Solution Structures of the Acyl Carrier Protein Domain from the Highly Reducing Type I Iterative Polyketide Synthase CalE8

Jackwee Lim1*, Rong Kong2*, Elavazhagan Murugan2, Chun Loong Ho2, Zhao-Xun Liang2*, Daiwen Yang1*
1 Department of Biological Sciences, National University of Singapore, Singapore, Singapore, 2 School of Biological Sciences, Nanyang Technological University, Singapore, Singapore

Abstract

Biosynthesis of the enediyne natural product calicheamicins γ1 in Micromonospora echinospora ssp. calichensis is initiated by the iterative polyketide synthase (PKS) CalE8. Recent studies showed that CalE8 produces highly conjugated polyprenes as potential biosynthetic intermediates and thus belongs to a family of highly-reducing (HR) type I iterative PKSs. We have determined the NMR structure of the ACP domain (meACP) of CalE8, which represents the first structure of a HR type I iterative PKS ACP domain. Featured by a distinct hydrophobic patch and a glutamate-residue rich acidic patch, meACP adopts a twisted three-helix bundle structure rather than the canonical four-helix bundle structure. The so-called ’recognition helix’ (s2) of meACP is less negatively charged than the typical type II ACPs. Although loop-2 exhibits greater conformational mobility than other regions of the protein with a missing short helix that can be observed in most ACPs, two bulky non-polar residues (Met992, Phe996) from loop-2 packed against the hydrophobic protein core seem to restrict large movement of the loop and impede the opening of the hydrophobic pocket for sequestering the acyl chains. NMR studies of the hydroxybutyryl- and octanoyl-meACP confirm that meACP is unable to sequester the hydrophobic chains in a well-defined central cavity. Instead, meACP seems to interact with the octanoyl tail through a distinct hydrophobic pocket without involving large conformational change of loop-2. NMR titration study of the interaction between meACP and the cognate thioesterase partner CalE7 further suggests that their interaction is likely through the binding of CalE7 to the meACP-tethered polyprene moiety rather than direct specific protein-protein interaction.

Citation: Lim J, Kong R, Murugan E, Ho CL, Liang Z-X, et al. (2011) Solution Structures of the Acyl Carrier Protein Domain from the Highly Reducing Type I Iterative Polyketide Synthase CalE8. PLoS ONE 6(6): e20549. doi:10.1371/journal.pone.0020549

Editor: Maria Gasset, Consejo Superior de Investigaciones Cientificas, Spain

Received: February 17, 2011; Accepted: May 3, 2011; Published: June 2, 2011

Copyright: © 2011 Lim et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work is supported by Singapore Ministry of Education (MOE) through an ARC grant to Z-X L (Grant Number: 06/1/22/19/464) and by a grant from Biomedical Research Council, Agency for Science, Technology and Research of Singapore (R154000373305) to DY. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: zxl@ntu.edu.sg (Z-XL); dbsydw@nus.edu.sg (DY)
† These authors contributed equally to this work.

Introduction

One of the most distinctive features of fatty acid, nonribosomal peptide and polyketide biosynthetic pathways is the utilization of the acyl carrier protein (ACP) or peptidyl carrier protein (PCP) for the shuttling of biosynthetic intermediates among various catalytic domains or proteins [1–5]. The small and highly dynamic ACPs or PCPs can be either free-standing proteins or integrated domains within a complex multidomain fatty acid synthase (FAS), polyketide synthase (PKS) or nonribosomal peptide synthase (NRPS). The free-standing ACPs from type II FAS pathways are the best studied ACP systems, with crystallographic and solution NMR studies showing that the type II FAS ACPs adopt a canonical four-helix bundle fold with a binding pocket for sequestering the growing fatty acid chain [6–8]. Meanwhile, although the ACP domains from the multidomain type I FASs adopt a similar overall structure, they do not seem to contain a pocket for binding and protecting the growing fatty acid chain [9–11]. Recent studies on the integrated ACP domain from the modular type I PKS DEBS and the discrete ACPs from type II PKSs have revealed the similarity in overall protein structure but salient differences in the binding of acyl chain between type I and type II PKS ACPs [12–17]. Subtle differences between the ACPs in local structure, surface electrostatic potentials and binding mode of biosynthetic intermediates have been also documented. Recent NMR studies further revealed functionally important protein dynamics in PCPs for the modulation of the interaction between the PCPs and the NRPS catalytic domains [3,18,19].

CalE8 is an iterative type I PKS that plays a central role in the early stage of the biosynthesis of the naturally occurring enediyne calicheamicin γ1 in Micromonospora echinospora ssp. calichensis. Distinct from the modular type I PKSs, CalE8 is composed of a single module consisting of several catalytic domains for the synthesis of the putative precursor of the 10-membered enediyne moiety [20–24]. The ACP domains of CalE8 and other enediyne PKSs share very low sequence homology with known ACPs [20,25,26]. In fact, the initial assignment of the ACP domain in CalE8 was associated with a great degree of uncertainty, not just...
because of the low sequence homology shared with other ACPs, but also because of the absence of the signature GX(H/D)S(L/I) motif conserved in many ACPs. We previously confirmed the identity of the ACP domain (meACP) in CalE8 through in vitro modification of the excised meACP by the phosphopantetheinyl transferase (PPTase) Sfp [27,28]. Shen and coworkers also demonstrated that the ACP domain of SgcE, a homolog of CalE8 from the biosynthetic pathway of the 9-membered enediyne natural product C-1027, can be phosphopantetheinylated at the predicted Ser site by mass spectrometry [29].

