A Journal of the Gesellschaft Deutscher Chemiker
Angewandte
GDCh
International Edition
Chemie
www.angewandte.org

Accepted Article

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To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201900771
Angew. Chem. 10.1002/ange.201900771

Link to VoR: http://dx.doi.org/10.1002/anie.201900771
http://dx.doi.org/10.1002/ange.201900771

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COMMUNICATION

Control mechanism for carbon chain length in polyunsaturated fatty acid synthases

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Abstract: Polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are essential fatty acids for humans. Microalgae and some marine bacteria synthesize these PUFAs with PUFA synthase from acetyl units. The PUFA synthases are composed of three to four subunits and each create a specific PUFA without undesirable byproducts even though the multiple catalytic domains in each huge subunit are very similar. However, detailed biosynthetic mechanisms for controlling final product profiles have been obscure. Here, we carefully dissected bacterial DHA and EPA synthases by *in vivo* and *in vitro* experiments. *In vivo* analysis showed that β-ketoacyl synthase (KS)/chain length factor-like domains in "C"-subunits of EPA and DHA synthases controlled DHA or EPA production. Furthermore, *in vitro* analysis with two KS domains in the "A" and "C" subunits (KS_A and KS_C) and acyl-ACP substrates showed that KS_A accepted short to medium chain substrates while KS_C accepted medium to long chain substrates. Unexpectedly, condensation from C_{18} to C_{20}, the last elongation step in EPA biosynthesis, was catalyzed by KS_A domains in both EPA and DHA synthases. Conversely, condensation from C_{20} to C_{22}, the last elongation step for DHA biosynthesis, was catalyzed by the KS_C domain in DHA synthase. Based on our results, we converted a microalgal DHA synthase into an EPA synthase.

Polyunsaturated fatty acids (PUFAs, Fig. 1A) such as docosahexaenoic acid (DHA; C22:6 ω3), eicosapentaenoic acid (EPA; C20:5 ω3), docosapentaenoic acid (DPAω6 or DPAω3; C22:5 ω6 or ω3), and arachidonic acid (ARA; C20:4 ω6) are essential fatty acids for humans and we ingest them from fish oils. However, because of the increased demand, commercial fermentative processes using microalgae, yeasts, and fungi have been developed to produce DHA, EPA, and ARA[1], respectively.

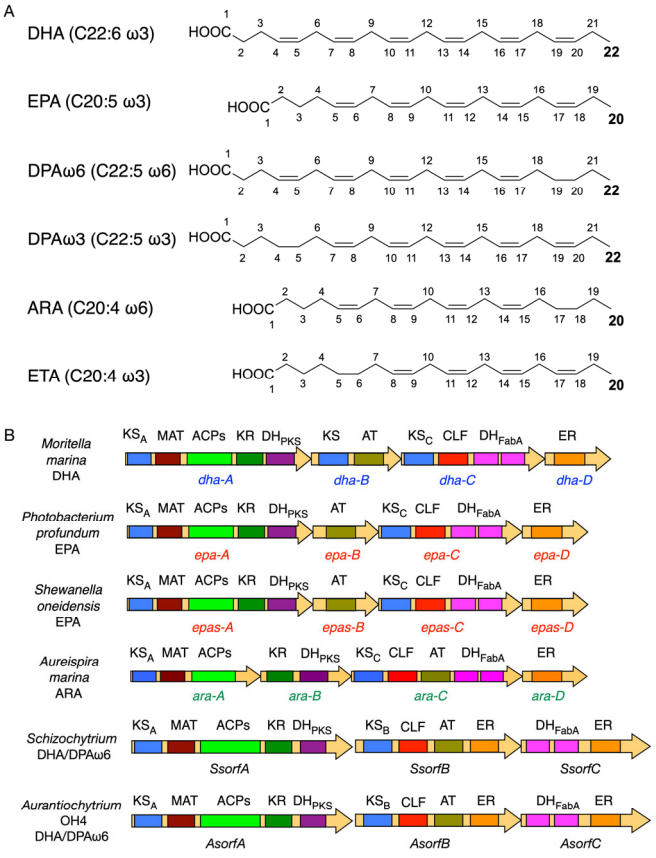

Figure 1. (A): Chemical structures of docosahexaenoic acid (DHA; C22:6 ω3), eicosapentaenoic acid (EPA; C20:5 ω3), docosapentaenoic acid (DPAω6 and DPAω3; C22:5 ω6 or ω3), arachidonic acid (ARA; C20:4 ω6), and eicosatetraenoic acid (ETA; C20:4 ω3). (B): Domain organizations of PUFA synthases. KS: β-ketoacyl synthase, MAT: malonyl CoA transacylase, ACP: acyl carrier protein, KR: β-ketoacyl reductase, DH_{PKS}: polyketide synthase-type dehydratase, AT: acyltrasferase, CLF: chain length factor-like domain, DH_{FabA}: FabA-type dehydratase, ER: enoyl reductase.

