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Characterization of the Regiochemistry and Cryptoregiochemistry of a *Caenorhabditis elegans* Fatty Acid Desaturase (*FAT-1*) Expressed in *Saccharomyces cerevisiae*[†]

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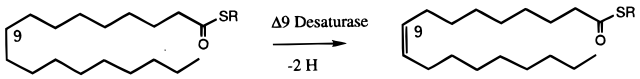
ABSTRACT: To characterize the fatty acid desaturase produced by the *fat-1* gene from the nematode *Caenorhabditis elegans*, the functional expression of this enzyme was effected in the yeast *Saccharomyces cerevisiae*. The GC-MS analysis of desaturated products derived from various fatty acids, including deuterium-labeled thia fatty acids supplied to growing cultures of transformed yeast, has defined the substrate requirements, regiochemistry, and cryptoregiochemistry of the enzyme. The desaturase acts on substrates of 16−20 carbons with a preference for ω-6 fatty acids, and its regioselectivity was confirmed to be that of an ω-3 desaturase. (ω-x refers to a double bond or desaturation between carbons x and $x+1$, counting from the methyl end of a fatty acid.) The primary deuterium kinetic isotope effects (KIEs) at C-15 and C-16 of a C18 fatty acid analogue were measured via competitive incubation experiments: While k_H/k_D at the ω-3 position was shown to be large (7.8 ± 0.4), essentially no KIE at the ω-2 position was observed ($k_H/k_D = 0.99 \pm 0.04$). This result indicates that ω-3 desaturation is initiated by an energetically difficult C−H bond cleavage at the carbon closer to the carboxyl terminus. The results are discussed in the context of a general model relating the structure and function of membrane-bound fatty acid desaturases featuring differing regioselectivities.

Fatty acid desaturases are non-heme iron-containing oxygen-dependent enzymes involved in regioselective introduction of double bonds in fatty acyl aliphatic chains *(1, 2)*. A prototypical desaturation reaction is the conversion of stearoyl thioester to its oleoyl counterpart. Two general classes of fatty acid desaturases have been identified. The soluble plant enzymes are plastid-localized, use acyl carrier protein substrates, and contain a carboxylate- and histidine-bound diiron active site *(3, 4)*. The membrane-bound desaturases are found in a wide range of taxa, use acyl-CoA or acyl-lipid substrates, and are thought to have a histidine-rich diiron active site *(5, 6)*.



Until very recently, information about the substrate specificities, regioselectivities, and mechanism of membrane-bound fatty acyl desaturases was quite limited *(1, 7, 8)*. This is largely due to difficulties in the isolation of active forms of such enzymes and their requirement for hydrophobic substrates and additional redox proteins (e.g., cytochrome b_5 reductase and cytochrome b_5). Three classes of regioselectivity have been observed for fatty acid desaturases: The Δ^x desaturases introduce a double bond x carbons from the carboxyl end; ω-x desaturases dehydrogenate x carbons from the methyl terminus; while $\nu+x$ desaturases use a preexisting double bond as a reference point and dehydrogenate x carbons from the nearest olefinic carbon *(9, 10)*.

In addition to their more apparent regioselectivities, fatty acid desaturases can also be characterized in terms of what has been labeled their cryptoregiochemistry (site of initial oxidation) *(11)*. That is, for all membrane-bound desaturases studied to date, large primary deuterium isotope effects have been observed only at the carbon proximal to the acyl end of the fatty acyl substrate *(11−15)*. These results have been interpreted within the context of a mechanistic model[1] *(16)* which features acyl-side C−H bond cleavage as the initial, rate-determining oxidation event (Scheme 1). Subsequent loss of the methyl-side hydrogen is fast and insensitive to isotopic substitution. Use of thia fatty acids as probes for oxidant

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[1] The intermediacy of a discrete carbon-centered radical is consistent with the KIE data; however, other alternatives including carbocations or organoiron intermediates remain a possibility as discussed previously *(14)*.

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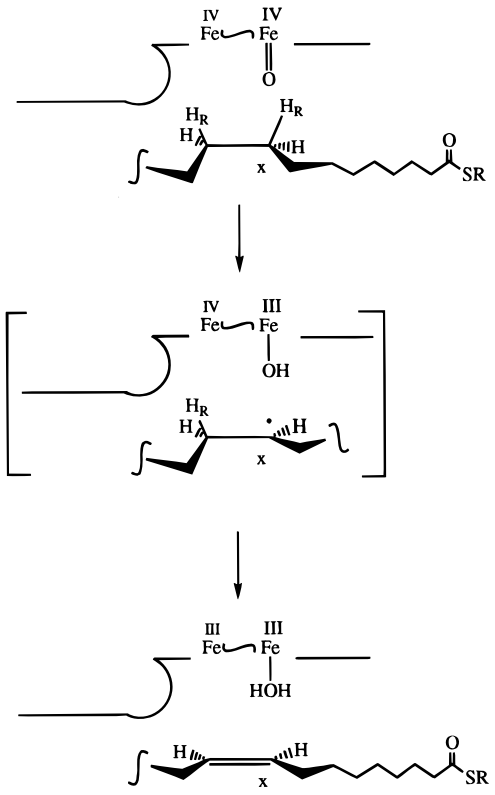
Scheme 1

