Molecular mechanism of substrate specificity for delta 6 desaturase from Mortierella alpina and Micromonas pusilla

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Abstract The ω6 and ω3 pathways are two major pathways in the biosynthesis of PUFAs. In both of these, delta 6 desaturase (FADS6) is a key bifunctional enzyme desaturating linoleic acid or α-linolenic acid. Microbial species have different propensity for accumulating ω6- or ω3-series PUFAs, which may be determined by the substrate preference of FADS6 enzyme. In the present study, we analyzed the molecular mechanism of FADS6 substrate specificity. FADS6 cDNAs were cloned from Mortierella alpina (ATCC 32222) and Micromonas pusilla (CCMP1545) that synthesized high levels of arachidonic acid and EPA, respectively. M. alpina FADS6 (MaFADS6-I) showed substrate preference for LA; whereas, M. pusilla FADS6 (MpFADS6) preferred ALA. To understand the structural basis of substrate specificity, MaFADS6-I and MpFADS6 sequences were divided into five sections and a domain swapping approach was used to examine the role of each section in substrate preference. Our results showed that sequences between the histidine boxes I and II played a pivotal role in substrate preference. Based on our domain swapping results, nine amino acid (aa) residues were targeted for further analysis by site-directed mutagenesis. G194L, E222S, M227K, and V399I/I400E substitutions interfered with substrate recognition, which suggests that the corresponding aa residues play an important role in this process. —Shi, H., H. Chen, Z. Gu, Y. Song, H. Zhang, W. Chen, and Y. Q. Chen. Molecular mechanism of substrate specificity for delta 6 desaturase from Mortierella alpina and Micromonas pusilla. J. Lipid Res. 2015. 56: 2309–2321.

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Humans can synthesize long-chain PUFAs from the essential fatty acids, linoleic acid [LA (18:2$^{9,12}$)] and α-linolenic acid [ALA (18:3$^{9,12,15}$)], obtained from diet. However, the conversion of ALA to EPA (20:5$^{4,7,10,13,16,19}$) is limited, and further transformation to DHA (22:5$^{4,7,10,13,16,19}$) is also quite low (1, 2). On the other hand, some microorganisms are able to accumulate high levels of EPA or DHA (5–7). This large disparity can be accounted for by key enzymes in their biosynthesis pathway of ALA to EPA/DHA (Fig. 1). Delta 6 desaturase (FADS6), a membrane-bound desaturase which converts LA to γ-linolenic acid [GLA (18:3$^{9,12,15}$)] or ALA to stearidonic acid [SDA (18:4$^{5,8,11,14}$)] by introducing a double bond between carbons 6 and 7 from the carboxyl end of the substrate, is the critical enzyme in this pathway (6, 7). Catalytic characteristics of FADS6 with the ALA substrate determine whether it can be further converted to EPA and DHA (8). Besides ALA, FADS6 can also convert LA to GLA in a parallel delta 6 pathway (9) (Fig. 1). This conversion is more conspicuous, especially in some strains producing high levels of GLA or arachidonic acid (AA [20:4$^{5,8,11,14}$]) (10, 11). Thus, the way in which FADS6 regulates its catalytic direction appears to be a key factor in determining whether LA can be further converted to ω-6 PUFAs or ALA to ω-3 PUFAs. In other words, FADS6 is a molecular switch that controls the ω-3/ω-6 PUFAs’ metabolic flux. Therefore, the substrate

Abbreviations: aa, amino acid; AA, arachidonic acid (20:4$^{5,8,11,14}$); ALA, α-linolenic acid (18:3$^{9,12,15}$); FADS6, delta 6 desaturase; FAME, fatty acid methyl ester; GLA, γ-linolenic acid (18:3$^{9,12,15}$); His, histidine; LA, linoleic acid (18:2$^{9,12}$); Ma, Mortierella alpina; MaFADS6, Mortierella alpina delta 6 desaturase; Mp, Micromonas pusilla; MpFADS6, Micromonas pusilla delta 6 desaturase; PI, preference index; SDA, stearidonic acid (18:4$^{5,8,11,14}$).

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The preference of FADS6 for LA and ALA is likely to be different in species which produce greatly divergent levels of \(\omega-3\) and \(\omega-6\) PUFAs.

For example, in a small number of species, especially in marine microbes, EPA or DHA production is much higher than production of \(\omega-6\) PUFAs, probably because their FADS6s preferentially use ALA (12). The catalytic efficiency of FADS6s from various species with LA and ALA substrates is summarized in supplementary Table 1. From the results, we found that Micromonas pusilla, a type of marine microalgae whose FADS6 (MpFADS6) appears to function as an acyl-CoA desaturase, has the highest preference for the \(\omega-3\) substrate (ALA) compared with other plants and fungi. Its conversion efficiency was found to be 4.9% in the \(\omega-6\) pool and 63% in the \(\omega-3\) pool (12). MpFADS6 has the highest conversion ratio of ALA/LA by far. Therefore, M. pusilla was used as one of the sources of FADS6 in our study.

