Identification of amino acid residues that determine the substrate specificity of mammalian membrane-bound front-end fatty acid desaturases

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Abstract Membrane-bound desaturases are physiologically and industrially important enzymes that are involved in the production of diverse fatty acids such as polyunsaturated fatty acids and their derivatives. Here, we identified amino acid residues that determine the substrate specificity of rat Δ6 desaturase (D6d) acting on linoleoyl-CoA by comparing its amino acid sequence with that of Δ5 desaturase (D5d), which converts dihomo-γ-linolenoyl-CoA. The N-terminal cytochrome b5-like domain was excluded as a determinant by domain swapping analysis. Substitution of eight amino acid residues (Ser209, Asn211, Arg216, Ser235, Leu236, Trp244, Gln245, and Val344) of D6d with the corresponding residues of D5d by site-directed mutagenesis switched the substrate specificity from linoleoyl-CoA to dihomo-γ-linolenoyl-CoA. In addition, replacement of Leu323 of D6d with Phe323 on the basis of the amino acid sequence of zebra fish Δ5/6 bifunctional desaturase was found to render D6d bifunctional. Homology modeling of D6d using recent crystal structure data of human stearoyl-CoA Δ9 desaturase revealed that Arg216, Trp244, Gln245, and Leu323 are located near the substrate-binding pocket. To our knowledge, this is the first report on the structural basis of the substrate specificity of a mammalian front-end fatty acid desaturase, which will aid in efficient production of value-added fatty acids.—Watanabe, K., M. Ohno, M. Taguchi, S. Kawamoto, K. Ono, and T. Aki. Identification of amino acid residues that determine the substrate specificity of mammalian membrane-bound front-end fatty acid desaturases. J. Lipid Res. 2016, 57: 89–99.

Supplementary key words site-directed mutagenesis • heterologous expression • mass spectrometry • homology modeling

Fatty acid desaturases are oxidases that introduce a double bond in the acyl chain of a fatty acid substrate by removing two hydrogens from adjacent carbon atoms using active oxygen. They comprise two types. Water-soluble desaturases are found in cyanobacteria and higher plants and act on the acyl chain bound to acyl carrier protein (ACP) (1), whereas membrane-bound desaturases from fungi, higher plants, and animals act on acyl-CoA or acyl-lipid substrates (2, 3). Some water-soluble enzymes such as castor Δ9 desaturase and ivy Δ4 desaturase are well characterized, and their crystal structures have revealed a molecular interaction between the ACP portion of the substrate and an amino acid located at the substrate-binding pocket of the enzyme, which could be the basis for change in the substrate specificity (4). The membrane-bound desaturases associate with endoplasmic reticulum membranes via two large hydrophobic domains that separate three hydrophilic clusters. The N-terminal hydrophilic region of some of these desaturases including mammalian Δ5 and Δ6 desaturases (D5d and D6d, respectively) and the C-terminal region of Saccharomyces cerevisiae Δ9 desaturase (OLE1p) contain a cytochrome b5-like heme-binding His-Pro-Gly-Gly (HPGG) motif. The histidine residue is indispensable for electron transfer from NADH-dependent cytochrome b5 reductase during the redox reaction (5, 6). Both this motif and that of diffused cytochrome b5 are necessary to fully express desaturase activity (7, 8). The other hydrophilic regions contain three histidine clusters (HXγ̅H, HXγ̅HH, and QXγ̅H) that form a catalytic center by coordinating nonheme diiron centers, and all of these histidine residues and the glutamine residue are essential for enzymatic activity (9, 10). D5d and D6d, as well as Δ4 desaturase, introduce a double bond at the respective Δ positions

Abbreviations: ACP, acyl carrier protein; ARA, arachidonic acid; D5d, Δ5 desaturase; D6d, Δ6 desaturase; DGLA, dihomo-γ-linolenic acid; DMOX, 4,4-dimethyloxazoline; FAME, fatty acid methyl ester; GLA, γ-linolenic acid; LA, linoleic acid; ΔD5/6d, zebra fish bifunctional Δ5/6 desaturase.
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of fatty acid substrates between the carboxyl group and a preexisting double bond; therefore, these enzymes are called “front-end” desaturases (11). They are distinct from desaturases of \(\omega-\alpha\) and \(\alpha-\alpha\) types that form double bonds at the methyl-terminal side.

