Biosynthesis of Docosahexaenoic Acid in *Euglena gracilis*: Biochemical and Molecular Evidence for the Involvement of a Δ4-Fatty Acyl Group Desaturase†

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ABSTRACT: Docosahexaenoic acid (DHA) can be synthesized via alternative routes from which only the ω3/ω6-pathways involve the action of a Δ4-fatty acid desaturase. We examined the suitability of *Euglena gracilis*, *Thraustochytrium* sp., *Schizochytrium* sp., and *Crypthecodinium cohnii* to serve as sources for cloning a cDNA encoding a Δ4-fatty acid desaturase. For this purpose we carried out in vivo labeling studies with radiolabeled C22 polyunsaturated fatty acid substrates. *Schizochytrium* sp. was unable to convert exogenously supplied [2-^{14}C]-docosapentaenoic acid (DPA, 22:5$^{\Delta7,10,13,16,19}$) to DHA, while *E. gracilis* and *Thraustochytrium* sp. carried out this desaturation very efficiently. Hydrogenation and α-oxidation of the labeled DHA isolated from these two organisms showed that it was the result of direct Δ4-desaturation and not of substrate breakdown and resynthesis. To clone the desaturase gene, a cDNA library of *E. gracilis* was subjected to mass sequencing. A full-length clone with highest homology to the Δ4-desaturase of *Thraustochytrium* sp. was isolated, and its function was verified by heterologous expression in yeast. The desaturase efficiently converted DPA to DHA. Analysis of the substrate specificity demonstrated that the enzyme activity was not limited to C22 fatty acids, since it also efficiently desaturated C16 fatty acids. The enzyme showed strict Δ4-regioselectivity and required the presence of a Δ7-double bond in the substrate. Positional analysis of phosphatidylcholine revealed that the proportion of the Δ4-desaturated products was up to 20 times higher in the *sn*-2 position than in the *sn*-1 position.

In recent years, much interest has been focused on the biosynthesis of ω3-long-chain polyunsaturated fatty acids (LCPUFAs)[1] such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). They are important components of the human diet because of their contribution to several aspects of health, including the development of the infant brain, the function of the eye, the synthesis of hormones and signaling molecules, and the prevention of cardiovascular diseases (*1*, *2*). Marine fish represent the major source of these nutritionally relevant fatty acids, which are not found in seed oils of higher plants. To create sustainable sources of LCPUFAs, attempts are underway to implement the biosynthetic pathways in annual oilseed crops such as linseed and rapeseed (*3*). EPA and DHA are found in substantial amounts in a variety of microorganisms including diatoms (*4*), dinoflagellates (*5*), some marine bacteria (*6*), and fungi (*7*). Therefore, these organisms are regarded as potential sources for the cloning of the genes encoding the enzymes required for LCPUFA biosynthesis.

Different routes of LCPUFA synthesis have been developed in nature (Figure 1). The production of EPA and DHA in marine bacteria, such as *Shewanella* sp., *Vibrio* sp., and *Photobacterium profundum*, relies on polyketide synthase (PKS) systems, which are encoded by large gene clusters of about 30 kb (*8*−*12*). Heterologous expression of the *Shewanella* PKS system resulted in EPA synthesis in *Escherichia coli* and *Synecchococcus*. The *Vibrio* gene cluster was able to complement selected ORFs of the *Shewanella* cluster and changed the final product to DHA [Patent WO 00/42195]. Since the oleaginous fungus *Schizochytrium* sp. has been shown to possess a gene cluster with significant similarity to the bacterial PKS systems (*13*), LCPUFA production via this pathway might not be restricted to prokaryotes.