In contrast, the EPA or DHA production is fairly low or even absent in numerous oleaginous species, while the level of GLA or AA is high (10). This suggests that their FADS6s may have preference for LA. The genus Mortierella has been extensively studied for its production of GLA (13) or AA (14–16). Some species of this genus are now used for the commercial production of single cell oil that is rich in AA (17). Among them, Mortierella alpina (ATCC 32222), whose genome has previously been characterized in our lab (18), has the ability to synthesize a wide range of PUFAs (up to 50% of its cell dry weight), including AA as high as 40% of total fatty acid. Therefore, even if MaFADS6 had equivalent catalytic efficiency toward LA and ALA, it would
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not result in an equal level of its products, GLA and SDA (or longer-chain PUFAs), because initial levels of ALA are low. So the high levels of GLA and AA in *M. alpina* do not automatically imply that MaFADS6 has a strong substrate preference for LA.

Recently, some genes encoding FADS6 have been cloned and sequenced from many organisms (13, 20–26), but little is known about their molecular mechanism of FADS6 substrate preference. This is mainly due to the lack of crystal structure information. In this work, we cloned the MaFADS6 (MaFADS6 is equivalent to MaFADS6-I) and MpFADS6 genes, and characterized their substrate preference in *Saccharomyces cerevisiae*. Furthermore, domains were swapped with each other and the recombinant chimeras were expressed in *S. cerevisiae* to determine key areas responsible for the substrate preference. Finally, site-directed mutagenesis within key areas was used to identify sites that are important for substrate recognition.

**MATERIALS AND METHODS**

**Strains and plasmids**

Wild-type *M. alpina* (ATCC 32222) was from our laboratory. The MpFADS6 gene was synthesized by Shanghai Sunny Biotechnology Company, Ltd. and *Escherichia coli* Top 10 was preserved in our laboratory. INVSc1 yeast strain (Invitrogen) was used for confirmation of the strain and expression in *Saccharomyces cerevisiae*. Plasmid pYES2/NT/C (Invitrogen) was used for FADS6 expression.

**Media and cultural conditions**

*Escherichia coli* Top 10 was used for plasmid construction and cultivated at 37°C on LB agar plates. SCU is synthetic minimal defined medium for yeast consisting of 0.67% yeast nitrogen base [without amino acids (aas) but with ammonium sulfate], 2% glucose or raffinose (2% galactose and 1% raffinose for induction), 0.01% (adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan, and uracil), 0.005% (aspartic acid, histidine (His), isoleucine, methionine, phenylalanine, proline, serine, tyrosine, and valine), and 2% agar (for plates). *M. alpina* was grown on potato dextrose agar medium at 28°C.

**RNA isolation and gene synthesis**

Approximately 1 g (fresh weight) of *M. alpina* protonemal tissue was ground to a fine powder under liquid nitrogen using a precooled mortar and pestle. Total RNA was isolated from cooled powder using the HYQspin™ Total RNA Kit (HG403-03), and 1 μg RNA was reverse-transcribed with the QuantScript RT kit (TIANGEN) according to the manufacturer’s instructions. The cDNA transcribed was used as a template for MaFADS6 amplification with primers. In this work, MaFADS6 is equivalent to MaFADS6-I, because the RNA transcriptional level of the MaFADS6-II isoform is quite low (16).

MpFADS6 gene was synthesized by Shanghai Sunny Biotechnology Company, Ltd. and subcloned into the vector pUC57 and transformed into DH5α.

**Primer design, PCR amplification, and sequence analysis for MaFADS6 and MpFADS6**

For MaFADS6 and MpFADS6 gene amplification, primers were synthesized based on MaFADS6 [DDBJ (DNA Data Bank of Japan) / EMBL (The European Molecular Biology Laboratory) / GenBank accession number ADAG00000000 ([first version]) and MpFADS6 gene sequences. The forward primers were FMA and FMp and the reverse primers were RMa and RMp (all primers are listed in Table 1). PCR was carried out in a total volume of 50 μl, with initial denaturation at 94°C for 4 min, amplification was performed in 30 cycles of 40 s at 94°C, 40 s at 55°C, and 2 min at 68°C, followed by a final extension at 68°C for another 5 min. Amplification products were fractionated on 1.0% agarose gels and subcloned into the pYES2 vector (Invitrogen) downstream of the GAL1 promoter to generate recombinant plasmids designated pYES2-MaFADS6 and pYES2-MpFADS6. The constructs were transformed into competent *E. coli* cells and positive clones were selected by colony PCR with T7 and pYES2-R primers. Positive clones were sequenced in both directions.