The substrate specificity and regioselectivity (double bond positioning) of membrane-bound desaturases are defined by the structural fitness and interface affinity between the fatty acid substrate, including CoA and the lipid carrier, and the substrate-binding pocket with its surrounding residues. Protein engineering has been applied to understand the structure-function relationship. For instance, domain swapping has been used to identify the regioselective sites of nematode \(\Delta 12\) and \(\omega 3\) desaturases (12), a region determining the substrate specificity of \textit{Aspergillus nidulans} \(\Delta 12\) and \(\omega 3\) desaturases (13), and a substrate recognition region of black currant \(\Delta 6\) fatty acid desaturase and \(\Delta 8\) sphingolipid desaturase (14). Site-directed mutagenesis based on amino acid sequence comparison has been used to identify amino acids participating in the substrate specificity of \textit{Mucor rouxii} D6d (15). \textit{Siganus canaliculatus} \(\Delta 4\) and \(\Delta 5d\)/\(\Delta 6d\) (16), and marine copepod \(\Delta 9\) desaturase (17). The regioselectivity of house cricket \(\Delta 12/\Delta 9\) desaturase was investigated using chemical mutagenesis and yeast complementation assays (18). Moreover, fatty acid-modifying enzymes with protein structures similar to but chemoselectivities different from, the fatty acid desaturases have been used to swap the function of \textit{Arabidopsis} oleate 12-desaturase and hydroxylase (19) and to alter the product partitioning between \textit{Crepis alpina} \(\Delta 12\) desaturase and acetylensane (20) and \textit{Monomorica conjugase} itself (21).

In this study, we aimed to elucidate the structural basis of the substrate specificity of \textit{Rattus norvegicus} \(\Delta 6d\) and \(\Delta 5d\) (22) by domain swapping and site-directed mutagenesis. The corresponding genes are positioned in a head-to-head configuration on the rat genome, suggesting a paralogous relationship (11). Although their primary structures are highly homologous, they are in charge of positioning) of membrane-bound desaturases are defned

\begin{align*}
\text{Materials and Methods}
\end{align*}

\textbf{Microorganisms, culture media, and reagents}

Transformants of \textit{Escherichia coli} DH5\(\alpha\) were grown in LB medium (0.5% yeast extract, 1% NaCl, 1% Bacto tryptone, 2% agar for plates) or 2\(\times\)YT medium (1.6% Bacto tryptone, 1% yeast extract, 0.5% NaCl) supplemented with ampicillin (50 \(\mu\)g/ml) at 37°C with rotary shaking at 160 rpm. Transformants of \textit{S. cerevisiae} INVSc1 (Invitrogen, Carlsbad, CA) were selected on SD agar plates (0.67% yeast nitrogen base, 0.19% yeast synthetic dropout medium without uracil, 2% \(\alpha\)-glucose, 2% agar) and cultivated in SCK medium (0.67% yeast nitrogen base, 0.19% yeast synthetic dropout medium without uracil, 4% raffinose, 0.1% Tergitol) or YPD medium (2\(\times\) polypeptone, 1% yeast extract, 2% \(\alpha\)-glucose) at 28°C and 160 rpm. Fatty acids were purchased from Sigma-Aldrich (St. Louis, MO) or Cayman Chemical (Ann Arbor, MI). Other guaranteed reagents were obtained from Nacalai Tesque (Kyoto, Japan), Sigma-Aldrich, Toyobo (Osaka, Japan), or Wako Chemicals (Osaka, Japan), unless otherwise indicated.

\textbf{Construction of plasmids carrying desaturase genes}

A FLAG DNA fragment was synthesized by PCR amplification with Takara Ex Taq (Takara, Kyoto, Japan) and the oligonucleotide primers FLAGf and FLAGr (Table 1), using 10 cycles of 95°C for 30 s, 50°C for 30 s, and 74°C for 30 s, without template. The fragment was cloned in pGEM-T Easy vector (Promega, Madison, WI) and transformed into \textit{E. coli} DH5\(\alpha\) (pGEM-FLAG). The rat D6d gene (DDB accession number AB021980) was amplified from stock plasmid with KOD-Dash DNA polymerase (Toyobo) and the primers 24aF+ and 24R+ (Table 1), using 30 cycles of 95°C for 30 s, 68°C for 2 s, and 74°C for 30 s, and was digested with \textit{Kpn}\(1\) and \textit{Xba}\(1\). The product was ligated into \textit{Kpn}\(1\)/\textit{Spd}-digested pGEM-FLAG and the plasmid was transformed into \textit{E. coli} DH5\(\alpha\) (pGEM-FLAG-D6d). The rat D5d gene (DDB accession number AB052085) was amplified using KOD polymerase (Toyobo), the primers rD5df and rD5dr (Table 1), and a rat liver cDNA library (Clontech Laboratories, Palo Alto, CA) under the same thermal cycling conditions as for D6d, and was ligated into pGEM-FLAG (pGEM-FLAG-D5d). The nucleotide sequences of all plasmids were determined using the DYE-dna Terminator cycle sequencing kit (GE Healthcare, Buckinghamshire, UK) or BigDye Terminator v3.1 cycle sequencing kit (Life Technologies, Carlsbad, CA) with \textit{T7}, \textit{SP6}, and other appropriate primers (Table 1) on an ABI PRISM 310 or 3130 \(\times\) 1 genetic analyzer (Life Technologies).