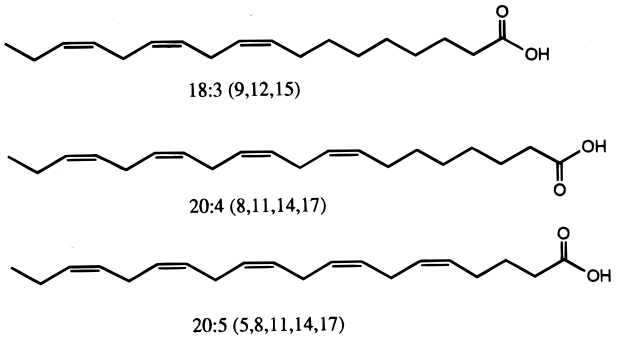


location with respect to substrate have also provided additional support for this scheme *(16)*. However, to date, the cryptoregiochemistry, i.e., the site of initial C−H bond cleavage, of an *ω-x* fatty acid desaturase has not been investigated.

ω-3 polyunsaturated fatty acids (PUFA)[2] are important structural components of membrane glycerolipids and serve as precursors to signaling molecules such as eicosanoids in animals and jasmonates in plants *(17, 18)*. Apart from vertebrates, a wide range of organisms are capable of *ω*-3 fatty acid desaturation in the course of producing various PUFA. The free-living nematode, *Caenorhabditis elegans*, accumulates significant amounts of eicosapentaenoic acid [20:5(5,8,11,14,17)] in addition to other *ω*-3 PUFA, by de novo biosynthesis or conversion of ingested fatty acids *(19−21)*. The gene encoding the fatty acid desaturase responsible for *ω*-3 fatty acid production in *C. elegans* (*fat-1*) was recently identified *(22)*. It was found to share 32−35% sequence identity, as well as conserved structural motifs, with plant extraplastidial Δ^{12} and *ω*-3 desaturases and is thus thought to share a common membrane topology and diiron active site with these enzymes *(5)*. Expression of the nematode gene in the plant *Arabidopsis thaliana* indicated that the gene product was capable of desaturating both 18 and 20 carbon fatty acids *(22)*.

There is currently considerable interest in understanding the structure−function relationships among the class of non-

[2] Abbreviations: FAME, fatty acid methyl ester(s); KIE, kinetic isotope effect; GC-MS, gas chromatography coupled to mass spectrometry; PUFA, polyunsaturated fatty acids; *X*:*Y*(*m, n, ...*), a fatty acid with *X* carbon atoms and *Y* cis double bonds at positions *m, n, ...*, where optional prefixes and suffixes indicate sulfur and deuterium substitutions and esters, for example, 16,16-d_2S18:2(9,15)Me is methyl [16,16-$^2H_2$]-7-thiaoctadeca-9,15-dienoate; *X*:*Y*OH(*mc, ..., th, nc, ...*), a derivative of *X*:*Y*(*m, n, ...*) with a hydroxy group at position *t*.



heme diiron oxidants to which the membrane-bound desaturases belong *(1)*. To broaden the scope of such studies *(8, 11, 14)*, a detailed analysis of the regio- and cryptoregiochemistry of an animal *ω*-3 desaturase was undertaken. This involved the first successful heterologous expression of the *C. elegans fat-1* gene in baker's yeast—an organism which has been shown *(23)* to provide a suitable eukaryotic environment for enzymes of this type *(24)*.

EXPERIMENTAL PROCEDURES

Materials. Nondeuterated fatty acids were obtained from Nu-Chek-Prep, Inc. (Elysian, MN). All fatty acids used were of known purity (typically >99%). Tergitol (type NP-40) and methanolic/HCl (3 M) were obtained from Sigma-Aldrich Canada LTD. (Oakville, Ontario, Canada). Methyl-7-thiastearate (S18:0Me), methyl [15,15-$^2H_2$]-7-thiastearate (15,15-d_2S18:0Me, [15,15-$^2H_2$]-**1**), and methyl [16,16-$^2H_2$]-7-thiastearate (16,16-d_2S18:0Me, [16,16-$^2H_2$]-**1**) were synthesized by procedures analogous to those previously described and purified by flash chromatography (Silica Gel, 4% EtOAc/hexanes) *(14)*. All spectral data obtained for these compounds were in accord with their assigned structure. The full details of the syntheses will be published elsewhere. The thia fatty acid substrates were >95−99% chemically pure based on GC-MS analysis. The two deuterated starting materials consisted entirely of dideuterated species as determined by GC-MS.

