

A Small Membrane-peripheral Region Close to the Active Center Determines Regioselectivity of Membrane-bound Fatty Acid Desaturases from *Aspergillus nidulans**

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Fatty acid desaturases catalyze the introduction of double bonds at specific positions of an acyl chain and are categorized according to their substrate specificity and regioselectivity. The current understanding of membrane-bound desaturases is based on mutant studies, biochemical topology analysis, and the comparison of related enzymes with divergent functionality. Because structural information is lacking, the principles of membrane-bound desaturase specificity are still not understood despite of substantial research efforts. Here we compare two membrane-bound fatty acid desaturases from *Aspergillus nidulans*: a strictly monofunctional oleoyl- Δ 12 desaturase and a processive bifunctional oleoyl- Δ 12/linoleoyl- ω 3 desaturase. The high similarities in the primary sequences of the enzymes provide an ideal starting point for the systematic analysis of factors determining substrate specificity and bifunctionality. Based on the most current topology models, both desaturases were divided into nine domains, and the domains of the monofunctional Δ 12 desaturase were systematically exchanged for their respective corresponding matches of the bifunctional sister enzyme. Catalytic capacities of hybrid enzymes were tested by heterologous expression in yeast, followed by biochemical characterization of the resulting fatty acid patterns. The individual exchange of two domains of a length of 18 or 49 amino acids each resulted in bifunctional Δ 12/ ω 3 activity of the previously monofunctional parental enzyme. Sufficient determinants of fatty acid desaturase substrate specificity and bifunctionality could, thus, be narrowed down to a membrane-peripheral region close to the catalytic site defined by conserved histidine-rich motifs in the topology model.

Metabolic processes are often controlled by the presence of highly specific enzymes. An interesting model to study enzyme specificity is a fatty acid desaturase, which introduces double bonds at specific positions into acyl chains (1). Desaturase enzymes differ in substrate- and regioselectivity (2). The strict substrate- and regiospecificities are of physiological relevance, because the major mechanism controlling the biophysical properties of a membrane, aside from changing its overall lipid class composition, is the modification of acyl chain length, position, and number of the double bonds in glycerolipids (3). Fatty acid desaturases may also control the acyl composition of seed storage lipids, which is of considerable economic interest. Despite the importance of desaturases, knowledge about the determinants of their enzymatic specificity is still in its infancy.

The solution of the three-dimensional crystal structure of the soluble castor bean and ivy stearyl-ACP²-desaturases has brought some insight into the structure/function relationship involved in the determination of specificity of soluble desaturase enzymes (4–6). However, the majority of desaturase enzymes reside within membranes, and up to now the principles of membrane-bound desaturase specificity are not understood. Moreover, structural information on membrane-bound desaturases is limited, so only hydropathy and topology analyses are available, predicting the formation of four transmembrane domains and either one or two membrane-peripheral protein domains (7–9). Three highly conserved histidine-rich motifs are essential for catalysis (1, 10), and it was proposed that these histidine clusters coordinate two iron atoms in the active site (11).

The clearly related genes for membrane-bound desaturases have developed a broad range of catalytic diversity of the encoded desaturase enzymes during evolution (12). Depending on the enzyme studied, introduction of a double bond can occur counting carbons from the carboxyl end or from the methyl terminus of the acyl chain. Other enzymes may use a pre-existing double bond as a reference point for subsequent desaturation (2). Also the carbon chain length (2) or the specific lipid

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) XP_658641 and XP_664808.

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² The abbreviations used are: ACP, acyl carrier protein; FAMES, fatty methyl esters; GMM, glucose minimal medium; ORF, open reading frame; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; ER, endoplasmic reticulum; DMOX, 4,4-dimethylloxazoline.

head group of the glycerolipid to which the substrate fatty acid is esterified can be distinguished (13). One conceivable model how changes in regiospecificity may have developed in evolution is by gene duplication and random accumulation of mutations in the catalytic portion of the enzymes to produce first a broader and then a different specificity (10, 12). Changes to an enzyme regiospecificity typically require between two and six specific alterations at key locations along the amino acid chain (6, 14). According to the described model the first step toward a new specificity of an enzyme is the accumulation of mutations in the corresponding gene, which result in decreased enzyme specificity and lead to broader substrate acceptance and/or formation of multiple products (12). The simplest case of multifunctionality is represented by bifunctional enzymes. Examples include the oleate 12-hydroxylase/desaturase from the flowering plant *Lesquerella fendleri* (15), a $\Delta 6$ acetylenase/desaturase from the moss *Ceratodon purpureus* (16), a $\Delta 5/6$ desaturase from the fish *Danio rerio* (17), an acyl-ACP desaturase from the flowering plant *Hedera helix*, which can synthesize 16- and 18-carbon monoene and diene products (18) and $\Delta 12/\omega 3$ desaturases from the fungi *Fusarium moniliforme*, *Fusarium graminearum*, and *Magnaporthe grisea* (19).

The determinants of altered desaturase specificity may be identified by the comparison of a strictly specific monofunctional to a bifunctional enzyme. Here we compare the specific An2 oleoyl- $\Delta 12$ desaturase (19) to the bifunctional An1 oleoyl- $\Delta 12$ /linoleoyl- $\omega 3$ desaturase from *Aspergillus nidulans*. Heterologous expression of the $\Delta 12/\omega 3$ desaturase gene in yeast and in *Arabidopsis* seeds demonstrated its broad $\omega 6$ substrate specificity and especially its bifunctionality, similar to that of reported bifunctional enzymes (19). By using a domain-swapping approach between the genes for the two enzymes, domains responsible for the broadened substrate specificity and altered regioselectivity were identified and reside in a small membrane-peripheral region in close proximity to the active site.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and DNA-modifying enzymes were obtained from MBI Fermentas. Standards of fatty acids as well as all other chemicals were from Sigma; methanol, hexane, 2-propyl alcohol (all HPLC grade) were from Baker. Fatty acids were either obtained from Cayman Chemicals or Larodan. Basic molecular biological and biochemical techniques were performed as described (20).

Cultivation of *A. nidulans*—*A. nidulans* (strain FGSC A4) was cultivated in glucose minimal medium (GMM) (21) at 37 °C. For vegetative growth cultures were cultivated in liquid GMM at 37 °C in the light and shaken vigorously at 220 rpm. For sexual growth cultures were incubated in liquid GMM at 37 °C in the dark. Mycelium was harvested by vacuum filtration through sterile filter paper.

Isolation and Cloning of Desaturase Genes—Total RNAs were extracted from mycelia obtained from vegetative growth or sexual development using TRIzol reagent (Invitrogen). First strand cDNA was synthesized from total RNA using M-MuLV Reverse Transcriptase (MBI Fermentas). The desaturase genes An2 (GenBankTM accession no. XP_658641) and An1 (GenBankTM accession no. XP_664808) were isolated by PCR ampli-

fication using the Expand High Fidelity polymerase (Roche Diagnostics) and a standard PCR protocol (2 min at 94 °C, 10 cycles of 30 s at 94 °C, 30 s at 55 °C, 60 s at 72 °C, followed by 15 cycles of 30 s at 94 °C, 30 s at 55 °C, and 60 s at 72 °C; and a terminal extension step of 5 min at 72 °C) with primers 5'-GAA TTCATGGCTGCAACTGCAACAACCC-3' (An2.forward)/5'-GGCGGCCGCTATTCCGCTTTGGCACCCCTTC-3' (An2.reverse), and (An1.forward) 5'-GAA TTC ATG GCC TCG GAT GCG GGC AAG-3'/(An1.reverse) 5'-GGCGGCCGCTTAGTTAGGCTTGGTCAGCTTAATG-3', respectively, introducing EcoRI sites upstream of the start codons and NotI sites downstream of the stop codons. The ORFs were cloned into pGEM-T (Promega) and moved as EcoRI/NotI fragments into the yeast expression vector pESC-HIS (Stratagene), yielding pESC-An2 and pESC-An1.