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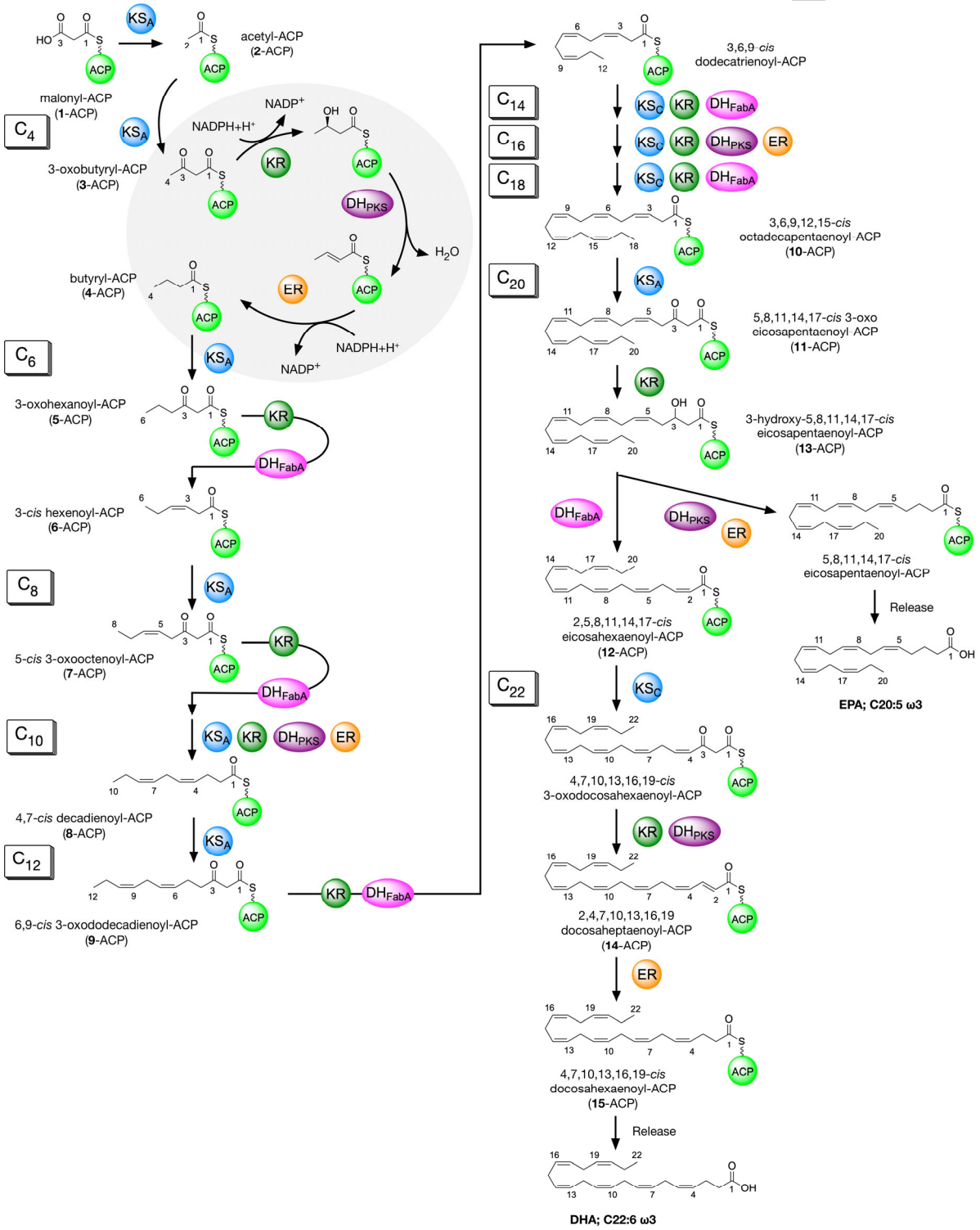

Figure 2. Proposed biosynthetic pathway of EPA and DHA synthases. The catalytic cycle in PUFA biosynthesis is schematically shown (gray circle).

