromatography step at neutral, high or low pH, increased salt concentration, or metal ion affinity chromatography following incubation with 6M urea were largely unsuccessful.

Anion exchange chromatography was utilized, in lieu of the size exclusion chromatography step, to achieve the separation following a linear salt gradient as shown in Figure 4. The fractions containing holo-ACP synthase (8-18) contained only holo-ACP synthase and no ACP as shown in Figure 5. Fractions 27-31 contained 15 mg of holo and acetyl-ACP (Table 1), but not holo-ACP synthase and are analyzed in Figure 6A. A portion of the holo-ACP pool shows the characteristic shift of 177 mass units, indicating the presence of His6-tag gluconoylation [20, 21].

We pursued a chemical method to measure the degree of phosphopantetheine modification in the holo-ACP preparation using natural features of apo and holo-ACP because

the MALDI-MS based method does not provide an accurate quantification.

Phosphopantetheinylation catalyzed by the holo-ACP synthase introduces a free thiol to holo-ACP that is not present in apo-ACP which also contains no cysteine residues. Thus, a measurement of the free thiol content in a holo-ACP prep using the thiol-reactive reagent DTNB divided by the protein concentration as measured with NMR is expected to provide the percentage of phosphopantetheinylation that is accessible to further modification by FabD, the malonyl-CoA:ACP transferase. We determined that a sample containing 1.000 ± 0.083 molar equivalents of ACP polypeptide reacted with 0.800 ± 0.029 molar equivalents of DTNB. This result indicates that $80.0 \pm 5.8\%$ of the ACP pool contains a free thiol.

Conversion to malonyl-ACP

Malonyl-ACP is formed in a malonyl-CoA dependent reaction catalyzed by the malonyl-CoA:ACP transacylase FabD. Purified *E. coli* FabD efficiently converted holo-ACP to malonyl-ACP as shown in Figure 6B. This reaction was performed at 0°C to prevent spontaneous decarboxylation, which occurs rapidly at higher temperatures. Previous techniques to monitor malonyl-ACP involve conformational sensitive gel electrophoresis [10]. This method provided insufficient resolution of holo and malonyl-ACP in our laboratory, so we optimized an existing MALDI-TOF method for high sensitivity detection [22].

It is curious to note that the levels of acetyl-ACP decrease during the reaction. *E. coli* FabD failed to catalyze a reaction with either acetyl-ACP or acetyl-CoA, suggesting a minor component in the reaction was responsible for depleting the acetyl-ACP pool. This unexpected side reaction was repeatable, and beneficial as it removed a contaminant to the malonyl-ACP preparation and occurred in a CoA dependent-manner. The acetyltransferase activity was removed from the ACP preparation by size exclusion chromatography using a Superdex 75 column (Figure 7).

Following conversion to malonyl-ACP, reaction components are removed with a final metal affinity chromatography step using a 1 mL nickel column. Malonyl-ACP elutes in the 250 mM imidazole fractions. In total, 15 mg of malonyl-ACP was recovered from 1 L of *E. coli* expression (Table 1).

Enzymatic analysis of malonyl-ACP

Malonyl-ACP is a viable substrate for the enzyme FabH as shown by a fluorometric assay that traps the product CoA using the fluorescent coumarin-based reagent, CPM (Figure 8) [16]. Furthermore, the initial reaction velocity increased linearly with respect to FabH concentration (Fig 8B). The assay used is an alternative to the reported radioactivity-based assays and we observed a FabH reaction rate of specific activity of 1 $\mu\text{mole CoA mg}^{-1} \text{ FabH min}^{-1}$. Radioactive assays for FabH monitoring [2- ^{14}C] malonyl-ACP production have shown a specific activity of 3 $\mu\text{moles CoA mg}^{-1} \text{ FabH min}^{-1}$ [9].

Conclusion

Here we demonstrate a complete strategy for holo- and malonyl-ACP production and characterization. Through co-expression with a modifying enzyme, holo-ACP was prepared with a single protein expression step followed by a rapid two-step column purification. High yields of malonyl-ACP were prepared following a single in vitro enzymatic conversion. The utility of these reagents to probe the enzymes of fatty acid biosynthesis was also presented and the fluorescent method reported serves as a viable alternative to radioactive assays. This expression and purification strategy is amenable to modifications, particularly with respect to alterations at the malonyl moiety because the FabD-catalyzed CoA-ACP thiol exchange is the final step in the process. This work provides new and accessible tools to probe fatty acid biosynthesis and substrates for enzymes designed as green catalysts for the renewable production of commodity or specialty chemicals.

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Figure Legends:

Figure 1. An excerpt from the fatty acid biosynthesis pathway highlights the steps of ACP modification. ACP and its various modified forms are highlighted. The enzymes that catalyze these reactions are indicated in ***bold***.

Figure 2. Purification of a holo-ACP:holo-ACP synthase complex using immobilized metal ion affinity chromatography. “Pre” and “post” refers to samples of bacteria harvested at the time of IPTG addition or at the end of the culture, respectively. “CL” refers to the lysate following centrifugation, and “FT” is the lysate following passage over the column. Washes “W” and elutions “E” are as described.

Figure 3. Size exclusion chromatography of the ACP:holo-ACP synthase complex. (A) Profiles of absorbency at 280 nm (*solid* line) and conductivity (*dashed* line) are shown from the purification using a Superdex S200 column. **(B)** Calibration curve for the S200 column using proteins of known mass.

Figure 4. ACP is efficiently separated from holo-ACP synthase using anion exchange chromatography. (A) Profiles of absorbency at 280 nm (*solid* line) and conductivity (*dashed* line) are shown from purification using an anion exchange column. **(B)** SDS PAGE analysis of the fractions using a 20% acrylamide gel. Fraction numbers corresponding to the column purification are indicated.

Figure 5. Analysis of holo-ACP synthase by mass spectrometry.

A MALDI-TOF spectrum of the holo-ACP-synthase following anion exchange purification. Three peaks shown for the synthase correspond to the $+2H^+$, $+3H^+$ and $+4H^+$ forms.

Figure 6. FabD efficiently converts holo-ACP to malonyl-ACP.(A) MALDI-TOF analysis of ACP fractions after anion exchange chromatography.(B) A MALDI-TOF spectrum following the FabD-catalyzed conversion of holo-ACP to malonyl-ACP. The $(+2H^+)$ ions are shown in each panel.

Figure 7. ACP elutes in a single peak from a Superdex75 size exclusion column. (A) Profiles of absorbency at 280 nm (*solid* line) and conductivity (*dashed* line) are shown. (B) SDS PAGE analysis of the S75 fractions using a 20% acrylamide gel. Fraction numbers corresponding to the column purification are indicated.

Figure 8. FabH efficiently utilizes malonyl-ACP. (A) The accumulation of CoA is plotted for various reactions with FabH, acetyl-CoA, and malonyl-ACP. Only early portions of the reaction are shown. (B) The rate of CoA formation increases linearly (*dotted* line) with FabH concentration using acetyl-CoA and malonyl-ACP as substrates. The result shown is representative of multiple, independent experiments.

Table 1. Purification and preparation of malonyl-ACP from a 1 L *E. coli* expression.

Stage	total protein (mg)
Clarified Lysate	206
Nickel Column	62
Q Column	15

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A

B

Figure 7

Figure 8