**Construction of chimeric genes by reciprocal section swapping**

According to the conserved regions of FADS6 (HPGG and three His boxes), MaFADS6 and MpFADS6 genes were divided into five regions (Fig. 3A). The corresponding regions of both enzymes were systematically exchanged to construct recombinant swap genes in order to determine enzymatic specificity of each fragment. Recombinant swap genes were generated by overlapping extension PCR with the primers listed in Table 1. In the first step, each fragment was amplified individually from the MaFADS6 and MpFADS6 cDNA template using primers specified in Table 1. In the second step, two adjacent fragments that had homologous arms were fused using upstream and downstream primers to produce double fragments if the recombinant swap gene had three fragments. Similarly, double fragments and a complementary fragment were fused by the same method (with the longer one generally selected). The amplification procedure included 10 min at 94°C, 2 min at 60°C, and 1 min per kilobase at 68°C, followed by 35 cycles of 50 s at 94°C, 50 s at 60°C, and 1 min per kilobase at 68°C, and a terminal extension step of 5 min at 68°C. Amplification products were fused in a subsequent PCR reaction, and the protocol used was the same as for the first step. The hybrid genes were digested with EcoRI/NotI and ligated into the pYES2 plasmid (Invitrogen) with a His tag sequence, and transformed into competent Top10 cells.

**Site-directed mutagenesis of MpFADS6**

For mutagenesis, oligonucleotide primers were used to introduce nucleotide substitutions into MpFADS6 using the Fast Site-Directed Mutation kit (TIANGEN) according to the manufacturer’s instructions. Seven mutants derived from the MpFADS6 gene were constructed, where aas were substituted with the corresponding residues in the MaFADS6 gene (V189L/Q190A, G194L, S197Q, Q209G, Q222S, M227K, and V399I/H400E). Mutation primers are listed in Table 1. All mutants were verified by colony PCR with T7 and pYES2-R primers. Positive mutants were sequenced in both directions.

**Yeast transformation, heterologous expression in *S. cerevisiae*, and determination of substrate preference for LA or ALA by measuring chimeric desaturase activity**

Constructs pYES2-chimera 1~10, pYES2-MaFADS6, and pYES2-MpFADS6 were transformed into *S. cerevisiae* using the lithium acetate transformation method (27). After SC-U plate selection, the recombinant yeasts were selected on uracil-deficient medium containing 1.0% (w/v) raffinose as the single carbon source. MaFADS6 and MpFADS6 genes were induced under transcriptional control of the GAL1 promoter containing 2% galactose for 12 h at 28°C.

The pellets were collected by centrifuging 2 ml culture at 6,000 g for 1 min. Yeast pellets were resuspended in 80 μl breaking
### Lipid extraction and fatty acid analysis

Lipids from an equivalent weight of freeze-dried cells and supernatant were extracted and methyl esterified as described previously (28). Fatty acid methyl esters (FAMEs) were analyzed by GC. Pentadecanoic acid (C15:0; NU-CHEK) and heicosanoic acid (C21:0; NU-CHEK) were added to the biomass samples as internal standards to quantify the fatty acid content. GC analysis was performed with a GC-2010 Shimadzu Company, Japan equipped with a flame ionization detector and a capillary DBWAX column (30 m x 0.25 mm, Agilent). The samples were measured with a split of 20:1 with the injector temperature set to 240°C. The column temperature was 180°C. FAMEs were identified by comparing with polyvinylidene fluoride membrane by electroblotting (150 mA, one for Western blotting analysis.

### TABLE 1. Primers used in this study

| Primer Name | Restriction Enzyme | Oligonucleotide Sequence (5’3’) | Function |
|-------------|-------------------|---------------------------------|----------|
| F-Ma        | Hind III          | GGCCCACGGCTTAATGGCTGCCTGCTGCCCAAGTGTTAGGAC | MaFADS6 amplification for expression in S. cerevisiae |
| R-Ma        | Xho I             | CCGGGGCTCTAGTTACTGCGGCTTACCCATCTTTGAGGAGG | MpFADS6 amplification for expression in S. cerevisiae |
| F-Mp        | Eco R I           | TACCGGAATTCATGGCCCGCCGAAGACGGA | Target genes insert detection for yeast expression construction |
| R-Mp        | Xba I             | TCTAGACCCGGGTACATGGCGGCTTCCCGCTTGCAGCG | Target genes insert detection for yeast expression construction |
| T7          | —                 | TAAATACGACTCACTATAGGG | Overlap extension PCR for ten kinds of fusion genes |
| pYES2.R     | —                 | TCCTGATAGACCGGATGTC | Target genes insert detection for yeast expression construction |
| FA          | Eco R I           | GGCCCCGAATTCATGGCCCGCCGAAAGAGC GA | Target genes insert detection for yeast expression construction |
| RA          | —                 | CACACTCCACCGGGATTGATTGTTGGGAA | Target genes insert detection for yeast expression construction |
| RB          | Not I             | CCAAGGAAAGAGAAAAAAGCCGCGCTTACCTGCGCTTACCCATCTTG | Target genes insert detection for yeast expression construction |
| FC          | Eco R I           | GGCCCCGAATTCATGGCCCGCCGAAAGAGC GA | Target genes insert detection for yeast expression construction |
| FD          | —                 | GTGCTATGGTTCCTGACATGGTTCGCACATCCAC | Target genes insert detection for yeast expression construction |
| RD          | —                 | ATGCAAAAATTCGTGCTGACCCCAACCGCA | Target genes insert detection for yeast expression construction |
| FF          | —                 | CAGCGATTTTTCATACCTGCTACCGGGG | Target genes insert detection for yeast expression construction |
| RF          | —                 | GTGCTATGGTTCCTGACATGGTTCGCACATCCAC | Target genes insert detection for yeast expression construction |
| RH          | —                 | ATGCAAAAATTCGTGCTGACCCCAACCGCA | Target genes insert detection for yeast expression construction |
| FK          | —                 | CAGCGATTTTTCATACCTGCTACCGGGG | Target genes insert detection for yeast expression construction |
| RR          | —                 | ATGCAAAAATTCGTGCTGACCCCAACCGCA | Target genes insert detection for yeast expression construction |
| FT          | —                 | CAGCGATTTTTCATACCTGCTACCGGGG | Target genes insert detection for yeast expression construction |