In microorganisms, PUFAs are biosynthesized by two pathways, the aerobic desaturase/elongase pathway and the anaerobic PUFA synthase pathway. In the former pathway, specific desaturases and elongases catalyze individual desaturation and elongation steps to synthesize PUFAs from oleic acid (C18:1 ω9)[1c]. In the latter pathway, PUFA synthases composed of huge enzyme complexes with multiple catalytic domains synthesize PUFAs[2].

The biosynthetic genes for DHA, EPA, and ARA have been identified in marine bacteria; *Moritella marina*, *Photobacterium profundum*, and *Aureispira marina*, respectively (Fig. 1B)[3]. In contrast, only DHA producers have been isolated from microalgae

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such as *Schizochytrium* sp. and *Aurantiochytrium* sp.[4]. The PUFA synthases identified in these organisms are composed of three or four subunits and possess similar domain structures including acyltransferase (AT), acyl carrier protein (ACP), malonyl CoA transacylase (MAT), β-ketoacyl synthase (KS), ketoacyl reductase (KR), dehydratase (DH), and enoyl reductase (ER) domains in a manner similar to fatty acid synthases (FASs) (Fig. 2). However, PUFA synthases have unique features distinct from FASs. First, PUFA synthases have multi-tandem ACP domains and we previously showed that PUFA productivities increased depending on the number of ACPs without profile changes[5]. Second, PUFA synthases possess two-types of dehydratase, polyketide synthase (PKS)-type dehydratase (DH_{PKS}) and FabA-type dehydratase (DH_{FabA}). We recently showed that ARA and EPA synthases utilized DH_{PKS} and DH_{FabA} depending on the carbon chain length for introduction of saturation or *cis* double bonds on growing acyl chains[6]. Third, PUFA synthases contain two ketoacyl synthase domains, KS_A and KS_C, in subunit "A" and "C", respectively, and a chain length factor (CLF)-like domain, which has similarity to the KS domain but has no conserved active residues (Fig. S1), exists next to the KS_C domain. To date, however, there are few reports on the function of these KS domains. Here, we dissected the PUFA biosynthetic machineries by *in vivo* and *in vitro* experiments using the EPA and DHA synthases. Moreover, we applied the obtained results to a microalgal DHA synthase and succeeded in conversion into an EPA synthase.

To find clues to how the enzymes control the carbon chain length of the final products, we employed *in vivo* gene exchange assays. The EPA synthase genes (*epa*) of *P. profundum* and DHA synthase genes (*dha*) of *M. marina*, both of which comprise four genes (*A* to *D*), were cloned into pDuet vectors with T7 promoters and introduced into *E. coli* for evaluation of PUFA profiles. The transformant harboring *epa-ABCD* genes produced EPA as the main product concomitant with significant amounts of DPAω3. The transformant harboring *dha-ABCD* genes also produced DHA as the main product with small amounts of eicosatetraenoic acids (ETA) (Figs. 3A and 3B). Because *P. profundum* and *M. marina* were shown to produce only EPA and DHA[7], DPAω3 and ETA were suggested to be by-products produced by the heterologous expression system. As shown in Fig. 1B, Epa-ABCD and Dha-ABCD have very similar domain structures, each EPA synthase gene was replaced with the corresponding DHA synthase gene. When *epa-A*, *-B* or *-D* was replaced with the corresponding *DHA* gene, the PUFA profiles (EPA production) were almost the same as that of the transformant expressing *epa-ABCD*, although the PUFA productivities tended to decrease. By replacing *epa-C* with *dha-C*, however, the major product was changed to DHA (Fig. 3A). Similarly, EPA was produced by replacing *dha-C* with *epa-C* (Fig. 3B). The same result was also obtained with the EPA synthase