Yeast Strain Construction. Copy DNA of the *FAT-1* gene of *C. elegans* was amplified from the clone pCe8 kindly provided by J. Browse (Washington State University) by PCR using the oligonucleotide primers Delta-15-5′-Cele (GATATGGTCGCTCATTCC) and Delta-15-3′-Cele (CACGGGATATTCTTTTACTTG) by standard methods with Vent polymerase (New England Biolabs) *(23, 25)*. The PCR product was ligated into pYES2.1 (Invitrogen), containing the galactose-inducible GAL1 promoter, to give the plasmid pDM015. The sequence of the insert of pDM015 was confirmed to be identical to that previously reported *(22)* and in the sense orientation relative to the GAL1 promoter, using the PRISM DyeDeoxy Terminator Cycle Sequencing System (Perkin-Elmer/Applied Biosystems) and a model 373 DNA sequencer (Applied Biosystems). The haploid *S. cerevisiae* strain INVSc2 (*MAT*α *his3-*Δ*200 ura3−167*; Invitrogen) was transformed with pDM015 by the method of Gietz et al. *(26)* and selected on minimal agar plates lacking uracil *(25)* to give the strain pDM015/INVSc2.

Growth and Biochemical Analysis of Transformed Yeast. To test various substrates of *FAT-1*, the pDM015/INVSc2 strain was grown in minimal media lacking uracil and

containing galactose (CM gal −ura), various fatty acids (100 mg/L unless otherwise stated), and Tergitol [type NP-40, 0.1% (v/v)] at 20 °C for 3 days and at 15 °C for 3 days. The plasmid vector pYES2.1 in the yeast strain INVSC2 was used as a control. For isotope effect experiments, yeast cultures were grown as described above except that 50% (w/w) mixtures of S18:0Me (**1**) with 15,15-d_2S18:0Me ([15,15-$^2H_2$]-**1**) or 16,16-d_2S18:0Me ([16,16-$^2H_2$]-**1**) were supplied.

Analytical Procedures. FAME were prepared from yeast cultures using methanolic HCl as described previously *(8)*. To facilitate GC-MS analysis in the isotope effect experiments, the FAME were fractionated on a Hewlett-Packard 1100 Series HPLC system (Hewlett-Packard, Palo Alto, CA) equipped with an auto-sampler, solvent degasser, quaternary pump, column heater, and diode array detector (monitoring at 205 and 254 nm) which were all controlled by HP Chemstation software. The samples were separated on a series of two 4.6 × 125 mm Whatman Partisphere C-18 reverse-phase columns (Whatman Inc., Clifton, NJ), eluting at a rate of 1 mL/min with 5 mL of acetonitrile followed by a 0−30% acetone gradient (15 mL). Fractions from 2 to 7 min (0.5 mL) were concentrated and analyzed by GC to confirm fractions containing thia fatty acids. These were pooled for MS analysis.

GC analysis of FAME was performed using a Hewlett-Packard 5890 GC equipped with an automatic injector. Data analysis was done with ChemServer software (Hewlett-Packard) and Target Compound Analysis Software (Thru-Put Systems Inc., Orlando, FL). Split injections (100:1) were used on 2 μL samples onto a 30 m × 0.253 mm DB-23 (0.25 μm film thickness) fused silica column with a helium flow of 1 mL/min, and the temperature program was isothermal 180 °C for 1 min, gradient 4 °C/min to 240 °C, and then isothermal at 240 °C for 15 min.

The position of newly introduced double bonds in desaturated products was determined by preparing the fatty acyl diethylamides as described previously *(27, 28)* followed by detailed GC-MS analyses.

