Domain Swapping Localizes the Structural Determinants of Regioselectivity in Membrane-bound Fatty Acid Desaturases of Caenorhabditis elegans

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Most fatty acid desaturases are members of a large superfamily of integral membrane, O₂-dependent, iron-containing enzymes that catalyze a variety of oxidative modifications to lipids. Sharing a similar primary structure and membrane topology, these enzymes are broadly categorized according to their positional specificity or regioselectivity, which designates the preferred position for substrate modification. To investigate the structural basis of regioselectivity in membrane-bound desaturases, the Caenorhabditis elegans ω-3 (FAT-1) and "Δ12" (FAT-2) desaturases were used as a model system. With the use of unnatural substrates, the regioselectivity of C. elegans FAT-2 was clearly defined as ω-3, i.e. it "measures" three carbons from an existing double bond. The structural basis for ω-3 and ω-regioselectivity was examined through construction and expression of chimeric DNA sequences based on FAT-1 and FAT-2. Each sequence was divided into seven domains, and were replaced with sequence from FAT-1. When tested by expression, the chimeras were constructed in which domain swapping introduced regioselectivity from ω-3 to ω. Structural determinants of regioselectivity in FAT-2 have been localized to two histidine boxes and a hydrophobic membrane spanning domain.

Fatty acid desaturases are enzymes that catalyze the oxygenation and nicosynthesis of fatty acids. They are classified into regio-specific superfamily of oxidative enzymes based on their fatty acid desaturases reference the double bonds at positions ranging from three carbons from the carboxyl terminus; GC, gas chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry.

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Given the nutritional and commercial importance of unsaturated fatty acids of various types (16), it is essential that we develop an understanding of the structure-function relationships of integral membrane fatty acid desaturases. These enzymes are involved in the biosynthesis of polyunsaturated fatty acids in C. elegans that range from 18:2(9,12) to 20:5(5,8,11,14,17) (17–20). The two enzymes that range from 18:2(9,12) to 20:5(5,8,11,14,17) (17–20). The two enzymes that share 51% amino acid sequence identity. As such, they are two of the most similar desaturases. Italicized sequence in the outlying primers indicates the position of conserved "histidine boxes" and are shown coordinating a putative diiron site (Fe).

EXPERIMENTAL PROCEDURES

Materials—Fatty acids (typically Nu-Chek Prep, Inc. (Elysian, MN), obtained from Sigma.

Preparation of fat-1 and fat-2 Constructs—Cloning of the C. elegans fat-1 gene was obtained by amplifying the sequence from plasmid pDM015 (21) using oligonucleotides fat-1start and fat-1end (Table I). The resulting fragment was gel-purified and cloned from plasmid pDM015 (21) using oligonucleotide primers fat-1start and fat-1end (Table I). The resulting fragment was gel-purified and cloned from plasmid pDM015 (21) using oligonucleotide primers fat-1start and fat-1end (Table I) restriction sites.

The temperature profile for a typical megaprimer fusion PCR was 94 °C for 30 s, 55 °C for 1 min, 72 °C for 4 min, 94 °C for 30 s, 72 °C for 3 min one time. PCR products were purified from a 1.2% agarose gel using the S. C. EasyComp transformation Kit (Invitrogen) with selection on uracil-deficient medium. For assessment of desaturase activity, recombinant yeast cells were grown to saturation in 50-mL cultures for 48–72 h at 28 °C on minimal medium (synthetic dropout) lacking uracil and supplemented with 2% galactose (24). INVSc1 yeast containing the empty plasmid vector pYES2.1 behind the galactose-inducible GAL1 promoter. The same chimeric constructs was confirmed by DNA sequencing.