A second strategy relies on the alternating action of desaturases and elongases requiring fatty acid substrates such as linoleic acid and α-linolenic acid to start with. They constitute the so-called ω6- and ω3-pathways for polyunsaturated fatty acid (PUFA) biosynthesis (Figure 1). Besides the main routes leading to arachidonic acid and DHA, another route in different organisms makes use of a Δ9-elongase and a Δ8-desaturase (*14*, *15*). To date, all genes coding for desaturases and elongases necessary to convert linolenic acid to EPA have been cloned from organisms such as *Physcomitrella patens* (*16*, *17*), *Mortierella alpina* (*18*−*21*), *Caeno-*

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[1] Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DTA, docosatetraenoic acid; PUFA, polyunsaturated fatty acid; LCPUFA, long-chain polyunsaturated fatty acid; PKS, polyketide synthase; ATCC, American Type Culture Collection; ORF, open reading frame; FAME, fatty acid methyl ester; FFA, free fatty acid; FID, flame-ionization detector; DMOX, 4,4-dimethyloxazoline; PC, phosphatidylcholine.

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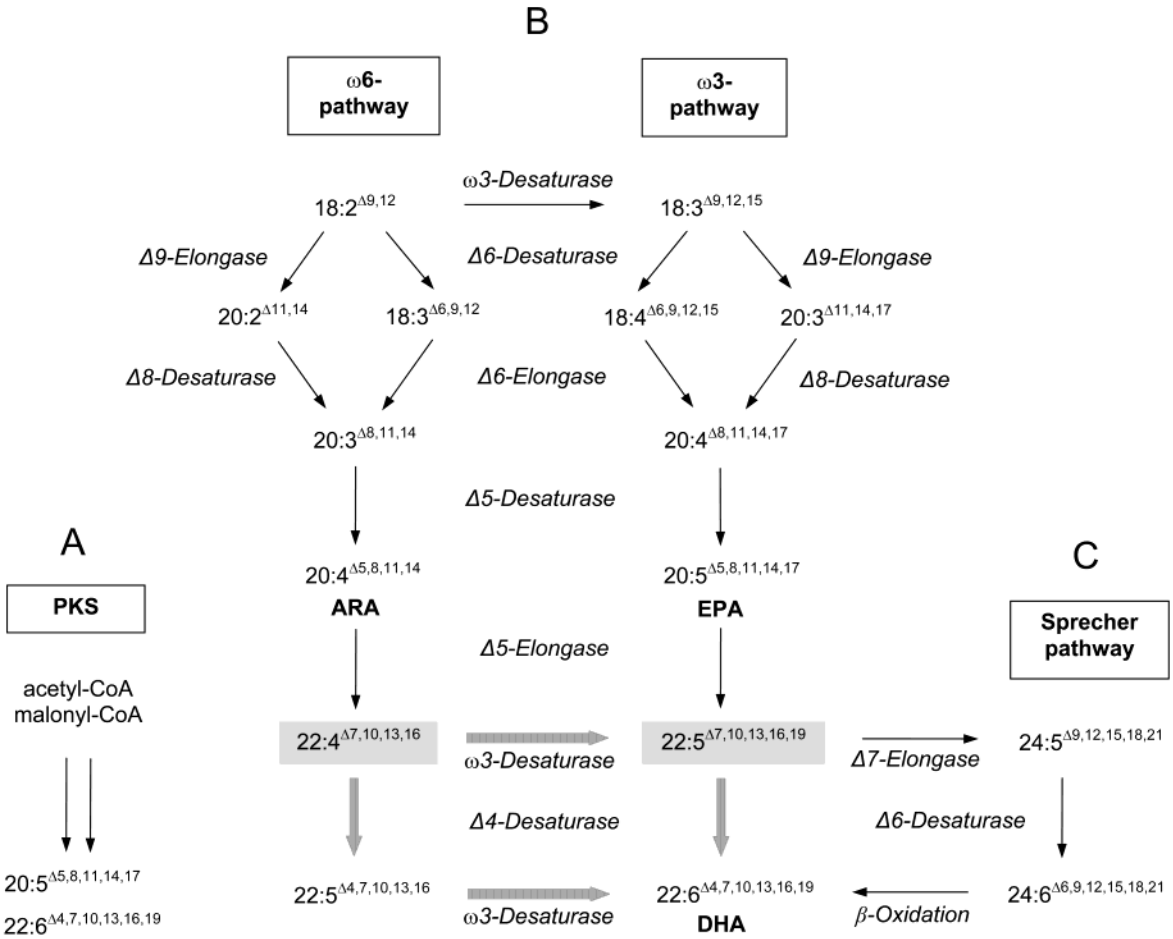