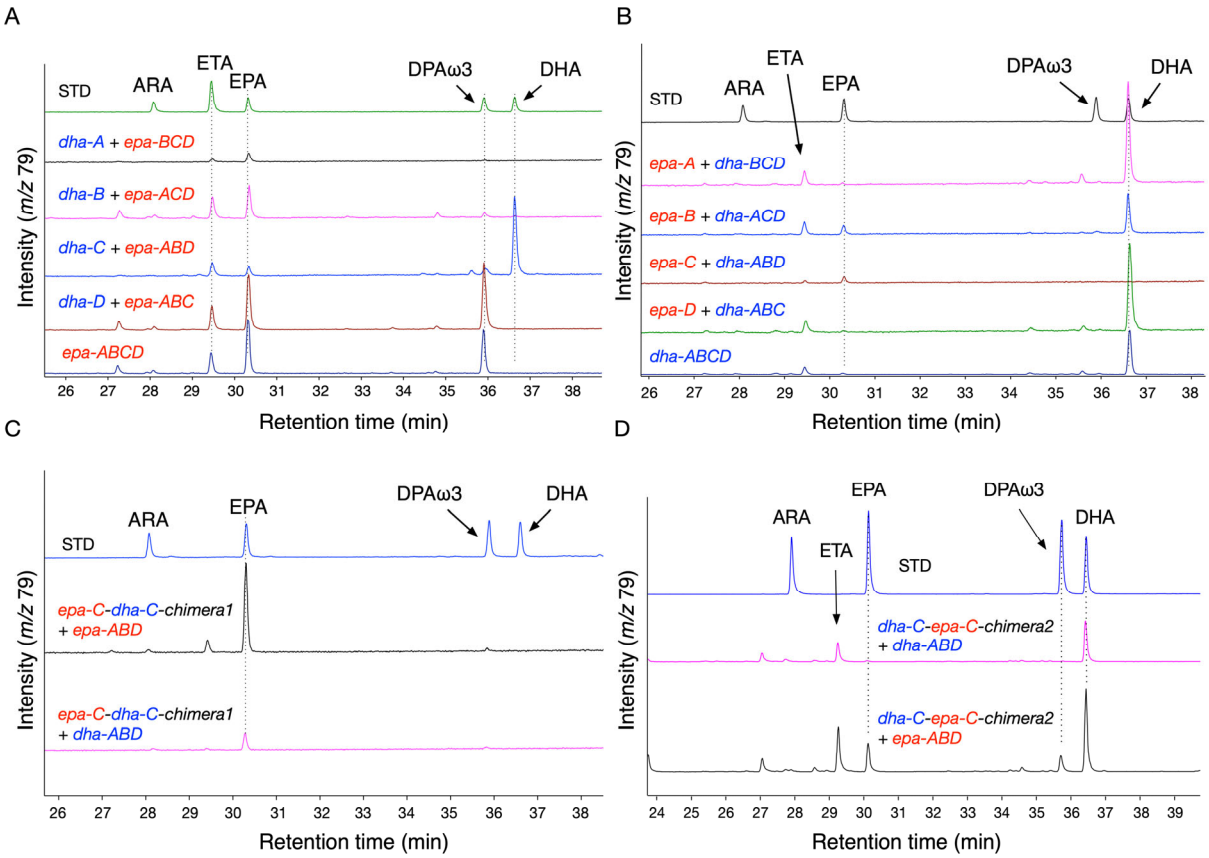

Figure 3. GC-MS analysis traced at *m/z* 79[5] of products produced in the gene replacement assay. (A) Gene replacement of *epa* genes with *dha* genes (from 2nd to 5th). Standards (top) and *epa-ABCD* (bottom). (B) Gene replacement of *dha* genes with *epa* genes (from 2nd to 5th). Standards (top) and *dha-ABCD* (bottom). (C) Co-expression of *epa-C-dha-C-chimera1* with *epa-ABD* (middle) or *dha-ABD* (bottom). Standards (top). (D) Co-expression of *dha-C-epa-C-chimera2* with *dha-ABD* (middle) or *epa-ABD* (bottom). Standards of methyl esters of ARA, eicosatetraenoic acid (ETA, C20:4 ω3), EPA (0.16 mM (A), 0.16 mM (B), 0.03 mM (C), 0.3 mM (D)), DPAω3, and DHA (top) were injected.

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gene (*epas-C*) of *Shewanella oneidensis* MR-1 (Fig. S2). These results suggested that the *"C"* gene is the key factor for controlling the carbon chain length of final products.

We next constructed chimeric *"C"* genes to narrow down the chain length control domain. The *dha-C* gene has an *Eco*RI site in its center region (Fig. S3) that divides the gene into two regions, the upstream KS_C/CLF-like domains and the downstream DH_{FabA} domains. Therefore, we artificially introduced an *Eco*RI site into the corresponding locus of the *epa-C* gene without amino acid changes in the translated enzymes. In the case of *epa-C-dha-C-chimera1*, EPA was produced by co-expression with *dha-ABD* (Fig. 3C). The transformant expressing *dha-C-epa-C-chimera2* together with *epa-ABD* produced both EPA and DHA (Fig. 3D). These results clearly indicated that the KS_C or CLF-like domain controlled the carbon chain length. To examine which domain is responsible for this control, we constructed dozens of chimeric genes in which the *dha-C* gene and *epa-C* gene were fused at different points between the KS_C domain and CLF-like domain. However, all the constructs lost PUFA productivity, suggesting that the quaternary structures of these regions are important.