According to the degree of reduction of keto groups in the final polyketide product, iterative type I PKSs have been classified into the non-reducing (NR), partially-reducing (PR) and highly-reducing (HR) families [21,30]. CalE8 belongs to the HR type I PKS family given that CalE8 and its homologs produce conjugated polyenes under both in vitro and in vivo conditions [22,23,31,32]. To date, only one structure of the iterative type I PKS ACP domain has been reported [33]. Crump and coworkers determined the solution structure of the ACP domain from the fungal norsolorinic acid synthase (NSAS), which belongs to the iterative NR PKS family [33]. Unlike the polyketide products of the iterative NR and PR PKSs that are cyclized immediately upon formation, the chemically labile conjugated polyenes produced by CalE8 seem to be stabilized by the protein to such an extent that they can be co-purified with the protein [23,24,34]. It is thus intriguing to speculate whether the ACP domain plays any role in protecting the conjugated polyenes. Here we report the NMR solution structure of the ACP domain (meACP) of CalE8, which represents the first structure of ACP domains from an HR iterative type I PKS. Studies on the apo, holo and acylated meACP suggest that the modifications do not alter the overall protein structure but affect the structure at a local level. Two-dimensional NMR spectra of meACP collected in the presence of thioesterase (CalE7) also provide some interesting insight into the transient nature of the interaction between meACP and the cognate protein partner.

Results

Overall structure of meACP

The ACP domain construct used in this study encompasses a 92 residue long (Ala925–Pro1016) fragment of CalE8. Previous studies have confirmed that the E. coli overexpressed meACP is in its apo form and can be modified by the PPTase Sfp to generate holo-meACP [27]. 98% of the residues of the recombinant apo-meACP were unambiguously assigned. Unassigned residues are the amino-terminal Met924 and Ala925. The only disordered segments are the unstructured termini that include Ala925 to Thr936 and Ala1015 to Pro1016 (Figure 2A). The structured region of meACP has a backbone r.m.s. deviation of 0.44 Å and no distance violation greater than 0.5 Å was observed in our structure calculation (Table 1). Ramachandran plot of the final ten structures shows that 98.9% of the residues fall in the allowed region (Table 1).

Overall, meACP assumes a globular fold of a twisted three-helix bundle (α1 [Ala935–Ala950], α2 [Ser971–Met984] and α3 [Val1001–

![Diagram of Polyketide Synthase Enzymes]

Figure 1. Three classes of type I iterative polyketide synthases (PKS). The proposed PKS products are tethered to the ACP domains. doi:10.1371/journal.pone.0020549.g001
and two well-defined long loops (loop-1 [Glu 951-Ser970] and loop-2 [Met 985-Thr1000]) (Figure 2B). The domain seems to be stabilized through the packing of the hydrophobic side chains of the residues originated from the three helices as well as the two loops. The estimated helical content of 44.6% based on 41 out of 92 residues, is significantly lower than that of other known ACPs, which usually have more than 50% of helical content [35]. Most characterized ACPs adopts a canonical four-helix bundle structure with helix \( \alpha_1 \) running almost anti-parallel to helices \( \alpha_2 \) and \( \alpha_4 \), as well as a short helix \( \alpha_3 \) within the loop-2 that connects the helices \( \alpha_2 \) and \( \alpha_4 \). In meACP, the region where the short helix should be located seems to be disordered and the lack of the local helical feature contributes to the low helical content of the protein. Some ACPs and PCPs have been reported to adopt two or more interconverting conformations [18,36]. But we did not observe a significant sub-population for meACP.

Table 1. Experimental restraints and structural statistics for ten lowest-energy NMR structures of apo-meACP out of 100 structures.

| NMR distance constraints                  |       |
|------------------------------------------|-------|
| Intra-residue                             | 628   |
| Sequential                                | 324   |
| Medium-range (1<|i-j|<5)                 | 702   |
| Long-range (|i-j|≥5)                     | 654   |
| Total                                     | 2308  |

| NMR dihedral restraints                  |       |
|------------------------------------------|-------|
| \( \Phi \)                               | 62    |
| \( \Psi \)                               | 63    |
| Total                                     | 125   |

| Structural statistics                    |       |
|------------------------------------------|-------|
| Violations per structure (residues Gly937-Arg1013) |       |
| NOE violation (Å)                        | 0.41±0.04 |
| Angle violation (')                      | 3.74±0.39 |
| TALOS >5.0                               | 0     |
| Max. dihedral angle violation (')        | 4.18   |
| Max. distance constraint violation (Å)   | 0.47   |

Ramachandran plot region (all residues) [%]

|                      |       |
|----------------------|-------|
| most favoured        | 76.3  |
| additionally allowed | 19.4  |
| generously allowed   | 3.2   |
| disallowed           | 1.1   |

Mean RMS deviation from the average coordinates (residues Gly937-Arg1013) (Å)

|                      |       |
|----------------------|-------|
| Backbone atoms (C\(^\alpha\), C', N, O) | 0.44±0.12 |
| All heavy atoms       | 1.10±0.11 |

Local structural features

A comparison with other ACP structures reveals some salient features of meACP. Despite the facts that meACP shares very low sequence homology with other ACPs and it lacks the signature GX(H/D)[S(L/I)] motif conserved in many ACPs (Figure 3A), the putative phosphopantetheine attachment site (Ser\(^{971}\)) is still located at the beginning of helix \( \alpha_2 \) (Figure 4). The orthodoxy GX(H/D)[S(L/I)] motif is replaced by a H\(^{968}\)MS\(^{970}\)I motif in meACP with His\(^{968}\) and Ser\(^{970}\) substituting the canonical Gly and Asp/His residues. The essential role of Ser\(^{971}\) was confirmed by the observation that S971A mutation completely abolished the activity of CalE8 (data not shown). The HMS\(^{970}\) triad constitutes part of loop-1 with the solvent-exposed side chains His\(^{968}\), instead of the typical Gly residue conserved among many characterized ACPs (Figure 4). In addition, Ser\(^{970}\) replaces an Asp residue commonly found in other type I and II ACPs. This Asp from \( B. \) subtilis FAS ACP forms a salt bridge with the Arg\(^{14}\) of ACP synthase (ACPS) [37]. Thus, considering that such residues preceding Ser\(^{971}\) have been known to affect protein-protein interaction in some ACPs and given the different physio-chemical property of the residues, a dissimilar protein surface of meACP may be involved in the recognition of some of its partners.
One of the common features of types II FAS or PKS ACPs is that they contain a helix $\alpha_2$ that is rich in acidic residues. The clustered negative charges on helix $\alpha_2$ are considered to be critical for the recognition of the free-standing ACPs by their protein partners. Helix $\alpha_2$ is therefore known as the universal ‘recognition helix’ in carrier proteins. Several acidic residues in helix $\alpha_2$ of type II ACPs can form salt bridges with positively charged residues on their protein partners [38]. However in type I FAS ACP domains the helix $\alpha_2$ seems to be less negatively charged, presumably because the specific domain-domain interaction driven by charged-charged interactions is less critical in the multidomain type I FAS system. In accordance with other type I ACPs, the helix $\alpha_2$ of meACP only contains a single acidic residue (Glu 980).