FIGURE 1: Alternative routes leading to EPA and DHA in microorganisms, plants, and animals. (A) Polyketide synthase (PKS) systems producing either EPA or DHA in marine bacteria (and possibly *Schizochytrium*). (B) The ω3- and ω6-pathways of LCPUFA biosynthesis, including the routes that use the Δ9-elongase and the Δ8-desaturase. (C) The Sprecher pathway operating in mammals. The ω3-desaturase(s) may accept further ω6-substrates in addition to the arrows included in the figure. It should be stressed that switching from the ω6- to the ω3-track is not a universal alternative. It occurs frequently in various lower eukaryotes (algae, fungi, mosses, worms), but it is not realized in mammalia which lack both the ω6- and the ω3-desaturases. The two radioactive substrates used for in vivo labeling are indicated by gray background. The enzymatic steps that were demonstrated in this work by in vivo labeling are indicated by bold arrows.

rhabditis elegans (*22*−*24*), the diatom *Phaeodactylum tricornutum* (*25*), and man (*26*−*28*). Yeasts expressing the Δ6-desaturase from *Borago officinalis* together with the Δ5-desaturase from *M. alpina* and the Δ6-elongase from *C. elegans* were able to convert exogenously supplied α-linolenic acid into EPA (*29*). The same observation was made with yeasts expressing the Δ5- and Δ6-desaturase from *P. tricornutum* together with the Δ6-elongase from *P. patens* (*25*). These results indicate that the desaturase/elongase strategy might be a useful approach for LCPUFA production in plants. Thus, the cloning of the genes involved in the final steps of DHA synthesis became the main task yet to be solved. Regarding these final steps in DHA biosynthesis, two alternatives exist. On one hand, the conversion of EPA to DHA requires chain elongation to C22 and the introduction of a double bond at the Δ4-position. On the other hand, Sprecher et al. demonstrated that DHA biosynthesis in rat liver does not involve a Δ4-desaturation step but a Δ6-desaturase acting on a C24 fatty acid substrate followed by β-oxidation (*30*). It was the cloning and functional verification of a Δ4-fatty acid desaturase from *Thraustochytrium* sp. that finally showed that both alternatives have evolved in parallel in different organisms (*31*).

The fatty acid profile of an organism does not seem to indicate which pathway(s) is(are) used for LCPUFA biosynthesis. *Thraustochytrium* sp., its close relative *Schizochytrium* sp., and the dinoflagellate *Crypthecodinium cohnii* accumulate as much as 50% DHA in their fatty acids but lack significant proportions of C20 and C22 intermediates (*5*, *32*, *33*). This might result either from an efficient channeling by a PKS system or from a very effective shuttling of the intermediates between fatty acid desaturases and elongases as in *P. tricornutum* (*25*). The fact that exogenously supplied long-chain fatty acids were incorporated into lipids but not converted to DHA or docosapentaenoic acid by *Schizochytrium* sp. points to a PKS system (*13*), while the cloning of a Δ5- and a Δ4-fatty acid desaturase from *Thraustochytrium* sp. supports the second alternative (*31*). This poses the question whether the PKS system and the desaturase/elongase pathway for PUFA biosynthesis might operate simultaneously in cells of this group of organisms. The alga *Euglena gracilis* differs from the above-mentioned organisms by the fact that it accumulates only minor proportions of DHA (about 2% of total fatty acids), while it displays a wide range of different fatty acids with significant proportions of C16−C22 PUFAs (*34*).

