Loading of malonyl-CoA onto tandem acetyl carrier protein domains of polyunsaturated fatty acid synthases

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ABSTRACT

Omega-3 polyunsaturated fatty acids (PUFA) are produced in some unicellular organisms, such as marine gammaproteobacteria, myxobacteria and thraustochytrids, by large enzyme complexes called PUFA synthases. These enzymatic complexes resemble bacterial antibiotic-producing proteins known as polyketide synthases (PKS). One of the PUFA synthase subunits is a conserved large protein (PfaA in marine proteobacteria) that contains three to nine tandem Acyl Carrier Protein domains as well as condensation and modification domains. In this work, a study of the PfaA architecture and its ability to initiate the synthesis by selecting malonyl units has been carried out. As a result, we have observed a self-acylation ability in tandem ACPs whose biochemical mechanism differ from the previously described for type II PKS. The acyltransferase domain of PfaA showed a high selectivity for malonyl-CoA that efficiently loads onto the ACPs domains. These results, together with the structural organization predicted for PfaA, suggest that this protein plays a key role at early stages of the anaerobic pathway of PUFA synthesis.

Polyunsaturated fatty acids (PUFA) are molecules with beneficial properties for human health synthesized among eukaryotic organisms by desaturation and elongation of shorter fatty acids (1, 2). However, it has been reported that the heterotrophic fungus-like thraustochytrids, some myxobacteria and some marine gamma-proteobacteria are able to de novo synthesize PUFAs from acyl-CoA precursors (3). These organisms accumulate PUFAs like eicosapentaenoic acid (EPA; 20:5) or docosahexaenoic acid (DHA; 22:6) by using modular proteins of the Pfa family, which show a modular organization similar to that of polyketide synthases (PKS) and fatty acid synthases (FAS) (4). Thus, current knowledge of PKS and FAS domains could help in the understanding of the mechanism by which PUFA synthases work. Pfa systems are formed by three (thraustochytrids and myxobacteria) or four polypeptides (gamma-proteobacteria). In marine bacteria like Moritella marina, that we used as a model, these proteins are named PfaA, PfaB, PfaC and PfaD (5). PfaD is predicted to contain a singular enoyl reductase (ER) domain (6), while PfaA, PfaB and PfaC are multimodular proteins, each of them formed by a series of domains that show sequence and structural homology with the ones present in PKS and FAS systems. These domains can be classified into condensing and modifier motifs according to their predicted biochemical function (7). The condensing domains are responsible for extending the fatty acid chain. They comprise acyl transferases (AT) that select the acyl building blocks, and keto synthases (KS) that polymerize these extender units by a condensation reaction. The fatty acid intermediates are held by the acyl carrier protein domains (ACP) that transfer them to the different catalytic domains. Thereupon the modifier domains, keto reductases (KR),
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dehydratases (DH) and enoyl reductases (ER), reduce intermediate keto groups to form partially or fully saturated fatty acid moieties. Unlike FAS systems, Pfa proteins have redundant domains (KS, AT, ACP and DH), whose roles are not currently clear, so there is a growing interest in understanding its biological function.

One of the first steps in FAS and PKS synthesis is the selection of extender blocks through the coordinated action of ACP and AT. For this process, ACP has to be previously activated with a 4'-phosphopantetheine prosthetic group (PPT) that acts as a linkage between the ACP active serine and the acyl group (8). ACP activation in PUFA synthases is performed by a specific 4'-phosphopantetheine transferase (PfaE in M. marina). Transfer of extender units to the terminal sulfhydryl group of PPT is carried out by an AT domain, at least in FAS and modular PKS (9). However, it has been reported the self-acylation ability of ACP in iterative PKS systems, being ACP capable of selecting the extender blocks in the absence of AT (10).

The main striking feature of Pfa systems is the presence of ACP domains in tandem, which can have from 3 to 9 repetitions with sequence identity ranging from 85 to 96% (11). A higher number of ACP domains within this tandem arrangement is directly related with improvements in the productivity of the system (12, 13). These tandem ACP domains are located in the PfaA polypeptide in all omega-3 PUFA synthases. PfaA shows a KS-AT condensing module at their N-terminal half, followed by the tandem ACPs and a KR-DH C-terminal domain (3).

The critical process for the polymerization of a polyketide or a fatty acid is the correct selection of the extender units through a collaborative process that involves AT and ACPs. Different extension units have been described in PKS synthesis (14), however FAS systems show less complexity, being primarily malonyl the extender unit of choice (15). For the selection of these building blocks the presence of specific AT is necessary. Pfa systems have two different AT domains, one in PfaA, and a second one in PfaB. This domain was reported to be involved in the determination of the final product of the omega-3 PUFA synthesis (16), but its specific biochemical activity has never been proven, neither has been clarify the differential activity of both AT domains.

In the present article we studied the loading of extender blocks onto the tandem ACPs of PfaA. The presence of two AT domains (PfaA AT and PfaB AT) that had not been fully characterized suggested us the need of a more comprehensive study. Here, we researched on the AT substrate preference, mechanism of action and interaction with the PfaA tandem ACPs, which are critical aspects to understand the omega-3 PUFA synthesis process by the Pfa family.

