A high yield optimized method for the production of acylated ACPs enabling the analysis of enzymes involved in \textit{P. falciparum} fatty acid biosynthesis

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\textbf{ABSTRACT}

The natural substrates of the enzymes involved in type-II fatty acid biosynthesis (FAS-II) are acylated acyl carrier proteins (acyl-ACPs). The state of the art method to produce acyl-ACPs involves the transfer of a phosphopantetheine moiety from CoA to apo-ACP by \textit{E. coli} holo-ACP synthase (EcACPS), yielding holo-ACP which subsequently becomes thioesterified with free fatty acids by the \textit{E. coli} acyl-ACP synthase (EcAAS). Alternatively, acyl-ACPs can be synthesized by direct transfer of acylated phosphopantetheine moieties from acyl-CoA to apo-ACP by means of EcACPS. The need for native substrates to characterize the FAS-II enzymes of \textit{P. falciparum} prompted us to investigate the potential and limit of the two methods to efficiently acylate \textit{P. falciparum} ACP (PfACP) with respect to chain length and β-modification and in preparative amounts. The EcAAS activity is found to be independent from the oxidation state at the β-position and accepts fatty acids as substrates with chain lengths starting from C8 to C20, whereas EcACPS accepts very efficiently acyl-CoAs with chain lengths up to C16, and with decreasing activity also longer chains (C18 to C20). Methods were developed to synthesize and purify preparative amounts of high quality natural substrates that are fully functional for the enzymes of the \textit{P. falciparum} FAS-II system.

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1. Introduction

Acyl carrier proteins (ACP) are small (~10 kDa), acidic proteins playing a central role in the type-II fatty acid biosynthesis (FAS-II) pathway as they shuttle the growing fatty acid chain to the enzymes involved in the pathway. ACPs are highly conserved between various organisms. Their structures consist exclusively of helical elements and comprise of a DSL-motif being located in a specific loop region [1,2]. To become functional, newly expressed ACP (called apo-ACP) needs to be activated to holo-ACP by the enzyme holo-ACP synthase (ACP5), which transfers the phosphopantetheine moiety of Coenzyme A (CoA) to the conserved serine residue of the DSL-motif of apo-ACP [3,4]. Together with malonyl-CoA, holo-ACP represents the starting material for the synthesis of malonyl-ACP which in combination with acetyl-CoA is needed for initiating fatty acid biosynthesis.

The type-II fatty acid biosynthesis system is typically found in prokaryotic organisms and plants. In 1998, a FAS-II system was discovered in the malaria parasite \textit{Plasmodium falciparum} (\textit{P. falciparum}) [5]. Being structurally and functionally different from the eukaryotic FAS-II system, it has not only generated much interest as a putative drug target [6], but has also uncovered its role in cell metabolism, in cell viability, and in the development of the various stages of the complex parasite life cycle [7]. The further analysis of the enzymes involved in parasite FAS-II with respect to their activity and substrate specificity is therefore of high interest.

Hitherto, the thorough characterization of the \textit{P. falciparum} FAS-II pathway has been hampered by the fact that it is difficult to obtain natural acyl-ACP substrates. Traditionally, the lack of commercially available acylated ACPs made it necessary to use acyl-CoAs as alternative substrates for the characterization of FAS-II enzymes of various organisms, including FAS-II of \textit{P. falciparum}. However, acyl-ACPs should be used for an accurate determination of kinetic parameters, as there is evidence that the FAS-II enzymes display different behavior with the acyl-CoA substrates [8]. Thus, an efficient method for the production of large amounts of pure and functional acyl-ACPs of all types and chain lengths is needed for an in-depth analysis of the \textit{P. falciparum} FAS-II pathway.

The chemical methods developed to produce acyl-ACPs suffer from drawbacks like polyacylation [9,10], suggesting the enzyme-directed synthesis of acyl-ACPs as the method of choice. The archetypes and
most widely used enzymes for the modification of ACPs are *E. coli* holo-ACP synthase (EcACPS) and *E. coli* acyl-ACP synthase (EcAAS), but holo-ACP synthases of other organisms (for a comprehensive review see [11]) and the acyl-ACP synthase of *Vibrio harveyi* [12,13] were shown to be valuable alternatives.