GC-MS analysis was done using a Fisons VG TRIO 2000 mass spectrometer (VG Analytical UK) controlled by Masslynx version 2.0 software, coupled to a GC 8000 Series gas chromatograph equipped with a 30 m × 0.253 mm DB-23 (0.25 μm film thickness) fused silica column (temperature program isothermal 200 °C for 1 min, gradient 10 °C/min to 240 °C, and then isothermal at 240 °C for 25 min). The deuterium content was measured using selected ion monitoring in the EI^+ mode (70 eV) of pertinent ion clusters with a cycle time of 0.1 s per channel [4 channels for S18:1(9)Me and 5 channels for S18:2(9,15)Me], corresponding to ca. 20−25 scans per peak. The integrated intensities of the individual ions were corrected for natural isotopic abundance, and the isotopic ratios were determined using these corrected intensities for the ions: *m*/*z* 316 (318 for d_2 analogue), M^+ (S18:0Me); *m*/*z* 152 (154 for d_2 analogue), $(CH_3{-}CH_2{-}CH_2{-}CH_2{-}(CH_2)_3{-}CH{=}CH{-}CH{=}CH_2)^+$ [S18:1(9)Me]; *m*/*z* 150 (151 for d_1 analogue), $(CH_3{-}CH_2{-}CH{=}CH{-}(CH_2)_3{-}CH{=}CH{-}CH{=}CH_2)^+$ [S18:2(9,15)Me].

RESULTS

Expression and Substrate Requirements of FAT-1 in Yeast. The successful functional expression of *C. elegans fat*-1 is demonstrated in Figure 1 with examples of GC analyses of fatty acid methyl esters (FAME) from yeast cultures: pDM015/INVSc2 cultures show a peak corresponding to 18:2(9,15) (Figure 1, panel A-2) which is not present in the pYES2.1/INVSc2 control strain (Figure 1, panel A-1). The retention time of the new peak matched that for the GC-MS-identified compound produced by yeast expressing the corresponding *B. napus Fad3 (8)*.

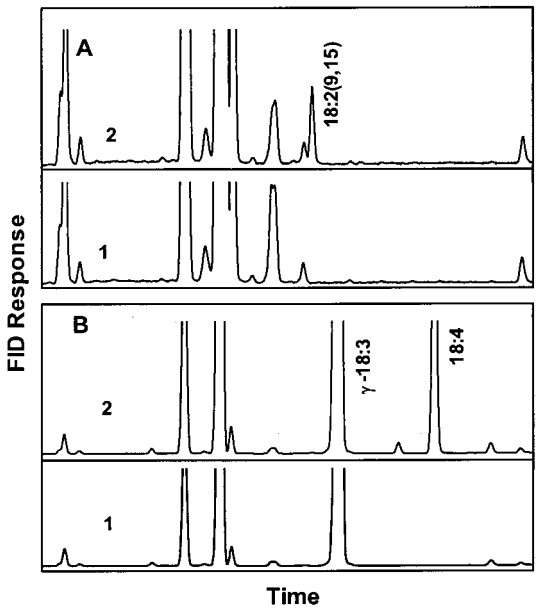


FIGURE 1: Analysis of fatty acids from yeast cultures expressing *C. elegans fat-1*. GC of FAME from yeast transformed with control plasmid pYES2.1 (1) or pDM015 containing *fat-1* (2) for cultures grown in media without additional fatty acid (A) or media containing 18:3(6,9,12) (B). For each pair of chromatograms, the FID signal corresponds to the same volume of yeast culture.

To gain some insight into the substrate requirements of *FAT-1* in a qualitative sense, a range of possible fatty acid substrates were supplied to yeast cultures expressing this enzyme. In conjunction with this, analysis of the desaturation products was undertaken to determine the regioselectivity of the enzyme (see below).

An example of the conversion of exogenous substrates is illustrated in Figure 1B for pDM015/INVSc2 and pYES-2.1/INVSc2 strains grown on media supplemented with 18:3(6,9,12). In this case, 18:4(6,9,12,15) was evident only for the cultures expressing the *C. elegans fat-1*. A complete list of fatty acid substrates tested and the products of desaturation for *fat-1* expressed in yeast is given in Table 1. Previous results obtained for the corresponding plant enzyme (*B. napus FAD3*) are included for the purposes of comparison. In each case, the appearance of the desaturated product was dependent on the presence of the *C. elegans* gene in the yeast strain.

Regioselectivity of FAT-1. The positions of the newly formed double bond in the products of desaturation derived from 16:1(11), 18:2(9,12), 20:2(11,14), and 20:3(8,11,14) were determined by GC-MS of the corresponding fatty acyl diethylamides *(27, 28)*. [In the case of 16:1(11), this substrate is actually formed by the in vivo elongation of exogenously supplied 14:1(9).] An example of this analysis is shown for the 16:2(11,13) product in Figure 2. For other products, retention times were compared to known standards or to GC-MS-identified compounds from the expression of the *B.*