Results and discussion
PfaA domain organization is conserved in all omega-3 PUFA synthases
Pfa gene clusters of marine bacteria like M. marina (Figure 1A) are formed by 3 genes that code for multimodular proteins (PfaA, PfaB and PfaC) and a fourth gene that codes for a single protein domain (PfaD). Like in PKS or FAS, the basic domain architecture contains keto synthase (KS), acyl transferase (AT), acyl carrier protein (ACP), keto reductase (KR) and dehydratase (DH) domains. Despite the general lack of biochemical evidences to assign a role to each redundant domain of the Pfa system, a general outline is normally assumed (4). An AT domain should load the malonyl extender units onto the ACPs, a KS domain adds these blocks to the growing chain by successive condensations and the KR, DH and ER domains modify the keto groups formed in each condensation to form the fatty acid unsaturation pattern (Figure 1B). Besides the redundancy of KS and DH domains in omega-3 PUFA synthases, the main difference between its biochemical pathways and that of PKS are the acyl precursors used during the synthesis as well as the characteristic cyclization patterns of PKS that involves specific enzymes.

To analyze the M. marina PfaA (mmPfaA) protein sequence and make a preliminary prediction of its domains, the InterPro online server (17) was used. The predicted domain organization of mmPfaA (Figure 1C) shows five different folding motifs along its 2,652 amino acids. From the N-terminal to the C-terminal domain the predictions were as follows: a KS domain (aa 29-487), an AT domain (aa 601-923), five ACP
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domains in a tandem arrangement with an overall sequence identity of 82% (aa 1255-1768), two KR domains (aa 1810-1992 and 2037-2303) and a DH domain (aa 2366-2642).

Structural predictions made with Phyre2 (18) by using close-related solved structures as templates (listed in Experimental Procedures) also support the identity of the defined domains (Figure 1C). We have found a duplication of the thiolase fold, typical of KS domains (19) represented in purple, and a ferredoxin-like subdomain (in grey) followed by an α/β hydrolase fold that corresponds to the AT domain (20, 21) represented in blue. Five repetitions of the four α-helices in an up-down-down topological arrangement that correspond to the ACPs (22) are represented in pink. Two repetitions of the Rossman fold (23) with a twisted, parallel β-sheet composed of seven β-strands flanked in both sides by eight α-helices that correspond to two KR domains represented in cyan and green and a repetition of the hot-dog fold (24) that corresponds to the DH domain represented in red.

Comparisons with characterized similar domains helped us to identify the residues presumably involved in catalysis. The KS active site is formed by the C229-H364-H404 triad involved in the condensation reaction while the AT active residue responsible of the extender unit binding was identified at S703 (Figure S1). The five serine active residues of the ACPs responsible for PPT binding were localized at S1293, S1395, S1496, S1597 and S1703, respectively (Figure S2). KR catalytic triad is formed by the K2195-S2219-Y2232 residues while N2236 is involved in the formation of the proton-wire (Figure S3). We have predicted the existence of two differentiated KR-like structures identified as KR’-KR, where KR’ is a pseudo-KR domain that lacks sequence homology with any described catalytic KR, but shows structural homology with KR domains of PKS type I and FAS systems. KR’ does not contain any of the catalytic residues conserved in KR domains (25, 26), indicating that this KR’ domain is a structural element of PfaA. This full KR’-KR domain is present in the marine bacteria analyzed but partially absent in myxobacteria and thraustochytrids. Within KR catalytic domain, the Asn/Lys catalytic dyad involved in proton replenishment (N2236 and K2195 in PfaA) are swapped with respect to KR domains from PKS type II (27). This swapping also occurs in mammalian FAS and PKS type I, which indicates an evolutionary relationship of the PfaA protein with FAS and PKS type I systems (Figure S4). Finally, H2379-D2563 (Figure S5) are predicted to be the active residues of the C-terminal DH domain involved in the reduction of the hydroxyl group generated at the keto reduction step. This C-terminal DH domain was not described in most of the previous publications that assume the existence of a larger KR domain (5, 7, 12, 16, 28). We have proved here the conservation of the residues conforming the active sites of all predicted domains by doing sequence alignments with distant homologs of FAS and PKS systems and also with homologous proteins of gammaproteobacteria, myxobacteria and thraustochytrids (Figures S1, S2, S3, S4 and S5), all of them previously reported omega-3 PUFA producers (29).

Thus, our bioinformatic analysis predicted a KS-AT-ACP(x5)-KR’-KR-DH organization in PfaA. This organization is conserved among PfaA-like proteins, also named Pfa1 in thraustochytrids, or Pfa2 in myxobacteria (3, 4) although the number of ACP domains is variable and the KR’ structural domain is absent in some systems. In fact, ACP repetitions can vary from three in Shewanella pealeana to nine in some thraustochytrids (11, 30). The conservation of this scheme indicates that this particular structural conformation may be essential for the proper function of the PUFA synthase protein complex.

Tandem ACPs of PfaA are self-acylated with malonyl-CoA

An essential role in polyketide or FA synthesis is performed by ACP domains. These domains determine the final product by selecting and carrying the extender units that will be condensed and modified by other enzymatic Pfa domains. PfaA-like proteins show the peculiarity of having a variable number of tandem ACP domains. Thus, we were interested in studying the specificity of these ACP domains within PUFA synthases to understand the synthesis process.

Based on the information obtained by in silico analysis, we have cloned into the expression vector pET29c a DNA fragment
coding for the predicted 5 tandem ACP domains of *M. marina* PfaA, as described in Experimental Procedures. Using this construction, pET29c::5ACP, we have purified these 5 tandem ACP domains in their apo form. The tandem ACPs construction was also co-expressed with mmPfaE, the phosphopantetheinyll transferase (PPTase) of *M. marina*. mmPfaE was cloned into an expression vector with a different antibiotic resistance (pET3a vector), so that we could directly purify the active ACP holo-form by coexpression of pET29c::5ACP and pET3a:PfaE plasmids (Figure 2A).