\textbf{Construction of chimeric desaturase genes}

DNA fragments corresponding to the N-terminal region (\textit{cyt}) and the central and C-terminal regions (\textit{des}) of D6d (D6cyt and D6des) and D5d (D5cyt and D5des) were amplified by PCR using KOD Dash, the template plasmids, and the following sets of oligo primers (Table 1): D6cyt (amino acids 1–154), 24aF+, and D6d-\(d6zebd5-20\) (Table 1) were designed to introduce nucleotide modifications of D6d catalyzes the conversion of linoleic acid (LA; 18:2 \(\Delta 9,12\)) into \(\gamma\)-linolenic acid (GLA; 18:3 \(\Delta 6,9,12\)) and stearidonic acid (18:4 \(\Delta 6,9,12,15\)) respectively, whereas D5d acts on dihomo-\(\gamma\)-linolenic acid (DGLA; 20:3 \(\Delta 8,11,14\)) and eicosatetraenoic acid (20:4 \(\Delta 8,11,14,17\)) to generate arachidonic acid (ARA; 20:4 \(\Delta 5,8,11,14\)) and eicosapentaenoic acid (20:5 \(\Delta 5,8,11,14,17\)), respectively. To identify and evaluate the amino acid residues important for substrate selection of D6d, we performed additional analyses on the basis of the primary sequence of zebra fish bi-functional \(\Delta 5/\Delta 6\) desaturase (ZD5/\(\Delta 6d\) (23)) and the recently reported crystal structure of human stearoyl-CoA (\(\Delta 9\)) desaturase (24, 25).
## TABLE 1. Oligonucleotide primers used in this study

| Primer | Nucleotide Sequence (5′ to 3′ Direction) | Purpose |
|--------|----------------------------------------|---------|
| a | Preparation of whole and partial regions of desaturases | FLAG tag |
| b | | N-terminal region of D6d |
| c | | Middle and C-terminal regions of D5d |
| d | | N-terminal region of D5d |
| e | Amino acid substitution of D6d with D5d | |
| f | Amino acid substitution of D5d with zD5/6d | |

### Preparation of whole and partial regions of desaturases

| Primer | Nucleotide Sequence (5′ to 3′ Direction) | Purpose |
|--------|----------------------------------------|---------|
| FLAGf | GCAAAGCTTAAGATGGACTATAAGGATGTAGTAC | FLAG tag |
| FLAGr | CGTGGTACCTTGACACTCATCATCGCTTAT | FLAG tag |
| 24aF+ | ACAGGTACCATGGGGAAGGGTACATCGG | D6d |
| 24R+ | GTCTCTAGATTCATTTGTGGAGGTAGGCATCC | D6d |
| D5df | CCCGGTACCATGGCTCCCGACCCGGTGCAGACCC | D5d |
| D5dr | CCCCTGCAGCTATTGGTGAAGGTAAGCATCCAGCC | D5d |
| D6d-cytr | GGCCGGGGAAGATGAGAGTACCGTACGG | N-terminal region of D6d |
| D6d-cytf | CCCTTCCGCGGCAATGGCTGGATTCCC | Middle and C-terminal regions of D6d |

### Amino acid substitution of D6d with D5d

| Primer | Nucleotide Sequence (5′ to 3′ Direction) | Purpose |
|--------|----------------------------------------|---------|
| d6d5-1 | ACCGGTCACCGCGCTCCCTTCGGCTGGTCTACCTCCC | F166V, V167L |
| d6d5-2 | ACAGGGCTTGGCTTCTTCTTCGCTGACATAGGGGT | A169S, S171V |
| d6d5-3 | GGCTACAACATGATTTTGGCCACCTTTCTGT | Y182F |
| d6d5-4 | GCCACCTTTCTGTCTTTAGCACCTCCATATGGAAC | Y188F, K189S, K190T |
| d6d5-5 | TTTCTGTCATACGGCCGTTCTGCTACCTCCC | I192T |
| d6d5-6 | TCCATATGGGAAAAAACCATCGGGAATACAACATCCAGCC | I196L, K199H |
| d6d5-7 | TCCCATATGGGAAAAAACCATCGGGAATACAACATCCAGCC | I196L, K199H |