* Underlined sequences indicate the additional restriction sites or mutation sites.
commercial FAME standards (SDA-ME and 37 component FAME mix; Supelco).

**Topology prediction**

Prediction of transmembrane helices and topology for MaFADS6 and MpFADS6 was performed with I-TASSER (http://zhanglab.ccmr.med.umich.edu/) (Fig. 6B). Both predicted topology models were in qualitative agreement with other membrane-bound enzymes.

**RESULTS**

**Cloning of MaFADS6 and MpFADS6 and characterization of their substrate specificity**

To characterize the substrate specificity of MaFADS6 and MpFADS6, a 1,374 bp fragment was amplified using FMA and RMA primers, and a 1,392 bp fragment was amplified using FMP and RPM primers (primers listed in Table 1). Successful expression of pYES2-MaFADS6 and pYES2-MpFADS6 was confirmed by Western blotting. They were incubated with 0.5 mM LA, 0.5 mM ALA, or 0.25 mM of each of them as fatty acid substrates for 12 h at 28°C, and the resulting fatty acid composition is listed in Table 2. When a single substrate was added, LA and ALA could each be catalyzed by MaFADS6 and their conversion rates were 45.6 ± 2.1% and 19.6 ± 1.3%, respectively. When LA and ALA were added at the same time, the LA conversion rate of MaFADS6 reached 58.4 ± 1.4%, but ALA conversion decreased to 2.0 ± 0.9%. These results show that MaFADS6 is capable of catalyzing LA and ALA conversion to GLA and SDA, respectively. However, when LA and ALA were present at the same time, MaFADS6 preferentially catalyzed LA to GLA. Similarly, MpFADS6 had a preference for the ALA substrate with a 66.5 ± 2.8% conversion rate, which is consistent with the results of Petrie et al. (12).

**Multiple sequence alignment among FADS6s**

The deduced aa sequences of MaFADS6 showed 20.35% identity with the aa sequence of MpFADS6. In order to determine the structural basis of ω-3/ω-6 substrate preference, multiple sequence alignment was performed among several FADS6 genes, arranged according to their substrate preference for LA or ALA (the closer to the top, the stronger preference for ALA; the closer to the bottom, the stronger preference for LA). The result of multiple sequence alignment among these FADS6s revealed that there were three conserved His-rich motifs, HDFLHH (Ma172-177) and HEGGH (Mp191-196), HDFLHH (Ma209-214) and HNKHH (Mp228-232), and QEHH (Ma395-399) and QVHH (Mp398-402) (Fig. 2). In addition, a cytochrome b5-like domain, HPGG, was found near the N terminus, which is required as an electron donor for fatty acid desaturation. Alignment and analysis of MaFADS6 and MpFADS6 sequences with other FADS6s indicated that the homology occurs mainly in the cytochrome b5-like domain and in the three conserved His-rich motif areas. We expected that FADS6s with ω-3 substrate preference should have a homologous site or fragment not present in FADS6s with ω-3 substrate preference, but our multiple sequence alignment results failed to uncover such a site or fragment.

**Construction and expression of fusion genes in yeast**

To identify which structural elements were functionally involved in substrate preference of MaFADS6 and MpFADS6, both enzymes were divided into five sections as follows: section 1, the N-terminal end region minus the HPGG domain; section 2, from the HPGG domain to His box I (including HPGG); section 3, from His box I to His box II (including His box I); section 4, from His box II to His box III (including His box II); and section 5, the C-terminal region including His box III (Fig. 3A). After expression in S. cerevisiae, 10 different chimeras were quantified by Western blotting analysis. Our results showed that none of the chimeras were clearly seen by SDS-PAGE, probably because the expression level of membrane-bound proteins in S. cerevisiae was too low to be observed with the naked eye. However, our Western blotting analysis showed that all chimeras were expressed.