EcACPS being promiscuous with respect to its substrates ACP and CoA, is able to catalyze in vitro, and in presence of CoA, the conversion of apo-ACP of a variety of organisms to holo-ACP, but can also generate short-chain acyl-ACPs (e.g. acetyl-ACP, malonyl-ACP, and crotonoyl-ACP) by using the corresponding acyl-CoAs and CoA analogs [11,14–17]. The use of saturated longer chain acyl-CoAs failed in one study [17], while it could be achieved in others [8,18–20].

Long chain acyl-ACPs are typically produced using EcAAS in a two-step reaction. In the first step holo-ACP is generated by EcACPS in presence of CoA, and then EcAAS is used to attach the free fatty acids to the terminal sulfhydryl group of the phosphopantetheine moiety of holo-ACP. EcAAS has been shown to accept various types of fatty acids as well as holo-ACPs of different organisms. This method has been applied in combination with saturated fatty acids [10,20–25], but also unsaturated (C16:1Δ9, C18:1Δ9) [26] and β-hydroxylated fatty acid chains [27,28]. However, the protocols usually include the detergent Triton X-100 to stabilize EcAAS, which renders difficult all downstream assays involving the final acyl-ACP substrates.

We along with others observed that *P. falciparum* acp gene (Gene ID: 947715) was PCR-amplified from *E. coli* genomic DNA using forward primer 5'-ATATACATTGGCAATATACGTTAGGACGGACGGATTTG-3' and reverse primer 5'-ATGAATCTTCTTCTTAATTTACGTTGCGGACAAGG-3’. After ligation into vector pET30, the protein was expressed and purified using a published protocol [8]. However, EcACPS was frozen (−20 °C) directly after metal chelate affinity chromatography, and the protein was only thawed and desalted (PD10) into desalting buffer (20 mM Tris, 200 mM NaCl, 20% glycerol, 3 mM DTT, pH 8.0) right before it was needed for the synthesis reaction.

The *E. coli* aus gene (Gene ID: 947315) ligated into vector pET28 (pAasH) was a kind gift of John Shanklin (Brookhaven National Laboratory, New York) and was applied as is for expression of EcAAS [22]. A one liter culture of C41(DE3) cells harboring the pAasH expression plasmid was grown over-night at 37 °C in TB, and expression was induced by addition of 0.4 mM IPTG. Six hours later, the cells were harvested by centrifugation. For the subsequent steps it was crucial to work with pre-cooled buffer solutions for washing and eluting the protein to maintain enzyme activity. The cells were taken up in lysis buffer composed of 96% buffer A (50 mM Tris, 20 mM MgCl2, 2% Triton X-100, 5 mM ATP, pH 8.0) and 4% buffer B (50 mM Tris, 20 mM MgCl2, 500 mM imidazole, 2% Triton X-100, 5 mM ATP, pH 8.0). ATP was always added shortly before use. After adding a small amount of DNase, the cells were disrupted by passing them twice through a French press. The lysate was clarified by centrifugation (20 min, 4 °C, 9800 rpm), and the supernatant was applied on a Ni-NTA column (2 ml volume) equilibrated with ice-cold lysis buffer. The column was washed with 10 ml lysis buffer, and after an additional wash step with a mixture of 90% buffer A and 10% buffer B, EcAAS was eluted with 10 ml of a mixture of 40% buffer A and 60% buffer B directly into 5 ml of ice-cold desalting buffer (50 mM Tris, 20 mM MgCl2, 20% glycerol, 2% Triton X-100, 5 mM ATP, pH 8.0). The protein was stored frozen (−20 °C) as 1 ml aliquots.