To investigate the mmACP substrate preference and test their possible ability to transfer and incorporate acyl groups, we incubated 20 μM of purified apo or holo-mm5ACP in 20μl reactions with 14C-radiolabeled malonyl-CoA or 14C-radiolabeled acetyl-CoA. Results (Figure 2B) indicate that the holo form of mm5ACP was able to perform self-acylation with malonyl-CoA (lane 3) but did not show activity against acetyl-CoA (lane 4). This catalytic activity associated to holo-ACPs has been extensively studied in the literature, being normally linked to PKS systems but not widespread in FAS (10, 31–34). A couple of examples of FAS proteins with self-acylation activity have also been described (10), specifically from *Plasmodium falciparum* and *Brassica napus*, being thus ACP self-acylation not exclusive from PKS systems. The self-malonylation ability of mm5ACP from PUFA synthases pointed out for the first time a functional homology of these domains with the ones present in PKS and other self-acylating systems. mm5ACP purified in the absence of PfaE, apo mm5ACP, did not react with any of the two substrates analyzed (lanes 1 and 2). Thus, mmPfaE seems to be essential for mm5ACP PPT binding and cannot be substituted by the *E. coli* homolog 4'-phosphopantetheinyll transferase (Acc. no. P37623).

We have demonstrated the ACP ability to be self-malonylated in its natural tandem arrangement form. This activity has also been reported for ACP domains of PKS II and some FAS systems. Unlike FAS systems, auto-malonylation activity is necessary in PKS II due to the absence of an associated AT domain (32). However, Pfa systems include one or two AT domains in their architecture, so, the presence of independent self-acylating ACPs in PUFA synthases was not foreseen. The ACPs self-malonylation ability proved here provides additional evidence of the evolutionary position of PUFA synthases between FAS and PKS systems. Besides that, the demonstrated ACP relative independence raises questions about the actual role of the *cis-* and *trans-* AT domains (PfaA AT and PfaB AT domains, respectively) that may not be essential to the synthesis process.

### PfaA AT domain promotes malonyl-CoA binding to ACPs

The core components required to produce a regular fatty acid condensation contain three domains, KS, AT and ACP, that perform one cycle of chain extension. An acetyl group or starter unit is attached to the cysteine thiol of the KS domain and the chain extender unit is selected by the AT and bound to a thiol residue of the ACPs PPT where is ready for condensation. Thus, AT is the domain responsible for selecting the molecules that will be polymerized to form the backbone of the fatty acid (Figure 1B). The extender unit is malonate in FAS, but can be malonate, methylmalonate, ethylmalonate or other extender units in PKSs. Although we have demonstrated that mmPfaA ACPs are able to perform self-malonylation we also checked if the mmPfaA AT domain stimulates this malonylation.

Since KS-AT di-domain usually functions as a tightly coordinated protein (25), both mmPfaA domains (mmKSAT) were initially included in this experiment as a single polypeptide. The concentration of proteins and substrates was experimentally established to improve the visualization of the bands. As described in experimental procedures, 5 μM mmKSAT, 20 μM mm5ACP and 20 μM 14C-radiolabeled malonyl-CoA or 14C-acetyl-CoA were incubated in a 20μl reaction volume. mmKSAT was able to bind covalently 14C-radiolabeled malonyl (Figure 2D, lane 2). However, under similar conditions 14C-acetyl binding to mmKSAT was not observed (Figure 2D, lane 1). The mmKSAT di-domain was subsequently incubated with both substrates in the presence of mm5ACP. When mmKSAT was mixed with mm5ACP and malonyl-CoA...
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(Figure 2D, lane 6), a stronger radioactive signal was detected, bound to mm5ACP, demonstrating that the mmKSAT di-domain transfers the malonyl extender units to the mmACPs. Quantification of the mm5ACP band intensity showed a significant increase of 3.5±0.68 times when mmKS-AT is added to the experiment (Figure 2E). 14C-radiolabeled acetyl was not transfer to ACP (Figure 2D, lane 5) and the presence of both 14C-radiolabeled acetyl and 14C-radiolabeled malonyl did not change the binding activity (lane 7).

To prove that this overloading effect was due to the activity of the AT domain within the mmKSAT di-domain, an inactive mutant for the KS active site (C229A) was used. We observed that this mutant was perfectly able to bind malonyl by itself (Figure 2F, lane 2) and transfer this malonyl to ACP, when present (lane 3). As expected, acetyl-CoA does not have any effect on the reaction (Figure 2D, lanes 1 and 4). As a complement to this experiment, we designed a construction carrying only the AT domain. The isolated AT domain (mmAT) was also able to bind malonyl but not acetyl (Figure 2D, lanes 6 and 5, respectively). Moreover, mmAT effectively loaded only the malonyl groups units, not the acetyl groups, onto mm5ACP (lanes 8 and 7), demonstrating structural and functional independence from its KS partner.

PfaA AT domain was highly specific for malonyl-CoA and able to load these extender units onto its adjacent ACPs. Despite the ACP self-malonylation activity, PfaA AT domain was needed to reach a higher level of malonyl-ACP (Figure 2D, lanes 4 and 6). This fact highlights that ACPs are not fully independent, thus the adjacent AT domain could be able to modulate the ACPs acylation state during the omega-3 PUFA synthesis. On the other hand, as the ACP domain is present from three to nine times in PfaA-like proteins, its stoichiometry in relation with the AT domain must be from 3:1 to 9:1, so the ACPs self-acylating ability may have emerged to drive high reaction rates, as was previously suggested in the literature in relation with its tandem organization (11–13).