### Amino acid substitution of D5d with D6d

| Primer | Nucleotide Sequence (5′ to 3′ Direction) | Purpose |
|--------|----------------------------------------|---------|
| d6d5-1 | ACCGGTCACCGCGCTCCCTTCGGCTGGTCTACCTCCC | F166V, V167L |
| d6d5-2 | ACAGGGCTTGGCTTCTTCTTCGCTGACATAGGGGT | A169S, S171V |
| d6d5-3 | GGCTACAACATGATTTTGGCCACCTTTCTGT | Y182F |
| d6d5-4 | GCCACCTTTCTGTCTTTAGCACCTCCATATGGAAC | Y188F, K189S, K190T |
| d6d5-5 | TTTCTGTCATACGGCCGTTCTGCTACCTCCC | I192T |
| d6d5-6 | TCCATATGGGAAAAAACCATCGGGAATACAACATCCAGCC | I196L, K199H |

### Amino acid substitution of D6d with zD5/6d

| Primer | Nucleotide Sequence (5′ to 3′ Direction) | Purpose |
|--------|----------------------------------------|---------|
| d6z5-1 | TCCATATGGGAAAAAACCATCGGGAATACAACATCCAGCC | I195L |
| d6z5-2 | TCCATATGGGAAAAAACCATCGGGAATACAACATCCAGCC | I195L |
| d6z5-3 | TCCATATGGGAAAAAACCATCGGGAATACAACATCCAGCC | I195L |

### Amino acid substitution of D6d with D5d

| Primer | Nucleotide Sequence (5′ to 3′ Direction) | Purpose |
|--------|----------------------------------------|---------|
| d6d5-1 | ACCGGTCACCGCGCTCCCTTCGGCTGGTCTACCTCCC | F166V, V167L |
| d6d5-2 | ACAGGGCTTGGCTTCTTCTTCGCTGACATAGGGGT | A169S, S171V |
| d6d5-3 | GGCTACAACATGATTTTGGCCACCTTTCTGT | Y182F |
| d6d5-4 | GCCACCTTTCTGTCTTTAGCACCTCCATATGGAAC | Y188F, K189S, K190T |
| d6d5-5 | TTTCTGTCATACGGCCGTTCTGCTACCTCCC | I192T |
| d6d5-6 | TCCATATGGGAAAAAACCATCGGGAATACAACATCCAGCC | I196L, K199H |

### Amino acid substitution of D5d with D6d

| Primer | Nucleotide Sequence (5′ to 3′ Direction) | Purpose |
|--------|----------------------------------------|---------|
| d6d5-1 | ACCGGTCACCGCGCTCCCTTCGGCTGGTCTACCTCCC | F166V, V167L |
| d6d5-2 | ACAGGGCTTGGCTTCTTCTTCGCTGACATAGGGGT | A169S, S171V |
| d6d5-3 | GGCTACAACATGATTTTGGCCACCTTTCTGT | Y182F |
| d6d5-4 | GCCACCTTTCTGTCTTTAGCACCTCCATATGGAAC | Y188F, K189S, K190T |
| d6d5-5 | TTTCTGTCATACGGCCGTTCTGCTACCTCCC | I192T |
| d6d5-6 | TCCATATGGGAAAAAACCATCGGGAATACAACATCCAGCC | I196L, K199H |
selected to check the nucleotide sequences of the cloned DNA fragments.

**Expression of desaturase genes in yeast**

The wild-type, chimera, and mutant desaturase genes were obtained by digestion of the pGEM-based plasmids with HindIII and EcoRI and were ligated into the yeast expression vector pYES2 (Invitrogen). The desaturase expression vectors were introduced into *S. cerevisiae* INVSc1 by using the lithium acetate method (26). Transformants were selected on uracil-deficient SD plates and cultivated at 28°C for 6 h with rotary shaking at 160 rpm in 15 ml of SCT medium supplemented with LA or DGLA at a concentration of 0.25 mM. After addition of galactose...

**TABLE 1. Continued.**

| Primer   | Nucleotide Sequence (5′ to 3′ Direction) | Purpose *a* |
|----------|-----------------------------------------|-------------|
| d6zebd5-10 | TCCAGTACCCGCTCTCATGACCATGATCAG         | I284F       |
| d6zebd5-11 | GCCATCGCACTATGGTTGTTCCCTCCTACA           | A305V       |
| d6zebd5-12 | TTGGGAGCTCCTTCTTCATACATTTGACATGTC       | F322L, L323F |
| d6zebd5-13 | GATGTCCCTACCTCTGTTGTCCTGAGAGCC         | L326V       |
| d6zebd5-14 | CAGATGACACAGCCTCCATGAGTTCAGTCTT         | V344F       |
| d6zebd5-15 | ATGGTCTGTACACCGCCAGCTGTCGTACCCAGCAG    | Y352N       |
| d6zebd5-16 | AAAGTGAGACGCTCCTCAATCGACTGTTGTC       | F370A       |
| d6zebd5-17 | TGCCCAAGCAACTATCAGAATGTGCCCC           | L396Y       |
| d6zebd5-18 | CTCGCGCAAGACTCGCATTAAATCAAGAGAAAGAG    | H410Y       |
| d6zebd5-19 | GCAAGATGCAGCATTAAATCAAGAGGAACCC        | E413K       |
| d6zebd5-20 | CCGTCGCTGACGCGCTCTACGGCAGATGTTGGTCGTC | L423F, L424A |

*a*Amino acids are indicated by single characters. Δ indicates a gap in the amino acid sequence alignment.