2.2. Cloning, expression and purification of proteins

The *acp* gene (Gene ID: 812677) sequence was PCR-amplified from a *P. falciparum* 3D7 gametocyte stage cDNA pSPORT plasmid library (kindly provided by Dr. T. Templeton, Weill Medical College of Cornell University) using forward primer 5'-TGTGTGTCATATAGTACTTTTGAATATATTTAAATTATAT-3' and reverse primer 5'-GGAATTC-TTATGCTTATTATTTTTCTATATAAT-3'. PfACP was expressed without its putative N-terminal signal- and translocation sequence, resulting in a protein consisting of residues 61–137, and purified using a similar protocol described earlier [8]. After isolation by metal chelate affinity chromatography, the N-terminal His6-tag was removed by thrombin digestion. The protein was concentrated, diluted with 20 mM Bis-Tris (pH 6.5) to achieve a final NaCl concentration of < 50 mM. The protein was applied to a 5 ml Hitrap Q- XL column to separate apo-PfACP and holo-PfACP and to remove the liberated purification tag. A linear NaCl gradient (600 ml, 2 ml/min) using a buffer containing 20 mM Bis-Tris and 1 M NaCl (pH 6.5) was applied. Fractions containing apo-PfACP were pooled and reapplied to the column. Three runs were necessary to complete the separation. The purity of apo-PfACP was verified by CS-PAGE. The pure fractions were concentrated (Centriprep, 3 kDa MWCO) and stored at −20 °C.

A typical acylation reaction (1 ml) consisted of 100 μM acyl-CoA for the production of C4:0- to C12:0- PiACP or 200 μM for the production of C14:0- to C20:0- PiACP, 250 μg of EcACPS, 1 mg apo-PiACP, 25 mM MgCl2 in 50 mM Tris (pH 7.5). The mixture was incubated at 37 °C for one hour. Holo-PiACP was generated applying the same conditions but using CoA instead of acyl-CoA. EcACPS was eliminated from the mixture by binding it to Ni-NTA agarose. To this end, a suspension of 100 μl 5 M NaCl solution and 100 μl Ni-NTA agarose were added to the 1 ml reaction mixture. The mixture was shaken for 5 min (30 osc/min), and then centrifuged to eliminate the affinity matrix. The
supernatant containing acyl-PfACP was collected, and the pelleted agarose was washed with 100 μl 50 mM Tris (pH 7.5) to recover remaining acyl-PfACP. The supernatant was pooled with the wash solution, filtered and concentrated (Amicon Ultra, 4.5 kDa MWCO). Finally the substrates were desalted and exchanged into the buffer needed for the enzyme assays (see section “Enzyme assays”) using PD10 desalting columns. The efficacy of the acylation reaction and the purity of the resulting acyl-PFACPs were confirmed by CS-PAGE. For time course experiments the acylation reaction was checked by taking samples at various time points after initiation of the reaction with EcACPS (5 s, 15 s, 30 s, 60 s, 2 min, 5 min, 10 min, 30 min and 60 min). The reaction was stopped by mixing the samples with 3-fold concentrated CS-PAGE sample buffer (150 mM Tris (pH 6.8), 3.75 M urea, 30% glycerol, 500 mM EDTA, 0.1% bromphenol blue). For all experiments only even numbered acyl-CoAs were used.

2.4. Preparation of acyl-PfACP substrates using EcAAS

A typical acylation reaction was carried out with 10 ml of holo-PfACP solution (prepared as described above) that was supplemented with 2 mg EcAAS, 2.5 mM fatty acid (dissolved in toluene at 250 mM), 0.4 M LiCl, 10 mM MgCl₂, 5 mM ATP, 3 mM DTT, and 2% Triton X-100. H₂O was added to give a final volume of 15 ml. The mixture was incubated over night at 25 °C to ensure complete conversion as judged by CS-PAGE. For time course experiments the acylation reaction was checked by taking samples at various time points after initiation of the reaction with EcACPS (5 s, 15 s, 30 s, 60 s, 2 min, 5 min, 10 min, 30 min and 60 min). The reaction was stopped by mixing the samples with 3-fold concentrated CS-PAGE sample buffer (150 mM Tris (pH 6.8), 3.75 M urea, 30% glycerol, 500 mM EDTA, 0.1% bromphenol blue). For all experiments only even numbered acyl-CoAs were used.