Figure 3. Comparison of meACP with other ACPs. (A) Multiple sequence alignment of meACP with selected type I and type II ACPs. The secondary structures of meACP (top) and B. subtilis FAS ACP (bottom) are shown. The phosphopantetheine attaching serine site is shaded in red. (B) Phylogenetic analysis of the type I and II ACPs from (A). doi:10.1371/journal.pone.0020549.g003

Figure 4. Conformation of the motif harboring the phosphopantetheine attaching serine in meACP and two other ACPs. The GX(H/D)S(L/I) motif in type I NSAS PKS ACP (PDB ID: 2KR5) (pink) and type II actinorhodin PKS ACP (PDB ID: 1AF8) (orange) and the HMSSI motif in meACP (grey) are shown as ribbons. The conserved Gly and Ser residues in GX(H/D)S(L/I) motif are shown as stick. The His and Ser residues of the HMSSI motif in meACP are also shown as stick.

doii:10.1371/journal.pone.0020549.g004

One of the common features of types II FAS or PKS ACPs is that they contain a helix $\alpha_2$ that is rich in acidic residues. The clustered negative charges on helix $\alpha_2$ are considered to be critical for the recognition of the free-standing ACPs by their protein partners. Helix $\alpha_2$ is therefore known as the universal ‘recognition helix’ in carrier proteins. Several acidic residues in helix $\alpha_2$ of type II ACPs can form salt bridges with positively charged residues on their protein partners [38]. However in type I FAS ACP domains the helix $\alpha_2$ seems to be less negatively charged, presumably because the specific domain-domain interaction driven by charged-charged interactions is less critical in the multidomain type I FAS system. In accordance with other type I ACPs, the helix $\alpha_2$ of meACP only contains a single acidic residue (Glu$^{980}$). The resemblance of meACP to type I ACPs is also consistent with the phylogenetic relationship of meACP with other carrier proteins (Figure 3B). In contrast to the relatively neutral helix $\alpha_2$, the adjacent loop-1 harbors a large number of highly charged residues including Glu$^{951}$, Glu$^{955}$, Arg$^{958}$, Asp$^{959}$, Asp$^{960}$, Arg$^{962}$, Asp$^{965}$, Asp$^{966}$, and His$^{968}$. The enrichment of the charged residues on loop-1 is not uncommon and has also been seen in some ACPs, such as the ones from the biosynthetic pathways of frenolicin and norsolorinic acid [33,39]. Given the proximity of loop-1 near the recognition helix $\alpha_2$, these charged residues might provide the
specific docking interface for other protein domains or stabilize the phosphopantetheine arm.

It has been suggested that loop-2, which contains a short helix for some ACPs, serves as a conformational switch in sequestering ACP domain-tethered acyl chains in type II FAS systems [5]. With the exceptions of act ACP and B. subtilis FAS ACP [6,16], all the characterized ACPs contain a short helix within loop-2. The short helix seems to be absent in meACP as well. In addition, in the case of type II ACPs, loop-2 is largely comprised of charged or small non-polar residues that allow the opening of a hydrophobic cleft for binding acyl intermediates (Figure 5A); whereas loop-2 of type I ACPs, such as NSAS ACP, usually contains bulky residues that are packed against the protein core to prevent the opening of the binding cleft (Figure 5B) [33]. In meACP, loop-2 also contains two hydrophobic residues (Met 992, Phe 996) with their bulky side chains packed against the hydrophobic core of the protein (Figure 5C). Although the packing of Met 992 and Phe 996 may restrict the conformational flexibility of loop-2, 1H-15N NOE plot of meACP indicates that the residues of loop-2 (Gly 986-Thr 1000) are still significantly more flexible than the residues of loop-1 and helices 2, 2, and 3 (Figure 5D). The observed conformational flexibility of loop-2 raises the tantalizing possibility that it may undergo a large conformational change to open a protein pocket for binding the PKS products.

Comparison between apo- and holo-meACP

ACP domain only becomes fully functional after conversion to holo protein with a phosphopantetheine group attached to the invariant Ser residue. To find out whether the presence of the phosphopantetheine group perturbs the meACP structure and

![Figure 5. Conformation mobility of loop-2.](image)

(A) Type II actinorhodin PKS ACP (PDB ID: 1AF8) that contain mainly small residues in loop-2. (B) Type I NSAS PKS ACP (PDB ID: 2KR5) with the two bulky residues (Phe 1766 and Phe 1771) highlighted. (C) meACP with the two bulky residues (Phe 996 and Met 992) packed against the hydrophobic pocket highlighted. (D). 1H-15N NOE plot of apo-meACP acquired at an 800 MHz spectrometer at 25°C. The positions of the helices are indicated.