**Table 2.** Fatty acid compositions of the total lipid contents of yeast transformants harboring the control plasmid (pYES2) and the recombinant plasmids (pYES2-MaFADS6 and pYES2-MpFADS6)

| Fatty Acid | pYES2 (control) | pYES2-MaFADS6 | pYES2-MpFADS6 |
|------------|-----------------|---------------|---------------|
|            | +LA +ALA +LA and +ALA | +LA +ALA +LA and +ALA | +LA +ALA +LA and +ALA |
| 16:0 (PA)  | 24.9 ± 0.1      | 23.6 ± 0.2    | 21.5 ± 0.0    |
| 16:1 (PA)  | 13.7 ± 0.3      | 12.6 ± 0.1    | 13.9 ± 0.2    |
| 18:0 (SA)  | 8.7 ± 0.1       | 9.2 ± 0.6     | 9.8 ± 0.8     |
| 18:1 (OA)  | 11.7 ± 0.8      | 10.3 ± 0.7    | 10.6 ± 0.4    |
| 18:2 (LA, ω-6) | 41.0 ± 0.6     | ND            | 21.0 ± 0.3    |
| 18:3 (ALA, ω-3) | ND            | 39.2 ± 0.1    | 23.2 ± 0.2    |
| 18:3 (GLA, ω-6) | ND            | ND            | 18.0 ± 0.5    |
| 18:4 (SDA, ω-3) | ND            | ND            | 7.0 ± 0.5     |
| LA conversion rate | —              | —             | 45.6 ± 2.1    |
| ALA conversion rate | —              | —             | 19.6 ± 1.3    |

| Fatty acid composition values are percentage by weight. ND, not detected. 16:0 (PA, palmitic acid); 16:1 (PA, palmitelaidic acid); 18:0 (SA, stearic acid); 18:1 (OA, oleic acid).
| Conversion rate = 100 × [product/(product + substrate)].
Fig. 2. Multiple sequence alignment of deduced aas for MaFADS6 (−10) and MpFADS6 (+13) with FADS6s from other species, including *Oncorhynchus mykiss* (+12), *Mantoniella squamata* (+11), *Cyprinus carpio* (+10), *Primula vialii* (+9), *Psetta maximus* (+8), *Sparus aurata* (+7), *Mucor rouxii* (+6), *Conidiobolus obscurus*
and their molecular mass was as predicted (Fig. 4A and data not shown). The relative expression level of every recombinant protein was fairly stable, with a range of 0.7–2.1 compared with MaFADS6, defined as 1 (Fig. 4B).

**Determination of substrate preference in 12 types of fusion FADS6 constructs**

For substrate preference determination, lipids from dried cells and supernatant were extracted to determine the conversion efficiency of each chimera (no fatty acids were detected in supernatant, data not shown). To account for differences in chimera expression, substrate conversion rates of all chimeras were converted to relative substrate conversion rates (Fig. 3C). Our analysis showed that the catalytic efficiency of chimera 3 (MaFADS6 aa172–208 replaced by MpFADS6 aa191–227) for ALA increased from 4.0 to 15.0%; whereas, the catalytic efficiency of chimera 3 for LA decreased from 73.9 to 24.0%; and, conversely, the efficiency of chimera 8 (MpFADS6 aa191–227 replaced by MaFADS6 aa172–208) for ALA decreased from 75.2 to 9.5%, but for LA increased from 5.2 to 16.3% compared with their corresponding wild-types. These results suggest that these 37 aas in section 3 are in part responsible for the substrate preference. Replacement of aa1–49 (chimera 1), aa50–171 (chimera 2), aa209–394 (chimera 4), or aa395–458 (chimera 5) in MaFADS6 caused partial or complete loss of activity. Similarly, replacement of aa1–68 (chimera 6), aa228–397 (chimera 9), or aa398–464 (chimera 10) in MpFADS6 resulted in partial or complete loss of activity. Replacement of aa69–190 (chimera 7) in MpFADS6, however, seemed to significantly decrease its activity toward ALA, while slightly increasing its activity toward LA (Fig. 3C).

**The effect of substrate concentration on the conversion rate**

To understand the effect of substrate concentration on the conversion rate, 0.125, 0.25, and 0.5 mM cis-LA/ALA were used in yeast cultures expressing active fatty acid desaturases, i.e., MaFADS6, MpFADS6, and chimeras 1, 3, and 5–9. Our results revealed that substrate concentration had little effect on the conversion rate in yeast expressing MaFADS6, MpFADS6, and chimera 5; whereas, it significantly augmented ALA conversion from 11.0 ± 1.7% to 19.2 ± 3.2% in chimera 3 and LA conversion from 8.7 ± 0.8% to 19.2 ± 0.7% in chimera 8. However, the substrate selectivity remained unchanged at any fatty acid concentrations (Table 3).

**Determination of substrate preference by mutating aas from MpFADS6**

To further understand the molecular mechanism of MpFADS6 substrate preference, we constructed a series of mutants in those 37 aas within section 3 and a double mutant within section 5. Targeted mutagenesis of V189L/Q190A, S197Q, and Q209G did not lead to major changes in substrate preference. However, the relative conversion rate of ALA was reduced almost by half in double mutant V991I/I400E and single mutants E222S and M227K (40.26, 31.42, and 31.61%, respectively) compared with MpFADS6 (71.37%). In addition, the relative conversion rate of ALA was significantly reduced to 6.50% in the presence of a G194L substitution. The relative conversion rate for LA remained low in all mutants (Fig. 5).