Surprisingly, and as a remarkable difference with FAS systems, we were not able to demonstrate acetyl binding with any of the domains analyzed. In all previously described models for FAS it is always necessary to prime the system with acetyl units to perform the first decarboxylative condensation (Wakil et al., 1983). Biosynthesis in bacterial aromatic PKS is initiated by priming acetate as starter unit, but some aromatic PKSs can use different starter units (Ray and Moore, 2016) or prepare their starter units through decarboxylation mechanisms (Szu et al., 2011). Because Pfa are hybrid systems located diffusely between FAS and PKS, it is difficult to speculate about the nature of this initiator system. Despite having defined the ability of mmAT to activate the ACPs with malonyl, a more comprehensive characterization of the system is needed to elucidate how the acetyl seed is incorporated to the nascent chain (in case such starter molecule is necessary).

A comparative study reveals evolutionary divergences between ACPs

To study the mmACP conserved residues that may be involved in the protein function, we have performed a multiple sequence alignment (Figure S2), that includes representative single ACP domains of PfaA-like proteins from gammaproteobacteria, myxobacteria and thraustochytrids. Three conserved motifs were found in the alignment. The active site motif containing the conserved serine involved in PPT binding, the “TGY” conserved motif and the “RT” dyad. The structure of each of the five mmACP domains is predicted to be a four-helix bundle (Figure 1C) like all the ACP structures solved so far. The three conserved motifs are located at the same side of the bundle with TGY and the active site between α1 and α2 and RT between α3 and α4 (Figure 3B). Based on this preliminary analysis we have compared the first ACP domain of M. marina (mmACP1; aa I2155-A1348) and other ACPs from marine bacteria with domains of homologous PKS and FAS systems, including the two exceptional examples with self-acylation activity (P. falciparum and B. napus) (Figure 3A). The results of this analysis revealed that mmACP1 T1272 of the “TGY” conserved motif maps at the position where PKS II homologs conserve a negatively charged D/E residue and FAS proteins show hydrophobic residues. We did not find any conservation pattern at the position of mmACP1 residue Y1274 of the “TGY” conserved motif. “RT” dyad residue T1323 of mmACP1 is fully conserved across all domains.
analyzed and the conservation of R1322 found in omega-3 PUFA producers was not observed in FAS nor PKS, having indistinctly neutral or charged residues in that position.

As a result of the previous analysis, the threonine and tyrosine of the “TGY” conserved motif and the “RT” dyad (Figure S2) were taken into consideration as possible residues involved in the acylation of the ACPs. WT holo mmACP1 as well as four holo mmACP1 constructions with alanine substitutions in these four residues (T1272, Y1274, R1322 and T1323) completely conserved among omega-3 PUFA producers (Figure 3B) were designed and purified to test their possible implication in the ACP malonylation process.

First the self-malonylation capacity of mmACP1 protein was analyzed. As shown in Figure S6A, mmACP1 was malonylated in the presence of 14C-radiolabeled malonylCoA as described for mm5ACP. Thus, mmACP self-malonylation activity does not depend on the tandem organization of mm5ACP, rather it seems to be an intrinsic property of each domain. As expected, apo mmACP1 did not react with malonylCoA. We have then assayed the self-malonylation of the four mmACP1 mutants (Figure S6A, lanes 3, 4, 5 and 6). All the four mutants showed similar ability to covalently bind malonyl by themselves with the same enzymatic efficiency (Figure S6B). Finally, this single mmACP1 domain and its four mutants were tested as substrates of the mmKSAT didomain in binding assays with 14C-radiolabeled malonyl and purified mmKSAT (Figure S6C). We have observed that mmACP1 and each of the four mutants were identically loaded with malonyl by mmKSAT (Figure S6D).

When the binding activity of mmACP1 and mm5ACP was compared in the presence of mmKSAT (Figure 4A), mm5ACP bound radioactive malonylCoA per mol was 6.6±1.7 times higher than in mmACP1 (Figure 4B). This result is consistent with a simultaneous occupation of the 5 ACP active sites, being each of the five ACP domains of mm5ACP able to be malonylated by PfaA AT.

The auto-acylation ability of ACPs was originally linked to the presence of several conserved Arginine residues that are poorly conserved amongst non-self-acylating FAS (33). Moreover, Y56 and R72 of the actinorhodin PKS were further described to be not only directly related to self-acylation capacity but with the ability to transfer the malonyl groups to other ACPs (32, 35). Finally, it was demonstrated that at least in PKS type II the self-malonylation was promoted by an acidic D/E residue and helped by some T residues (10). All these amino acids are located at the putative binding clefts of the ACPs and more specifically within the disordered loops connecting the alpha helices, that are presumably involved in the protein-protein and protein-substrate recognition processes (10). Despite our efforts to find homologous residues conserved among omega-3 PUFA producers, we could not identify any of them involved in catalysis. These results lead us to think that we are in front of a substantially different mechanism from the one described for PKS or FAS proteins, so there should exist evolutionary divergences between Pfa self-acylation mechanisms and other self-acylating systems. To analyze this divergent process, a phylogenetic tree was made (Figure 3C), including the sequences used in the multiple sequence alignment of Figure 3A. It can be noticed an evolutionary proximity between all the analyzed FAS ACPs, that are close related to the ones from Pfa systems, both distant from PKSII. These results evidence either a loss of the self-acylation ability for most of the FAS systems during evolution or an alternative emergence of this capacity within PUFA systems.