Gel Electrophoresis and Western Blotting—Yeast cells were disrupted using a French press. Proteins from disrupted cells were solubilized in SDS sample buffer (containing 125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.2 mM diithiothreitol, 0.001% bromophenol blue), incubated at 90 °C for 5 min, and analyzed by SDS-PAGE on 4–15% polyacrylamide gradient gels. Protein bands were visualized by staining with Coomassie Blue stain reagent (Ferriin) or electroblotted to polyvinylidene difluoride membranes (Amersham Biosciences) for Western blot analysis. Blots were blocked with 1% skim milk and 0.1% Tween 20 in phosphate-buffered saline and then incubated with primary antibody. For this, an antibody from a rabbit immunized with a synthetic oligopeptide (NH2-FLRGGTQDIDR-COOH, corresponding to amino acid sequence shared by FAT-1 (amino acids 297–307) and PAT-2 (amino acids 268–278)) conjugated to keyhole limpet hemocyanin was used. After the primary antibody incubation, the membrane was washed with phosphate-buffered saline containing 0.1% Tween 20 and then incubated with peroxidase-conjugated goat anti-rabbit IgG (Am-

TABLE I

| Primer | Sequence |
|--------|----------|
| Δ3Are | AATCCTTGATAAGATATTGATT |
| BSrev | CTGTGCACAGTGACGGACACAC |
| 3Arev | AGTAAAACATATTGTGTCATG |
| 3Erev | TCCCTGGTTCGATAAGAAAG |
| 5prev | TTGGTGAGAAAGATGGATGGGACAC |
| 3Brev | GATCTTCATTCATTTGTCGTC |
| 3Cfor | GGGCCCACTACCTCACAATAAT |
| 3Drev | CAAATAGGTAACAATG |
| Δ12Arev | GTCTTGCCCCCAGTCATTTG |
| Δ12Brev | CATGAGACGCAGTGACCACACACAA |
| Δ12Crev | GAAGTCCTCCCTTACCTTTC |
| Δ12Drev | APACCTTCAGTTGACATG |
| Δ12Erev | AGATATAGTGGATAT |
| Δ12Erev | ATAGGCGCAAGTG |
| Δ12Frev | ATGTTAGTGATATC |
| Δ12Grev | TATACCAAGGAT |
| Δ12Hrev | CGTGGCAAGT |

FIG. 1. Topological model of membrane-bound fatty acid desaturases such as FAT-1 and its division into seven domains (A–G, see "Results" for an explanation of the divisions). Division boundaries are indicated by dashed bars. H1, H2, and H3 depict the location of conserved "histidine boxes" and are shown coordinating a putative diiron site (Fe).
ersham Biosciences) and visualized by enhanced chemiluminescence (ECL, Amersham Biosciences) using x-ray film.

**Fatty Acid Analysis**

GC-flame ionization detection analysis of fatty acid methyl esters was performed as described previously (22). For GC/MS analysis, fatty acid methyl esters of interest were partially purified from the total fatty acid methyl ester fraction by HPLC using an Agilent 1100 Series HPLC system with the fraction collector collecting 0.5-ml fractions from 212.5-cm Whatman Partisphere C18 columns connected in series and using a linear solvent gradient starting at 90% acetonitrile, 10% water with increasing acetone to 30% in 20 ml.

The fractions containing the fatty acids of interest were saponified (21) and derivatized to diethylamides according to Nilsson and Liljenberg (25). The diethylamide reaction products were further purified by applying the reaction mixture to a short silica gel column (0.5 ml), washing with 10 column volumes of dichloromethane, and eluting the diethylamides with 10 column volumes of acetone. The acetone was removed under a nitrogen stream, and the samples were dissolved in 0.5 ml of dichloromethane for GC/MS analysis.

GC/MS analysis of the diethylamides was accomplished using an Agilent 5973 mass selective detector coupled to an Agilent 6890N gas chromatograph using G1701DA MSD Chemstation software (for instrument control and data analysis) and equipped with a 30-m × 0.25-mm DB-23 column with 0.25-µm film thickness (J&W Scientific). The chromatograph conditions included a split injection (20:1) onto the column using a helium flow of 0.4 ml/min, an initial temperature of 160 °C for 1 min, and a subsequent temperature ramp of 4 °C/min to 240 °C. The mass selective detector was run under standard electron impact conditions (70 eV), scanning an effective m/z range of 10–450 at 3.32 scans/s.