**DISCUSSION**

Our result indicates that the 37 aas between the His boxes I and II (Fig. 6) are in part responsible for the substrate preference (Fig. 3). Comparing sequences between the group with preference index (PI) greater than 1 and that with PI smaller than −1 within this 37-aa segment, four positions show clear differences, namely 190Q, 194G, 197S, and 209Q/H in the PI >1 group, and 170A, 175L, 178Q, and 190G are the corresponding aa residues in the same position in the PI <1 group. Among the single mutants corresponding to these sites, G194L displayed the largest decrease in ALA conversion rate. In the topological model of the FADS6, 194G is located within His box I, suggesting that 194G is a key residue in His box I that influences the ALA conversion to SDA. In the single mutant G194L, steric bulk presented by the larger Leu residue may cause the substrate to bind more loosely. Interestingly, it has been reported that sequences between His boxes I and II are involved in the regioselectivity of ω-3 and ω-6 fatty acid desaturases from *Pichia pastoris* (29) and *Aspergillus nidulans* (30). It has also been shown that this segment is implicated in fatty acid carbon length preference in FADS6 from *Mucor rouxii*, and the 213S and 218K residues, which correspond to the 222E and 227M residues from MpFADS6 mutants, may be critical (31). According to the predicted structural model, residues 222 and 227 are located right upstream of the second conserved His box. These two positions were also found to be important for ALA catalysis, but to a lower extent than 194G. Thus, these two residues are unlikely to be located near the binding site of the substrate or to have direct interactions with the ALA substrate. None of

(+) Primula farinosa (+4), Acanthopagrus schlegeli (+3), *Thalassiosira pseudonana* (+2), *Glossomastix chrysoplasta* (+1), *Ostreococcus tauri* (+1), *Phanodactylum tricornutum* (+2), *Parachloris incisa* (+3), *Ceratodon purpureus* (+4), *Rhizopus arrhizus* (+5), *Cunninghamella echinulata* (+6), *Pythium irregulare* (+7), *Mortierella fungus* 1S-4 (+8), and *M. alpina* W15 (+9) using ClustalX. The three conserved His-rich motifs and a cytochrome b5-like domain are underlined in red. Black arrows indicate domain boundaries for reciprocal section swapping. “+” and “−” species denominations are identical to those in supplementary Table 1.
Fig. 3. A: Skeleton map of the aa sequences of each FADS6, including division points. Dotted lines show the points where MpFADS6 (black box) and MaFADS6 (white box) were divided into five sections. The braces indicate trans-membrane sequences of FADS6s. B: Map of each chimeric protein constructed by reciprocal section swapping. Regions in white boxes originated from MaFADS6 and regions in black boxes originated from MpFADS6. All primer sequences are listed in Table 1. Substrate conversion efficiency of each chimera was classified into three groups: ND, not detected; +, 1–10%; ++, 11–30%; +++ , 31–100%. C: The relative substrate conversion rate of each recombinant protein expressed in *S. cerevisiae*, determined by adding 0.25 mM LA and 0.25 mM ALA after induction with 2% galactose. Relative substrate conversion rate = 100 × [product/(product + substrate)]/Density_{WB}/Density_{SDS-PAGE}. WB, Western blot. Histograms were established as the means of three independent samples (three transformants in SC-U plates) and error bars represent standard deviations. The + signs above the bars represent relative conversion rate determined from this study, and are as shown in (B).
Mechanism of substrate specificity for delta 6 desaturase

Because all known membrane-bound fatty acid desaturases contain the characteristic three His-box motifs, sequences between His boxes I and II may be a key

the single or double mutants increased LA conversion rate, which suggests that the substrate preference of MpFADS6 may be dependent on several aa residues.

Fig. 4. A: SDS-PAGE and Western blotting of recombinant gene expression in *S. cerevisiae*. M, marker; 01, pYES2 control vector; 02~04, pYES2-MaFADS6; 05~07, pYES2-MpFADS6; 08~10, chimera 1; 11~13, chimera 2. The arrow in the SDS-PAGE gel and the Western blot indicate the scanning region by Quantity One computational software; the Adjust Volume Value in Western blot and SDS-PAGE, in function of the amount of each chimeric protein, was determined by Density 

WB (WB, Western blot) and DensitySDS-PAGE. (There were no FADS6s bands in SDS-PAGE because the level of membrane-bound proteins expressed by *S. cerevisiae* was too low to see in SDS-PAGE.) B: Relative amount of expressing each recombinant protein in *S. cerevisiae*. Data was acquired using Quantity One. Relative amount value was acquired by DensityWB/DensitySDS-PAGE. Amount of MaFADS6 was defined as 1.
These residues were not very relevant for enzymatic activity of ALA conversion approximately by half, suggesting that the corresponding sites of MaFADS6. This decreased created by substituting residues 399V and 400I near the disrupt their function. Therefore, a double-mutant was several key sites at the junction of these two sections may our interpretation of this result is that the combination of common and no product was detected for these mutants.