Apo mmACP1 was also purified to analyze the efficiency of the phosphopantetheinylation by PfaE. Using High Definition Mass Spectrometry, as described in Experimental Procedures, we observed in the sample containing the purified apo mmACP1 a main peak of 11,352 Da corresponding to the theoretical molecular weight of mmACP1 (Figure S7). A main peak of 11,692Da, theoretical molecular weight of mmACP1 plus PPT, was found in the sample containing holo mmACP1. The presence of apo ACP was not detected in the holo ACP sample (Figure S7), thus confirming that mmPfaE is the phosphopantetheinylase (PPTase) of mmACP and that the reaction is 100 % effective.

mmPfaE modeling by Phyre2 predicted a typical PPTase structure like that of the Bacillus subtilis PPTase named Spf (PDB Accession.
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Number 1QR0) (Figure S8). In fact, Trujillo et al. (30) showed that Photobacterium profundum mmACP homolog is effectively phosphopantetheinylated by B. subtilis Spf. In that work they also detected some in vivo phosphopantetheinylation by the E. coli PPTase, reaction that was not detected in our experiments. Spf interacts with the second helix of ACP by residues around its first (α4 in Figure S8) and third helix (α6) (36). Then the Mg\(^{2+}\) ion coordinated by residues D107, E109 and E151 catalyzes the PPTase reaction. As shown in Figure S8, while the catalytic residues are conserved in mmPfaE as well as in E. coli, residues involved in ACP interaction are not 100% conserved. These differences could be responsible for the necessity of a specific PfaE protein in PUFA synthases.

PfaA AT is the main malonyltransferase domain within the Pfa cluster

Together with the AT domain of PfaA, there is another putative AT domain in the pfa cluster of M. marina, the AT domain of PfaB. PfaB is a 883 aa protein that determines the final product in the EPA/DHA biosynthesis (16), although the molecular mechanism is unknown. Its sequence contains a structural pseudo-KS motif in DHA-producers like M. marina that is absent in EPA-producers like Shewanella baltica, and an AT domain at the C-terminal position (Figure S9).

PfaB activity and specificity was studied using the same assay than the one used for the PfaA KS-AT didomain. When PfaB was incubated with \(^{14}\)C-radiolabeled acetyl-CoA or malonyl-CoA (Figure 5B, lanes 1 and 2, respectively), the presence of a radioactive acyl-PfaB covalent complex was not detected. When PfaB was incubated with \(^{14}\)C-malonyl-CoA in the presence of ACPs, an increase in the intensity of the malonyl-ACP band was detected (lane 5). However, PfaB concentration used for this experiment was 50 μM; 5 times higher than that used for KSAT PfaA in the previous experiment, suggesting that PfaB may show a lower affinity for malonyl-CoA. As with PfaA, no activity against acetyl-CoA was detected (lane 4) and no changes in the binding pattern in the presence of both substrates either (lane 6). Self malonylation of ACPs was again included in the experiment as a positive control (lane 3).

In order to measure the different activity shown by the AT domains of PfaA and PfaB against malonyl-CoA, a titration experiment was performed. Concentration of \(^{14}\)C-radiolabeled malonyl-CoA was fixed to 20 μM while concentration of the AT domain was progressively increased from 2 nM to 2 μM. The amount of malonyl-ACP was measured with a scintillation counter (Figure 5C). This resulted in a dissociation constant (Kd) of 16.6±3.8 nM for PfaA and 365.6±209 nM for PfaB and a maximum reaction rate of 2802±130 CPM for PfaA and 122±20.8 CPM for PfaB (Figure 5C). Thus, the Kd is significantly lower for PfaA so its affinity for malonyl-CoA is higher, pointing out its role in the loading of the malonyl extender units.

The XAXH motif within acyltransferases, close to the active site, has been identified as the main determinant of substrate specificity, being HAFH and YASH the most common variations, showing preference for malonyl (MAT) or methylmalonyl (MMAT) respectively (9, 37, 38). A multiple sequence alignment was performed comparing representative MAT and MMAT domains from PKS systems with PfaA and PfaB proteins from omega-3 PUFA synthases (Figure 6). Conservation of a [AG]AFH motif characteristic of MAT domains was observed in PfaA proteins, while a [NT][IM][ST] motif was found in PfaB proteins, indicating a sequence divergence that may promote the specificity for other substrates different from malonyl-CoA. In addition, the histidine residue of the GHSxGE motif, characteristic of MAT proteins and involved in the stabilization of the malonyl-enzyme intermediate (39) is conserved in PfaA, but is replaced by tyrosine in PfaB. These motifs are in agreement with the different in vitro affinity for malonyl of KSAT PfaA and PfaB.

We have demonstrated here that PfaB, when highly concentrated, showed a detectable capacity to select and load malonyl units onto the ACPs. Considering the trans-AT nature of PfaB, the AT redundancy, its reduced ability to load malonyl and its previous description as final product determinant (16), we think that the natural substrate of PfaB should be other molecule, probably involved at the last stages of the PUFA synthesis.
In summary, through a bioinformatic analysis we have completed the characterization of mmPfaA, defining an KS-AT-ACP(x5)-KR'-KR-DH organization that seems conserved in all Pfa systems. Due to the presence of ACPs with ability to be malonylated in parallel, the existence of an AT domain selector of malonyl units, the presence of a KS domain (whose catalysis has not been demonstrated yet) and a KR-DH modifier domain, we propose PfaA as a preparative protein for the omega-3 PUFA synthesis. The PfaA role would be the generation of mature extender units that will be eventually polymerized by the rest of Pfa proteins.