**RESULTS**

**Regioselectivity of the C. elegans “Δ12” Desaturase**—To further characterize the regioselective mode of FAT-2, INVS$c1$ yeast expressing pSAS050 were grown in culture with commercially available Δ10 monoenoic fatty acids. The use of 17:1(10) and 19:1(10) as substrates and analysis of the corresponding FAT-2 products allows the discrimination between the Δ12, ω-6, and ω-3 types of regioselectivity. The fatty acid composition of the final culture was assessed by GC analysis and compared with yeast cultures expressing an empty plasmid grown in parallel. HPLC fractionation and GC analysis of fatty acids from the pSAS050 (FAT-2)-expressing strain with exogenously added 17:1(10) or 19:1(10) revealed peaks not present in the empty vector control strain. In the case of cultures supplied with 17:1(10), the major new GC peak coeluted with 18:0 and was revealed upon fractionation by HPLC. GC/MS analysis of the HPLC-purified peaks identified them as the Δ3 desaturation products 17:2(10,13) and 19:2(10, 13). Fig. 2 shows a mass spectrum of the 17:1(10) desaturation product.
the 19:1(10) desaturation product is an ene-1,3-diol with double bonds at the A10 and A13 positions (data not shown). The above double bond assignments are supported by MS peak intensity spectra obtained under the same conditions (data not shown). Also the product of 19:1(10) desaturation was found to be insensitive to the dienophile 4-methyl-1,2,4-triazoline-3,5-dione under conditions for which conjugated fatty acids react (data not shown). The latter observation confirms that desaturation by FAT-2 did not occur at the A10 position for 19:1(10).

Chimera Construction and Heterologous Expression—All of the integral membrane fatty acid desaturases are thought to share the same membrane topology and overall three-dimensional structure (but see Ref. 13). This is particularly true of *C. elegans* FAT-1 and FAT-2 since they share 51% amino acid sequence identity. Furthermore, in replacing amino acid sequence domains of one of these enzymes with the corresponding domains from the other enzyme, we would expect, at least in some cases, to maintain this general topology and structure as well as (possibly altered) desaturase activity. With this in mind, we set out to investigate the structural elements that determine regioselectivity in the two enzymes through domain swapping experiments.

Transition points for chimera construction were chosen roughly on a structural basis as follows. Using the computer program TopPred II (26) to predict the location of the transmembrane helices in FAT-1 and FAT-2, a membrane topological model was constructed based on earlier proposals (9, 11, 12) (Fig. 1). For chimeric sequence construction, four divisions were made in the locations where the sequence was predicted to cross the membrane interface on the cytosolic side. Other divisions were placed after the second conserved histidine cluster, and another was placed after the third histidine cluster. These transition points divide the desaturases into seven broad structural domains: A, the amino-terminal region (amino acids 1–78, FAT-1 numbering); B, the first set of putative transmembrane helices (amino acids 79–122); C, the conserved His boxes H1 and H2 and an intervening relatively hydrophobic segment (amino acids 123–174); D, a second relatively hydrophobic segment (amino acids 175–232); E, the second set of putative transmembrane helices
Determinants of Regioselectivity in Fatty Acid Desaturases

Fig. 5. Product accumulation in single domain chimeric C. elegans fatty acid desaturases expressing various recombinant sequences. Yeast cultures expressing various recombinant sequences were supplied with exogenous fatty acid substrates, and desaturation product accumulation as a percentage of total cellular fatty acid is shown in A and B. Chimeras were named according to the identity of the parental sequence and the letter designator of the domain introduced from the other parent. For example, FAT-1 sequence with domain D replaced by domain D from FAT-2 is denoted by FAT-1/D/H9275.