doi:10.1371/journal.pone.0020549.g005
dynamics, the _holo_ form of the protein was prepared by incubating _meACP_ with coenzyme A and the PPTase Sfp. Comparing the HSQC spectra of _apo-_ and _holo-_ _meACP_, we found that the overall structures of the two forms are essentially the same (Figure 6). Nonetheless, Ser971 exhibits a large shift from the _apo_ form (1H: 9.28 ppm, 15N: 116.80 ppm) to the _holo_ form (1H: 9.42 ppm, 15N: 116.50 ppm) or a combined chemical shift change of 0.31 ppm. The presence of the phosphopantetheine group also clearly affects backbone amides of the neighboring residues that include Asp965 and Ser970 from loop-1, Ile972, Gly975 and Val978 from helix α and Thr994, Thr998, Ala999 and Thr1000 from loop-2 (Figure 7A and 7G). These perturbed residues are mostly located at protein surface in the vicinity of Ser971 (with the exception of Val978 that is probably due to a subtle conformational change in helix α2). The observation indicates that the _meACP_-attached phosphopantetheinyl group is most likely to be exposed to solvent and accessible to other catalytic domains.

**Interaction of acylated moiety with _meACP_**

One of the most interesting findings about PKS/FAS and NRPS is the presence of a ligand binding pocket on the surface of type II ACPs or PCPs for the binding of the growing intermediates. For example, the sequestering of aliphatic segment of decanoate (10:0) or stearate (18:0) in the binding pocket of spinach ACP induces striking perturbations of HSQC peaks [40,41]. On the other hand, hexanoyl (6:0) or palmitoyl (16:0) chain attached to type I rat FAS ACP showed little changes of HSQC peaks, suggesting that the rat FAS ACP does not bind the aliphatic chain [9].

Given the observed conformational flexibility of loop-2 in _meACP_, we asked whether type I _meACP_ can also bind the chemically labile polyene intermediates in the same hydrophobic pocket that accommodates the side chains of Met992 and Phe996. Loading _meACP_ with the highly conjugated polyenes is impractical given the chemical reactivity of the polyenes. Instead, we used hydroxybutyryl- and octanoyl- groups to mimic the aliphatic chains of the polyene products. Overall, only minor perturbations in 1H-15N HSQC spectrum were observed between the _holo-_ and _acylated-_ _meACPs_, suggesting that residues lining the putative binding pocket are not disturbed (Figure 6). However, although only negligible differences between the _holo-_ and _hydroxybutyryl-_ _meACP_ (combined chemical shift differences <0.05 ppm) were observed, extending the acyl chain from hydroxybutyryl to octanoyl causes greater and more extensive chemical shift changes (0.05–0.20 ppm) (Figure 7B-F). With a chemical shift change cutoff of 0.05 ppm, eight residues that include Ser971, Gly975, Arg983, Ala991, Thr1000, Ala1007 and Arg1011 exhibit greater perturbation in octanoyl-_meACP_ than hydroxybutyryl-_meACP_. Note that the three residues Ser971, Gly975 and Thr1000 are affected by both phosphopantetheinylation and extension of the acyl chain, indicating that elongation of acyl chain may also alter the position or conformation of the phosphopantetheinyl moiety. These perturbed residues of octanoyl-_meACP_ are mapped on the protein and most of them are located on helix α2, loop-2 and as well as helix α3 (Figure 7H).

![Figure 6. Overlaid 1H-15N HSQC spectra of _apo-_ _meACP_ and its derivatives. _apo-_ _meACP_ (black), _holo-_ _meACP_ (A, yellow), hydroxybutyryl-_ _meACP_ (B, green) and octanoyl-_ _meACP_ (C, red) acquired at 15°C. The change of chemical shifts for the residues Ser971 and Gly975 from _apo_, _holo_, hydroxybutyryl to octanoyl-_meACP_ are indicated by the black arrows. doi:10.1371/journal.pone.0020549.g006](https://www.plosone.org/doi/10.1371/journal.pone.0020549.g006)
Figure 7. Chemical shift perturbations ($\Delta\delta$) of meACP caused by phosphopantetheinylation and acylation. Combined chemical shift change plots of apo-meACP versus (A) holo-meACP, (B) hydroxybutyryl-meACP and (C) octanoyl-meACP. Combined chemical shift change plots of (D) hydroxybutyryl-meACP versus holo-meACP, (E) octanoyl-meACP versus holo-meACP and (F) octanoyl-meACP versus hydroxybutyryl-meACP. A combined chemical shift cut-off of 0.05 ppm is shown as the dashed line. The residue number and the positions of the helices ($\alpha_1$, $\alpha_2$ and $\alpha_3$) are also indicated. (G) Ribbon representation of apo-meACP with the positions of the perturbed residues upon phosphopantetheinylation are highlighted (combined chemical shift cut-off $>0.05$ ppm (yellow sphere) and $>0.10$ ppm (red sphere)). (H) Ribbon representation of apo-meACP with the positions of the perturbed residues based on (F) upon acylation by octanoyl moiety are highlighted (combined chemical shift cut-off $>0.05$ ppm (blue sphere)). doi:10.1371/journal.pone.0020549.g007
Interaction between meACP and the thioesterase CalE7