**Fig. 1.** Alignment of the amino acid sequences of rat D6d and D5d and zebra fish zD5/6d. Site-directed mutagenesis was applied to create mutations at the sites shown with a white background using oligonucleotide primers indicated by respective numbers above (d6d5-1–d6d5-35) and below (d6zebd5-1–d6zebd5-20) the alignment. Conserved histidine clusters are indicated by bold letters. Asterisks indicate mutation sites that altered the substrate specificity from D6d-type to D5d-type; “#” indicates the mutation site that gave rise to Δ5/6 bifunctionality of D6d.
(2%, w/v) and further cultivation for another 16 h, yeast cells were recovered by centrifugation for fatty acid and protein analyses.

Fatty acid analysis

The yeast cells from ~15 ml of broth were washed with distilled water and then vigorously vortexed in 2 ml of chloroform-methanol (2:1, v/v) plus 0.5 ml of distilled water. The chloroform phase was recovered by centrifugation, and methanolation of total lipid was carried out by adding 1 ml of 10% methanolic hydrochloric acid (Tokyo Kasei, Tokyo, Japan) and heating at 60°C for 2 h. After evaporation of the solvents, fatty acid methyl esters (FAMEs) were extracted twice and dissolved in hexane. Fatty acid composition was determined using a gas chromatographic system (GC-17A and GC-2014; Shimadzu, Kyoto, Japan) equipped with a capillary column (TC-70, 0.25 mm × 30 m, GL Sciences, Tokyo, Japan; or Omegawax 250, 0.25 mm × 30 m, Sigma-Aldrich), a split injector (split ratio at 1:20–25; 270°C), and a flame ionization detector (270°C). The temperature of the column oven was maintained at 180°C (TC-70) or raised from 210°C to 225°C at 0.5°C/min (Omegawax 250). FAMEs were identified by comparing their retention time with those of the 37-Component FAME mix (Supelco, Bellefonte, PA) and by analyzing their molecular mass using MS. For GC/MS analysis, total lipid or FAME extracts were dissolved in 0.5 ml of 2-amino-2-methyl-1-propanol preheated at 75°C and were heated at 180°C for 24 h to form 4,4-dimethyloctadecane (DMOX) derivatives of fatty acids. After cooling to 75°C and adding 2 ml of distilled water preheated at 75°C, the DMOX derivatives were extracted several times with n-hexane/dichloromethane (2:3, v/v), dehydrated with anhydrous sodium sulfate, and analyzed on a GC/MS system consisting of a gas chromatograph (7890A, Agilent Technologies) equipped with a ZB-1HT Inferno capillary column (0.25 mm × 30 m; Phenomenex, Torrance, CA) and an electron ionization mass spectrometer (70 eV, JMS-T100GCV; JEOL, Tokyo, Japan). The enzymatic activity of the desaturase expressed in yeast was evaluated using the conversion ratio, which was determined as the ratio of the amount of product to the sum of the amounts of substrate and product and was expressed as a percentage.

SDS-PAGE and Western blotting

Yeast cells recovered from 1 ml of broth were washed with distilled water and suspended in 0.1 ml of 50 mM Tris-HCl (pH 7.5) containing 4 µl EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). An equivalent volume of glass beads (0.5 mm in diameter) was added to disrupt the cells by eight rounds of vortexing for 30 s and chilling on ice for 30 s. The homogenate was centrifuged at 5,000 g for 10 min and the supernatant was subjected to SDS-PAGE (27). The proteins separated in the gel were transferred to an Immobilon membrane (Merck Millipore, Darmstadt, Germany) using a semidy blotter. The membrane was blocked by immersing in 5% skim milk in PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na 2 HPO 4 , 1.76 mM KH 2 PO 4 , 0.05% was blocked by immersing in 5% skim milk in PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na 2 HPO 4 , 1.76 mM KH 2 PO 4 , 0.05% was blocked by immersing in 5% skim milk in PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na 2 HPO 4 , 1.76 mM KH 2 PO 4 , 0.05%). Statistical analysis

All experiments were performed at least twice. Student’s t-test was used to compare experimental values between groups where applicable. P < 0.05 was considered significant.