Expression levels of the various wild type and chimeric fatty acid desaturases were determined by immunoblotting as indicated in Fig. 4. The most part, within a given group of chimeras, desaturase protein levels in yeast cells were similar or somewhat reduced compared with the corresponding wild type enzymes. Thus for single domain chimeras with an ω-3 background, it is estimated from Fig. 4A that expression levels were 30–100% of the wild type FAT-1, and ω-3:D is no exception. Chimeras with a “Δ12” background showed a slightly wider range of expression with Δ12:B, Δ12:C, and Δ12:D levels being reduced relative to wild type FAT-2 and the other chimeras. Again the chimera with the G domain swap was expressed at levels comparable to the corresponding wild type enzyme. The double domain swap enzymes were expressed at levels that were comparable or slightly reduced compared with the wild type levels (Fig. 4, C and D). Given the aforementioned expression patterns, it was possible to compare semiquantitatively and qualitatively the activities of the various enzymes in yeast cells.

Functional Characterization of Single Domain Swap Chimeras—Yeast is an ideal host for heterologous expression in the study of membrane-bound desaturases and related lipid-modifying enzymes (27). Because the polyunsaturated products of the ω-3 and “Δ12” desaturases are not metabolized further, the final levels of product in the total lipid extract may be used as a semiquantitative indicator of enzyme function (21, 28).

The ω-3 fatty acid desaturases are expressed in yeast cells and therefore the ω-3 fatty acids are not metabolized further. The desaturation products are detected as a percentage of total cellular fatty acid.

The ω-3 fatty acid desaturase activities were assayed in yeast cells expressing the wild type and chimera sequences. The desaturase activities were assayed as a percentage of total cellular fatty acid.

Fig. 6. Regioselectivity changes in two domain swap chimeric C. elegans fatty acid desaturases. Yeast cultures expressing wild type and “D+G” chimeras were grown without fatty acid supplementation (16:1(9)) or supplied with exogenous fatty acids indicated. Desaturation product accumulation as a percentage of total cellular fatty acid is shown. A. FAT-1 (white) and 16:1(9) (gray). B. FAT-2 (white) and 16:1(9) (gray). Means and S.D. of three experiments are indicated.
D

FAT-1: [sequence]
FAT-2: [sequence]

G

FAT-1: [sequence]
FAT-2: [sequence]

Fig. 7. Amino acid sequence alignment of C. elegans FAT-1 and FAT-2 demonstrating sequence variations in domains D and G. Black and gray backgrounds indicate identical and similar amino acids, respectively.

FAT-2 that allows better conformity with Kozak consensus sequences (22, 29).

The fatty acid desaturase activities of the single domain swap chimeras are shown in Fig. 5. All chimeras containing a single substituted domain were catalytically active and retained the regioselectivity of their parent desaturase. None of the ω-3 chimeras showed ω-3 activity with 16:1(9) or 18:1(9) (data not shown). For wild type FAT-1, as well as that of related ω-3 desaturase, the ω-3 desaturase activity on the substrates 18:2(9,12) and 18:3(6,9,12) (yielding 18:3(9,12,15) and 18:4(6,9,12,15), respectively) showed the same general trends (Fig. 5A). However, there are some notable differences in relative activities for the two substrates. Yeast cells expressing wild type FAT-1 accumulated more product from 18:2(9,12) than from 18:3(6,9,12). Yeast expressing most chimeras gave product accumulations relative to wild type enzyme. The single domains derived from FAT-2 sequence showed reduced activity for the two substrates. Yeast cells expressing wild type FAT-1 accumulated more product from 18:2(9,12) than from 18:3(6,9,12). Yeast expressing most chimeras showed product accumulations that were equal to or higher than the wild type ω-3 enzyme. Single domains derived from ω-3 domains showed 20% of wild type levels with both substrates.