Such a diversity is unlikely to result from the action of PKS systems. The cloning of a Δ8-desaturase from *E. gracilis* may be seen as additional evidence for the presence of a LCPUFA biosynthetic pathway that involves desaturases and elongases (*14*). Therefore, we decided to use in vivo studies with radiolabeled fatty acid substrates to examine the biosynthetic route of DHA production in *Thraustochytrium* sp., *Schizochytrium* sp., *E. gracilis*, and *C. cohnii* and by this evaluate the suitability of these organisms as sources for the cloning of a Δ4-fatty acid desaturase gene. *E. gracilis* was shown to be a suitable source for gene cloning, and a cDNA clone coding for a Δ4-fatty acid desaturase was isolated and functionally characterized.

EXPERIMENTAL PROCEDURES

Chemicals. Restriction enzymes, polymerases, and DNA-modifying enzymes were purchased from New England Biolabs (Frankfurt, Germany). Commercially available free fatty acids were supplied from SIGMA (Taufkirchen, Germany). Hexadecatrienoic acid ($16:3^{\Delta7,10,13}$) was part of a fatty acid mixture isolated from monogalactosyl diacylglycerol of *Aquilegia vulgaris*. $16:1^{\Delta7}$ was synthesized as described elsewhere (*35*).

Synthesis of Radiolabeled Compounds. [2-^{14}C]$^{\Delta7,10,13,16,19}$-Docosapentaenoic Acid. [2-^{14}C]-Dimethylmalonate (191 mg (1.45 mmol); 40.7 mCi/mmol; Perkin-Elmer, Rodgau-Jugesheim, Germany) was added at rt to a solution of 1.47 mmol sodium ethylate in 730 μL of ethanol. The mixture was stirred for 5 min at rt, and then 254 mg (0.949 mmol) of 1-bromo-eicosa$^{\Delta5,8,11,14,17}$-pentaene was added at 50 °C. After being stirred for 3 h at 50 °C and 10 h at rt, the reaction mixture was quenched with 10 mL of water and extracted three times with 20 mL of pentane. The organic phase was separated and dried over sodium sulfate. Concentration in vacuo yielded 730 mg of crude [2-^{14}C]-2-eicosa$^{\Delta5,8,11,14,17}$-pentaenyl-malonic acid dimethylester as a yellow oil that was used in the next step without further purification. The crude [2-^{14}C]-2-eicosa$^{\Delta5,8,11,14,17}$-pentaenyl-malonic acid dimethylester was dissolved in 100 mL THF, added to 30 mL of 1 M LiOH, and stirred for 10 h at rt in the dark. The THF was removed under reduced pressure. After addition of 30 mL of water, the clear solution was extracted with 50 mL of hexane which was discarded. The aqueous phase was acidified with potassium hydrogen sulfate to pH 5 and extracted four times with ether. The organic phase was dried over sodium sulfate and concentrated in vacuo to yield 172 mg crude of [2-^{14}C]-2-eicosa$^{\Delta5,8,11,14,17}$-pentaenyl-malonic acid as yellowish oil. The crude [2-^{14}C]-2-eicosa$^{\Delta5,8,11,14,17}$-pentaenyl-malonic acid was refluxed in 6 mL of acetic acid together with some crystals of 2,6-di-*tert*-butyl-4-methyl-phenol for 20 h. The formation of [2-^{14}C]$^{\Delta7,10,13,16,19}$-docosapentaenoic acid in solution was shown by ^{1}H NMR spectroscopy. Radiochemical purity as measured by radio-HPLC was 84% (Figure 2). Total radiochemical yield was 22%.

[2-^{14}C]$^{\Delta7,10,13,16}$-Docosatetraenoic Acid. Starting from 1-bromo-eicosa$^{\Delta5,8,11,14}$-tetraene, [2-^{14}C]$^{\Delta7,10,13,16}$-docosatetraenoic acid was synthesized by the same procedure described for [2-^{14}C]$^{\Delta7,10,13,16,19}$-docosapentaenoic acid in 9% total yield and 65% radiochemical purity (Figure 3).