Preparation of $\Delta 12$ - and $\Delta 12/\omega 3$ Desaturase Constructs—The ORFs of An2 and An1 desaturases were modified using the Expand High Fidelity polymerase (Roche Diagnostics) and a standard PCR protocol (see above) with primers: 5'-GGGAA-TTCGCTATGGCCTCGGATGCGGGCAAG-3' (An2for)/5'-GTCAGTTCGAGGTTAGGCTTGGTCAGCTT-3' (An2rev) and (An1for) 5'-GGGAATTCGCTATGGCTGCAACTGCAACAACC-3'/(An1rev) 5'-GTCAGTTCGAGTTCGCTTTGGCACCCCTTCTT-3', respectively, introducing EcoRI sites upstream of the start codons (in boldface) and removing the stop codons, respectively, introducing XhoI sites downstream of the terminal triplets. The ORFs were cloned into the amplification plasmid pGEM-T (Promega) and moved as EcoRI/XhoI fragments into the yeast expression vector pYES2/CT (Invitrogen) in-frame with the sequence encoding a C-terminal V5-tag, yielding pYES2/CT-An2 and pYES2/CT-An1.

Cloning and Vector Construction for Production of Transgenic *Arabidopsis* Plants—The cDNA ORF of An $\Delta 12/\omega 3$ was moved as an EcoRI/NotI-fragment into the Gateway Entry vector pUC18-Entry (22). The insert was introduced by Gateway cloning (Invitrogen) into the binary plasmid pCAMBIA modified for resistance to ammonium glufosinate and seed-specific expression using the USP promoter (22). The construct was transformed into chemically competent *Agrobacterium* (strain EH105), and introduced into *Arabidopsis thaliana* ecotype Columbia by floral dipping (23). T3-seeds were collected from individual T2 plants resistant to ammonium glufosinate and analyzed individually by GC.

Engineering Hybrid Genes—According to results from hydrophathy analysis, the desaturases An2 and An1 were divided into nine domains, A₁-I₁, and A₂-I₂, respectively (see Fig. 2, A and B), that were systematically exchanged between the two desaturase proteins to delineate determinants of enzymatic specificity. Domain swap constructs between An2 and An1 were generated by overlap extension PCR (24). In a first step, domains A₁-I₁ were amplified individually from an An1 cDNA template with overlapping ends using primers specified in Table 1. Similarly, domains A₂-I₂ were amplified from An2 cDNA (see Table 1). cDNAs encoding the respective truncated acceptor desaturase domains were amplified from An2 and An1 cDNA using primers also listed in Table 1. Amplifications were carried out with Expand High Fidelity polymerase (Roche Diagnostics) and a protocol of 2 min at 94 °C, 10 cycles of 15 s at

Active Site Determinant of Fatty Acid Desaturase

TABLE 1

Oligonucleotide primers used to construct hybrid genes of *A. nidulans* bifunctional $\Delta 12/\omega 3$ -desaturases (An1) and monofunctional $\Delta 12$ (An2) fatty acid desaturases

Domains A–I were amplified individually from An1 and An2 cDNA templates with overlapping ends.

Primer	Sequence
Bifunctional $\Delta 12/\omega 3$-Desaturase, An1	
An1for	5'-GGGAATTCGCTATGGCTGCAACTGCAACAACC-3'
An2 ₁ rev	5'-GCGAGATCACGGAAGACGTAGGCAGAGGAAA-3'
An1B ₁ for	5'-TCAGGAGTCTGTCTATGTCTCGTCCGCGATCTCC-3'
An1B ₁ rev	5'-TGAGCAAGAACCACAAATGATTCGGTGCC-3'
An1C ₁ for	5'-GGTCTGTTCCGGTACCGGTATCTGGATCCTC-3'
An1C ₁ rev	5'-GATCCAGCCAACAGTGTCTGTTCCACGTTTG-3'
An1D ₁ for	5'-ACTTCCAAGGTGCTCAACGATGTCGTCCGGA-3'
An1D ₁ rev	5'-CTTGCCGTGAGAGATCTTCCAGCTGAAA-3'
An1E ₁ for	5'-GTCCCTATTTCTCTCGTGGAAAGATCACGCA-3'
An1E ₁ rev	5'-CAGGATGGAGTGGTGGCGACGGGCGTGTCTC-3'
An1F ₁ for	5'-GAGACCCCTTGGCCACTTACAACCTTATTAGCCTA-3'
An1F ₁ rev	5'-TGTCGTGACCGGTAAACGTACAGAGATACATCTG-3'
An1G ₁ for	5'-CCCTGTACCTGCCACTTTTACGTTAGGCCC-3'
An1G ₁ rev	5'-GGCGGTGATGGCGAGGCCGAAATCGCAATCAG-3'
An1H ₁ for	5'-TCATCATTCTGATGATATCGACCTGGGGTTGCTG-3'
An1H ₁ rev	5'-GCTGGTAGTGGGGGAGAGTGGGGTGTGTGTGG-3'
An1I ₁ for	5'-GCCATCACCTACCTCCAGCACACACACCCCT-3'
An1rev	5'-GTCACFCGAGTTCGGCTTTGGCACCCCTTCT-3'
Monofunctional $\Delta 12$-Desaturase, An2	
An2for	5'-GGGAATTCGCTATGGCCCTCGGATGCGGGCAAG-3'
An2A ₁ rev	5'-GGAGATCGCGGACGACATAGGACAGACTCCTGA-3'
An2B ₁ for	5'-TTTCCCTCTGCTACGCTCTCCGTGATCTCCG-3'
An2B ₁ rev	5'-GAGGATCCAGATACCGGTACCGAACAGCCA-3'
An2C ₁ for	5'-GGCACCGGAATCATTTGGGTCTTGCTCA-3'
An2C ₁ rev	5'-TCCGACCACATCGTTGAGCACCTTGGAAAGT-3'
An2D ₁ for	5'-CAAACGTGGAACGACACTGTTGGCTGGATC-3'
An2D ₁ rev	5'-TGGCGTATCTTCCACGAGAAAATAGGGGAC-3'
An2E ₁ for	5'-TTTCAGCTGGAAGATCTCTCACGGCAAG-3'
An2E ₁ rev	5'-GCTAATAAGTTGTAAGTGGCCAAGGGGGTCTC-3'
An2F ₁ for	5'-GAGGACACGCGCCGTCGCCACCCACTCCATCCTG-3'
An2F ₁ rev	5'-GGCGCTAACGTAAAAGGTGAGCAGGTACAAGGG-3'
An2G ₁ for	5'-CAGATGTATCTCTGTAACGTTACCGGTCACGACA-3'
An2G ₁ rev	5'-CAGCAACCCAGGTGATATCACTCAGATTTCATGA-3'
An2H ₁ for	5'-CTGATTGCGATTTCCGGGCTCGCCATCACCGCC-3'
An2H ₁ rev	5'-AGGGGTGTGTGTGCTGGAGGTAGGTGATGGC-3'
An2I ₁ for	5'-CCACACACACCCACTCTCCACCCACTACACAGC-3'
An2rev	5'-GTCACFCGAGTTAGGCTTGGTCAGCTT-3'

94 °C, 30 s at 68 °C, 70 s at 72 °C, followed by 20 cycles of 15 s at 94 °C, 30 s at 70 °C, and 70 s at 72 °C; and a terminal extension step of 7 min at 72 °C. Amplicons were fused in a subsequent PCR reaction, using primers An2for/An2rev and An1for/An1rev, respectively, and the protocol specified for the first step, generating the hybrid gene constructs of the An2- and An1 desaturases indicated in Table 1. The hybrid genes were cloned into the amplification plasmid pGEM-T (Promega) and moved as EcoRI/XhoI fragments into the yeast expression vector pYES2/CT (Invitrogen) in-frame with the sequence encoding a C-terminal V5 tag.