Because the functions of the KS_A domain in the "A" subunit and the KS_c domain in the "C" subunit are still unclear, we carried out *in vitro* experiments with truncated KS enzymes and acyl-ACP substrates (Fig. S4). We tried to express a truncated enzyme containing only the KS_A or the KS_C domain, but no recombinant enzyme was obtained. Therefore, the former and the latter domain were co-expressed with a MAT and CLF-like domain, respectively, and four truncated enzymes, Epa-KS_A-MAT (Epa-KS_A), Epa-KS_C-CLF (Epa-KS_C), Dha-KS_A-MAT (Dha-KS_A), and Dha-KS_C-CLF (Dha-KS_C), were successfully obtained as soluble enzymes (Fig. S5). We prepared acyl-ACP substrates using the previously constructed single *apo*-ACP of EPA synthase of *Shewanella oneidensis* MR-1 and phosphopantetheinyl transferase Sfp[6]. The first elongation reaction in EPA and DHA biosynthesis is perhaps the condensation of malonyl-ACP (**1**-ACP) and acetyl-ACP (**2**-ACP). However, it was recently reported that malonyl-CoA, but not acetyl-CoA, could be loaded on the five tandem ACP domains of *M. marina*[8]. We therefore examined whether acetyl-ACP (**2**-ACP) could be accepted by the KS domains. When acetyl-ACP (**2**-ACP) and malonyl-ACP (**1**-ACP) were incubated with Epa-KS_A or Dha-KS_A, 3-oxobutyryl-ACP (**3**-ACP) was clearly detected by LC-MS, while no product was formed with the KS_C enzymes (Figs. 4A and S6). We then used only malonyl-ACP (**1**-ACP) as the substrate and incubated it with the KS_A domain. As shown in Fig. S7, 3-oxobutyryl-ACP (**3**-ACP) was clearly detected, suggesting that acetyl-ACP (**2**-ACP) formed from malonyl-ACP (**1**-ACP) via a decarboxylation reaction would be used for the condensation reaction.

We then examined the subsequent elongation steps. When malonyl-ACP (**1**-ACP) and butyryl-ACP (**4**-ACP) or 3-*cis* hexenoyl-ACP (**6**-ACP) were used as substrates, the KS_A enzymes showed high activities to form 3-oxohexanoyl-ACP (**5**-ACP) and 5-*cis* 3-oxooctenoyl-ACP (**7**-ACP, Figs. 4B and S8) while the KS_C enzymes weakly catalyzed these reactions. We next examined the functions of the two KS domains during middle to late biosynthetic stages. However, none of the predicted