Cultures. *E. gracilis* strain 1224-5/3 was obtained from the Sammlung für Algenkulturen Göttingen (SAG) and grown in medium II (*36*), except that the concentration of $(NH_4)_2HPO_4$ was increased to 3.8 mM. The cultures were shaken (100 rpm) at 23 °C under a light (35 μE s^{-1} m^{-2})/dark interval of 8 h/16 h, respectively. *Thraustochytrium* sp. ATCC strain 26185 and *Schizochytrium* sp. ATCC strain 20888 were shaken (180 rpm) in reconstituted saltwater medium (*32*) with 2% glucose and 0.2% yeast extract at 30 °C in the dark. Cultures of *Crypthecodinium cohnii* ATCC strain 30556 were shaken (100 rpm) at 23 °C in ATCC medium 460 A2E6 complemented with 2% glucose.

In Vivo Labeling Studies. Cultures of *Thraustochytrium* sp. and *Schizochytrium* sp. (2 days old) were diluted 1:100 in 3−5 mL of fresh medium and grown at 30 °C to an OD_{600} of 0.9−1.2, before 0.5−10 μCi of the ^{14}C-labeled fatty acid substrate was added. The cultures were incubated with shaking (100 rpm) at rt for an additional 24 h. Cells of a growing culture of *E. gracilis* (5×10^6 cells) and of *Crypthecodinium cohnii* (2×10^7 cells) were harvested and resuspended in 3 mL of fresh medium before 0.5−10 μCi of the radiolabeled fatty acid substrate was added. The cultures were shaken at rt for 24 h. In the case of *E. gracilis*, constant light (10 μE s^{-1} m^{-2}) was applied. All cultures were harvested and washed with 100 mM of $NaHCO_3$. Total lipids were extracted with chloroform/methanol/H_2O (*37*) and saponified with 0.5 mL of 0.5 M aqueous KOH and 1 mL of methanol for 2 h at 80 °C. After acidification with HCl, free fatty acids (FFAs) were extracted into petroleum ether. Alternatively, the lipids were incubated with 0.5 M of sulfuric acid in methanol containing 2% dimethoxypropane at 80 °C for 1 h and then extracted with petroleum ether and washed once with 100 mM of $NaHCO_3$ and once with H_2O to obtain fatty acid methyl esters (FAMEs).

HPLC Analysis. FAMEs and FFAs were separated by reversed-phase HPLC using appropriate unlabeled reference substances. The chromatography was carried out using the SpectraSYSTEM (Thermo Separation Products, Inc., San Jose, CA), an ODS Hypersil RP18 column (5 μm, 25 cm × 4.6 cm, Bischoff, Leonberg, Germany), and an isocratic elution with methanol/acetonitrile/H_2O, 9:1:1.3 as solvent for FAMEs and methanol/acetonitrile/H_2O/acetic acid, 9:1:1.3:0.01 as solvent for FFAs at a flow rate of 1 mL/min. FAMEs and FFAs were detected at 210 nm (UV6000LP, Thermo Separation Products, Inc.), whereas radioactivity was measured with a RAMONA Star monitor (Raytest, Sprockhöfel, Germany). Bromophenacyl esters were separated with a solvent gradient (5 min in 92% methanol/acetonitrile, 9:1 and 8% water, then 45 min in 100% methanol/acetonitrile, 9:1) at a flow rate of 1 mL/min and detected at 254 nm.