**Experimental procedures**

**Domain prediction and structural modelling**

InterPro online tool (40) was used to predict the signatures of the domains by scanning a database of previously known protein signatures with our query proteins. Jpred (41) was used to predict the secondary structure elements of the entire polypeptides. The 3D structures of the different domains were predicted by homology modeling using the Phyre2 server (18). The crystal structures used as templates for each domain were as follows: the mammalian fatty acid synthase (mFAS) (PDB Accession Number 2VZ8) for the mmKS-AT didomain; the ACP of Mycobacterium tuberculosis (1KLP) for the mmACP1; the KR from Saccharopolyspora spinose (4IMP) for the mmKR domain; the dehydratase domain from CurF module of curacin polyketide synthase (3KG6) for the mmDH domain. Images of the resulting models were generated using the program Pymol (pymol.org).

**Sequence analysis software and public gene expression data sets**

All protein and nucleic acid sequences were obtained from the public databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Protein database searches were performed using BLASTP (42), available on the NCBI web site. Multiple sequence alignments were made using the software T-coffee (43) (http://www.ebi.ac.uk/Tools/msa/tcofffee/) and plotted using the program ESPript 3.0 (44) (http://escriptb.ibcp.fr/ESPript/ESPript/).

Phylogenetic analysis of the protein sequences was performed in phylogeny.fr (45). Sequences were aligned using MUSCLE (v3.8.31). Gaps and not-well aligned regions were removed by using Gblocks (c0.91b). Dendrograms were constructed by using the maximum likelihood method included in PhyML (v3.1/3.0 aLRT). Reliability for internal branch was set using bootstrapping method (100 replicates). Dendrogram was drawn with TreeDyn (v198.3).

**Strains and culture conditions**

The *Escherichia coli* strain DH5α was employed for cloning procedures and *E. coli* strain BL21 (DE3) for protein expression. Liquid cultures were prepared in flasks containing ¼ volume LB medium (Pronadisa, Spain) supplemented with either kanamycin sulphate (Sigma Aldrich, St. Louis, MO) at a final concentration of 50 μg/ml or/and ampicillin (Sigma Aldrich, St. Louis, MO) at 100 μg/ml and incubated at 37°C and 180 rpm. For solid media culture LA was used (LB medium supplemented with 1.5% (w/v) agar; Pronadisa, Spain).

**DNA manipulation and plasmid construction**

The pDHA plasmid DNA containing *pfa* genes from *M. marina*, used as template to clone the different constructions, was kindly provided by Orikasa’s lab (46). All DNA fragments were amplified by chain polymerase reaction (PCR) with oligonucleotides purchased from Sigma-Genosys (Sigma-Aldrich, St. Louis, MO) and the high-fidelity DNA polymerase Phusion (Thermo fisher, EEUU). All constructions were carried out using the isothermal assembly Gibson method (47).

DNA was analyzed by agarose gel electrophoresis (1% w/v agarose in TBE buffer) in a horizontal running system (BioRad, Hercules, CA), using Hyperladder I (Biolabs, UK) as molecular weight marker and 0.05 mg/ml SYBR Safe (Invitrogen, Life Technologies, Waltham, MA) for staining. Quantity One software (BioRad, Hercules, CA) was used to visualize the images taken with a Gel Doc 2000 UV system. Plasmid DNA isolation was performed by using the QIAprep Spin Miniprep kit (QIAGEN, Germany). PCR
products were purified with the GenElute PCR Clean-Up kit (Sigma-Aldrich, St. Louis, MO) or extracted from the agarose gels by using the GenElute Gel Extraction kit (Sigma-Aldrich, St. Louis, MO). DNA concentration was determined at 260 nm with a Nano-Drop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA). Microdialized (Millipore GS, 0.05 μm pore size) isothermal assembly reactions were transformed into competent *E. coli* DH5α or BL21 (DE3) cells by electroporation using 0.2 cm Gene Pulser cuvettes (BioRad, Hercules, CA) in a MicroPulser™ electroporator (BioRad, Hercules, CA). The polymerase Biotaq (Bioline, London, UK) was used for PCR verification of the genetic constructions.

**Expression and purification**

*E. coli* BL21 (DE3) competent aliquots were individually transformed with plasmid constructions pET29c::KS-AT, pET29c::KS*-AT, pET29c::AT, pET29c::PfaB, pET29c::5ACP or pET29c::1ACP. Plasmids pET29c::5ACP or pET29c::1ACP and their mutants were also co-transformed with pET3α::PfaE to produce the holo-ACP proteins. Individual transformant colonies were grown in 2 l flasks containing 1 l LB supplemented with kanamycin (and ampicillin when coexpressing pET3α::PfaE) at 37°C and induced with 0.2 mM IPTG when OD600 was approximately 0.6. After induction, incubation temperature was decreased to 15°C in all cases but the ACPs constructions that were expressed at 18°C. Cell cultures were collected 18 hours post-induction by centrifugation at 5,500 g for 15 minutes at 4˚C and pellets were stored at -80˚C until analyzed. For purification, pellets were resuspended in buffer A (NaCl 300 mM, Tris-HCl 50 mM pH 7.5, imidazole 20 mM and PMSF 1 mM), sonicated and clarified by ultracentrifugation at 100,000 g for 15 minutes at 4˚C. Supernatants were loaded onto a HisTrap HP column (GE Healthcare, 5 ml) previously equilibrated with buffer B (NaCl 150 mM, Tris-HCl 50 mM pH 7.5, 1 mM EDTA). Purified proteins were stored at -80°C in glycerol 5% for further experiments.