RESULTS

The N-terminal region of desaturase is not involved in substrate specificity

To examine the involvement of the N-terminal hydrophilic regions of D6d and D5d, including the cytochrome b 6 -like domain, in the substrate specificity of both desaturases, chimeras D5cyt-D6des and D6cyt-D5des were constructed and expressed in yeast in the presence of LA or DGLA. The results indicated that D5cyt-D6des and D6cyt-D6des converted LA into GLA, but did not act on DGLA, whereas D6cyt-D5des and D5cyt-D5des generated ARA from DGLA, but did not use LA as a substrate (Table 2). No other fatty acids, except spontaneous ones, were detected in all cases (data not shown). These results indicated that the N-terminal domains of both enzymes do not determine the specificity toward the corresponding substrates. However, the rate of conversion by D5cyt-D6des (8%) was substantially lower than that by D6cyt-D6des (40%), suggesting that a specific interaction between the N-terminal region and the central and C-terminal regions may contribute to maximum activity of D6d through conformational stabilization of the enzyme.

Identification of amino acids responsible for D6d activity

The amino acid sequence homology between D6d and D5d was 66% (67/101 amino acids) in the central hydrophilic region (hydrophilic region II) and 75% (91/124) in the C-terminal region (hydrophilic region III). To identify the amino acids involved in substrate specificity, site-directed mutagenesis was applied to the 67 nonidentical amino acids, which had been organized into 35 groups of 1–3 amino acid substitutions as depicted in Fig. 1. Multi site-directed mutagenesis using mixtures of three or four groups of oligonucleotide primers (d6d5-1–d6d5-35; Table 1) resulted in the generation of an array of mutant D6d genes encoding enzymes in which various (numbers of) amino acids were substituted with the corresponding D5d residues (Fig. 2). The mutant genes were individually expressed in S. cerevisiae in the presence of DGLA. Despite the successful expression of mutant proteins, none of the mutants generated ARA at a detectable level (data not shown). A series of expression experiments was then performed in the presence of LA to see whether the D6d activity of the mutants had been changed. As shown in Fig. 2, the D6d activity of four mutants constructed using the primer sets d6d5-1/11/25 (introducing the mutations F166V+V167L, K444Q, R451K, and E454K) was attenuated substantially compared with wild-type D6d activity. These results suggested that the N-terminal region of D6d is not involved in substrate specificity.

Table 2. Substrate specificity of chimeric desaturases

| Desaturase     | Rate of Substrate Conversion (%) |
|---------------|---------------------------------|
|               | LA to GLA | DGLA to ARA |
| D6cyt-D6des   | 40        | 0           |
| D5cyt-D6des   | 0         | 0           |
| D6cyt-D5des   | 8.0       | 0           |
| D5cyt-D5des   | 0         | 45          |

Substitute specificity of mammalian fatty acid desaturases
L247V+Y249L, F356V+S358T, Q415E+E416S) was significantly lower than that of intact D6d. Further analysis of the mutants generated by using fewer or single primer(s) revealed that the amino acid change(s) introduced by each of the primers d6d5-7 (S209P+N211S), d6d5-8/31 (R216M, Q415E+E416S), d6d5-10/35 (K234N+S235M+L236Δ, K444Q), and d6d5-11 (Δ238P+Δ239L) yielded decreased or null D6d activity. Because the single primer mutations d6d5-31 and d6d5-35 did not affect the D6d activity, the decreases in the activity by the mutations d6d5-8/31 and d6d5-10/35 could be due to the mutations by d6d5-8 and d6d5-10, respectively.

Switching the substrate specificity of D6d

As expected, a D6d mutant made by using a mixture of the four primers d6d5-7, 8, 10, and 11 showed neither D6d nor D5d activity. Additional mutations were introduced into this mutant using several sets of primers randomly selected from d6d5-1 to d6d5-35, and D5d activity of the resultant mutants was investigated using DGLA substrate. Fig. 3D shows that one D6d mutant, namely, D6d-Z2, made using the primers d6d5-13 (E243K+W244V+Q245L) and d6d5-21 (V344P+E346H) in addition to d6d5-7/8/10/11 (S209P+N211S, R216M, K234N+S235M+L236Δ, Δ238P+Δ239L), gave a peak with a retention time similar to that of ARA (peak 6, Fig. 3D) on the chromatogram. By GC/MS analysis, D6d-Z2 yielded de-...
that conferred D5d activity to D6d in this study were compared with the corresponding residues in desaturases from various vertebrates (23, 28–34) as shown in Fig. 6. Most of the amino acids at positions 209, 211, 216, 236, and 245 of rat D6d and D5d are conserved among D6d and D5d from most vertebrates, whereas those at positions 244 and 323 are variable. It is possible that these conserved amino acids cooperatively contribute to substrate recognition.