α-*Oxidation of Radiolabeled Fatty Acids Isolated from in Vivo Labeling Experiments*. DHA from two to three labeling experiments with *E. gracilis* or *Thraustochytrium* sp. using [2-^{14}C]-docosapentaenoic acid as substrate was isolated as free fatty acid by repetitive reversed-phase HPLC runs and pooled. The DHA was dissolved in methanol and hydrogenated by agitation in the presence of platinum(IV) oxide and hydrogen gas for 4 h at rt. The resulting behenic acid (C22:0) was dried and redissolved in acetone (*38*). In the case of *Thraustochytrium* sp., it was divided into 4−6 aliquots because of the large quantity of the resulting C22:0 and its limited solubility in acetone. In the case of *E. gracilis*, 100 μg of unlabeled C22:0 free fatty acid was added to the labeled fatty acid. $KMnO_4$ (22 mg) was added to 1 mL

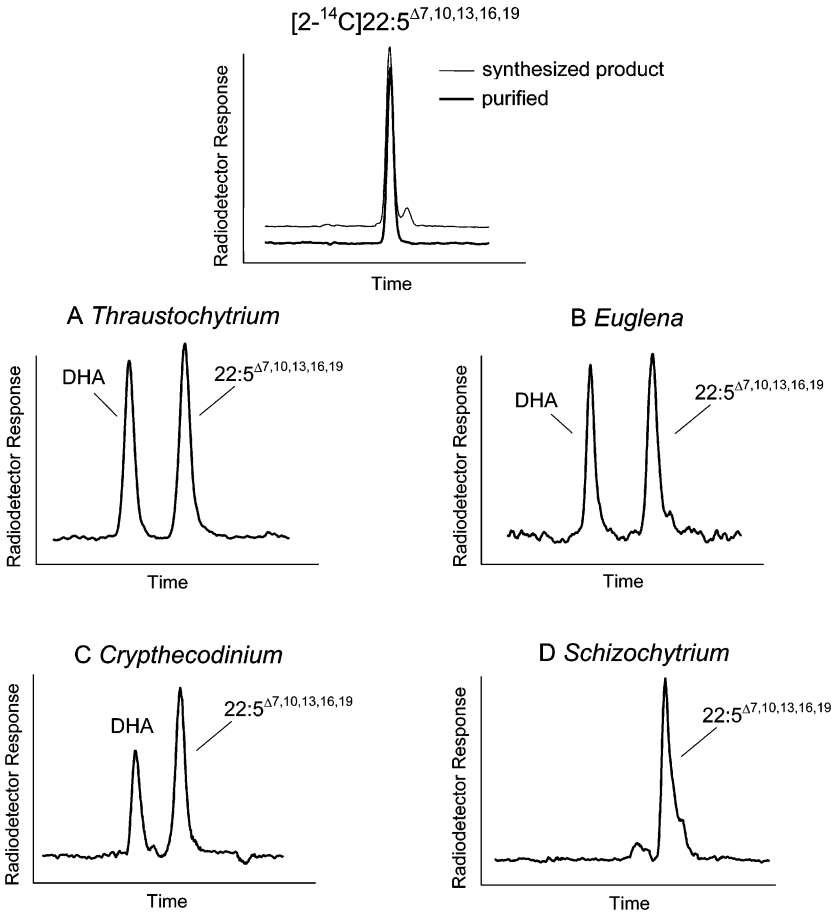

FIGURE 2: In vivo labeling studies on the desaturation of [2-^{14}C]22:5$^{\Delta7,10,13,16,19}$ by various organisms. *Thraustochytrium* sp. (A), *E. gracilis* (B), *C. cohnii* (C), and *Schizochytrium* sp. (D) were incubated for 24 h with the radiolabeled pentaenoic fatty acid substrate. Total fatty acids were obtained by saponification of cell pellets (*E. gracilis*, *C. cohnii*) or total lipids (*Thraustochytrium* sp., *Schizochytrium* sp.) and separated by reversed-phase radio-HPLC. The new peaks in A, B, and C eluting before the substrate peak have the retention time of DHA, whereas in D no DHA was observed. The upper chromatogram represents the labeled substrate before and after purification by HPLC.

