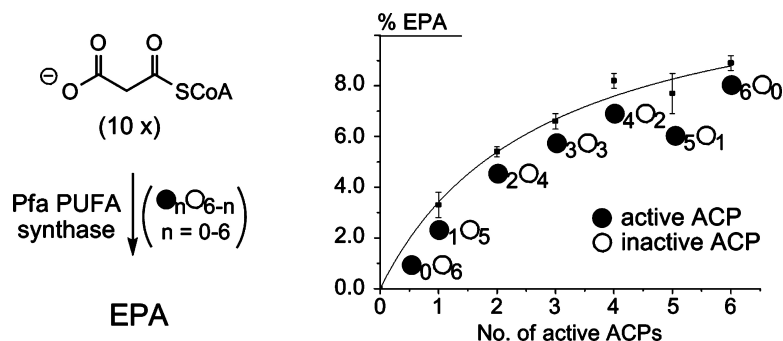Communication

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The Role of Tandem Acyl Carrier Protein Domains in Polyunsaturated Fatty Acid Biosynthesis

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Polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6$\omega\omega$3) (Figure 1a), are essential to human health and nutrition, providing important visual, mental, and cardiovascular benefits throughout life.[1] While fish and fish oil products are the traditional sources of PUFAs, they possess a number of problems including poor taste, instability, and variability.[2a] In addition, this natural source of PUFAs has been endangered due to ocean pollution and overfishing.[2b] Although methods for the total synthesis of PUFAs are available, they are not practical for industrial scale production.[3] In contrast, microbial fermentation has recently emerged as an attractive alternative for PUFA production.[4]

PUFA biosynthesis can occur by the elongation and desaturation of preexisting fatty acids or de novo, from small precursor molecules, by a specialized "PUFA synthase".[5] These multi-subunit PUFA synthase enzymes have been identified in a number of marine bacteria as well as in the eukaryotic Thraustochytrids.[5,6] The minimal number of genes required for PUFA biosynthesis has been determined by expression of those genes in heterologous hosts;[7] however, our understanding of the enzymology and biochemistry of the PUFA synthase remains rudimentary. PUFA synthases contain many unprecedented features including the presence of multiple acyl carrier protein (ACP) domains, as exemplified by the PfaA subunit with six ACPs from *Shewanella japonica*,[6e] the Orf8 subunit with five ACPs from *Moritella marina* (Figure 1b),[6c] and the OrfA subunit with nine ACPs from the marine protist *Schizochytrium*.[5]

Most fatty acid synthases (FASs) and polyketide synthases (PKSs) are characterized by a single ACP for each cycle of chain elongation.[8] ACPs, either as a domain (type I) or a discrete protein (type II), contain a conserved Ser residue that is post-translationally modified with the phosphopantetheinyl moiety of CoA by a phosphopantetheinyl transferase (PPTase). This modification provides a free thiol for tethering the starter and extender units and channeling the intermediates during fatty acid and polyketide biosynthesis.[9] The multiple ACP domain architecture of PUFA synthases is therefore in striking contrast to the current paradigm of FAS and PKS structure and mechanism. In this report, we expressed the *S. japonica pfaA/B/C/D/E* genes in *Escherichia coli* to produce EPA and docosapentaenoic acid (DPA, 22:5ω3) (Figure 1). Utilizing the *S. japonica* genes as a model for PUFA synthases, we describe here in vivo and in vitro characterization of each ACP of the PfaA subunit of the PUFA synthase. Our findings shed new light into the role of ACPs and their important contributions to PUFA biosynthesis.

First we confirmed that each of the bioinformatics-predicted ACPs can be phosphopantetheinylated by a PPTase in vitro. Genes encoding each of the PfaA-ACPs were cloned into pETDuet-1, and *pfaA-ACP1*
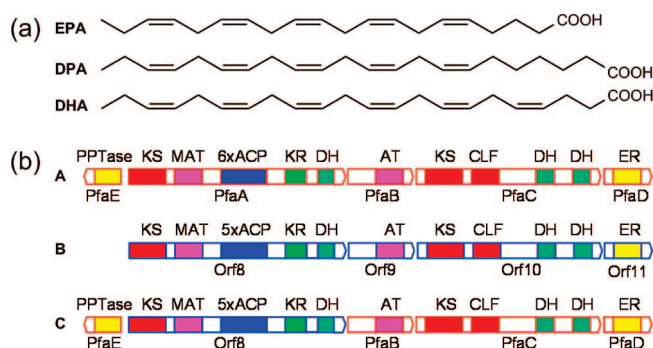