2.5. Enzyme assays

The functionality of the resulting acyl-PFACPs was investigated using three enzymes of the P. falciparum FAS-II pathway. All experiments were carried out in triplicates. Control reactions were run exactly the same way but omitting the enzyme under investigation.

PFACPs acylated with saturated fatty acids were assayed with PfFabBF using a similar method published elsewhere [8]. The reaction consisted of 30 μM malonyl-PfACP, 100 μM acyl-PfACP, 5 μg of PfFabG, 300 mM NaCl, 80 μM NADPH and 3 mM DTT in 20 mM NaH₂PO₄ (pH 7.5) in a total volume of 100 μl. The reaction was started with the addition of 3 μg of PfFabBF [8], and the decrease in absorption at 340 nm was monitored for 1 min.

PfFabZ was used to test β-hydroxybutyryl-PfACP (OH-C₄-PfACP). The reaction consisted of 30 μM substrate in a mixture containing 5 μg PfFabl, 80 μM NADH, 150 mM NaCl and 20 mM Hepes (pH 7.0) in a total volume of 100 μl. The reaction was started with the addition of 5 μg of PfFabZ [40]. The decrease in NADH absorption was monitored for 1 min.

Enoylacyl-PFACPs were tested with PfFabl using the same reaction mixture as described above for β-hydroxybutyryl-PfACP (OH-C₄-PfACP), except that PfFabZ was omitted and the reaction was started by adding 5 μg of PfFabl [41].

3. Results

3.1. Expression and purification of proteins

Recombinant expression of PfACP typically yielded 35–45 mg of highly pure protein as deduced from Tris-Tricine SDS-PAGE (Fig. 1, panel A). When PfACP is expressed as described, apo- and holo-PfACP are formed (Fig. 1, panel B, and lane 1 of panel C). Attempts to increase the amount of apo-PFACP by varying expression temperature and using different growth media failed, thus apo-PFACP was separated from the other forms by anion exchange chromatography. This was possible due to the negatively charged phosphate group of the phosphopantetheine moiety which caused holo- and acyl-PFACPs to elute at slightly higher NaCl concentrations than apo-PFACP. Dimerized holo-PFACP, the oxidized form of holo-PFACP, was found to elute at higher NaCl concentrations than apo-PFACP. (B) Results of apo- and holo-PFACP separation by anion exchange chromatography using a HiTrap Q XL column. The solid line represents the elution pattern of the first run using a linear NaCl-gradient, yielding incompletely separated apo-PFACP and holo-PFACP. The fractions corresponding to the peak eluting between 35 and 65 ml were pooled, concentrated and re-purified on the same column (dashed line). The fractions containing apo-PFACP were pooled again for a third run (dotted line) to achieve complete separation and pure apo-PFACP. (C) CS-PAGE analysis of fractions eluted during the first run of anion exchange chromatography (corresponding to solid line in panel B). Lane 1, Tris-Tricine SDS-PAGE pure PfACP (see panel A, lane 7) before anion exchange chromatography; lane 2–10, representative samples taken from fractions corresponding to ml 45–75 of the first run (solid line in panel B).
concentrations than monomeric holo-PfACP, thus quantitative dimerization of holo-PfACP could have a favorable effect on the separation of apo-PfACP. Subsequent attempts to increase the amount of oxidized PfACP either failed or resulted in poor quality PfACP (data not shown). Nevertheless, complete separation was achieved by applying a shallow gradient (120 CVs) and three subsequent rounds of pooling and reloading of the apo-PfACP containing fractions (Fig. 1, panel B and C). This procedure routinely yielded around 20 mg of the desired highly pure apo-PfACP out of one liter bacterial culture. The purity and yield of the apo-PfACP was estimated by SDS-PAGE and gel densitometry. As a matter of fact, increasing the urea concentration improved the resolution of apo- and holo-PfACP while it had no further influence on the migration pattern of acyl-PfACPs with various chain lengths.