Table 1: Conversion of Exogenous Fatty Acids by the Yeast Strain pDM015/INVSc2 Expressing *C. elegans fat-1*[a]

substrate	substrate accumulation [% (w/w) of total fatty acids]		product	identification[b]	product accumulation [% (w/w) of total fatty acids]	
	C. elegans (this paper)	*B. napus* *(8)*			*C. elegans* (this paper)	*B. napus* *(8)*
16:1(11)[c]	47	44	16:2(11,13)	GC-MS	0.18	0.14
16:1(9)[d]	44	23	16:2(9,13)	RTF	<0.002	0.048
18:1(9)	52	57	18:2(9,15)	RTF	0.24	0.21
18:2(9,12)	33	49	18:3(9,12,15)	GC-MS, RTS	4.1	1.3
18:3(6,9,12)	23	47	18:4(6,9,12,15)	RTS	4.2	0.35
20:1(11)	2.4	7.3	20:2(11,17)	RTF	<0.002	0.016
20:2(11,14)	4.4	3.4	20:3(11,14,17)	GC-MS, RTS	0.18	0.18
20:3(8,11,14)	16	16	20:4(8,11,14,17)	GC-MS, RTS	1.0	0.1
20:4(5,8,11,14)	7.4	29	20:5(5,8,11,14,17)	RTS	0.14	0.21
18:1-OH(9c,12h)	6.6	10	18:2-OH(9c,12h,15c)	RTS	0.06	0.16

[a] See Experimental Procedures for culture conditions. Values are the means of two experiments each with duplicate cultures. The values for substrate and product accumulation for *B. napus FAD3 (8)* are shown for comparison. For control experiments using the pYES2/INVSc2 strain, with the exception of 20:3(8,11,14)- and 20:4(5,8,11,14)-supplied cultures, no significant peaks were detected at the retention time of the desaturation product. In the case of 20:3(8,11,14) and 20:4(5,8,11,14), the area of the GC peak in the pYES2/INVSc2 control culture due to substrate impurity was subtracted from the peak area for the corresponding product found in the pDM015/INVSc2 cultures. Despite incorporation levels of 0.7−2.4%, ω-3 desaturation products were not detected above 0.002% for 14:1(9), 18:1(13), 20:0, and 22:2(13,16). [b] Products were screened for and identified by GC-MS; comparison of retention times was with authentic all-cis standards (RTS) or GC-MS-identified compounds from yeast expressing *B. napus Fad3* (RTF). [c] Derived from in vivo elongation of supplied 14:1(9). [d] Endogenous substrate; no fatty acid added to medium.

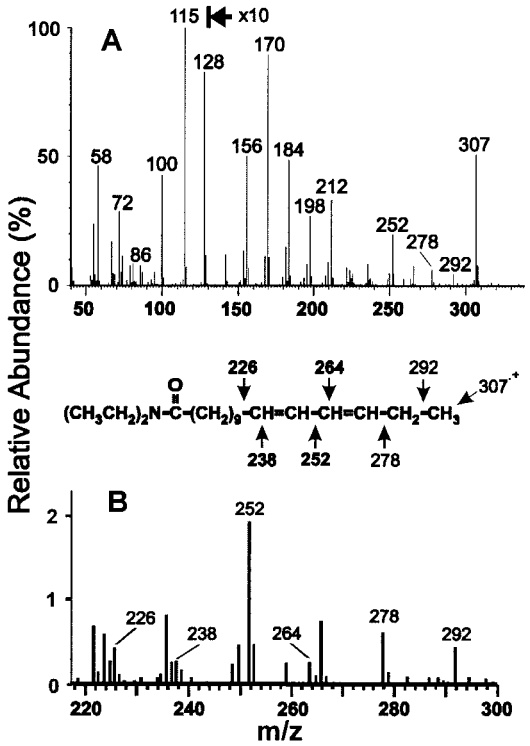


FIGURE 2: GC-MS analysis of the diethylamide derivative product [16:2(11,13)] of yeast cultures supplied with 14:1(9). (A) *m*/*z* from 0 to 340; (B) *m*/*z* 220 to 300. Diagnostic amide-containing fragments are indicated by *m*/*z* values and arrows. See Experimental Procedures for culture conditions.

napus Fad3 (8). The standards used were all-cis compounds, and retention times typically matched to within 1 s in a 6−25 min GC run. Under the same chromatographic conditions, positional and stereochemical isomers of olefinic fatty acids are resolved by up to 1 min. On the other hand, the stereochemistry of the ω-3 double bond in 16:2(11,13), 18:2(9,15), 20:2(11,17), and 18:2OH(9c,12h,15c) was not determined, although the latter compound coeluted with the natural product (densipolic acid) of seed oils of the genus *Lesquerella (29, 30)*. Based on the data presented here and elsewhere *(20, 22)*, it seems reasonable to conclude that *C. elegans FAT-1* catalyzes the introduction of a cis double bond at the ω-3 position of a wide range of mono- and polyunsaturated fatty acid derivatives. This regiochemistry is also confirmed by the conversion of 15,15-d_2S18:1(9) and 16,16-d_2S18:1(9) to the corresponding d_1S18:2(9,15) isotopomers (see below).