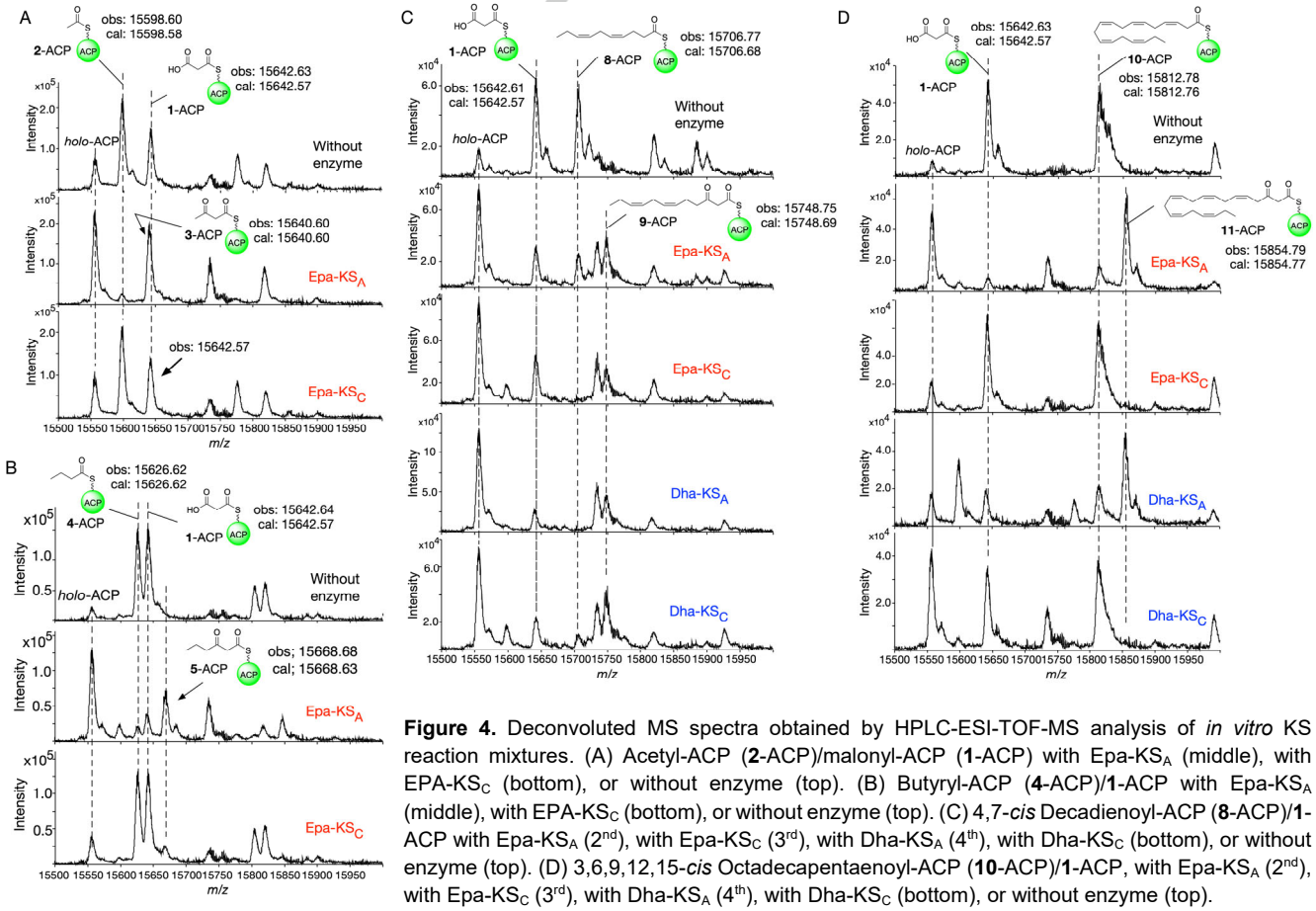

Figure 4. Deconvoluted MS spectra obtained by HPLC-ESI-TOF-MS analysis of *in vitro* KS reaction mixtures. (A) Acetyl-ACP (**2**-ACP)/malonyl-ACP (**1**-ACP) with Epa-KS_A (middle), with EPA-KS_C (bottom), or without enzyme (top). (B) Butyryl-ACP (**4**-ACP)/**1**-ACP with Epa-KS_A (middle), with EPA-KS_C (bottom), or without enzyme (top). (C) 4,7-*cis* Decadienoyl-ACP (**8**-ACP)/**1**-ACP with Epa-KS_A (2^{nd}), with Epa-KS_C (3^{rd}), with Dha-KS_A (4^{th}), with Dha-KS_C (bottom), or without enzyme (top). (D) 3,6,9,12,15-*cis* Octadecapentaenoyl-ACP (**10**-ACP)/**1**-ACP, with Epa-KS_A (2^{nd}), with Epa-KS_C (3^{rd}), with Dha-KS_A (4^{th}), with Dha-KS_C (bottom), or without enzyme (top).

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substrates were commercially available and their chemical synthesis was also difficult. Because 4,7-*cis* decadienoyl-ACP (**8**-ACP) and 3,6,9,12,15-*cis* octadecapentaenoyl-ACP (**10**-ACP) were the sole substrates that we could chemically synthesize (Fig. S4), we also performed *in vivo* analysis besides the *in vitro* assay. When 4,7-*cis* decadienoyl-ACP (**8**-ACP) and malonyl-ACP (**1**-ACP) were used as substrates, Epa-KS_A and Epa-KS_C showed almost the same activities and formed a 6,9-*cis* 3-oxododecadienoyl-ACP (**9**-ACP, Fig. 4C). Similarly, both Dha-KS_A and Dha-KS_C showed the same activities (Fig. 4C). To get more information about the role of the KS_C domain, we then constructed two mutated enzymes, Epa-C-KS^0 and Dha-C-KS^0, in which the catalytic Cys residues in the KS_C domains were mutated to Ala (Fig. S1), and co-expressed each with *epa-ABD*. In the case of *epa-C-KS^0* expression, α-linoleic acid (ALA; C18:3 ω3) and 7,10,13-*cis* hexadecatrienoic acid (C16:3 ω3) were produced as major and minor products, respectively (Figs. S9 and S10). Similarly, DHA production was completely abolished and ALA was produced as the major product with *dha-C-KS^0* (Fig. S9). Considering that ALA was produced as the major product in both cases, the products including ALA were shunt products probably formed by chain elongation from C12:3 ω3 by the KS_A domain. Therefore, the KS_C domain was suggested to catalyze the intrinsic chain elongation during the middle biosynthetic stage.