meACP domain must interact with the ketoacyl synthase (KS), acyltransferase (AT), Ketaoreductase (KR), dehydratase (DH) and PPTase domains of CalE8 for the synthesis of the polyene product as well as the thioesterase CalE7 for hydrolytic release of the product. It would be interesting to know the molecular basis of the interaction between meACP and the catalytic domain of CalE8 as well as CalE7. However, cloning and expression of the stand-alone catalytic domains of CalE8 failed to produce soluble proteins. We were only able to examine the interaction between meACP and the hot-dog fold thioesterase CalE7 in an attempt to map out the recognition surface of meACP for CalE7. When we first determined the crystal structure of CalE7, a few charged residues at the entrance of the substrate-binding tunnel were speculated to be involved in binding meACP [42]. However, when the thioesterase CalE7 was titrated into the 15N-labeled apo- and holo-meACP protein solution for NMR measurement, no HSQC peak was perturbed in both chemical shifts and intensity even when the ratio of CalE7 to meACP reached 2.5:1. This indicates that the interaction between CalE7 and meACP is too transient or weak to be detected (Figure S1 and S2). The transient nature of the CalE7 and meACP interaction is further supported by the observation that none of the charged residues at the substrate-binding channel is conserved in the homologous DynE7 protein [42]. In conjunction with the observation that a catalytic incompetent mutant of CalE7 (but not the wild type CalE7) can form stable 1:1 complex with CalE8 with the conjugated polyene products remaining attached to the ACP domain [42], it is reasonable to believe that the interaction between meACP and CalE7 is rather weak and the recognition is through the binding of the nascent polyene product in the hydrophobic substrate channel of CalE7. The weak transient interaction will be further explored using paramagnetic relaxation enhancement approaches.

Discussion

As the first structure determined for the ACP domain of a highly-reducing (HR) iterative type I PKS, meACP shares extremely low sequence homology with other known ACPs, including the ACP domain of the non-reducing (NR) iterative type I PKS NSAS (12% identity). meACP also features an unusual HMSS1 motif (or H(L/M)(S/T/N)(I/L) for meACP homology) harboring the putative Ser971 phosphopantetheinylation site, in contrast to the more common GX(H/D)(S/L/I) motif seen in other ACPs. We have previously performed site-directed mutagenesis to show that Ser971, but not Ser970, is required for the enzymatic function of CalE8 (data not shown). The specificity towards Ser971 must be determined by the location of the residue and its proximity to the CoA substrate in the meACP-PPTase complex. As there is a ~7.0 Å distance between the two hydroxyl groups for the two Serine residues, it is unlikely for the PPTase domain to modify the wrong Serine (Ser970).

Despite the low sequence homology and the uncommon HMSS1 motif, meACP adopts a helix-bundle structure that is highly similar to other ACPs with the exception of the absence of the short helix within loop-2. As a result of the missing short helix, the structure of meACP is best described as a twisted three-helix bundle instead of the canonical four helix-bundle structure. In addition, the relatively shortage of negative charges on the ‘recognition helix’ (a2) of meACP is consistent with the observations for other type I FAS and PKS ACPs, presumably resulted from the lack of evolutionary selection pressure on type I ACP domains for specific domain-domain interactions within the multidomain FAS or PKS protein.

It is well known that loop-2 of type II FAS and PKS ACPs generally consists of charged and less bulky residues to confer great conformational mobility to the loop; whereas type I ACPs tend to contain bulky hydrophobic residues on the loop to restrict the mobility of the loop through hydrophobic interaction with the protein core. In light of this, meACP harbors two non-polar residues (Met992 and Phe996) with the side chains packed against the protein core. Similar hydrophobic interactions were recently reported for the ACP domains of the rat type I FAS, the non-reducing (NR) type I iterative PKS NSAS and the type I modular PKS DEBS [9,33]. The protection of the intermediates is considered to be particularly important for type II FASs and PKSs because the free-standing ACPs need to transport the intermediates from one protein to another in the cytoplasm. The conformational mobility of loop-2 has been suggested to be crucial for the opening of a hydrophobic cleft for sequestering and protecting acyl intermediates. Such protection, however, is less critical for type I FASs and PKSs with the integrated ACP domain and intermediates probably already shielded from solvent by the surrounding catalytic domains. The structural observations thus seem to reinforce the view that restricted conformational mobility of loop-2 resulted from interaction of the non-polar residues may be a common feature for the ACP domains of the multidomain type I FAS and type I iterative PKS.

Despite the hydrophobic interaction between Met992 and Phe996 with the protein core, the H15N NOE plot revealed relatively greater conformational flexibility for loop-2 than loop-1 and the three helices (Figure 5D). This observation first raised the question whether loop-2 of meACP still can undergo conformation change to create a transient protein pocket for binding the highly hydrophobic polyene products. Attachment of 4′-phosphopantetheine and the additional hydroxysterbutyl group to the Ser971 site has little effect on the overall protein fold, despite minor local perturbations restricted to residues in the vicinity of Ser971 (Figure 7). These observations suggest that the phosphopantetheine and hydroxysterbutyl moieties are unlikely to be bound in a protein pocket and thus remain in the bulk solvent. In contrast, the NMR spectra of the octanoyl-meACP show more extensive perturbations, with the perturbed residues distributed in a region flanked by helix 2, loop-2 and helix 3 (Figure 7H). The magnitude of the perturbation is considered to be relatively small compared to that for the type II ACPs that sequester the acyl chain through a significant conformational change [13,43]. Hence, the results do not seem to support that meACP is able to undergo dramatic conformational change and sequester the PKS product in a well-defined protein pocket. Instead, examination of the electrostatic potential surface of meACP reveals two distinct patches, a unique hydrophobic patch and an acidic patch unlike other ACPs currently deposited in Protein Data Bank (PDB) (Figure 8). The rather common charged patch encompassing the recognition helix 2 observed in other ACPs, is replaced by an unusual hydrophobic patch in meACP (Figure 8E). meACP also features an acidic patch that consists mainly Glu residues, including Glu940 and Glu948 from helix 2, Glu941 and Glu953 from loop-1, Glu980 from helix 2, and Glu957, Glu959, Glu1012 and Glu1014 from helix 3 (Figure 8I and Figure 9). No such hydrophobic or acidic patch has been observed in other type I ACPs, such as the rat FAS ACP and NSAS PKS ACP or type II ACPs such as actinorhodin PKS ACP (Figure 8) [33]. Given the location of the hydrophilic patch and the observed NMR perturbations with the eight residues in the distinct hydrophobic surface of meACP, it is feasible that the non-polar octanoyl chain may interact with hydrophobic patch to avoid the unfavorable solvation of the hydrophobic groups. Such hydrophobic interac-
Figure 8. Embedded ribbon representation and electrostatic potential surface of ACPs. Type I meACP (A, E and I), rat FAS ACP (B, F and J, PDB ID: 2PNG), NSAS PKS ACP (C, G and K, PDB ID: 2KR5) and Type II actinorhodin PKS ACP (D, H and L, PDB ID: 1AF8). The invariant Ser is shown as blue stick and labeled. The protein surfaces are colored as white (hydrophobic), blue (basic) and red (acidic) with the same electrostatic potential scale. doi:10.1371/journal.pone.0020549.g008