aliquots in acetone, and the mixture was sonicated in a water bath at 54 °C for 12 h. The reaction mixture was acidified with 1 mL of 5 N H_2SO_4 before $Na_2S_2O_5$ was added until the solution became clear. The resulting fatty acids were extracted with diethyl ether, dried, and purified by TLC on silica 60 plates (Merck, Darmstadt, Germany) with diethyl ether/petroleum ether/acetic acid (8:2:0.01) as solvent. Radioactivity was detected with a phosphorimager (Fujifilm BAS-1000) and analyzed with PC BAS 2.09g software. The FFA zones were scraped off and extracted once using chloroform/methanol, 2:1 and once using 2-propanol. For derivatization to bromophenacyl esters, according to Halgunset et al. (*39*), the residue was dried, mixed with dimethyl formamide (160 μL), *p*-bromophenacyl bromide (20 μL), and diisopropylethylamine (20 μL), and heated to 65 °C for 1 h. The bromophenacyl ester derivatives were extracted with diethyl ether, washed with 0.1 M K_2HPO_4 (pH 7), purified on a silica TLC plate (chloroform/petroleum ether 8:2), and recovered from the scrapings by extracting once with chloroform/methanol (2:1) and once with 2-propanol. Reversed-phase radio-HPLC with UV monitoring at 254 nm was used for analysis.

Isolation of E. gracilis Δ4-Desaturase cDNA. A λ ZAP cDNA library made from *E. gracilis* strain Z (SAG strain 1224-5/25) was kindly provided by Prof. Dr. Martin (University of Düsseldorf, Germany) and in vivo mass excised. Plasmid clones were prepared using a Qiagen DNA preparation robot (Qiagen, Hilden) and subjected to random sequencing by the chain-termination method using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Weiterstadt, Germany). A single clone showed similarity to PUFA desaturases and was used as template for PCR amplification of a digoxigenin-labeled DNA probe to be used for the screening of the cDNA library. About 2×10^5 plaques were screened according to the manufacturer's instructions. Two rounds of rescreening led to the isolation of one cDNA clone of sufficient length, which was sequenced on both strands.

Heterologous Expression in Yeast. The ORF was amplified by PCR for subcloning. The PCR primers included restriction sites adjacent to the start and stop codon and contained a yeast consensus sequence for enhanced translation in front of the start codon (*40*). The PCR product was cloned into the *Kpn*I and *Xho*I sites of the yeast expression vector pYES2 (Invitrogen, Karlsruhe, Germany), yielding pEGD4. *Saccharomyces cerevisiae* strain 334 (*41*) was transformed with the plasmid DNA by electroporation (1500 V) and selected on minimal plates without uracil. Cells harboring the plasmid were grown in minimal medium lacking uracil with 0.2% raffinose. The medium (5 mL) was inoculated with a preculture (2 days old) to an OD_{600} of 0.05. After 6 h incubation at 30 °C, 300 μM of fatty acid substrate (or 100 μM 16:1$^{\Delta7}$) was added, and expression was induced with 2% galactose. The cells were further grown at 15 °C for 4