On the contrary, MaFADS6 is less stringent in section 5, disrupted, which caused its inactivation. Therefore the section 2 of MpFADS6 plays a part in catalysis of ALA. In fact, we did not detect conversion rate of chimera 2 for ALA should be higher than the wild-type MpFADS6, suggesting that MpFADS6 plays a part in catalysis of ALA, and thus, the catalytic center of FADS6, comprised by three His boxes and two ferric ions, should interact with and desaturate the three-dimensional structure of chimera 2 was disrupted, which caused its inactivation. Therefore the sec-

The substrate preference can be further analyzed in terms of the molecular structure of cis-LA and cis-ALA. Figure 6A illustrates the C=C double-bond structure in the ω-3 position of cis-ALA and the C=C bond in the same position of cis-LA. We reasoned that the molecular mechanism of substrate preference for MaFADS6 and MpFADS6 may be their binding with the corresponding position of the respective substrate. It is likely that the catalytic areas of two enzymes are in common because, except for the ω-3 position substrate, overall molecular structure is identical. We propose the following model (Fig. 6B) based on the structures of stearoyl-CoA desaturase reported recently (32, 33): there are four membrane-spanning helices (1, 2, 9, and 10) that are connected through two short endoplasmic reticulum lumen loops, and two membrane-embedding helices (3, 13). Key aas between His boxes I and II and the catalytic center of FADS6, comprised by three His boxes and two ferric ions, should interact with and desaturate the sixth and seventh carbon positions.