Figure 9. Ribbon representation of apo-meACP with either hydrophobic or acidic face. The hydrophobic and Glu-Asp acidic faces are shown with highlighted spheres colored red (Glu) and orange (Asp). doi:10.1371/journal.pone.0020549.g009
tion may play a significant role in the stabilization of unsaturated polyene intermediates during chain elongation. Alternatively, the hydrophobic patch could be conserved for domain-domain interaction. The latter remains a possibility considering that this region also encompasses the helix 21 that is most likely to be involved in protein-protein interaction.

The biosynthetic mechanisms for the 10- and 9-membered endomycin moieties and the precise function of CalE8 and its homologs are currently under intensive investigation. The first structure of the HR iterative PKS ACP domain presented here not only reveals some interesting structure features that reinforce some of the views held for type I FAS and PKS ACPs, but also discloses some unique characteristics of the meACP domain. The lack of a strong negatively charged helix 21 and the moderate conformational flexibility of loop-2 are consistent with the role of the meACP domain in shuttling acyl intermediates within the catalytic chamber of the multidomain PKS CalE8. The absence of the short helix within loop-2 is rare but not totally unprecedented. The interaction between the acyl aliphatic chain and the hydrophobic patch is distinct from the well-established interaction between acyl chains and the well-defined protein pocket. Although the biological significance of the hydrophobic patch and highly acidic patch remain to be established, the hydrophobic patch may be involved in the interaction with other catalytic domain, or play a role by interacting and stabilizing the intermediates and products of CalE8. Lastly, the lack of significant binding between meACP and its cognate protein partner CalE7 indicates that specific strong protein-protein recognition is not crucial for CalE7 to bind and perform the hydrolytic cleavage on the product of CalE8.

Materials and Methods

Protein cloning, expression and purification

The cloning of meACP domain has also been described previously [27]. The ACP and catalytic domains of CalE8 were cloned into compatible vectors and transformed into BL21 cells for expression. Seed culture of BL21 cells containing the respective plasmids were grown overnight before being inoculated into large scale cultures. The cultures were allowed to grow at 37°C and 200 rpm until the OD600nm reached 0.6. Induction was done with IPTG at 0.4 mM concentration. Cells were harvested 20 hours later after being left to grow at 16°C and 160 rpm. The cell pellet was then resuspended in lysis buffer (50 mM HEPES at pH 7.5, 300 mM NaCl, 5% glycerol, 5 mM β-mercaptoethanol). Subsequently the cells were lysed by sonication. The supernatant obtained after high speed centrifugation was applied onto Ni²⁺-NTA column for purification with a stepped gradient. The final eluent containing the recombinant protein was applied onto gel-filtration column Superdex75 or Superdex200 depending on the size of the individual protein for further purification in a buffer suitable for NMR experiments (50 mM NaH₂PO₄/Na₂HPO₄ at pH 6.85, 50 mM NaCl, 1 mM DTT). The fractions containing the protein were pooled and concentrated. After that, the proteins solutions were stored away in the −80°C freezer for future NMR analysis. The same expression and purification protocols were used to prepare ¹⁵N, ¹³C-labeled meACP protein by using an M9 medium containing ¹⁵N-isotopic ammonium chloride and ¹³C isotopic glucose. The labeled protein obtained from gel filtration was further purified with a Mono Q column. A refined gradient was employed using buffer A (50 mM NaH₂PO₄/Na₂HPO₄, 1 mM DTT) and buffer B (50 mM NaH₂PO₄/Na₂HPO₄, 1 M NaCl, 1 mM DTT). The salt gradient increased gradually from 0% to 40% of B in 50 ml. The majority of the meACP did not bind to the column and was eluted at early stage of the gradient. The fractions containing meACP were pooled and concentrated for NMR as well as acylation experiments. The thioesterase CalE7 were cloned, expressed and purified as described [23,34]. The PPTase Sfp used for modification of meACP was prepared according to the established procedure [27].

Preparation of holo- and acylated-meACP

The reaction for the production of holo- and hydroxybutyryl-meACPs were summarized as follows: 400 μl of ACP (12 mg/ml), 100 μl of Sfp (68 mg/ml), 40 μl of CoA or acyl-CoA (50 mM) and 10 μl of MgCl₂ (1 M) were mixed together in 1 ml of reaction buffer (100 mM Tris at pH 8.2, 300 mM NaCl, 1 mM DTT). The reaction was carried out at 30°C for 8–12 hours. The progress of the conversion was monitored by HPLC analysis using an eclipse XDB RP C8 column at 2 hour intervals. The gradient employed was from 10% of acetonitrile to 90% acetonitrile in half an hour. Elution of meACP and Sfp were monitored at the wavelength of 220 nm. Upon completion of the reaction, the mixture was desalted with NaH₂PO₄/Na₂HPO₄ buffer without NaCl to bring down the NaCl concentration to ~50 mM. Aforementioned refined gradient was employed using MonoQ for the separation of holo- or acylated-meACP from Sfp. A gel filtration step with Superdex-75 column was added to further improve the purity of the holo- and hydroxybutyryl-meACP. For the production of octanoyl-meACP, the reaction buffer was modified to have the following composition: 50 mM NaH₂PO₄/Na₂HPO₄ at pH 7.0, 200 mM NaCl, and 1 mM DTT. The duration of the reaction was prolonged to 16 to 20 hours at 25°C and the conversion rate for the modifications is >95%.