Figure 1. (a) Structures of EPA, DHA, and DPA. (b) Organization of selected PUFA biosynthetic gene clusters: A, *Shewanella japonica* (EPA/DPA producer); B, *Moritella marina* (DHA producer); and C, the hybrid *S. japonica* and *M. marina* PUFA cluster constructed in this study.
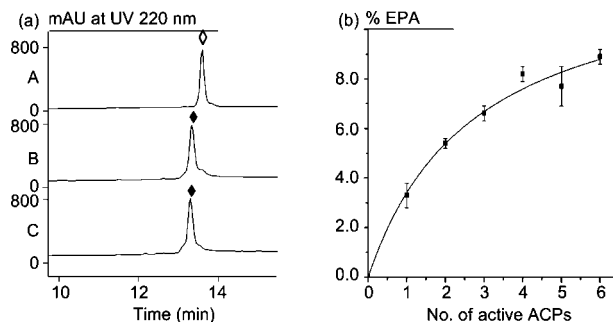

Figure 2. (a) HPLC analysis of apo- and holo-ACPs as exemplified by PfaA-ACP1: (A) apo-ACP1 by expression of *pfaA-ACP1* in *E. coli* BL21(DE3); (B) holo-ACP1 by coexpression of *pfaA-ACP1* with *pfaE* in *E. coli*; and (C) holo-ACP1 by Svp-catalyzed phosphopantetheinylation of apo-ACP1 in vitro (C). ◇, apo-ACP1; ◆, holo-ACP1. (b) Relationship between EPA production and number of active PfaA-ACPs.

was also cloned into pET28a (Table S1). The resultant plasmids were introduced into *E. coli* BL21(DE3) for the overproduction of each ACP as a N-His$_6$-tagged protein. After affinity chromatography on Ni^{2+}-NTA resin (Figure S1), each of the purified ACPs was eluted as a single peak upon HPLC and confirmed to be the apo-ACP by ESI-MS. In vitro phosphopantetheinylation was carried out by incubating the apo-ACPs with CoA in the presence of the known promiscuous PPTase Svp,[9b] and the resultant holo-ACPs were confirmed by HPLC and ESI-MS analyses (Figure 2a and Table S2), thus providing the first evidence that these domains are indeed ACPs.

Second we established PfaE as a dedicated PPTase for the PfaA PUFA synthase by phosphopantetheinylating the PfaA-ACPs in vivo. The *pfaE* gene, whose deduced product resembles the PPTase family of enzymes,[9a] is clustered within the *pfa* PUFA synthase locus (Figure 1b) and has previously been shown to be essential for PUFA production in vivo.[7b] To provide direct evidence that PfaE can phosphopantetheinylate each of the PfaA-ACPs, genes encoding the PfaA-ACPs were

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cloned together with *pfaE* into pETDuet-1 to generate the coexpression plasmids (Table S1). Under the conditions identical to that used for expression of *pfaA-ACPs* alone, introduction of the coexpression plasmids into *E. coli* BL21(DE3) resulted in the production of each ACP as a N-His$_6$-tagged protein with concomitant production of PfaE. The overproduced PfaA-ACPs were similarly purified, and HPLC and ESI-MS analyses confirmed that each of the ACPs was produced in the holo-form (Figure 2a and Table S2). Taken together, the in vitro and in vivo results unambiguously established each of the six *pfaA-ACPs* encoding a bona fide ACP and provided the first direct experimental evidence supporting PfaE as a PPTase dedicated to PUFA biosynthesis.