### Table 3. Fatty acid compositions of yeast transformants expressing the wild-type and chimera fatty acid desaturases

| Fatty Acid | FADS6 | Ma | Mp | 1 | 3 | 5 | 6 | 7 | 8 | 9 |
|------------|-------|----|----|---|---|---|---|---|---|---|
| cis-LA/ALA (0.125 mM) |       |    |    |   |   |   |   |   |   |   |
| 18:2 (LA, ω-6) | 7.0 ± 0.7 | 14.0 ± 0.3 | 10.0 ± 0.3 | 10.6 ± 0.5 | 10.2 ± 1.0 | 15.7 ± 1.7 | 15.1 ± 0.5 | 13.4 ± 0.3 | 14.8 ± 0.6 |
| 18:3 (ALA, ω-3) | 16.3 ± 0.3 | 6.3 ± 0.3 | 13.5 ± 0.6 | 12.5 ± 0.3 | 14.0 ± 1.0 | 14.2 ± 1.3 | 13.9 ± 1.0 | 14.6 ± 1.4 | 14.3 ± 1.1 |
| 18:3 (GLA, ω-6) | 8.5 ± 0.7 | 0.3 ± 0.1 | 4.0 ± 0.6 | 4.0 ± 0.2 | 3.0 ± 0.1 | 0.0 ± 0.0 | 1.2 ± 0.1 | 1.9 ± 0.1 | 0.5 ± 0.0 |
| 18:3 (SDA, ω-3) | 0.2 ± 0.0 | 0.7 ± 0.9 | 0.2 ± 0.0 | 0.9 ± 0.3 | 0.1 ± 0.0 | 1.1 ± 0.2 | 1.5 ± 0.3 | 1.4 ± 0.2 | 1.3 ± 0.3 |
| LA conversion rate | 54.9 ± 2.5 | 2.0 ± 0.4 | 0.0 ± 0.0 | 11.0 ± 1.7 | 0.0 ± 0.0 | 7.4 ± 1.3 | 7.2 ± 0.9 | 7.1 ± 0.3 | 8.6 ± 1.1 |
| ALA conversion rate | 1.5 ± 0.3 | 55.4 ± 1.7 | 14.9 ± 0.2 | 15.3 ± 2.5 | 15.3 ± 2.9 | 24.5 ± 1.1 | 22 ± 0.4 | 21.9 ± 0.3 | 23.7 ± 0.5 |
| cis-LA/ALA (0.25 mM) |       |    |    |   |   |   |   |   |   |   |
| 18:2 (LA, ω-6) | 9.1 ± 2.0 | 20.4 ± 0.7 | 25.0 ± 0.4 | 21.1 ± 0.6 | 21.3 ± 1.3 | 21.1 ± 1.1 | 22.5 ± 0.4 | 23.5 ± 1.3 | 22.9 ± 0.4 |
| 18:3 (ALA, ω-3) | 25.9 ± 1.5 | 9.5 ± 0.1 | 7.6 ± 0.1 | 6.6 ± 0.7 | 10.7 ± 2.4 | 0.0 ± 0.0 | 2.5 ± 0.2 | 2.4 ± 0.3 | 1.4 ± 0.3 |
| 18:3 (GLA, ω-6) | 13.8 ± 2.0 | 13.0 ± 0.5 | 0.0 ± 0.0 | 5.0 ± 0.1 | 0.0 ± 0.0 | 3.9 ± 0.1 | 3.9 ± 0.4 | 4.1 ± 0.3 | 3.9 ± 0.3 |
| 18:3 (SDA, ω-3) | 1.1 ± 0.3 | 16.4 ± 2.4 | 35.7 ± 0.2 | 29.8 ± 1.4 | 23.2 ± 2.5 | 0.0 ± 0.0 | 14.7 ± 1.3 | 16.8 ± 1.0 | 5.6 ± 1.1 |
| LA conversion rate | 60.5 ± 2.0 | 61.2 ± 2.0 | 0.0 ± 0.0 | 19.0 ± 0.4 | 0.0 ± 0.0 | 15.3 ± 0.8 | 10.1 ± 0.9 | 10.0 ± 1.2 | 14.6 ± 0.8 |
| ALA conversion rate | 2.1 ± 0.9 | 63.2 ± 2.9 | 19.1 ± 0.4 | 19.8 ± 4.6 | 19.7 ± 3.0 | 32.8 ± 0.7 | 30.5 ± 0.7 | 32.2 ± 1.3 | 32.2 ± 0.3 |
| cis-LA/ALA (0.5 mM) |       |    |    |   |   |   |   |   |   |   |
| 18:2 (LA, ω-6) | 11.0 ± 3.7 | 26.4 ± 1.2 | 36.1 ± 1.3 | 29.3 ± 1.3 | 28.8 ± 1.6 | 28.1 ± 1.1 | 28.4 ± 0.6 | 27.8 ± 0.3 | 31.3 ± 0.2 |
| 18:3 (ALA, ω-3) | 35.2 ± 2.7 | 12.3 ± 0.4 | 10.5 ± 0.2 | 8.5 ± 1.4 | 18.0 ± 4.7 | 0.0 ± 0.0 | 5.6 ± 0.6 | 6.2 ± 0.1 | 1.7 ± 0.1 |
| 18:3 (GLA, ω-6) | 18.4 ± 3.2 | 21 ± 0.9 | 0.0 ± 0.0 | 7.7 ± 0.3 | 0.5 ± 0.1 | 6.3 ± 0.2 | 3.1 ± 0.3 | 3.1 ± 0.3 | 6.1 ± 0.1 |
| 18:3 (SDA, ω-3) | 1.2 ± 0.5 | 24.3 ± 4.5 | 35.5 ± 0.0 | 30.0 ± 2.2 | 26.1 ± 3.0 | 0.0 ± 0.0 | 15.4 ± 1.6 | 19.2 ± 0.7 | 6.1 ± 0.2 |
| LA conversion rate | 63.3 ± 4.5 | 7.1 ± 2.7 | 0.0 ± 0.0 | 19.2 ± 3.2 | 1.1 ± 0.5 | 18.3 ± 0.0 | 9.9 ± 1.1 | 10.2 ± 0.8 | 16.4 ± 0.1 |
| ALA conversion rate | 3.2 ± 1.1 | 66.1 ± 3.5 | 19.8 ± 4.6 | 19.7 ± 3.0 | 32.8 ± 0.7 | 30.5 ± 0.7 | 32.2 ± 1.3 | 32.2 ± 0.3 | 66.1 ± 3.5 |

Fatty acid composition values are percentage by weight. ND, not detected.

*Conversion rate = 100 × (product/(product + substrate)).

†Chimeras 2, 4, and 10, which did not have a LA or ALA conversion rate when the substrate concentration was 0.25 mM cis-LA/ALA were not determined.
Fig. 5. A: A partial comparison of deduced FADS6 aas between the His boxes I and II for 23 species listed in supplementary Table 1. Black lines indicate the boundaries of the five sections in MaFADS6 and MpFADS6. His clusters 1, 2, and 3 are indicated by bars under the sequences. Arrows below the alignment indicate the aa positions that were selected for site-directed mutagenesis. B: Relative substrate conversion rate of each mutant. LA (0.25 mM) and ALA (0.25 mM) were added in yeast cultures after induction with 2% galactose. Substrate conversion rate = 100 × [product/(product + substrate)]/[Density WB /Density SDS-PAGE]. WB, Western blot. Histograms represent the means of three replicates and error bars represent standard deviations. Substrate conversion efficiency of each chimera for ALA was classified into three groups: +, 0–10%; ++, 11–40%; ++++, 41–100%.
Fig. 6. A: The molecular structure of cis-LA, cis-ALA, cis-GLA, and cis-SDA. B: The predicted topology model of FADS6. Four transmembrane α-helices (1, 2, 9, and 10) span the endoplasmic reticulum lumen (ER lumen). The other polypeptides extend into the cytoplasm. Two ferric ions are bound at the center of the cytoplasmic domain. According to this model, the pocket shape should be different between MaFADS6 and MpFADS6 in order to accommodate the ω end of LA and ALA. The chemical structural formulas are ALA in red and CoA in blue.
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