NMR spectroscopy

All NMR experiments were performed on an 800 MHz NMR spectrometer (Bruker) unless otherwise indicated. All NMR samples including ACPs and CalE7 were prepared in a buffer with 50 mM NaH₂PO₄/Na₂HPO₄ at pH 6.85, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% D₂O. Because the meACP protein has weak UV absorbance at 280 nm due to the lack of Trp residues, the protein concentrations were determined by Bradford assay at 595 nm. To determine the structure of apo-meACP, we collected the following NMR spectra on a ~1 mM ¹³C,¹⁵N-labeled protein sample: 2D ¹H-¹⁵N HSQC, 2D ¹H-¹³C HSQC, 3D HN/CO/CA, 3D HNCA, 3D MQ/HCCH-TOSY [44] and 4D timeshared ¹³C/¹⁵N edited-NOESY [45]. To measure the heteronuclear NOEs, two spectra without and with proton saturation were measured on a ~1 mM ¹³C/¹⁵N-labeled sample with a saturation delay of 4 s and recycle delay of 4 s by the inverse-detected 2D NMR method. Proton saturation was achieved by a train of 120° pulses spaced at 5 ms.

In the thioesterase CalE7 titration experiments, an initial concentrated unlabeled CalE7 protein solution (0.8 mM) was added to ¹³N-labeled apo-meACP (0.3 mM) or ¹⁵N-labeled holo-meACP (0.3 mM). The samples were first allowed to incubate for 10 min prior to NMR data collection. TROSY-HSQCIs were recorded at three CalE7 concentrations (0 mM, 0.22 mM and 0.38 mM) in the same ACP buffer till a final [meACP] of 0.15 mM or molar ratio [CalE7]: [meACP] of 2.5:1. All data were processed with NMRpipe and analyzed with NMRPipe and an extension XYZ4D [http://yangdw.science.nus.edu.sg/Software&Scripts/XYZ4D/index.htm]. NMRSpy was recently developed to facilitate resonance assignment with the 4D NOESY-based strategy [46].

¹H-¹³C HSQCIs of apo-, holo-, hydroxybutyryl- and octanoyl-meACP (0.1 mM) were acquired at 15°C instead of 25°C on an 800 MHz NMR spectrometer. This allows us to observe Ser²⁷⁸ which was predominantly weak at higher temperature (>20°C using 0.1 mM
of acylated ACPs. The combined chemical shift perturbation was calculated using equation 1:

\[ \Delta_{\text{ppm}} = \left( \left( \Delta\delta_{\text{HN}} \right)^2 + \left( \Delta\delta_{\text{N}} / 7 \right)^2 \right)^{0.5} \]

where \( \Delta\delta_{\text{HN}} \) and \( \Delta\delta_{\text{N}} \) are the respective differences of \(^1\text{H} \) and \(^{15}\text{N} \) chemical shifts between different forms of meACP: apo-meACP, holo-meACP or acylated-meACP (hydroxybutyryl- and octanoyl-meACP).

Structure calculation

NOE restraints were obtained from the timeshared 4D \(^{13}\text{C} /^{15}\text{N} \)-edited NOEY (containing \(^1\text{H} \), \(^{13}\text{C} \), \(^{15}\text{N} \)-edited, \(^{13}\text{C} \), \(^{15}\text{N} \)-edited subspectra) using NMRspy. Backbone torsional angle restraints, \( \Phi \) and \( \Psi \) were obtained from chemical shift data using TALOS [47]. Ambiguous NOEs were assigned with the iterative structure calculation method using Cyana 2.1 [48]. The final 10 lowest energy structures out of 100 calculated were selected. The quality of the structure was assessed using PROCHECK [49].

The NMR assignment data is deposited in the BioMagResBank under BMRB accession number 17355. The coordinates of the ensemble of 10 structures have been deposited in the Protein Data Bank (PDB ID: 2LF9).

Supporting Information

Figure S1 \(^1\text{H}-^{15}\text{N} \) HSQC of \(^{15}\text{N} \)-labeled apo-meACP titrated against unlabeled CalE7. The titration is performed at 25°C in monomeric apo-meACP: CalE7 molar ratio of (A) 1:0 (black) (B) 1:1 (blue) (C) 1:2.5 (red) and (D) an overlaid spectrum between (A) and (C).

Figure S2 \(^1\text{H}-^{15}\text{N} \) HSQC of \(^{15}\text{N} \)-labeled holo-meACP titrated against unlabeled CalE7. The titration is performed at 25°C till monomeric holo-meACP: CalE7 molar ratio of (A) 1:0 (black) (B) 1:1 (blue) (C) 1:2.5 (red) and (D) an overlaid spectrum between (A) and (C).

Author Contributions

Conceived and designed the experiments: DY Z-XL. Performed the experiments: JL RK EM CLH. Analyzed the data: JL RK. Wrote the paper: JL Z-XL RK DY.