Results from the “ω-3” chimeras revealed structure-function relationships for integral membrane proteins, which includes most fatty acid desaturases. Sequence comparisons, functional characterization, and spectroscopic results are consistent with essential amino acid coordination of a diiron center at the front-end of these enzymes (10, 30). Eight highly conserved histidine residues have been shown to be required for function in the exception of one that is replaced by glutamine in some “front-end” desaturases, which introduce double bonds near C-1 in the substrate (3, 31). Shanklin and colleagues (32) have identified a number of individual amino acids that are important for the chemoselectivity of the FAD2-like enzymes, which tend to have both desaturase and hydroxylase activities to varying degrees. A domain swapping study was undertaken by Napier and colleagues (33) to investigate the regioselectivity and substrate specificity of two front-end desaturases. This work hinted at the importance of the carboxyl terminus of desaturases in determining specific regiochemical and/or substrate characteristics of these enzymes. Additionally the inactivity of a carboxyl-terminal deletion mutant of the Bacillus subtilis Δ5 desaturase supports the importance of domain G in desaturase function (13).

In this work, two regioselectively distinguishable desaturases were chosen that have a high degree of sequence similarity. While the substrate classes of FAT-1 and FAT-2 are not known precisely, it has been suggested that they are both acyl-CoA desaturases (18). Their natural substrates are similar in length and have double bonds at C(x-3) where x is the position of the incipient double bond, i.e. the “Δ12” desaturase acts on a Δ9 substrate, and the ω-3 desaturase acts on ω-6 substrates. However, the enzymes differ in both regioselectivity and ω-3 desaturase function (13).
ity and regioselective mode; FAT-2 “measures” from a double bond (as indicated by its activity on 17:1(10) and 19:1(10)), and FAT-1 measures from the methyl end of the substrate. The r+3 regioselectivity of FAT-2 determined by this study is very similar to that found for the plant oleate desaturase (FAD2) (14).

Our initial approach to investigating the structural determinants of desaturase regioselectivity was to construct single domain chimeric enzymes in which one domain in a wild type enzyme was replaced with the domain sequence of the other enzyme. It is useful to consider the possible activity of a given example of such a chimeric enzyme. It may be inactive. It may be active and retain the regioselectivity of the parent desaturase, suggesting that the domain is functionally interchangeable but not solely responsible for regioselectivity. The regioselectivity could correspond to that of the introduced domain, suggesting that it is the major determinant of positional specificity. Other possibilities are that the chimeric enzyme could have both regioselectivities or a novel regioselectivity such as ω-4, for example.

In the case presented for the C. elegans FAT-1 and FAT-2, single domain chimeras of both enzymes show activity with retention of parental positional specificity. The conclusion to be drawn from this is that no single domain is either necessary or sufficient to confer regioselectivity on the enzyme. This leaves the possibility that two or more domains act in concert to determine regioselectivity. In the interest of avoiding a complex search of two-domain chimeras “space,” we attempted to rationalize the single domain chimera results that indicate that domain G was somehow important. Replacing both domains of a second domain might result in restoration of positional specificities between the two domains. Most of the times the chimeras were inactive. Clearly there is limited activity of most domains. The chimera in which the D and G domains were simultaneously swapped had results corresponding to the identity of the D and G domains, therefore, these two domains may contribute a large part to the determination of ω-4 and “Δ12” desaturases of G in C. elegans desaturases. Further study is required to identify the unique features of domain D and G in the carboxyl terminus of desaturase.

The results from our work and prior others support this observation to show that domain D may be an important determinant of regioselectivity.

Domain D is important for both ω-4 and “Δ12” positional substrate specificity. Alteration of this region in the DCD histidine region near the second histidine box in the consensus background changes the relative activity on linoleic acid and γ-linolenic acid (Fig. 5A). This and its importance in regioselectivity suggest that domain D may be intimately involved in binding and positioning the substrate relative to the active site.

It is notable that none of the putative transmembrane domains are implicated in the determination of regioselectivity and that both D and G domains are thought to be on the same side of the endoplasmic reticulum. While neither domain contains the putative active site histidine boxes, domain D is immediately adjacent to the second His box, and domain G is immediately adjacent to the third His box.

Inspection of alignment of domains D and G for FAT-1 and FAT-2 reveals a total of 63 amino acid differences (Fig. 7). Further work is in progress to define more specifically the amino acid residues that are important for regioselectivity.

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