Expression in *Saccharomyces cerevisiae*—Transformation, selection, and growth of transgenic yeast cells were performed as described (25). Expression of desaturases was induced by supplementing galactose (2%) to the media. If fatty acids were added (at a concentration of 0.02% (w/v)), the media was additionally supplemented with Igepal CA 630 (Nonidet P-40') from Fluka (Sigma-Aldrich) at a concentration of 0.2% (w/v). Cultures were maintained at 16 °C for 10 days with shaking (140 rpm) to A_{600} densities of 10–20. Lipid analysis of transgenic yeast cells was performed after harvesting and lyophilization of 100-ml cultures supplemented with linoleic acid or without additional fatty acids. Absolute fatty acid accumulation varied with expression levels between experiments and with different

expression vectors (data not shown) and, therefore, specific enzyme activities cannot be directly compared, allowing only for qualitative evaluation of altered enzyme specificities.

Growth conditions of chimeric enzymes: Expression cultures were grown at 30 °C in minimal medium lacking uracil, but containing 2% (w/v) galactose. When these cultures had reached an OD_{600} of 0.5–0.8, they were transferred to room temperature (23–25 °C). After 96 h at 23–25 °C, cells were harvested by centrifugation at 1200 × *g* for 5 min, and pellets were washed once with sterile doubled-distilled H₂O before being used for fatty acid analysis. The host strain transformed with the empty vector was used as a negative control in all experiments.

Total and Esterified Fatty Acid Analysis—For the analysis of esterified fatty acids, 25 ml of sedimented and lyophilized yeast cultures were homogenized in derivatization solution. Fatty acids were transesterified with sodium methoxide in methanol or methanol/toluol solution (26). Fatty acid methyl esters (FAMES) were then analyzed by gas-liquid chromatography. For the determination and verification of the position of double bond positions in fatty acids, fatty acid methyl esters were converted into their 4,4-dimethylloxazoline derivatives as described (27) and analyzed mass spectrometrically according to Ref. 27, using the 6890 Gas Chromatograph/5973 Mass Selective Detector system (Agilent). For the analysis of total fatty acids, FAMES were prepared from sedimented cell pellets by direct transmethylation with methanol containing 2% (v/v) dimethoxypropane and 2.75% (v/v) of sulfuric acid. After 1 h at 80 °C, 0.2 ml of 5 M NaCl was added, and FAMES were extracted with 2 ml of hexane.

Lipid Analysis and Positional Analysis—Extraction and separation of phospholipids by TLC were performed as described (25). After extraction of the phospholipids from the silica matrix 1/10 of each sample was transesterified with sodium methoxide and analyzed by GC. In the rest of the sample, the positional distribution of fatty acids was determined by dissolving the phospholipids in 900 μ l of Tris-buffer (40 mM Tris-HCl, pH 7.2; 0.2% v/v Triton 100; 50 mM boric acid) by sonication. 100 units of *Rhizopus arrhizus* lipase (Fluka) were added, and the digestion conducted for 2 h at 37 °C. 100 μ l of acetic acid was added to stop the reaction, and the aqueous phase was extracted with chloroform and methanol (28). The resulting organic phase was dried under nitrogen stream and dissolved in 800 μ l of methanol. 400 μ l of each sample was transesterified with sodium methoxide to convert lysophospholipids to FAMES. 6.5 μ l of trimethylsilyldiazomethane was added to the rest of each sample to convert free fatty acids into their corresponding methyl esters, shaken for 30 min at room temperature, and 0.2 μ l of acetic acid was added to degrade remaining trimethylsilyldiazomethane. Samples were dried under nitrogen stream. The resulting FAMES were dissolved in acetonitrile and analyzed by GC.

GC analysis was performed with an Agilent GC 6890 system coupled with an FID detector equipped with a capillary HP INNOWAX column (30 m × 0.32 mm; 0.5 μ m coating thickness; Agilent, Germany). Helium was used as a carrier gas (1 ml min⁻¹). Samples were injected at 220 °C. The temperature gradient was 150 °C for 1 min, 150–200 °C at 15 °C min⁻¹, 200–

250 °C at 2 °C min⁻¹, and 250 °C for 10 min. Fatty acids were identified according to authentic standards. GC/MS analysis was carried out using an Agilent 5973 Network mass selective detector connected to an Agilent 6890 gas chromatograph equipped with a capillary DB-23 column (30 m × 0.25 mm; 0.25- μ m coating thickness; J&W Scientific, Agilent). Helium was used as a carrier gas (1 ml min⁻¹). Samples were injected at 220 °C. The temperature gradient was 150 °C for 1 min, 150–200 °C at 4 °C min⁻¹, 200–250 °C at 5 °C min⁻¹, and 250 °C for 10 min. The detector was set to an electron energy of 70 eV, an ion source temperature of 230 °C, and a transfer line temperature of 260 °C.

RESULTS AND DISCUSSION

Identification of Genes Encoding *A. nidulans* Membrane-bound Desaturases—To characterize and identify new desaturases from fungi, we searched the *A. nidulans* genome sequence (29) databases with known query desaturase sequences from plants. Two sequences with similarity to known plant desaturases were identified. The genomic sequence information was used to design primers to amplify putative fatty acid desaturases from cDNA prepared from *A. nidulans* cultures in vegetative and sexual growth states. Two cDNA fragments were successfully amplified, cloned, and sequenced. The resulting deduced full ORF of the first sequence, *An2* (GenBank™ accession no. XP_658641), encodes a polypeptide of 426 amino acids and is largely identical with the annotated gene *odeA* (GenBank™ accession no. AF262955) with the exception of six additional encoded amino acids at the C terminus of *An2*. The gene *odeA* has been suggested to encode a fatty acid Δ 12 desaturase according to knock-out studies in *A. nidulans* (21).

The second sequence identified, *An1* (GenBank™ accession no. XP_664808) encodes a polypeptide of 394 amino acids. Sequencing of the cloned cDNA fragments for *An1* revealed *An1* to be longer than the predicted and published sequence from the genome data base because of insertion of eight amino acids following amino acid position 13. The longer sequence has been delineated before and associated with a putative fatty acid ω 3-desaturase in a patent (30).

Classification of *An2* and *An1* Sequences in the Phylogenetic System—Pairs of Δ 12 desaturase-like proteins similar to that found in *A. nidulans* have been reported from several fungal species, and the relationship between these fungal enzymes has led Damude *et al.* (19) to establish two subfamilies for monofunctional and bifunctional enzymes, respectively; hence the description of *An2* and *An1*. The polypeptides deduced from the *An2* and *An1* cDNA sequences share 45% identity. The deduced *An2* protein is most similar to representatives of the group of monofunctional desaturases (19), with the highest degree of similarity to the putative Δ 12 desaturase from *Neurospora crassa* (GenBank™ accession no. XP_959528, 70% identity), followed by the putative Δ 12 desaturases from *M. grisea* (GenBank™ accession no. XP_365283, 69% identity) and from *F. moniliforme* (GenBank™ accession no. ABB88515, 63% identity). Similarity of the deduced *An2* protein toward bifunctional enzymes from the species mentioned above is around 45% identity. The situation for the deduced *An1* protein from *A. nidulans* is reversed in that with \sim 60% identity in all