References

1. Smith S, Tsai SC (2007) The type I fatty acid and polyketide synthases: a tale of two megasynthases. Natural Product Reports 24: 1041–1072.
2. Mercier AC, Burkart MD (2007) The ubiquitous carrier protein—a window to metabolic biosynthesis. Nat Prod Rep 24: 750–771.
3. Jenni S, Leibundgut M, Boehringer D, Frick C, Mikolaask M, et al. (2007) Structure of fungal fatty acid synthase and implications for iterative substrate shuttling. Science 316: 254–261.
4. White SW, Zheng J, Zhang VM, Rock CO (2005) The structural biology of type I fatty acid biosynthesis. Annu Rev Biochem 74: 791–831.
5. Roujeinikova A, Simon WJ, Gilroy J, Rice DW, Rafferty JB, et al. (2007) The crystal structure of the actIII actinorhodin polyketide reductase: Proposed mechanism for ACP and polyketide binding. Structure 12: 1865–1875.
6. Xu GY, Tam A, Lin L, Hixon J, Fritz CC, et al. (2001) Solution structure of Pseudomonas Aeruginosa. Protein Expr Purif 71: 132–138.
7. Lai JR, Koglin A, Walsh CT (2006) Carrier protein structure and recognition in polyketide and nonribosomal peptide biosynthesis. Biochemistry 45: 14890–14879.
8. Kelly KL, Krumm C, Mowat DR, Barry LL, Angus SL, et al. (2008) A mammalian type I fatty acid synthase acyl carrier protein domain does not sequester acyl chains. J Biol Chem 283: 518–528.
9. Reed MC, Schweizer M, Szafrańska AE, Arthur CG, Nicholson TP, et al. (2003) The type I rat fatty acid synthase ACP shows structural homology and analogous biochemical properties to type II ACPs. Organic & Biomolecular Chemistry 1: 463–471.
10. Leibundgut M, Jenni S, Frick C, Ban N (2007) Structural basis for substrate delivery by acyl carrier protein in the yeast fatty acid synthase. Science 316: 280–290.
11. Alexandraki YY, Liu GW, Cane DE, Puglia JD, Khodai C (2007) Solution structure and proposed domain domain recognition interface of an acyl carrier protein domain from a modular polyketide synthase. Protein Sci 16: 2093–2107.
12. Evans SE, Williams C, Kirby CJ, Crosby J, et al. (2004) The structural basis of the acyl carrier protein: Strategy and characterization of the acyl carrier protein domain does not sequester acyl chains. J Biol Chem 283: 518–528.
13. Reed MC, Schweizer M, Szafrańska AE, Arthur CG, Nicholson TP, et al. (2003) The type I rat fatty acid synthase ACP shows structural homology and analogous biochemical properties to type II ACPs. Organic & Biomolecular Chemistry 1: 463–471.
14. Hadfield AT, Limplin C, Tearlasm W, Simpson TJ, Crosby J, et al. (2004) The crystal structure of the actIII acyl carrier protein from Streptomyces rimosus. Biochem Soc Trans 32: 273–276.
15. Crump MP, Crosby J, Dempsey CE, Parkinso JNA, Murray M, Hopwood DA, et al. (1996) Conserved secondary structure in the acyl carrier protein from Streptomyces coelicolor A3(2). Biochemistry 36: 6000–6008.
35. Kim Y, Prestegard JH (1990) Refinement of the NMR structures for acyl carrier protein with scalar coupling data. Proteins 8: 377–385.
36. Sharma AK, Sharma SK, Surolía A, Surolía N, Sarma SP (2006) Solution structures of conformationally equilibrium forms of holo-acyl carrier protein (ACP) from Plasmodium falciparum provides insight into the mechanism of activation of ACPs. Biochemistry 45: 6904–6916.
37. Parris KD, Lin L, Tam A, Mathew R, Hixon J, et al. (2000) Crystal structures of substrate binding to Bacillus subtilis holo-(acyl carrier protein) synthase reveal a novel trimeric arrangement of molecules resulting in three active sites. Structure 8: 883–885.
38. Arthur CJ, Williams C, Pottage K, Ploskon E, Findlow SC, et al. (2009) Structure and malonyl CoA-ACP transacylase binding of streptomyces coelicolor fatty acid synthase acyl carrier protein. ACS Chem Biol 4: 625–636.
39. Li Q, Khosla C, Puglisi JD, Liu CW (2003) Solution structure and backbone dynamics of the holo form of the frenolicin acyl carrier protein. Biochemistry 42: 4648–4657.
40. Zornetzer GA, Fox BG, Markley JL (2006) Solution structures of spinach acyl carrier protein with decanoate and stearate. Biochemistry 45: 5217–5227.
41. Zornetzer GA, Tanem J, Fox BG, Markley JL (2010) The Length of the Bound Fatty Acid Influences the Dynamics of the Acyl Carrier Protein and the Stability of the Thioester Bond. Biochemistry 49: 470–477.
42. Liew CW, Sharff A, Kotaka M, Kong R, Sun H, et al. (2010) Induced-fit upon ligand binding revealed by crystal structures of the hot-dog fold thioesterase in dynemicin biosynthesis. J Mol Biol 404: 291–306.
43. Ploskon E, Arthur CJ, Kanari ALP, Wattana-amorn P, Williams C, et al. (2010) Recognition of intermediate functionality by acyl carrier protein over a complete cycle of fatty acid biosynthesis. Chem Biol 17: 776–785.
44. Yang DW, Zheng Y, Liu DJ, Wyss DF (2004) Sequence-specific assignments of methyl groups in high-molecular weight proteins. Journal of the American Chemical Society 126: 3710–3711.
45. Xu YQ, Long D, Yang DW (2007) Rapid data collection for protein structure determination by NMR spectroscopy. Journal of the American Chemical Society 129: 7722–7723.
46. Xu YQ, Zheng Y, Fan JS, Yang DW (2006) A new strategy for structure determination of large proteins in solution without deuteration. Nature Methods 3: 931–937.
47. Cornilescu G, Delaglio F, Bax A (1999) Protein backbone angle restraints from searching a database for chemical shift and sequence homology. Journal of Biomolecular NMR 13: 289–302.
48. Herrmann T, Guntert P, Wuthrich K (2002) Protein NMR structure determination with automated NOE assignment using the new software CANDID and the torsion angle dynamics algorithm DYANA. Journal of Molecular Biology 319: 209–227.
49. Laskowski RA, Macarthur MW, Moss DS, Thornton JM (1993) Procheck - a Program to Check the Stereochemical Quality of Protein Structures. Journal of Applied Crystallography 26: 283–291.