in all tissues examined (leaves, seeds and corolla), 
desB and desD are inactive in these tissues, and 
desE is expressed in leaves only. desC appears to be 
a pseudogene with several deletions and insertions. 
These results, however, do not explain the re-
main 40% 18:2 in the seed lipid fraction of homozygous desA mutants. It is possible that (i) 
the RACE experiments do not give conclusive 
information on the fad2 expression patterns, (ii) 
there are still other seed-specific Petunia fad2 
genes responsible for the 40% 18:2, (iii) in the 
mutant lines the desA function is taken over by one 
or more of the other fad2 genes, or (iv) in Petunia, 
50% of the total 18:2 in seed lipids is synthesized 
in the chloroplast by the action of the plastidial 
-6 desaturase. This latter possibility is, however, 
different from the situation in Arabidopsis where 
the plastidial -6 desaturase does not contribute 
to oleate desaturation in seeds [3]. Further ex-
perimentation will be required to discriminate 
between these possibilities.

References
1 Koes, R., Souer, E., van Houwelingen, A., Mur, L., Spelt, C., 
Quattrocchio, F., Wing, J., Oppedijk, B., Ahmed, S., Maes, T. 
2 Verwoert, I., Hersmus, B., Nijkamp, H., van Haaren, M., Koes, 
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Substrate specificity, regioselectivity and cryptoregiochemistry of plant and animal -3 fatty acid desaturases
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Abstract
In order to define the substrate requirements, 
regiochemistry and cryptoregiochemistry of the 
-3 fatty acid desaturases involved in poly-
unsaturated fatty acid formation, the genes Fad3

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and fat-1 from Brassica napus and the nematode 
Caenorhabditis elegans respectively were expressed 
in baker's yeast (Saccharomyces cerevisiae). Various 
fatty acids, including deuterium-labelled thia-
fatty acids, were supplied to growing cultures of 
transformed yeast. The results from GC-MS 
analysis of the desaturated products indicate that both 
the plant and animal desaturases act on 
unsaturated substrates of 16–20 carbons with a
preference for ω-6-unsaturated fatty acids. The regioselectivities of both enzymes were confirmed to be that of ω-3 desaturases. The primary deuterium kinetic isotope effects at C-15 and C-16 of a C_{ω} fatty acid analogue were measured via competitive incubation experiments. Whereas \( k_{d}/k_{o} \) at the ω-3 position was shown to be large, essentially no kinetic isotope effect at the ω-2 position was observed for the plant or the nematode enzymes. These results indicate that ω-3 desaturation is initiated by an energetically difficult C–H bond cleavage at the carbon closer to the carboxyl terminus. These results will be discussed in the context of a general model relating the structure and function of membrane-bound fatty acid desaturases featuring different regioselectivities.

**Introduction**

Until very recently, information about the substrate specificities, regioselectivities and mechanism of membrane-bound fatty acyl desaturases was quite limited [1–3]. 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. Three classes of regioselectivity have been observed for fatty acid desaturases: the Δ^+ desaturases introduce a double bond \( x \) carbons from the carboxyl end; \( ω-x \) desaturases dehydrogenate \( x \) carbons from the methyl terminus; while \( ω+x \) desaturases use a pre-existing double bond as a reference point and dehydrogenate \( x \) carbons from the nearest olefinic carbon [4,5].

In addition to their more apparent regioselectivities, fatty acid desaturases can also be characterized in terms of what has been labelled their cryptoregiochemistry (site of initial oxidation) [6]. That is, for all membrane-bound desaturases studied to date, large primary deuterium isotope effects have been observed at the carbon proximal to the acyl end of the fatty acyl substrate [6–10]. These observations have been interpreted within the context of a mechanistic model [11], which features acyl-side C–H bond cleavage as the initial, rate-determining oxidation event. In order to broaden the scope of such studies [6] to include \( ω-x \)-type desaturases [3,9], a detailed analysis of the substrate requirements, regio- and cryptoregio-chemistry of two ω-3 desaturases, one from a plant, Brassica napus [4], and one from an animal, Caenorhabditis elegans [12], was undertaken.