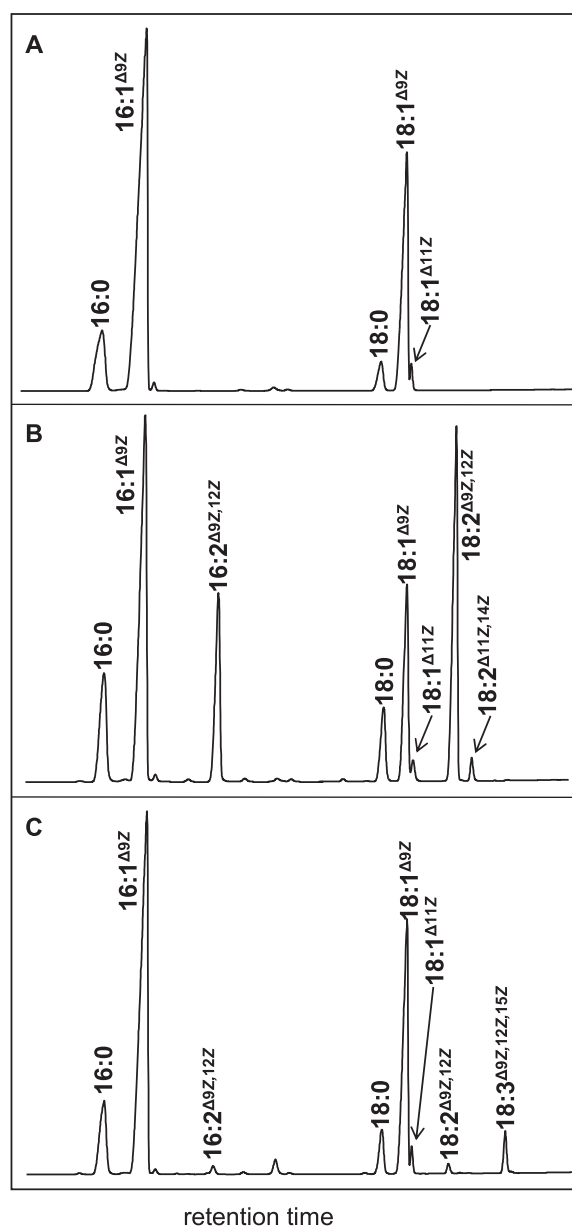


FIGURE 1. GC/FID analysis of fatty acid methyl esters isolated from yeast cells expressing *An1*. The lipids were extracted from lyophilized yeast cells and esterified fatty acids were transmethylated and analyzed by GC/FID as described under "Experimental Procedures." A shows the chromatogram of the control: yeast cells transformed with the empty vector pESC-HIS; B shows the GC/FID analysis of fatty acid methyl esters from yeast cells transformed with the plasmid pESC-An2, and C shows the results from yeast cells transformed with the plasmid pESC-An1.

cases, the *An1* sequence is most similar to representatives of the bifunctional desaturase group (19). Similarity between the deduced *An1* protein and monofunctional enzymes from the other species mentioned is less pronounced with values between 42 and 52% identity.

Functional Characterization of *An2* and *An1* in *S. cerevisiae*—Sequence analyses and data base searches indicate a close relationship between the two enzymes. However, only experimental data can validate such functional annotations. To confirm the substrate specificities of the putative desaturases encoded by the full-length cDNA clones of *An2* and *An1*, the open reading frames of both cDNAs were

TABLE 2

Substrate specificity of An1

Yeast cells transformed with pESC-An1 were supplemented with different fatty acids. The lipids were extracted from lyophilized yeast cells, esterified fatty acids were transmethylated, and GC/FID analysis of fatty acid methyl esters isolated from these yeast cultures was performed as described under "Experimental Procedures." All fatty acids were characterized by co-elution of authentic standards, and their identity was verified by GC/MS analysis of their corresponding DMOX derivatives.

Fatty acids detected	Supplemented fatty acids				
		18:2 ^{Δ9,12}	18:3 ^{Δ6,9,12}	20:3 ^{Δ8,11,14}	20:4 ^{Δ5,8,11,14}
	% ^a	% ^a	% ^a	% ^a	% ^a
16:0	11.5 ± 0.5	14.6 ± 2.2	13.7 ± 0.8	11.9 ± 0.3	12.2 ± 0.2
16:1 ^{Δ9}	52.7 ± 3	44.1 ± 3.8	40.0 ± 1.54	53.1 ± 0.6	42.3 ± 6.7
16:2 ^{Δ9,12}	0.8 ± 0	0.6 ± 0	0.6 ± 0.1	0.6 ± 0	0.6 ± 0.1
18:0	5.0 ± 0.5	4.8 ± 0.2	4.6 ± 0.5	4.2 ± 0.1	4.5 ± 0.9
18:1 ^{Δ9}	26.9 ± 1.1	42.2 ± 1.0	20.4 ± 1.5	27.0 ± 0.4	18.8 ± 3.9
18:2 ^{Δ9,12}	0.7 ± 0.1	6.3 ± 2.6	0.6 ± 0.17	0.5 ± 0	0.6 ± 0.1
18:3 ^{Δ6,9,12}			10.9 ± 5.6		
18:3 ^{Δ9,12,15}	3.2 ± 0.3	6.0 ± 1.0	2.0 ± 0.6	2.0 ± 0	2.5 ± 0.3
18:4 ^{Δ6,9,12,15}			7.4 ± 1.3		
20:3 ^{Δ8,11,14}				0.6 ± 0.1	
20:4 ^{Δ5,8,11,14}					16.4 ± 8.5
20:4 ^{Δ8,11,14,17}				0.2 ± 0.1	
20:5 ^{Δ5,8,11,14,17}					3.2 ± 0.5
	<i>n</i> = 3	<i>n</i> = 7	<i>n</i> = 5	<i>n</i> = 4	<i>n</i> = 3

^a Amount of each fatty acid was expressed as relative ratio of all esterified fatty acids. Data represent the mean values of given number of independent experiments, and the S.D. is given.

cloned into the yeast expression vector pESC-HIS, yielding the plasmids pESC-An2 and pESC-An1, respectively. The clones were individually expressed in the *S. cerevisiae* strain INVSc1. As a control, yeast was also transformed with the corresponding empty vector (Fig. 1A). With expression of An2 in yeast, three new fatty acid products were observed. These fatty acids were identified as 16:2^{Δ9,12}, 18:2^{Δ9,12}, and 18:2^{Δ11,14} (Fig. 1, A versus B) by GC/MS analysis of their 4,4-dimethylloxazoline (DMOX)-derivatives (data not shown). When the desaturation efficiency with endogenous yeast fatty acids was determined for the transgenic cultures, linolenic acid was produced up to 25% of total fatty acids representing an average desaturation efficiency of 64% whereas 16:2^{Δ9,12} was produced only up to 13%, which corresponds to an average conversion efficiency of endogenous 16:1^{Δ9} of 28%. 18:2^{Δ11,14} the result from elongation of 16:2^{Δ9,12} was produced up to 0.9% of the total fatty acids. Overall, 45% of monoenoic fatty acids present in yeast were converted to dienoic fatty acids.

Fatty acid analysis of yeast cultures expressing An1 resulted in the identification of three additional fatty acids, namely 16:2^{Δ9,12}, 18:2^{Δ9,12}, and 18:3^{Δ9,12,15}, beside the endogenous fatty acids (Fig. 1, A versus C). The production of 18:2^{Δ9,12} and 18:3^{Δ9,12,15} indicates bifunctional activity of An1, which introduced double bonds, first at the Δ12 and then processively at the Δ15 position. These results expand previous observations (30), which described An1 as a strict ω3-desaturase by functional characterization in yeast. Our findings are, however, in accordance with previous results (19). In addition to the fatty acid products listed above, the An1 enzyme produced 16:2^{Δ9,12} up to 0.8% of total fatty acids in our experiments (Table 2). For further analysis of An1 substrate and product specificity, yeast cultures were supplemented with various fatty acids (Table 2), and the conversion of the exogenous substrates was monitored. Various ω6-fatty acids like γ-linolenic acid (18:3^{Δ6,9,12}), dihomogamma-linolenic acid (20:3^{Δ8,11,14}), or arachidonic acid (20:4^{Δ5,8,11,14}) were accepted as substrates and transformed into the corresponding ω3-fatty acids (Table 2). In all experiments,

TABLE 3

Average conversion efficiencies of substrate to product by An1

Yeast cells transformed with pESC-An1 were supplemented with different fatty acids. After extraction, FAMES were analysed by GC/FID as described under "Experimental Procedures." Conversion rates are calculated from absolute amounts of fatty acids (FID signal): 100 × product/(substrate and product). Data represent the mean values of a given number of independent experiments (*n*).