### 3.2. Acylation of apo-PfACP by EcACPS

We found that the substrate acceptance of EcACPS is not limited to short chain acyl-CoAs (Fig. 3, panel A), and that acyl-CoAs with chain lengths up to 18 carbons could be used to generate the corresponding acyl-PfACPs (Fig. 3, panel B). Under the conditions applied, the acylation reaction was complete within 5 min when acyl-CoAs with chain lengths up to 12 carbons were used (Fig. 3, panel C). The transfer of the acyl-phosphopantetheine group from C14:0-CoA was less efficient, and a weak band corresponding to apo-PfACP was still present after an incubation time of 60 min (Fig. 3, panel D). Efficacy decreased with longer chains (C16:0-CoA and C18:0-CoA) as only about 50% of the apo-PfACP were acylated after 1 h. Nevertheless, acylation to produce C14:0-PfACP to C18:0-PfACP could be driven to completion within 60 min when doubling the initial acyl-CoA concentration (Fig. 3, panel B, lanes 7–9). However, it was not possible to achieve complete transfer of C20:0-CoA (Fig. 3, panel B, lane 10). Subsequent purification of the acyl-PfACP products did not lead to a significant decomposition or loss of protein.

Completeness of reaction and the purity of the acyl-PfACPs were confirmed by CS-PAGE. Interestingly, the migration of short chain acyl-ACP in CS-PAGE exhibited some characteristics that were specific for the corresponding acyl-ACPs. While butyryl-PfACP (C4:0-PfACP) usually migrated as a single band with a faint tailing (Fig. 3, panel A, lane 2) or as single band only (Fig. 3, panel B, lane 2), the other short chain acyl-ACPs appeared as two bands. For crotonoyl-PfACP (C4:1-PfACP) both bands showed equal intensity (Fig. 3, panel A, lane 3), while for malonyl-PfACP the upper band migrated with higher intensity (Fig. 3, panel A, lane 4). For β-hydroxybutyryl-PfACP (OH-C4-PfACP) a weak band remained at the level of the second band of the other acyl-ACPs, whereas the second band migrated faster as all others and with high intensity (Fig. 3, panel A, lane 5). In contrast acetoacetyl-PfACP seemed to migrate as a single band but with some tailing (Fig. 3, panel A, lane 6). In contrast acetooctyl-PfACP and β-hydroxybutyryl-PfACP (OH-C4-PfACP) a weak band remained at the level of the second band of the other acyl-ACPs, whereas the second band migrated faster as all others and with high intensity (Fig. 3, panel A, lane 5). Interestingly, this behavior was independent of the urea concentration in the CS-gels and could only be observed if fully (i.e. overnight) polymerized gels were used. Subsequent mass spectrometric analyses confirmed identity and homogeneity of short chain acyl-ACPs (Table 2).

Regarding the longer chain acyl-ACPs, only minor differences in the migration pattern could be observed which did not allow distinguishing them by their migration front regardless of the gel composition. As a matter of fact, increasing the urea concentration improved the resolution of apo- and holo-PfACP while it had no further influence on the migration pattern of acyl-ACPs with various chain lengths.

### 3.3. Acylation of holo-PfACP by EcAAS

The EcAAS method was able to attach saturated fatty acids starting from C8:0 to C16:0 to holo-PfACP within 8 h, while for C18:0 and C20:0 the reactions were not complete unless they were incubated over...
night at 25 °C. A representative example for the production of C14:0-PfACP is given in Fig. 4 (panel A). In addition to the well documented efficacy for acylating saturated fatty acids, we found that EcAAS is also capable of transferring unsaturated (2-enoyl) fatty acids (Fig. 4, panel B) and β-hydroxylated fatty acids to holo-PfACP (Fig. 4, panel C), the latter with slightly reduced efficacy. Amongst the 2-enoyl fatty acids tried to attach to holo-PfACP, C4:1 (crotonic acid) was not accepted as substrate, and C6:1 was only transferred with difficulty, but C8:1 to C16:1 fatty acids were esterified to completion when incubated overnight (Fig. 4, panel D).