**Materials and methods**

The methods were described previously [3,13]. Methyl 7-thiastearet (S18:0Me), methyl [15,15-\(^{2}\)H_{2}]7-thiastearet and methyl [16,16-\(^{2}\)H_{2}]7-thiastearet were synthesized by procedures analogous to those previously described [9].

**Results and discussion**

In order to gain some insight into the substrate requirements of *C. elegans* FAT-1 and *B. napus* FAD3 in a qualitative sense, a range of possible fatty acid substrates were supplied to yeast cultures expressing these enzymes. In conjunction with this, analysis of the desaturation products was undertaken to determine the regioselectivity of the enzyme. The fatty acid substrates tested and accumulation of their desaturation products are shown in Figure 1. In each case, appearance of an ω-3 desaturated product was dependent on the presence of the animal or plant desaturase gene in the yeast strain. Some marked differences in substrate conversion were observed between FAT-1 and FAD3, e.g. for 18:3(6,9,12) and 20:3(8,11,14) [where the form \( X:Y(m,n,...) \) is a fatty acid with \( X \) carbon atoms and \( Y \) cis double bonds at positions \( m,n,... \); however, due to the in vitro design of the experiments, it is not clear if these variations are entirely due to differences in active-site architecture. The positions of the newly formed double bond in the products of desaturation derived from most of the substrates were determined by GC-MS of the corresponding fatty acyl diethylamides [14,15]. For other products, retention times were compared with known standards. With respect to substrate requirements and regiochemistry, the above results indicate that both the *C. elegans* FAT-1 and the *B. napus* FAD3 have the following properties: (i) ω-3 regioselectivity, (ii) the ability to desaturate unsaturated substrates in the 16–20 carbon range, (iii) 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 (iv) a relative insensitivity to the presence of double bonds proximal to the acyl end of the substrate.

To investigate the cryptoregiochemistry (site of initial oxidation) of *C. elegans* FAT-1, the primary deuterium kinetic isotope effects were measured for the individual C–H-bond cleavages at C-15 and C-16 of a C_{18} fatty acid analogue. This approach involves measuring the d_{15}/d_{16} ratio of...
olefinic product derived by desaturation of a 1:1 mixture of d_{10} and a regiospecifically didesaturated (CD₂) substrate. To simplify the syntheses of the deuterium-labelled substrates, 7-thia-fatty acid analogues were used and the native yeast Δ⁹ desaturase was recruited to introduce, in situ, the requisite double bond at the 9,10 position [6]. 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. Identification of the thia fatty acid methyl esters and isotope ratio measurements were carried out by GC-MS.

For *C. elegans* FAT-1, a large primary deuterium kinetic isotope effect (k_H/k_D = 7.8 ± 0.4) was found at the carbon closer to the carboxyl group (C-15) while C-H-bond cleavage at C-16 was found to be essentially insensitive to deuterium substitution (k_H/k_D = 0.99 ± 0.04). Similar results have been found recently for *B. napus* FAD3 (P. S. Covello and P. H. Buist, unpublished work).

It is interesting to compare the results for ω-3 desaturases with those for other types of desaturase. Despite the difference in regioselectivity when compared with Δ⁹ desaturases, the ω-3 desaturases display cryptoregiochemistry which matches the Δ⁹ 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 di-iron binding site, supports the suggestion that these enzymes belong to one topological family [2,16]. The three distinct types of regioselectivity may 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 at-

**Figure 1**

Conversion of exogenous fatty acids by the yeast strains pRS131/INVSc2 and pDM015/INVSc2 expressing *C. elegans* FAT-1 (black bars) and *B. napus* FAD3 (hatched bars), respectively.

Values are the means of two experiments each with duplicate cultures. 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. Products were screened for and identified by GC-MS and/or comparison of retention times with authentic all-cis standards.

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tempted to portray this in a generalized model as shown in Figure 2. For $\Delta^s$ desaturases, the distance between the acyl-group-binding domain and the putative non-haem iron oxo species is essentially determined by the number of intervening methylene groups. For the $\omega + x$ and $\omega - x$ 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 ($\omega + x$), or relative to the methyl terminus ($\omega - x$). Additional active-site features may also influence desaturase activity: the data for the plant and nematode $\omega - 3$ 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 $\omega - 6$ position strongly affects the activity of the enzyme [Figure 1, compare 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 elaborate our topological model.

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References

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