Cryptoregiochemistry of FAT-1. To investigate the cryptoregiochemistry (site of initial oxidation) of *FAT-1*, the primary deuterium KIE's were measured for the individual C−H bond cleavages at C-15 and C-16 of a C-18 fatty acid analogue (see the introduction). This was accomplished using a convenient in vivo methodology similar to that employed previously *(14)*. This approach involves measuring the d_1/d_0 ratio of olefinic product derived by desaturation of a 1:1 mixture of d_0 and a regiospecifically dideuterated (CD_2) substrate. To simplify the syntheses of the deuterium-labeled substrates, 7-thia-fatty acid analogues[3] were used, and the native yeast Δ^9 desaturase was recruited to introduce, in situ, the requisite double bond at the 9,10-position *(11)* (Scheme 2). A preliminary experiment was carried out using d_0-methyl 7-thiastearate **1** to establish conditions for optimal incorporation and substrate conversion. A typical gas chromatogram of FAME derived from pDM015/INVSc2 cultures supplied with S18:0Me is shown in Figure 3. New peaks with retention times in the range expected for S18:1(9)Me **2** and S18:2(9,15)Me **3** were well resolved from other components. (Due to the vertical scale, the resolution of S18:0 and S18:1 peaks is obscured in Figure 3.) The levels of thia analogues accumulated by pDM015/INVSc2 cultures were typically 5%, 6%, and 0.005−0.05% for S18:0Me, S18:1(9)Me, and S18:2(9,15)Me, respectively. Because of the low levels of accumulation of S18:2(9,15) in the yeast cultures, HPLC was used to provide samples enriched in this product and the S18:1(9) intermediate. An example of this fractionation is shown in Figure 4. Identification of the thia fatty acid methyl

[3] The use of thia fatty acids also allows us to distinguish between products of supplied deuterated fatty acids and endogenous compounds in the GC-MS analysis. It is known *(14)* that the substitution of a C-7 methylene group with a sulfur atom in the substrate does not change the regiochemistry of double bond introduction.

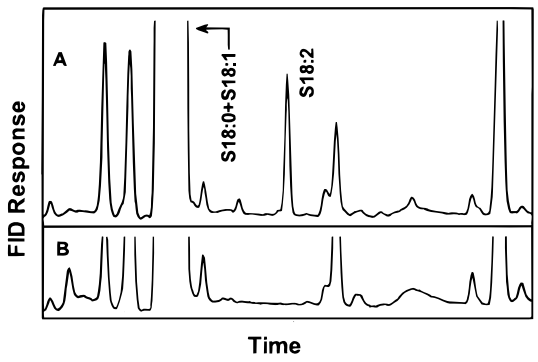


FIGURE 3: GC analysis of FAME derived from pDM015/INVSc2 yeast cultures supplied with undeuterated methyl 7-thiastearate. The dienoic product is indicated as S18:2(9,15). See Experimental Procedures for GC-MS parameters and culture conditions.

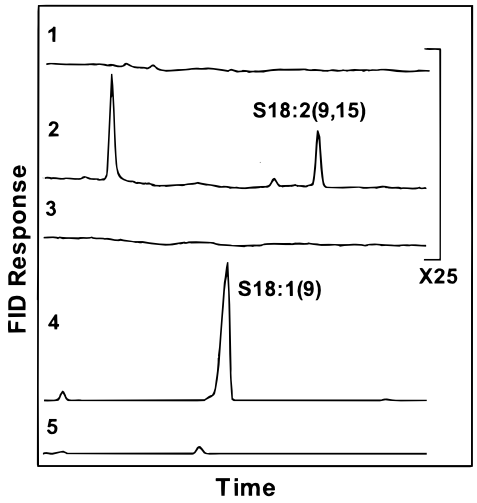
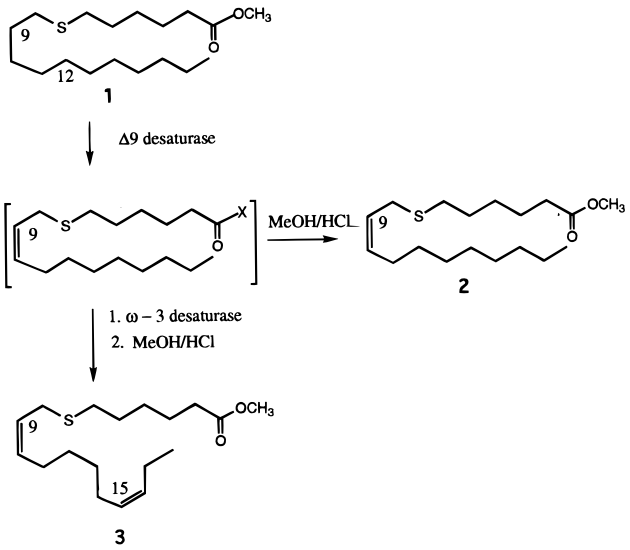