It is reasonable to assume that the substrate specificity and positioning are determined by the electric charge and polarity of the particular desaturase amino acids, which affect their affinity to the acyl chain and carrier portion of the substrate, and by the depth and angle of substrate insertion into the binding pocket (21). To evaluate the above-mentioned results of the protein engineering analysis, homology modeling of D6d was carried out on the basis of the recently reported crystal structure of human SCD1 (Protein Data Bank ID 4YMK) (24), using the structure prediction program Phyre2 (35). Amino acids at positions 235 and 344 are highly conserved among D6d and D5d from most vertebrates, whereas those at positions 244 and 323 are variable. It is possible that these conserved amino acids cooperatively contribute to substrate recognition.

Structure-function relationship

To explore the molecular evolution and functional divergence of front-end fatty acid desaturases, the mutations F322L/L323F (mutant 2C-2), I326V (4C-2), and E413K (D6d-25m) to the respective backgrounds (Table 3). Further analysis of single mutants for each of these four amino acids introduced into wild-type D6d demonstrated that only L323F led to the generation of ARA from DGLA at a conversion rate of 2.3% (Fig. 5 and supplementary Figure 3). Because addition of the mutations I326V and I326V/E413K to L323F did not significantly increase D5d activity, amino acids other than those might contribute to the full activity of D6d-25m.

Fig. 4. The D5d activity of point mutants obtained from D6d-Z2. A: Each of D5d-amino acid changes or the gap in D6d-Z2 was restored to its D6d counterpart by site-directed mutagenesis using oligonucleotide primers (d6d5-36–d6d5-48; Table 1). The mutants were expressed in yeast in the presence of DGLA, and the generation of ARA was detected by GC. D5d activity was determined as the conversion rate of DGLA to ARA and shown as the average value relative to that of D6d-Z2, together with the standard deviation (n = 3). B: D6d-mut8 carrying eight D5d-type amino acids was expressed in yeast in the presence of DGLA (peak 5), and the generation of ARA (peak 6) was detected by GC. Other peaks in B are 16:0 (peak 1), 16:1 Δ9 (peak 2), 18:0 (peak 3), and 18:1 Δ9 (peak 4).
bond (38) and an epoxy group (39), respectively. Elucida-
tion of the molecular basis of substrate recognition and 
regio- and chemoselectivity of the enzymes enables us to 
design new bioactive lipids and to produce them effi-
ciently. In this study, rat D6d and D5d with highly homolo-
gous primary structures were used as a model to identify 
the sites critical for their mutually exclusive substrate 
specificity.

Heterologous expression analysis of chimeric enzymes 
of D6d and D5d, in which the cytochrome 
b5 -like domains 
were swapped, demonstrated that these domains do not 
contribute to substrate recognition (Table 2). However, 
the D6d activity of the chimera D5cyt-D6des was signifi-
cantly lower than that of intact D6d, suggesting that the 
cytochrome 
b5 -like domain might be necessary for full ac-
tivity of D6d, but not D5d. Therefore, a D6d mutant with 
D5d activity (equivalent to D6cyt-D5des) would be prefer-
able over the reverse to detect declined desaturase activity. 
Indeed, the D6d-based mutant D6d-mut8 barely showed 
D5d activity (Fig. 4B), whereas the introduction of muta-
tions at the corresponding sites in D5d did not result in 
the generation of detectable D6d product (data not 
shown). Moreover, given that the distance from the car-
boxyl group of the fatty acid substrate to the position to be 
desaturated is considered to be larger in D6d than in D5d, 
the substrate range of D5d is expected to be wider than 
that of D6d (40). Thus, site-directed mutagenesis was ap-
plied to the D6d gene to readily observe successful conver-
sion of substrate specificity.

We assumed that structural differences between D6d 
and D5d due to some of the 67 nonconserved amino 
acid residues of R216, W244, and Q245, located near the 
substrate-binding pocket (Fig. 7A), are considered to 
form hydrogen bonds with the pantothenic acid portion 
and the carbonyl group of acyl-CoA substrate, according 
to the findings for SCD1. Substitution of these amino ac-
ids with the corresponding D5d residues (M, V, and L, 
respectively) that do not form hydrogen bonds might al-
ter the substrate-binding strength (Fig. 7B). Simultane-
ously, the substitutions R216M and W244V seem to cancel 
the steric hindrance just around the threshold of the 
pocket, allowing the substrate acyl chain to be inserted 
much deeper, resulting in the introduction of a carbon-
carbon bond at the Δ5 position close to the catalytic site. 
L323 was predicted to be situated at the bottom of the 
substrate-binding pocket. The mutation L323F, which 
conferred Δ5/6 bifunctionality to D6d but might not al-
ter the position of catalytic site nor deform the threshold 
of the pocket, would strengthen the hydrophobic affinity 
with the methyl terminus of the substrate acyl chain and/or create more space for insertion of the acyl chain (Fig. 7C).