Substrate	Product	Conversion rate	<i>n</i>
16:1 ^{Δ9} ^a	16:2 ^{Δ9,12}	1.5	3
18:1 ^{Δ9} ^a	18:2 ^{Δ9,12}	13	3
18:2 ^{Δ9,12} ^a	18:3 ^{Δ9,12,15}	60	3
18:2 ^{Δ9,12}	18:3 ^{Δ9,12,15}	79	6
18:3 ^{Δ6,9,12}	18:4 ^{Δ6,9,12,15}	48	4
20:3 ^{Δ8,11,14}	20:4 ^{Δ8,11,14,17}	23	4
20:4 ^{Δ5,8,11,14}	20:5 ^{Δ5,8,11,14,17}	15	2

^a Fatty acids not supplemented to the media.

expression of the bifunctional An1-enzyme resulted also in the production of minor amounts of 16:2^{Δ9,12} derived from endogenous 16:1^{Δ9}. The conversion efficiencies of An1 for different fatty acids are summarized in Table 3. All fatty acid products were characterized by co-elution with authentic standards, and their identities were additionally verified by GC/MS analysis of their corresponding DMOX derivatives (data not shown). The results indicate that An1 preferentially converts 18-carbon fatty acids to the corresponding ω3-fatty acids, followed in preference by 20-carbon fatty acids. Together these results establish An1 as a processive bifunctional Δ12/ω3 desaturase capable of introducing a further double bond into linoleic acid produced in the first step and generating α-linolenic acid (18:3^{Δ9,12,15}) from 18:1^{Δ9}.

Based on the data presented, the An1 enzyme can be categorized with a group of bifunctional desaturases described recently (19). Our experiments show clearly that with 79% versus 13% conversion the introduction of the second double bond at the ω3 position is catalyzed more efficiently than that at the Δ12 position, and that 16:1^{Δ9} is a very poor substrate for An1 (1.5% conversion, Table 3). Besides the Δ12 desaturase activity, ω3-functionality of various fungal bifunctional enzymes was implied before (19), but not supported by experimental evidence. To test whether An1 and An2 exhibited Δ or ω regio-

TABLE 4

Positional distribution of fatty acids on phospholipids in INVSc1 cells expressing An2

Yeast cells transformed with pESC-An2 were cultivated for 10 days at 16 °C. Lipids were extracted from yeast cells, and phospholipids were separated by thin layer chromatography and scraped out. Each phospholipid sample was treated with lipase, lipids, and free fatty acids were extracted followed by transmethylation of esterified fatty acids and methylation of free fatty acids. Fatty acid methyl esters were analyzed by GC/FID and characterized by authentic standards. Desat. (%) was calculated as (products × 100)/(educts + products) using values corresponding to percent of total fatty acids.

Fatty acid	Total fatty acids	PC			PE			PS		
		Total	sn-1	sn-2	Total	sn-1	sn-2	Total	sn-1	sn-2
16:0	13.6 ± 0.4 ^a	15.9 ± 1.3	21.3 ± 3.7	15.8 ± 2.2	19.2 ± 2.7	19.8 ± 4.6	19.2 ± 1.8	27.3 ± 0.6	20.8 ± 4.5	28.4 ± 5.1
16:1 ^{Δ9}	40.8 ± 3.7	36.2 ± 7.0	15.4 ± 4.6	28.8 ± 8.6	36.1 ± 4.7	21.0 ± 5.2	32.1 ± 5.8	17.7 ± 2.8	11.0 ± 7.1	9.9 ± 3.5
16:2 ^{Δ9,12}	7.1 ± 2.3	7.2 ± 2.9	1.7 ± 1.8	6.1 ± 1.4	5.5 ± 2.3	2.0 ± 2.9	4.6 ± 2.1	4.2 ± 1.5	ND ^b	2.1 ± 1.2
18:0	7.6 ± 0.5	15.5 ± 3.3	25.9 ± 3.4	19.8 ± 3.4	12.3 ± 2.9	22.5 ± 4.2	15.8 ± 2.7	23.6 ± 2.3	23.2 ± 6.1	32.6 ± 5.6
18:1 ^{Δ9}	16.3 ± 3.0	12.8 ± 2.7	20.5 ± 4.6	14.1 ± 3.7	14.0 ± 4.4	24.8 ± 7.4	14.6 ± 3.4	15.0 ± 3.6	27.5 ± 8.0	11.5 ± 3.2
18:2 ^{Δ9,12}	14.0 ± 4.0	13.2 ± 4.1	15.5 ± 7.7	15.1 ± 5.2	13.0 ± 3.7	10.4 ± 2.9	12.3 ± 4.1	12.2 ± 3.4	15.3 ± 10.1	11.7 ± 4.2
18:2 ^{Δ11,14}	0.5 ± 0.2	0.5 ± 0.2	ND	ND	0.5 ± 0.1	ND	ND	0.8 ± 0.0	ND	ND
Desat. (%)	27.4	30	32.4	33.1	27.3	21.3	26.6	34.1	28.4	39.2

^a Data are the mean values of five independent experiments and the S.D. is given. Data are presented as % total fatty acid content in the sample ± S.D.

^b ND, not detectable.

TABLE 5

Positional distribution of fatty acids on phospholipids in yeast cells expressing An1

Yeast cells transformed with pESC-An1 were cultivated for 10 days at 16 °C. Lipids were extracted from yeast cells, phospholipids were separated by thin layer chromatography and scraped out. Each phospholipid sample was treated with lipase and lipids and free fatty acids were extracted followed by transmethylation of esterified fatty acids and methylation of free fatty acids. Fatty acid methyl esters were analyzed by GC/FID and characterized by authentic standards.

Fatty acid	Total fatty acids	PC			PE			PS		
		Total	sn-1	sn-2	Total	sn-1	sn-2	Total	sn-1	sn-2
16:0	13.6 ± 0.3 ^a	14.2 ± 0.2	16.7 ± 1.2	13.4 ± 0.3	18.1 ± 0.6	17.1 ± 1.3	17.8 ± 1.0	27.2 ± 1.8	27.1 ± 3.4	30.5 ± 0.6
16:1 ^{Δ9}	50.5 ± 0.1	53.2 ± 2.8	25.8 ± 4.0	50.6 ± 2.2	42.0 ± 2.5	17.4 ± 1.9	40.9 ± 2.4	18.8 ± 2.4	8.8 ± 2.1	10.3 ± 2.9
16:2 ^{Δ9,12}	0.6 ± 0.0	ND ^b	ND	ND	ND	ND	ND	ND	ND	ND
18:0	6.8 ± 0.1	10.9 ± 1.2	20.7 ± 4.3	12.8 ± 1.0	10.7 ± 1.9	21.8 ± 4.3	13.8 ± 2.5	28.5 ± 3.9	33.9 ± 5.4	37.5 ± 3.2
18:1 ^{Δ9}	25.5 ± 0.5	18.6 ± 1.4	35.5 ± 1.9	16.9 ± 0.9	27.1 ± 1.3	42.8 ± 3.4	23.6 ± 1.7	23.5 ± 3.2	30.0 ± 6.0	17.8 ± 1.7
18:2 ^{Δ9,12}	0.6 ± 0.0	ND	ND	1.4 ± 0.1	ND	ND	1.8 ± 0.1	ND	ND	3.0 ± 0.8
18:3 ^{Δ9,12,15}	2.3 ± 0.1	2.9 ± 0.3	1.3 ± 0.9	4.4 ± 2.4	2.0 ± 0.6	0.9 ± 0.5	2.1 ± 0.3	2.0 ± 0.6	ND	0.8 ± 0.5

^a Data are presented as % of total fatty acid content in the sample ± S.D. Data are the mean values of six independent experiments.