FIGURE 4: HPLC fractionation of FAME from pDM015/INVSc2 yeast cultures supplied with S18:0Me. Gas chromatograms of HPLC fractions from 3−3.5 min (1), 3.5−4 (2), 4−4.5 min (3), 4.5−5 min (4), and 5.5−6 min (5) are shown. See Experimental Procedures for analytical procedures.

Scheme 2



esters was confirmed by GC-MS; the mass spectrum of S18:2(9,15)Me **3** is shown in Figure 5. The ions with *m*/*z* of 150 and 183 correspond to fragments arising from cleavage on either side of the sulfur atom and are indicative of a

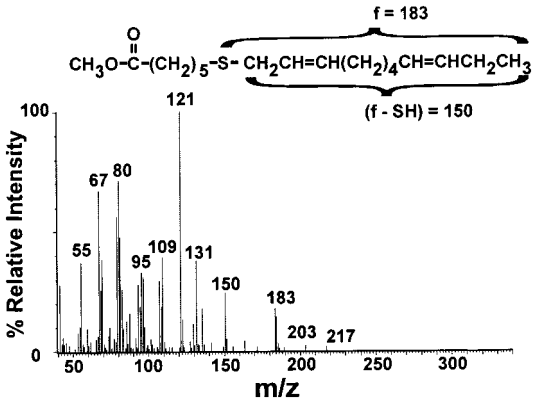


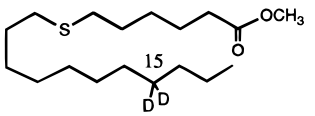
FIGURE 5: GC-MS analysis of the dienoic FAME product of pDM015/INVSc2 cultures supplied with S18:0Me. The molecular fragments corresponding to $m/z = 150$ are indicated by braces. See Experimental Procedures for culture conditions.

Table 2: Intermolecular Kinetic Isotope Effects on the ω-3 Desaturation of 7-Thia-9-octadecenoate Catalyzed by *C. elegans FAT-1*[a]

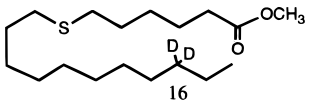
FAME mixture supplied	isotope effect
15,15-d_2S18:0Me/S18:0Me	7.8 ± 0.4
16,16-d_2S18:0Me/S18:0Me	0.99 ± 0.04

[a] Assays and intermolecular isotope effect calculations were performed as described under Experimental Procedures. Values are the means and standard deviations of separate calculations for 4−5 cultures.

dienoic acid. Our assignment of the double bond positions in the desaturated products **2** and **3** was substantiated by the pattern of deuterium loss in the KIE experiments discussed below and is also consistent with the known regiochemistry of 18:0 and 18:1(9) desaturation as it occurs in this transformed yeast system (Table 1).



[15,15-$^2H_2$]-**1**



[16,16-$^2H_2$]-**1**

The conditions used for the trial incubations with nondeuterated substrate were employed for the KIE experiments which were carried out by supplying yeast cultures expressing the nematode desaturase with a 1:1 mixture of d_0- and 15,15-d_2-methyl 7-thiastearate and a 1:1 mixture of d_0- and 16,16-d_2-methyl 7-thiastearate. The biosynthetic products—methyl 7-thiaoleate **2** and methyl 7-thiaisolinoleate **3**—were examined by GC-MS, and the product kinetic isotope effects were calculated using the ratio: [% d_0S18:2(9,15)Me/% d_1S18:2(9,15)Me]/[% d_0S18:1(9)Me/% d_2S18:1(9)Me]. [As anticipated, the isotopic content of the thiaoleate intermediate (S18:1(9)Me **2**) was essentially the same as that of the thiastearate starting material (**1**).] The details of the GC-MS analysis are given under Experimental Procedures.

The results of the intermolecular deuterium kinetic isotope effect measurements on the ω-3 desaturase reaction are displayed in Table 2. A large primary deuterium kinetic isotope effect ($k_H/k_D = 7.8 \pm 0.4$) manifests itself at the carbon closer to the carboxyl group (C-15) while C−H bond cleavage at C-16 is essentially insensitive to deuterium substitution ($k_H/k_D = 0.99 \pm 0.04$).