The hydrophobic Calbiosorb resin was investigated as a means to eliminate Triton X-100 from the acyl-ACP solution after the reaction. Triton X-100 is a detergent used in the EcAAS reaction mixture to stabilize the enzyme. Indeed, Calbiosorb treatment efficiently removed the detergent while maintaining the integrity of acyl-ACPs (Fig. 4, panel E), making it possible to skip the extensive washing step with 80% isopropanol used in the traditional method. However, some minor loss of acyl-PfACP during workup could be observed.

### 3.4. Enzyme assays

The acyl-PfACPs produced by the EcAAS and the EcACPS method were analyzed for functionality in standard activity assays. Indeed, saturated fatty acids were readily recognized by PfFabBF (Fig. 5, panel A), 2-enoylacyl-PfACP served as substrate for PfFabI (Fig. 5, panel B), and OH-C4-PfACP was transformed by PfFabZ (Fig. 5, panel C).

### 4. Discussion

The exclusive use of natural substrates for biochemical characterization of FAS-II enzymes has very rarely been applied to date, which is most likely due to the lack of commercial availability and the lack of efficient protocols to produce preparative amounts of such substrates. However, it is more than reasonable to use native substrates to analyze FAS-II enzymes. On the one hand, it is known that β-ketoacyl-ACP synthases, enzymes catalyzing the synthase step in chain elongation, only display little activity with acyl-CoAs [42,43], thus only function with natural substrates. On the other hand, our recent work has shown
that kinetic parameters determined with artificial (acyl-CoA based) substrates display different characteristics compared to their corresponding natural substrates [8]. Indeed, the unique β-ketoacyl-ACP synthase in *P. falciparum* (PfFabBF) uses acyl-CoAs as substrates with only very low activity while it readily elongates acyl-PIACPs, and it exhibits a very different behavior with acyl-PIACP compared to acyl-CoAs [8]. The requirement of large quantities of natural substrates prompted us to investigate the potential of the known methods and production protocols based on EcAAS and the EcACPS, aiming at finding and developing the most efficient enzyme based system to generate acylated PfACPs of high quality and at preparative amounts.

EcACPS allows the synthesis of acyl-PIACPs using acyl-CoAs with chain lengths up to 18 carbons, but with decreasing efficacy for chains longer than 12 carbons, indicating that the relaxed substrate specificity at the distal end of the phosphopantetheinyl moiety is disturbed with longer chain lengths and has reached its limit with acyl chains exceeding 18 carbons. This finding is in contrast to a recent study showing that EcACPS can only transfer short chain CoAs to *E. coli* apo-ACP [17], and it suggests that the protein-protein interactions between EcACPS and PfACP is different and hence in favor of long chain phosphopantetheinyl transfer compared to *E. coli* apo-ACP.

Interestingly, the oxidation state of the acyl-chain attached to the CoA does not play an important role for transfer by EcACPS as crotonoyl-CoA, β-hydroxybutyryl-CoA and acetoacetyl-CoA are accepted as substrates. Thus, although our investigation was limited to commercially available acyl-CoA derivatives (i.e. C4:0-CoA to C20:0-CoA, crotonoyl-CoA, malonyl-CoA, crotonoyl-CoA and β-hydroxybutyryl-CoA), we conclude that very likely EcACPS also accepts long chain β-hydroxyacyl-CoAs, 2-enoylacyl-CoAs and β-ketoacyl-CoAs as substrates. Despite the limited availability of acyl-CoAs, their chemical or enzymatic synthesis is well documented [44–46], and its use will increase considerably the variety of acyl-PIACPs that can be produced by this method. Taken together, the simple and fast reaction, combined with the simple purification of products, make acyl-PIACP synthesis with EcACPS the method of choice when corresponding acyl-CoAs are available.