^b ND, not detectable.

TABLE 6

Altered seed oil fatty acid composition of *Arabidopsis* plants expressing An1

Data represent the mean mol% of total fatty acids of single seed analyses ($n = 10-20$) ± S.D. and are given for five individuals, respectively, of three independent transgenic lines expressing An1. As a control, 40 individual seeds from plants expressing the pCAMBIA plasmid carrying no desaturase insert were analyzed.

Fatty acid	16:0	18:0	18:1 ^{Δ9}	18:2 ^{Δ9,12}	18:3 ^{Δ9,12,15}	20:1	Ratio (18:3/18:2)
Control	6.6 ± 0.4	3.1 ± 0.5	12.5 ± 0.2	27.4 ± 0.8	21.5 ± 1.5	15.2 ± 1.0	0.82 ± 0.01
Line 1							
1-1	7.5 ± 0.6	3.6 ± 0.3	12.5 ± 0.4	22.2 ± 1.1	26.0 ± 0.8	16.5 ± 0.8	1.16 ± 0.08
1-2	7.3 ± 0.7	3.5 ± 0.6	10.2 ± 0.8	22.8 ± 0.8	27.7 ± 2.2	15.9 ± 1.4	1.31 ± 0.05
1-3	7.1 ± 0.2	3.1 ± 0.2	10.9 ± 0.3	23.2 ± 0.7	24.5 ± 1.1	15.9 ± 1.0	1.10 ± 0.08
1-4	7.1 ± 0.8	2.8 ± 0.2	10.0 ± 0.7	23.0 ± 1.1	27.8 ± 1.3	14.2 ± 1.4	1.25 ± 0.01
1-5	6.8 ± 0.3	3.2 ± 0.6	12.2 ± 2.6	22.1 ± 2.6	26.3 ± 2.0	14.1 ± 0.9	1.17 ± 0.07
Line 2							
2-1	6.9 ± 0.2	2.9 ± 0.2	10.6 ± 0.8	28.4 ± 0.7	21.6 ± 0.4	15.3 ± 0.2	0.76 ± 0.07
2-2	6.9 ± 0.3	2.9 ± 0.2	10.6 ± 0.4	25.7 ± 0.8	22.41 ± 0.8	15.1 ± 0.8	0.92 ± 0.07
2-3	7.7 ± 0.8	3.6 ± 0.6	10.8 ± 1.6	25.4 ± 1.2	25.7 ± 0.7	13.9 ± 0.4	1.01 ± 0.03
2-4	7.0 ± 0.4	2.9 ± 0.2	10.6 ± 0.5	25.9 ± 0.8	24.8 ± 1.1	14.9 ± 1.3	0.95 ± 0.02
2-5	7.1 ± 0.3	3.3 ± 0.2	12.7 ± 0.9	24.8 ± 0.4	23.0 ± 0.9	17.8 ± 0.8	0.97 ± 0.05
Line 3							
3-1	8.2 ± 0.2	4.7 ± 0.6	12.3 ± 1.0	23.2 ± 1.5	27.1 ± 1.6	14.5 ± 0.8	1.33 ± 0.9
3-2	6.6 ± 0.4	2.8 ± 0.2	11.7 ± 0.9	24.7 ± 0.5	25.9 ± 1.2	15.9 ± 1.1	1.05 ± 0.05
3-3	6.2 ± 0.2	3.0 ± 0.2	13.4 ± 0.7	23.8 ± 0.1	21.8 ± 0.9	16.2 ± 1.4	0.92 ± 0.04
3-4	7.6 ± 0.5	4.6 ± 1.0	12.2 ± 0.9	22.6 ± 1.4	26.7 ± 1.8	14.2 ± 0.6	1.2 ± 0.04
3-5	7.1 ± 0.3	3.2 ± 0.3	12.7 ± 0.9	24.8 ± 0.4	23.0 ± 0.9	14.2 ± 2.0	0.93 ± 0.05

specificities, conversion of yeast-endogenous and of supplied diagnostic fatty acid substrates was analyzed. When conversion of yeast-endogenous fatty acids by An1 was monitored, 18:1^{Δ9} was converted first to 18:2^{Δ9,12} and further to 18:3^{Δ9,12,15}. At the same time, 16:1^{Δ9} was converted to 16:2^{Δ9,12} indicating An1 has Δ12 specificity for the initial desaturation step. Supplementation of yeast cultures expressing An1 with various substrates indicates that the alternative desaturation reaction occurs at the ω3 position, as evident from the conversion of dihomo-γ-linolenic acid (20:3^{Δ8,11,14}) to 20:4^{Δ8,11,14,17} and of arachidonic

acid (20:4^{Δ5,8,11,14}) to eicosapentaenoic acid (20:5^{Δ5,8,11,14,17}). Our data prove that the bifunctional An1-desaturase exhibits mixed Δ12/ω3 regiospecificities. These findings are consistent with the phylogenetic analyses by Damude *et al.* (19), which grouped the An1 sequence with bifunctional desaturases from other fungi. However, our findings contrast with results from a patent (31), in which An1 as well as a bifunctional desaturase from *N. crassa* were characterized as pure ω3-desaturases. The substrate specificities exhibited by An1 suggest a close relationship of An1 with the Fm1 bifunctional desaturase recently characterized