DISCUSSION

With respect to substrate requirements and regiochemistry, the above results indicate that the *C. elegans FAT-1* has the following properties: (1) ω-3 regioselectivity, (2) the ability to desaturate unsaturated substrates in the 16−20 carbon range, (3) a preference for substrates with ω-6 double bonds, but the ability to desaturate substrates with ω-6 hydroxyl groups or ω-9 or ω-5 double bonds, and (4) a relative insensitivity to the presence of double bonds proximal to the acyl end of the substrate. These characteristics are generally similar to those found previously using a plant expression system and a more limited set of substrates *(22)*. In addition, we have found that the substrate specificity of the nematode enzyme is qualitatively quite similar to that of the *B. napus* extraplastidial ω-3 desaturase *(8)*. Since all of the data in Table 1 are consistent with the *C. elegans FAT-1* being described as ω-3 as opposed to ν+3 or Δ^{15}, it was of particular interest to probe the site of the initial oxidation event (cryptoregiochemistry of this enzyme) since all previous studies of this type have involved enzymes of the Δ^x type (or a combined $\Delta^x/\upsilon+x$ type); i.e., the double bond is introduced at a specific distance from the acyl end of the fatty acid *(14)*. In each of these cases, a large isotope effect was only observed for C−H bond cleavage at the carbon (x) nearest the acyl side of the incipient double bond. These include a protist Δ^6 desaturase *(13)*,[4] yeast Δ^9 desaturase *(11, 12)*, an insect Δ^{11} desaturase *(15)*, and a plant extraplastidial Δ^{12} desaturase [a combined $\Delta^x/\upsilon+x$ type enzyme *(7, 14)*]. This study presents the first kinetic isotope effect determinations for a desaturase that measure from the methyl end of the substrate. Interestingly, despite the difference in regioselectivity, the *fat-1* gene product displays cryptoregiochemistry which matches the Δ^x enzymes in the sense that the site of the first (energetically difficult and hence isotopically sensitive) C−H cleavage is at the carbon closer to the acyl group. This common cryptoregiochemical theme together with the fact that all membrane-bound fatty acid desaturases share a degree of similarity with respect to stereochemistry, amino acid sequence, hydrophobicity profile, and a putative diiron binding site supports the suggestion that these enzymes belong to one topological family *(1, 2)*. The three distinct types of regioselectivity would simply reflect differences in the position of the active site relative to the features of the substrate binding pocket associated with substrate recognition. We have attempted to portray this in a generalized model as shown in Figure 6. For Δ^x desaturases, the distance between the acyl group-binding domain and the putative non-heme iron oxo species is essentially determined by the number of intervening methylene groups. For the $\nu+x$ and ω-x enzymes, this acyl group-binding site is less constrained; instead, the position of the incipient double bond is determined relative to an existing double bond ($\nu+x$), or relative to the methyl terminus (ω-x). Additional active site features may also influence desaturase activity: the data for the plant and nematode ω-3 desaturases indicate that while regioselectivity of double bond introduction is primarily determined by the distance of the iron oxidant to the methyl terminus, a double bond at the ω-6 position strongly affects the activity of the enzyme [Table 1, cf. results for 20:1(11) and 20:2(11,14) substrates]. Further experiments designed to probe the structure−function relationships of the membrane-bound desaturases are required to test the accuracy of our topological model.

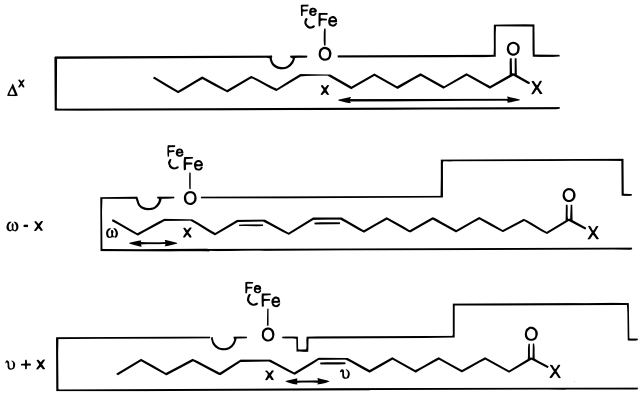


FIGURE 6: Model representing fatty acid desaturases with three different regioselectivities.

[4] We have recently confirmed the conclusions of ref *13* by carrying out KIE determinations (Fauconnot, L., and Buist, P. H., unpublished results).

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SUPPORTING INFORMATION AVAILABLE

GC-MS data for the desaturation products of 18:2(9,12), 20:2(11,14), and 20:3(8,11,14) (3 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

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