**DISCUSSION**

On the basis of the structural similarity of the enzymes, 
the desaturase family is also considered to include hydrox-
ylase that produces hydroxyl fatty acids such as plant sur-
face coating wax (36), conjugase that produces conjugated 
fatty acids with anticarcinogenesis activity (37), and acety-
lenase and epoxidase that produce fatty acids with a triple 

| Substitution | 1C-1 | 1C-2 | 1C-3 | 2C-1 | 2C-2 | 2C-3 | 2C-4 | 3C-1 | 3C-2 | 3C-3 | 4C-1 | 4C-2 | 4C-3 | 4C-4 | 5C-1 | D6d-25m |
|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| N156T | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | D5d activity (%) 16.3 17.4 13.7 5.3 11.6 10.0 13.5 13.0 13.0 16.0 13.8 11.0 21.3 21.3 26.8 26.3 |
| A305V | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | D6d activity (%) 16.3 17.4 13.7 5.3 11.6 10.0 13.5 13.0 13.0 16.0 13.8 11.0 21.3 21.3 26.8 26.3 |
| Y182F | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | D5d and D6d activities were represented as conversion rate from substrates (18:2 Δ9,12 for D6d and 20:3 Δ8,11,14 for D5d) to products (18:3 Δ6,9,12 for D6d and 20:4 Δ5,8,11,14 for D5d, respectively). The plus sign (+) indicates mutation points of each D6d mutant carrying zD5/6d-type amino acids.
acids in their hydrophilic regions (Fig. 1) would confer altered substrate specificity. To our knowledge, this study is the first to identify specific positions that are involved in alteration of the substrate selectivity of a mammalian front-end fatty acid desaturase. On the basis of heterologous expression analyses of a series of D6d mutants, we identified eight mutations (S209P, N211S, R216M, S235M, L236Δ, W244V, Q245L, and V344P) that abolished D6d activity from D6d but conferred D5d activity (Fig. 4B). It is obvious that several amino acid residues are necessary to determine the substrate specificity as well as to support maximum enzymatic activity. The K218 residue of *M. rouxii* D6d has been reported to be involved in binding of the substrate (15); however, mutation of the corresponding amino acid in rat, R216, did not yield D5d activity (data not shown). Compared with the D6d-Z2 mutant carrying 12 mutations and with D6d activity 4.6% that of the wild-type D6d (Fig. 3), the D6d-mut8 mutant carrying eight mutations showed much lower activity (1.4%; Fig. 4B), and the D6d product was not detected in the double mutant R216M/W244V (data not shown). The fact that all of the restored mutants shown in Fig. 4A retained D5d activity suggested that more than two critical amino acid residues exist in each group of mutations introduced with the primers d6d5-7/8/10/11 and d6d5-13/21.

The mammalian D6d and D5d and zebra fish zD5/6d might have evolved from a common ancestor enzyme (33). Site-directed mutagenesis targeting the residues identical between D5d and zD5/6d but not D6d resulted in the generation of a mutant D6d-25m possessing bifunctional activity (Table 3) and pinpointed L323F as responsible for providing D5d activity to D6d (Fig. 5). However, the mutation L323F was overlooked in the first mutagenesis experiment based on the sequence comparison of only D5d and D6d and the use of multimutagenic primers. This might be because the D5d activity of the D6d-L323F mutant was below the detection limit and/or the other amino acid mutations introduced by the primer (d6d5-18; V321C+F322L+L323F) counteracted its effect. By using two different approaches in the mutagenesis experiments, a broad-horizon search was achieved, resulting in the determination of the amino acid residues responsible for both switching and adding the substrate specificity of D6d.

In addition, the predicted D6d tertiary structure supported our findings at least in part on the molecular basis of substrate specificity of the fatty acid desaturases. This knowledge will largely contribute to furthering our understanding of the structure-function relationship and the molecular evolution of the desaturase family and to generating structurally and functionally novel fatty acyl compounds for industrial applications.

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*Fig. 5.* Conversion of ARA from DGLA by desaturase mutants with point mutation(s). A: D6d-F322L. B: D6d-L323F. C: D6d-I326V. D: D6d-E413K. E: D6d-L323F-I326V. F: D6d-L323F-I326V-E413K. G: D6d-25m. H: Intact D6d. Each mutant was expressed in yeast in the presence of DGLA (peak 1), and the generation of ARA (peak 2) was detected by GC.

*Fig. 6.* Comparison of the amino acid residues involved in the substrate specificity of rat D6d with corresponding residues in desaturases from various vertebrates. Amino acid residues identical to those in rat D6d (top row) and D5d (bottom row) are indicated with white and black backgrounds, respectively, and other amino acid residues are shown with a gray background.
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