Active Site Determinant of Fatty Acid Desaturase

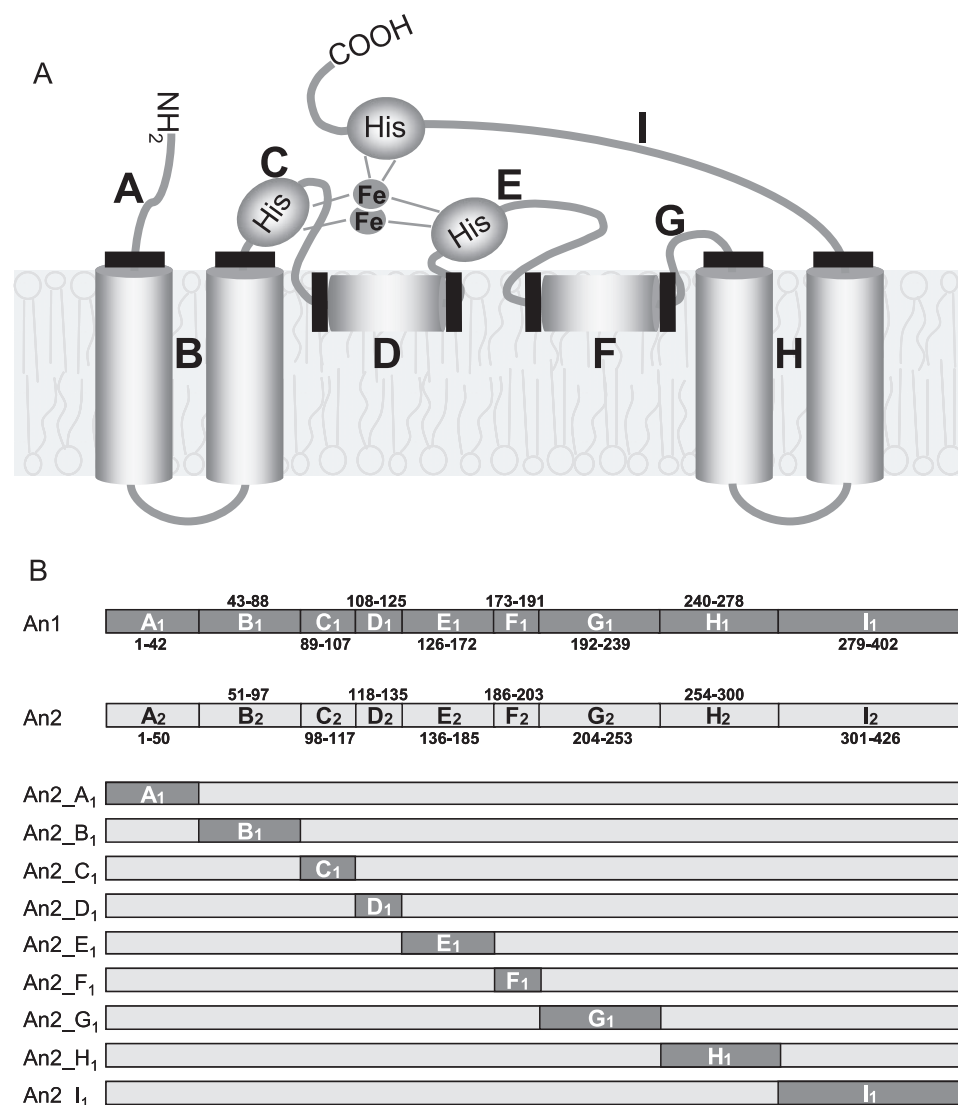


FIGURE 2. Assignment of desaturase domains. A, topology model proposed for membrane-bound fatty acid desaturases. The results from hydropathy analysis of An2 and An1 correspond with known topology models of membrane-bound desaturases (27). On the basis of these results, the desaturases An2 and An1 were divided into nine domains, A–I. The An1 and An2 fusion joints are indicated by black bars. B, scheme of cDNA chimeras of An2 and An1 used in this study. Single domains of the monofunctional $\Delta 12$ -desaturase, An2, (A₂–I₂) were substituted with the corresponding regions of the bifunctional $\Delta 12/\omega 3$ -desaturase, An1, (A₁–I₁).

from fungi (19); however, desaturation of 16-carbon fatty acids also indicates similarities to a bifunctional desaturase from *Acanthamoeba castellanii* (32).

Characterization of An1 and An2 Lipid Substrate Specificity—The lipid substrate specificities of front end desaturases from different organisms have been previously investigated (33); however, data on $\omega 3$ - and $\Delta 12$ desaturases are scarce. To investigate the preferred lipid substrates and positional specificity of the two desaturases from *A. nidulans*, phospholipids were extracted from yeast cultures expressing either An1 or An2. Individual lipid classes were isolated by TLC, and their fatty acid composition was analyzed. Positional specificities of An1 and An2 were tested by treating phospholipids with a *sn*-2-specific lipase and analyzing the hydrolysates.

The results for An2 (Table 4) show neither a definite preference for any acyl-lipid substrate nor a preferred position at the glycerol backbone. At an average of 24% conversion for both

positions in PE compared with an average of 34% in PC or of 33% in PI/PS the desaturation efficiency for monounsaturated fatty acids in PE was slightly lower than that for PC or PI/PS. The conversion of 16:1 $\Delta 9$ to 16:2 $\Delta 9,12$ may indicate a slight preference for the *sn*-2 position in PE, PC, and PI/PS with conversion efficiencies of ~10% at the *sn*-1 position up to about 20% at the *sn*-2 position. No such preference was found for the conversion of 18:1 $\Delta 9$ to 18:2 $\Delta 9,12$ although oleic acid is the preferred substrate of An2. These results may indicate an effect of *sn*-positional substrate presentation with less-than-optimal acyl substrates.

In contrast to An2, An1 showed a preference for acyl chains esterified to PC with a conversion of more than 20% of the total esterified fatty acids, followed by PE (10%) and PI/PS (Table 5). The *sn*-2 position was clearly preferred in all cases, with up to 6-fold more efficient conversion of 18:1 $\Delta 9$ esterified to the *sn*-2 position of PC compared with that of 18:1 $\Delta 9$ in the *sn*-1 position of the same lipid (Table 5). Similar patterns of substrate preferences have recently been described for the bifunctional desaturase from *A. castellanii* (32), again pointing to some similarities of the desaturases.

Effects on Fatty Acid Composition of *Arabidopsis* Seed Oil with Expression of An1—To evaluate whether the bifunctional An1 enzyme can be

utilized in optimizing the engineering of plant seed oil composition, the functionality of the bifunctional enzyme was tested in plants. To this end, the seed oil composition of T3 seeds of plants stably expressing An1 under a seed-specific promoter was compared with that of seeds from BASTA-resistant plants transformed with the pCAMBIA-plasmid carrying no insert. The amount of 18:2 $\Delta 9,12$ and 18:3 $\Delta 9,12,15$ products in seeds from the transgenic and control plants were determined, and the respective product ratios (18:3/18:2) are given in Table 6. The 18:3 $\Delta 9,12,15$ production in plants expressing An1 was significantly elevated in comparison to the control plants changing the 18:3/18:2 ratio from 0.82 to up to 1.33. This indicates relevant functionality of the An1 enzyme in plants.

The values for An1 are lower than those obtained for a bifunctional $\Delta 12/\omega 3$ desaturase from *F. moniliforme* that was expressed in soybean embryos and changed the 18:3/18:2 ratio from 0.4 to up to 18 (19), but the overall fatty acid profile of this

tissue is different in comparison to that of *Arabidopsis* seeds, and therefore the results may be difficult to compare. Moreover, the 18:1^{A9} to 18:2^{A9,12} ratio was unaltered in *Arabidopsis* seeds expressing An1. Whether this effect was due to reduced bifunctional action of conversion of An1 enzyme or the product of endogenous $\Delta 12$ desaturase activity remains unclear. However, because significant production of 18:3^{A9,12,15} can be attributed to An1 it may be concluded that at least part of the 18:2^{A9,12} formed was also the product of An1 and that the enzyme acted processively in plants.

From the characterization of the *A. nidulans* An1 and An2 enzymes and from observations made by others it is clear that a number of fungal species may have developed and retained a pair of coexisting fatty acid desaturase genes that are similar in sequence, but encode enzymes differing in their biosynthetic capabilities. The close relation of the two functional categories proposed by Damude *et al.* (19) represented by An1 and An2 in *A. nidulans* suggests an early gene duplication, likely of a gene for an originally monofunctional $\Delta 12$ desaturase, which removed selective pressure on the other desaturase gene and allowed for the accumulation of mutations leading to broader substrate specificity as a potential first step in the emergence of a gene for a new enzyme activity.