We next carried out an *in vitro* experiment with 3,6,9,12,15-*cis* octadecapentaenoyl-ACP (**10**-ACP) as the substrate to investigate the final biosynthetic reaction of EPA. When Epa-KS_A and Dha-KS_A were used as catalysts, the estimated 5,8,11,14,17-*cis* 3-oxoeicosapentaenoyl-ACP (**11**-ACP) was detected by LC-MS while Epa-KS_C and Dha-KS_C showed no activity (Fig. 4D), indicating that the KS_A domain again participated in the last chain elongation in EPA biosynthesis. To investigate the final chain elongation in DHA biosynthesis, we employed a combination enzyme assay (Fig. 5A) because preparation of the intrinsic substrate, 2,5,8,11,14,17-*cis* eicosahexaenoyl-ACP (**12**-ACP), was difficult. After addition of Epa-KR-DH_{PKS} into the abovementioned reaction mixture using 3,6,9,12,15-*cis* octadecapentaenoyl-ACP (**10**-ACP) and Dha-KS_A, a product whose molecular weight was identical to the estimated product, 3-hydroxy-5,8,11,14,17-*cis* eicosapentaenoyl-ACP (**13**-ACP), was detected. By further addition of Epa-DH_{FabA} and Dha-KS_C, a plausible 2,4,7,10,13,16,19-docosaheptaenoyl-ACP (**14**-ACP, Fig. 5B) was also detected, suggesting that the KS_C domain in Dha-C catalyzed the chain elongation from C_{20} to C_{22}.

As mentioned above, the difference between DHA and EPA biosynthesis was brought about by the substrate specificity of the KS_C/CLF-like domain against C_{20}-ACP substrates. Only the KS_C domain of DHA synthase catalyzed the chain elongation, although the corresponding amino acid sequence in EPA synthase is very similar. This fact prompted us to try to convert a microalgal DHA biosynthetic enzyme into an EPA biosynthetic enzyme. We used the DHA biosynthetic enzymes AsOrfABC of *Aurantiochytrium* sp. OH4, which has been used as a practical DHA producer and produced 44 g/L DHA[4b]. For *AsorfA*, we utilized *SsorfA* of *Schizochytrium* sp. because we were unable to clone *AsorfA*, probably due to its large size, and because *AsorfA* and *SsorfA* have the same domain structures. Three DHA biosynthetic genes were heterologously expressed in *E. coli* and co-production of DHA (67%) and DPAω6 (33%) was confirmed (Fig. S11). Then, we randomly introduced a mutation into the region corresponding to the KS_B or CLF-like domain in the *AsorfB* gene by error-prone PCR. In random screening, we isolated no EPA producer with the CLF-like domain as a template even though more than 3,000 mutants were screened. In contrast, we successfully obtained a KS_B domain mutant designated K01 that produced EPA as a minor product (EPA/DHA = 0.18) and possessed one mutation (F230L), after screening 2,000 mutants. Therefore, we used the K01 gene as a template and obtained a mutant (K02) producing increased EPA (EPA/DHA = 0.30) with an additional mutation (N65L). Using the same strategy, a mutant (K03) that had an additional mutation (I231T) and produced EPA as the major product (EPA/DHA = 1.07) was successfully isolated (Fig. S11). Thus, we were able to alter the product profiles through three amino acid substitutions, N65L, F230L, and I231T.