Third, by interrogating each of the PfaA-ACPs via site-directed mutagenesis, we revealed that each of the tandem ACPs is functionally equivalent for PUFA biosynthesis, but the number of ACPs controls the PUFA titer. The six PfaA-ACP domains are highly homologous with sequence identity ranging from 85 to 96%, and each ACP contains the LGIDS motif (Figure S2) with the Ser residue known to be the site of phosphopantetheinylation to form holo-ACP, the functional form of ACP for polyketide and fatty acid biosynthesis.[9] To study the roles of the six PfaA-ACPs in PUFA biosynthesis, we constructed 16 Ser-to-Ala mutants to eliminate the post-translational modification and hence abolish the utilization of the respective ACP during PUFA biosynthesis (Table S1). The relationship of PUFA production with each ACP mutant was investigated by expressing the *pfaBCDE* in *E. coli* BLR(DE3) along with either the ACP-mutated *pfaA* or the wild-type *pfaA* as a control. PfaA (or one of 16 ACP-mutated *pfaA*)/*pfaE*, *pfaB*/*pfaD*, and *pfaC* were cloned into pETDuet-1, pACYCDuet-1, and pCOLADuet-1, respectively, to generate 17 sets of three-plasmid-expression systems. PUFA production in *E. coli* BLR(DE3) that harbored each set of the three-plasmid-expression systems was quantified by GC−MS analysis using authentic EPA and DPA as standards. Expression of *pfaABCDE* in *E. coli* BLR(DE3) typically yielded cells accumulating ∼8.9% EPA and with an EPA to DPA ratio of ∼6:1 (Table S4).

Introduction of mutations to all six ACPs of PfaA completely abolished PUFA production, confirming that PUFA biosynthesis is an ACP-dependent process. In contrast, introduction of mutation to each of the six ACPs individually yielded strains whose PUFA titers were comparable to that of the wild-type, suggesting that the ACPs are functionally equivalent to support PUFA biosynthesis and demonstrating that no single ACP plays a unique and essential role in PUFA synthesis. Fermentations of the two quintuple Ser-to-Ala mutants (ACP12345m and ACP23456m), which provided the PUFA synthase with only one functional ACP, respectively, were then performed, and both mutants were competent in PUFA biosynthesis, although yield was significantly reduced to ∼37% of the wild-type. As summarized in Figure 2b and Table S3, EPA titer generally decreased relative to the number of functional ACPs in the *pfaA* expression constructs, with six single ACP mutants, one double ACP mutant, three triple ACP mutants, three quadruple ACP mutants, and two quintuple ACP mutants giving an average of 87% (five active ACPs), 92% (four active ACPs), 74% (three active ACPs), 61% (two active ACPs), and 37% (one active ACP), respectively, compared to the wild-type. In addition, the mutants with the same number of active ACPs produced nearly the same amount of EPA regardless of the location of inactive ACPs, and the PUFA ratio (EPA to DPA) was nearly identical for all of the combinations (Table S3). Taken together, these data suggest that the ACPs are functionally equivalent regardless of their physical location within the PfaA subunit, but the overall PUFA titer directly depends on total number of active ACPs.

Finally we demonstrated that our findings on the role of tandem ACPs from PfaA most likely are general for other PUFA synthases by constructing a hybrid PUFA synthase. The PUFA synthase gene sets, by which *M. marina* produces DHA and *S. japonica* produces EPA and DPA, have almost identical domain organizations and genetic architectures except that Orf8, the PfaA homologue of *M. marina*, contains five tandem ACPs (Figure 1b). An *S. japonica−M. marina* hybrid PUFA synthase was constructed by replacing *pfaA* with its homologue *orf8* from *M. marina* (Figure 1b), and the resultant construct was introduced into *E. coli* BLR(DE3) for PUFA production. Growth of this hybrid PUFA synthase system resulted in production of EPA and DPA, in a ratio and level similar to that of the wild-type Pfa PUFA synthase but not DHA (Table S4). On the basis of all above results, we concluded that the determinant(s) of final PUFA products is mediated by other synthase subunits and is ACP-independent.

In summary, the characterization of tandem ACPs in PfaA represents an important advance in our understanding of PUFA synthase, as well as the roles of multiple ACPs in FASs and PKSs. These findings set the stage to interrogate other domains and subunits of PUFA synthase for their roles in controlling the final PUFA products and could potentially be exploited to improve PUFA production.

Supporting Information Available: Full experimental details for overproduction of PfaA-ACPs, phosphopantetheinylation of ACPs in vivo and in vitro, HPLC and ESI-MS analyses of ACPs, GC−MS analysis of fatty acids, construction of PfaA-ACP point mutants, and engineering of the hybrid PUFA synthase. This material is available free of charge via the Internet at http://pubs.acs.org.

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