Assignment of Desaturase Functional Domains Based on Secondary Structure Prediction and Topology Modeling—The processive activity of the bifunctional An1 desaturase may provide a shortcut in the production of 18:3^{A9,12,15} and potentially of other $\omega 3$ -fatty acids, and in future crop plants bifunctional $\Delta 12/\omega 3$ desaturases may shift long chain fatty acid synthesis toward higher levels of $\omega 3$ fatty acids. For this reason, the determinants of fatty acid desaturase bifunctionality and processivity were investigated in the present study. The existence of two closely related fatty acid desaturase enzymes with different catalytic properties provided the opportunity to identify determinants of substrate specificity and regioselectivity. Our experimental approach was to determine protein domains of the $\Delta 12/\omega 3$ bifunctional desaturase, An1, sufficient to install bifunctionality in the frame of the monofunctional $\Delta 12$ desaturase, An2. Based on secondary structure predictions using TMHMM (34) and PredictProtein (35), we identified the sequence stretches of An2 and An1 corresponding to the membrane-spanning and cytosolic domains previously postulated for membrane-bound fatty acid desaturases (7–9, 36). In analogy to previous studies, the sequences of An2 and An1 were divided into the consecutive domains A through I, as follows (Fig. 2A): domain A is equivalent to the cytosolic N-terminal region. B contains two transmembrane helices connected by a short ER luminal loop. Domain C is comprised of the subsequent short cytosolic loop containing the first histidine box contributing to the catalytic site. Domain D enfolds the first peripheral membrane-associated segment. E forms the second short cytosolic loop and contains the second histidine box. F comprises the second peripheral membrane-associated region. G enfolds the third short cytosolic loop. H forms the second set of transmembrane helices connected by a short ER-luminal loop, and domain I is comprised of the cytosolic C terminus containing the third histidine motif.

TABLE 7**Fatty acid products attributable to the expression of the hybrid desaturase enzymes**

Desaturation product accumulation as a percentage of total cellular fatty acids is shown. Yeast strains expressing heterologous genes as shown: An2, *A. nidulans* monofunctional $\Delta 12$ -desaturase; An1, *A. nidulans* bifunctional $\Delta 12/\omega 3$ -desaturase; An2_A₁-I₁, Hybrid genes, termed An2, according to their parental sequence, followed by a letter designating the domain introduced from the bifunctional An1 desaturase.

Desaturase	18:2 ^{A9,12}	18:3 ^{A9,12,15}	Ratio (18:3/18:2)
Vector control	ND ^a	ND	ND
An2	9.30 ± 1.2 ^b	ND	ND
An1	0.17 ± 0.02	0.10 ± 0.004	0.54 ± 0.03
An2_A ₁	0.02 ± 0.01	ND	ND
An2_B ₁	0.02 ± 0.01	ND	ND
An2_C ₁	0.09 ± 0.003	ND	ND
An2_D ₁	5.80 ± 1.1	0.26 ± 0.03	0.05 ± 0.02
An2_E ₁	0.13 ± 0.02	0.03 ± 0.003	0.24 ± 0.04
An2_F ₁	0.06 ± 0.001	ND	ND
An2_G ₂	0.03 ± 0.01	ND	ND
An2_H ₁	0.14 ± 0.05	ND	ND
An2_I ₁	0.02 ± 0.002	ND	ND

^a ND, not detectable.

^b Values represent the mean of 2–5 independent experiments, S.D. is given.

Functional Characterization of Desaturase Chimeras of An2 and An1—The domains A₂–I₂ of the monofunctional $\Delta 12$ desaturase, An2, were systematically exchanged for their respective corresponding matches of the bifunctional An1 desaturase. The hybrid genes were termed An2, according to their parental sequence, followed by a letter designating the domain introduced from the bifunctional An1 desaturase (Fig. 2B). After expression of the hybrid proteins in yeast it was tested whether the hybrid enzymes were still active, and whether the specificities of the hybrid enzymes were altered from that of the parental An2 enzyme. As a negative control yeast cells were transformed with pYES2/CT plasmid containing no insert. When the recombinant yeast cells were analyzed for new fatty acid products attributable to the expression of the hybrid desaturase enzymes, all hybrid enzymes, An2_A₁–I₁, were active and showed the regioselectivity of their parental desaturase, however with somewhat reduced 18:2 product accumulation (Table 7). Reduced 18:2 accumulation may be due to the use of a different expression vector (pYES instead of pESC). It is also possible that part of the reduction in activity is a consequence of less-than-optimal folding of the hybrid An2 proteins harboring domains of An1. However, most of the hybrid constructs did not show bifunctional activity and, thus, no 18:3^{A9,12,15} accumulation. Importantly, two of the nine chimeric enzymes, An2_D₁ and An2_E₁ had additionally gained bifunctional $\Delta 12/\omega 3$ activity and, thus, exhibited additional regioselectivity, as shown in Fig. 3. The domains D and E of the An1 enzyme (spanning amino acids 108–125 and 126–172, respectively) were thus identified as determinants sufficient to cause bifunctionality in the monofunctional An2 desaturase enzyme. Domain D represents a relatively hydrophobic region near to the active site of the desaturase protein, assuming the topology model shown in Fig. 2A. Domain E comprises the second cytosolic loop and contains the second histidine box. Both domains, D (18 amino acids) and E (49 amino acids), are either near to the active site or are directly involved in forming the active side of the desaturase enzyme. Our result leads to the suggestion that both domains are involved in binding and positioning the fatty acid substrate relative to the active side. The

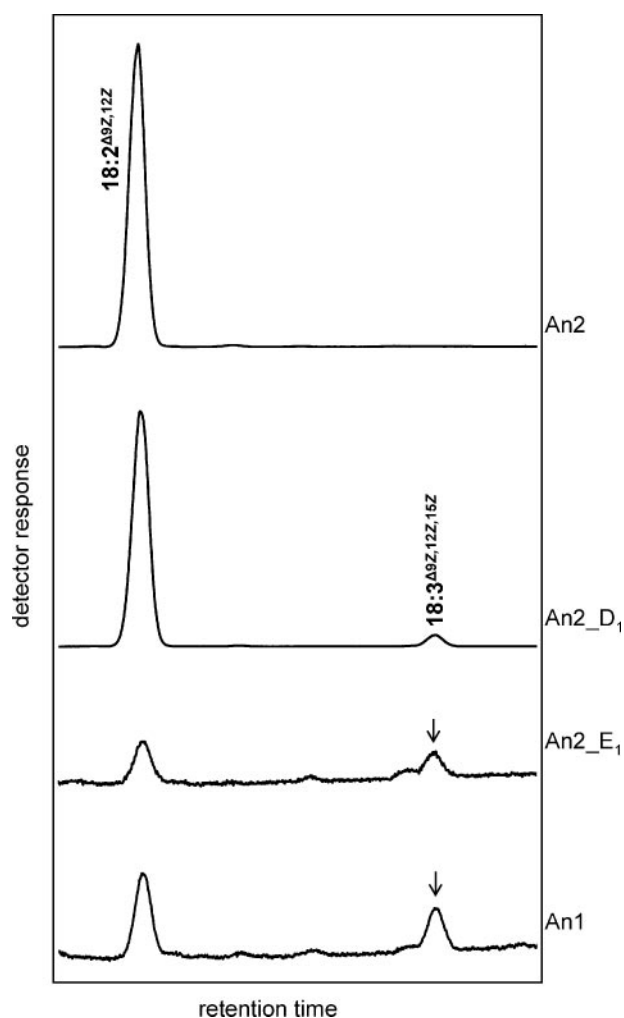


FIGURE 3. The hybrid desaturase enzymes An2_D₁ω₃ and An2_E₁ω₃ had gained bifunctional Δ¹²/ω₃ activity. Fatty acid profiles of yeast expressing wild-type enzymes and the hybrid desaturase proteins An2_D₁ω₃ and An2_E₁ω₃. An1, *A. nidulans* bifunctional Δ¹²/ω₃-desaturase; An2, *A. nidulans* monofunctional Δ¹²-desaturase; chimeric enzymes, An2_D₁ and An2_E₁, termed An2, according to their parental sequence, followed by a letter designating the domain introduced from the bifunctional An1 desaturase.

observation that domains putatively responsible for bifunctionality are positioned adjacent to each other prompted us to test the effects of exchanging a stretch spanning both domains, D and E, but this hybrid enzyme was inactive and showed no detectable desaturation products. Thus further work is needed to identify those amino acids that directly determine the bifunctionality of this unique group of enzymes.

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