The EcAAS reaction proved to be a valuable tool for the production of longer chain acyl-ACPs when only the fatty acid form is available. While long incubation times (8–16 h at 25 °C) are required to achieve complete acylation of the holo-ACP, the oxidation state of the fatty acyl chain does not influence the reaction efficiency, and all forms of acyl-ACP can be obtained. Moreover, the improved EcAAS preparation led to the robust setup of the reaction proposed in this work, and product purification using Calbiosorb resin significantly avoids degradation of the acyl-ACPs during workup. Nevertheless, a small loss of the protein may occur, most likely due to adsorption of the acyl chain of acyl-PIACPs to the resin.

The wide commercial availability of free fatty acids of various chain lengths makes this method very valuable for the synthesis of acylated PfACPs. Due to the fact that β-hydroxyacyl fatty acids are mostly sold as racemate, the EcAAS method will produce racemic β-hydroxyacyl-PfACPs. This may limit their use in kinetic measurements using PfFabZ because PfFabZ, like the homologous enzymes in other organisms, is stereoselective and only accepts the R-enantiomer as substrate [47]. However, as long as the S-enantiomer does not influence enzyme activity, the racemic substrate can still be used for assays. The production of longer chain β-ketoacyl-ACPs may be challenging using the EcAAS method since β-keto fatty acids are known to be chemically unstable, and they are, with the exception of acetooacetic acid, not commercially available.

To overcome these issues, PfACPs acylated with saturated fatty acids (Cn:0-PfACP), produced either using the EcAAS or the EcACPS method, could be converted quantitatively to β-ketoacyl-PfACPs by means of the FAS-II enzyme PfFabBF and malonyl-PfACP. If needed, the holo-PfACP formed during the PfFabBF reaction can be separated from the β-ketoacyl-ACP by hydrophobic chromatography [21]. The simultaneous incubation of Cn:0-PfACP with PfFabBF and PfFabG (in presence of malonyl-PfACP) will yield β-hydroxyacyl-PfACPs. Remarkably, this method will furnish the preferred R-enantiomer of the β-hydroxyacyl-PfACP produced. The production of preparative amounts of 2-enoylacyl-ACPs out of β-hydroxyacyl-PfACPs is may be less efficient due to the fact that the PfFabZ reaction is reversible with the reverse reaction being favored over the forward reaction [48,49], and the resulting mixture of 2-enoylacyl-ACP and β-hydroxyacyl-PfACP would need to be separated.

The natural substrates (acyl-PIACPs) tested in subsequent assays were readily recognized by the corresponding enzymes (PfFabBF, PfFabI, PfFabZ) involved in FAS-II of *P. falciparum*, confirming that both the EcAAS and the EcACPS method presented in this work have to capacity to produce pure and preparative amounts of acyl-PIACPs with full functionality in standard activity assays.

### 5. Conclusion

In this report we have shown that PfACP can be acylated very efficiently using two enzyme based methods, and we have improved acylation and purification protocols which are now capable to yield preparative amounts of a wide range of high quality natural substrates.
that are readily recognized by the corresponding enzymes in the *P. falciparum* fatty acid biosynthesis pathway. Based on the fast reaction, simple experimental setup, combined with the straightforward purification, we propose the EcAAS methods as the method of choice for the synthesis of acyl-PACP if the corresponding acyl-CoAs are available. However, the EcAAS methods remains a very valuable tool if only free fatty acids are available, and our improved protocols provide the means to prepare them fast and at high quality. For the first time we have the opportunity to characterize kinetics as well as substrate specificity without the need to use acyl-CoAs as surrogates, which will increase our understanding of *P. falciparum* fatty acid biosynthesis considerably.

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Appendix A. Transparency document

Transparency data associated with this article can be found in the online version at [http://dx.doi.org/10.1016/j.bbrep.2016.09.017](http://dx.doi.org/10.1016/j.bbrep.2016.09.017).

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