**Protein intact mass determination**

40 μg of purified apo or holo-mmACP1 proteins were desalted using C4 and C18 Micro SpinColumn™ (Harvard Apparatus). Eluted samples were dried in a Speed Vac (Thermo Scientific) and resuspended in 25 μl of 50% acetonitrile, 0.25% formic acid. The protein was directly injected into a SYNAPT HDMS mass spectrometer (Waters) and MS spectra were manually acquired in the m/z range 500-1700. Protein intact mass was determined by MaxEnt1 software (Waters) and default deconvolution parameters were used. Mass ranges were selected based on available protein sequence information and software was set to iterate to convergence. Experimentally obtained masses were matched to mmACP protein amino acid sequence, with or without 4'-phosphopantetheine, using the BioLynx tool embedded in MassLynx 4.1 software (Waters).

**Radioactive binding assays**

To investigate the enzymatic activity of the different AT and ACP domains *in vitro* the selected protein combinations were incubated with radiolabeled substrates and analyzed by SDS-PAGE and autoradiography (radio-SDS-PAGE). The reaction mixture (20 μL) contained 20 μM [14C]-malonyl-CoA (55 Ci/mol) or 20 μM [14C]-Acetyl-CoA (29 Ci/mol), 20 μM of the ACPs and, according to the experiment, 5-50 μM of the AT domain in a buffer containing 50 mM Tris pH 7.5, 150 mM NaCl and 0.5 mM EDTA. Following incubation at room temperature for 30 min, the reaction was quenched with 10 μL of protein loading buffer (0.1% bromophenol blue, 1% SDS, 40% glycerol), and loaded onto a 12% acrylamide SDS gel. After electrophoresis the gels were stained with Coomassie to visualize the proteins, dried and subjected to autoradiography to detect the radioactive substrates. The procedure was repeated in triplicate for each reaction.