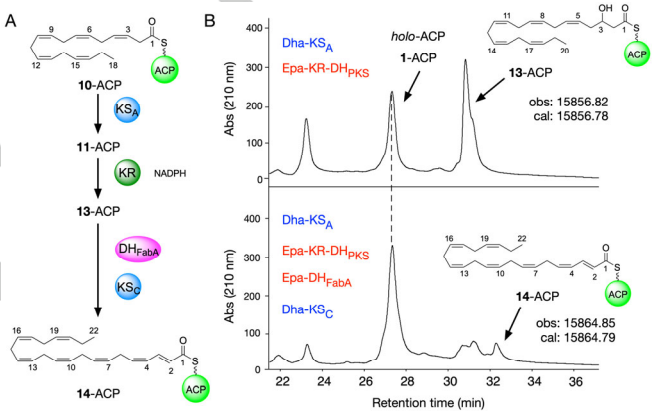

Figure 5. *In vitro* combination reactions. (A) Reaction scheme of *in vitro* combination reactions. (B) HPLC analysis (UV 210 nm) of *in vitro* combination reactions using 3,6,9,12,15-*cis* octadecapentaenoyl-ACP (**10**-ACP), Dha-KS_A, and Epa-KR-DH_{PKS} (top), plus Epa-DH_{FabA} and Dha-KS_C (bottom).

We recently showed that ARA and EPA synthases utilized DH_{PKS} and DH_{FabA} depending on the carbon chain length for introduction of saturation or *cis* double bonds on growing acyl chains[6]. As for the control mechanism of the carbon chain length, Orikasa *et al*., reported that "B" subunits, Dha-B and Epa-B, were key enzymes for determining the final product, DHA or EPA[9]. However, in this study, we unveiled the control mechanism of the carbon chain length, especially the mechanism involved in creating DHA and EPA.

The condensation of 3,6,9,12,15-*cis* octadecapentaenoyl-ACP (**10**-ACP), the last condensation in EPA biosynthesis, was catalyzed by the KS_A domain in both DHA and EPA biosynthesis. In EPA biosynthesis, a 5,8,11,14,17-*cis* 3-oxoeicosapentaenoyl-ACP (**11**-ACP) intermediate would be released after the reactions catalyzed by the KR, DH_{PKS}, and ER domains. Conversely, 2,5,8,11,14,17-*cis* eicosahexaenoyl-ACP (**12**-ACP) formed by KR and DH_{FabA} domains would be used as the substrate of the KS_C domain to form 4,7,10,13,16,19-*cis* docosahexaenoyl-ACP (**15**-ACP) in DHA biosynthesis (Fig. 2).

We also converted the microalgal DHA synthase to EPA synthase based on the obtained results. The K03 mutant possessing triple mutations, N65L, F230L, and I231T, produced EPA as the major product. Although we cannot provide a mechanism for substrate recognition, the F230 and I231 residues

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in the mutated enzyme were suggested to be located in an α-helix forming a cavity with the catalytic Cys residue by model building studies[10] with the crystal structure of mammalian fatty acid synthase[11] (PDB, 2VZ8), which has 25% identity with our enzyme (Fig. S12). Therefore, the helix region would be essential for strict substrate recognition.

Acknowledgements

This study was supported in part by Grants-in-Aid for Research on Innovative Areas from MEXT, Japan (JSPS KAKENHI Grant Number 16H06452 to T.D.) and Grants-in-Aid for Scientific Research from JSPS (15H03110 and 18H03937) to T.D. We thank Robbie Leiws, MSc, from Edanz Group for editing a draft of this manuscript.

Keywords: polyunsaturated fatty acid synthase • β-ketoacyl synthase • biosynthesis • carbon chain length

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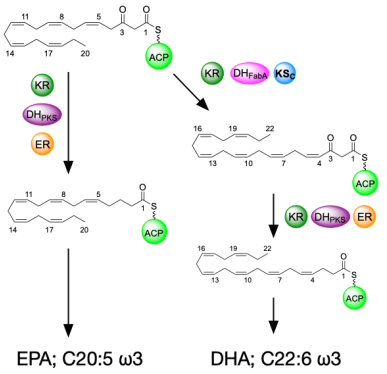
COMMUNICATION

Eicosapentaenoic acid (EPA; C20) and docosahexaenoic acid (DHA; C22) synthase complexes possess two β-ketoacyl synthase domains (KS_A and KS_C). We revealed that KS_A and KS_C function at early and late biosynthetic steps, respectively. In the last steps, however, KS_A catalyzed C_{18} to C_{20} elongation in both EPA and DHA biosynthesis while KS_C catalyzed C_{20} to C_{22} elongation in DHA biosynthesis, showing that KS_C domains determine the chain length.



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