**Radioactive titration experiments**

Superdex 75 GL10_30 column (Amersham Pharmacia Biotech, UK) previously equilibrated with buffer B (NaCl 150 mM, Tris-HCl 50 mM pH 7.5, 1 mM EDTA). Purified proteins were stored at -80°C in glycerol 5% for further experiments.
Each titration assay vial contained 20 μM [14C]-malonyl-CoA, 20 μM ACP and 2-2000 mM of the AT domain analyzed in a buffer containing 50 mM Tris pH 7.5, 150 mM NaCl and 0.5 mM EDTA in a final volume of 20 μl. The reaction mixture was incubated at room temperature for 5 min and stopped by the addition of 160 μl of ice-cold 10% trichloroacetic acid (TCA). The samples were incubated on ice for 10 min, and centrifuged at 10,000 rpm for 10 min. Pellet was washed with 10% TCA twice and finally resuspended in 60 μl of 4% SDS in 20 mM NaOH. The suspension was combined with 5 ml of scintillation cocktail and analyzed with a Packard Tri-Carb liquid scintillation counter. The experiments were performed in triplicate.
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Figure 1. PfaA analysis and general biochemical route. (A) General scheme of the pfa genetic cluster of Moritella marina. Each domain is represented with a specific color. Domain regions correspond to keto synthases (KS), acyltransferases (AT), acyl carrier proteins (ACP), keto reductases (KR), dehydratases (DH) and enoyl reductase (ER). PfaA that will be further analyzed is squared with a dashed line. (B) Overview of the biochemical route for omega-3 PUFA synthesis. The dashed arrows represent the beginning of a new synthesis cycle. (C) PfaA domain organization. Prediction of folding domains within the PfaA protein sequence made with Interpro. Each domain is represented with a specific color. Structural predictions made with Phyre2 of each domain are shown in the lower panel using the same color code. The first and last amino acid of each domain prediction is indicated in the figure. Only the structure of one of the five ACPs has been represented as they are similar.
Figure 2. Ligand binding assays of mm5ACP, mmKS-AT and mmAT domains. (A) 12% SDS-PAGE gel containing 20 µg of purified mm5ACP in its holo- (lane 1) and apo- (lane 2) forms. (B) 20 µM mm5ACP incubated with [14C]-malonyl-CoA and [14C]-acetyl-CoA and analyzed by 12% radio-SDS-PAGE. Lane 1: apo-5ACP+Malonyl-CoA; 2: apo-5ACP+Acetyl-CoA; 3: holo-5ACP+Malonyl-CoA; 4: holo-5ACP+Acetyl-CoA. (C) 12% SDS-PAGE gel containing purified mmKS-AT (lane 1), holo-mm5ACP (lane 2), mmKS*(C229A)-AT (lane 3) and mmAT (lane 4). (D) Same domains incubated with [14C]-malonyl-CoA and [14C]-acetyl-CoA, and analyzed by 12% radio-SDS-PAGE. Lanes 1: mmKS-AT+ Acetyl-CoA; 2: mmKS-AT+Malonyl-CoA; 3: holo-5ACP+Acetyl-CoA; 4: holo-5ACP+Malonyl-CoA; 5: holo-mm5ACP+mmKS-AT+Malonyl-CoA; 6: holo-mm5ACP+mmKS-AT+Malonyl-CoA; 7: holo-mm5ACP+mmKS-AT+Acetyl-CoA+Malonyl-CoA; (E) Quantification of the protein bands intensities of holo-mm5ACP+Malonyl-CoA (D, lane 4) and holo-mm5ACP+mmKS-AT+Malonyl-CoA (D, lane 6) by densitometric scanning, normalized with respect to the molarity of ACP in each sample. (F) mmKS(C229A)-AT mutant and mmAT isolated domain were incubated with [14C]-malonyl-CoA and [14C]-acetyl-CoA, and analyzed by 12% radio-SDS-PAGE. Lanes 1: mmKS(C229A)-AT+Malonyl-CoA; 2: mmKS(C229A)-AT+Acetyl-CoA; 3: holo-mm5ACP+mmKS(C229A)-AT+Malonyl-CoA; 4: holo-mm5ACP+mmKS(C229A)-AT+Malonyl-CoA+Acetyl-CoA; 5: mmAT+Acetyl-CoA; 6: mmAT+Malonyl-CoA; 7: holo-mm5ACP+mmAT+Acetyl-CoA; 8: holo-mm5ACP+mmAT+Malonyl-CoA. Samples from individual gels are separated by vertical lines. Protein mapping positions are indicated in A and C by black arrows.
**Figure 3.** Comparative analysis of representative ACP domains from different organisms. (A) Multiple sequence alignment of representative ACPs from PKSII, omega-3 PUFA syntheses and FAS. Alignment names correspond to actinorhodin PKSII ACP (Act) from *Streptomyces coelicolor* (UniProt Acc. no. Q02054), oxytetracycline PKSII ACP (Oxyt) from *Streptomyces rimosus* (Acc. no. P43677), frenolicin PKSII ACP (Fren) from *Streptomyces roseofulvus* (Acc. no. Q54996), PfaA ACP1 (Morit) from *M. marina* (Acc. no. Q9RA21), Pfa ACP1 (Enhy) from *Enhygromyxa salina* (Acc. no. A0A0C2CTM9), Pfa1 ACP1 (Schizo) from *Schizochytrium ATCC 20888* (Acc. no. AAK72879), ACP (Ecoli) from *E. coli* FAS (Acc. no. P0A6A8) and ACP (Strept) from *Streptomyces coelicolor* (Accession no. P72393), ACP (Plasm) from *Plasmodium falciparum* (Accession no. O77077) and ACP (Brass) from *Brassica napus* (Accession no. P17650). Identical residues are shown in white on a red background, while similar residues are shown in red. The position of the “D” motif, characteristic of self-acylating ACPs, has been marked with a black arrow. Left panel indicates with a color code the type of organism in each sequence. Position of α-helices is represented with grey boxes at the bottom of the alignment. (B) Predicted structure of mmACP1 from *M. marina*. The four residues selected for the alanine screening are highlighted in green and the active serine in red. (C) Phylogram representation of all the previous sequences, showing evolutive divergence between them.
Figure 4. Ligand binding assays of mmACP1 and mm5ACP with mmKS-AT. (A) 12% radio-SDS-PAGE gel containing 2.2 µg of mmKS-AT and mmACP1 0.32, 0.64, 1.6 or 3.2 µg (lanes 1,2,3 and 4) or mm5ACP 0.8, 0.8, 2.0 or 4.0 µg (lanes 5,6,7 and 8) incubated 1 hour with [14C]-malonyl-CoA. Protein mapping positions are indicated by black arrows. (B) Quantification of the protein bands intensities by densitometric scanning, normalized with respect to the molarity of ACP in each sample.
Figure 5. Binding assays of mmPfaB and mm5ACP. (A) 12% SDS-PAGE containing the purified mmPfaB (1) and holo-mm5ACPs proteins (2). Protein mapping positions are indicated with black arrows. (B) mmPfaB and holo-mm5ACPs incubated with radiolabeled substrates [14C]-malonyl-CoA and [14C]-acetyl-CoA and analyzed by 12% radio-SDS-PAGE. Lane 1: mmPfaB+Acetyl-CoA; 2: mmPfaB+Malonyl-CoA; 3: holo-mm5ACP+Malonyl-CoA; 4: holo-mm5ACP+mmPfaB+Acetyl-CoA; 5: holo-mm5ACP+mmPfaB+Malonyl-CoA; 6: holo-mm5ACP+mmPfaB+Acetyl-CoA+Malonyl-CoA. (C) Determination of ATs dissociation constants. The AT concentration dependence of [14C]-malonyl-ACP formation was measured by titration experiments. Concentration of protein (PfaA or PfaB AT domains) was progressively increased and the reaction product (malonyl-mm5ACP) was measured with a scintillation counter.
Alignment of representative AT protein regions from PKS and omega-3 PUFA synthases. Sequences correspond to epothilone PKS AT2 domain (Epot) from *Sorangium cellulosum* (Acc. no. Q9KIZ8), dynemicin PKS DynE8 AT domain (Ene) from *Micromonospora chersina* (Acc. no. Q84HI8), DEBS ATs 1 and 6 (DEBS1 and DEBS6) from *Saccharopolyspora erythraea* (Acc. no. Q03131 and Q03132), PfaA AT domains (Mor_A, Col_A and Phot_A) from *M. marina*, *Colwellia psychrerythraea* and *Photobacterium profundum* (Acc. no. Q9RA21, Q47ZG8 and Q93CG7, respectively), PfaB AT domains (Mor_B, Col_B and Phot_B) from *M. marina*, *Colwellia psychrerythraea* and *Photobacterium profundum* (Acc. no. Q9RA20, Q47ZG9 and Q93CG7, respectively). Identical residues are shown in white on a red background, while similar residues are shown in red. The position of the “S” catalytic motif has been marked with a black arrow, the XAXH motif, determinant of specificity was marked with orange arrows and the histidine of the GHSxGE motif was marked with a grey arrow. The color legend indicates the selectivity against malonyl (MAT) or methylmalonyl (MMAT) of the previously known domains.
Loading of malonyl-CoA onto tandem acetyl carrier protein domains of polyunsaturated fatty acid synthases
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