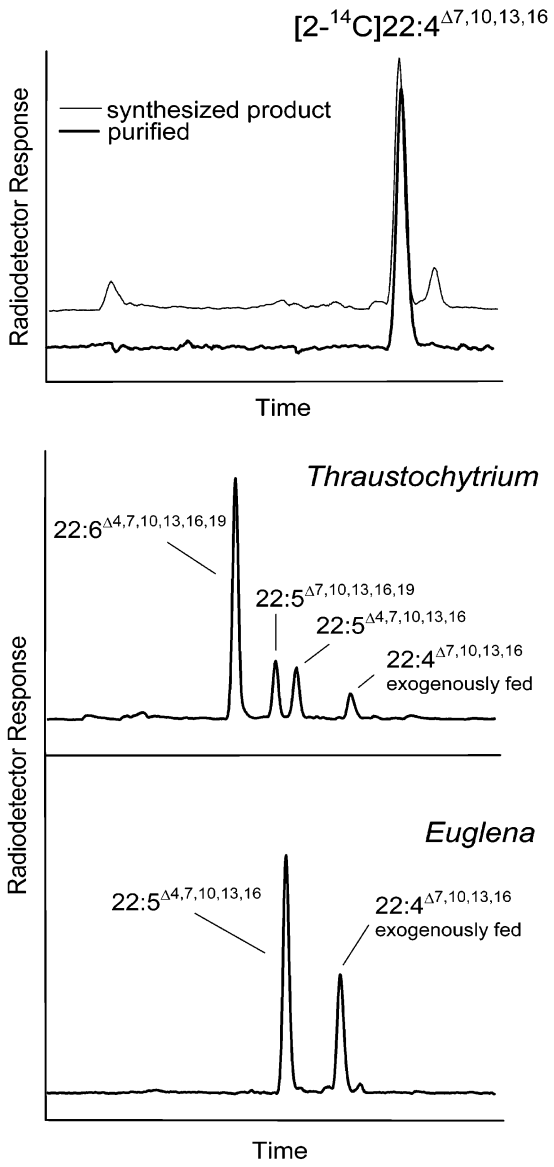

FIGURE 3: In vivo labeling studies on the desaturation of [2-^{14}C]-22:4$^{\Delta 7,10,13,16}$. *Thraustochytrium* sp. and *E. gracilis* were incubated for 24 h with the radiolabeled tetraenoic fatty acid substrate. FAMEs were isolated from total cells and separated by reversed-phase radio-HPLC. The upper chromatogram shows the labeled substrate before and after purification by HPLC.

days, harvested, washed with 100 mM of $NaHCO_3$, and used for fatty acid analysis by gas−liquid chromatography (GLC).

Isolation and Positional Analysis of Phosphatidylcholine from Transgenic Yeast. Medium without uracil (100 mL) was inoculated to an OD_{600} of 0.05 with a yeast culture (2 days old) harboring the plasmid pEGD4 and incubated for 6 h at 30 °C. Subsequently, gene expression was induced with 2% galactose, and 16:1$^{\Delta 7}$ or 22:4$^{\Delta 7,10,13,16}$ was added to a final concentration of 100 or 300 μM, respectively. The culture was further incubated at 15 °C for 5 days, and the cells were collected and washed twice with 100 mM of $NaHCO_3$. An aliquot of the cells was subjected to transmethylation to obtain FAMEs for GLC analysis. Lipids were extracted from the remaining cells and separated on silica TLC plates (chloroform/methanol/acetic acid 65:25:8). Positional analysis of phosphatidylcholine was carried out as described elsewhere (*42*) with lipase from *Rhizopus arrhizus delemar* (SIGMA, Taufkirchen, Germany).

Fatty Acid Analysis by GLC. FAMEs prepared from cell pellets or lipids and 4,4-dimethyloxazoline (DMOX) derivatives (*43*) were analyzed by GLC using a Hewlett−Packard 6850 gas chromatograph (Hewlett−Packard, Palo Alto, CA) equipped with a polar capillary column (ZB-Wax, 30 m × 0.32 mm internal diameter, 0.25 μm film, Torrance, CA) and a flame-ionization detector (FID). Data were processed using the HP Chem Station Rev, A0603. FAMEs were identified by comparison with reference substances or by GLC/MS of DMOX derivatives as described before (*44*).

RESULTS

In Vivo Labeling Studies. We used four different DHA-accumulating organisms for in vivo studies with radiolabeled fatty acids of different structures to select a suitable organism for the cloning of the cDNA encoding a Δ4-fatty acid desaturase. *E. gracilis*, *Thraustochytrium* sp., and *Crypthecodinium cohnii* were able to convert [2-^{14}C]-docosapentaenoic acid (DPA, 22:5$^{\Delta 7,10,13,16,19}$) into DHA, although with different levels of efficiency. After 24 h of incubation, about half of the DPA taken up by *E. gracilis* and *Thraustochytrium* sp. was recovered as DHA. *C. cohnii* was less effective in producing labeled DHA compared to *E. gracilis* and *Thraustochytrium* sp. (Figure 2), whereas *Schizochytrium* sp. was not able to convert DPA into DHA under the same conditions. The ability of *Thraustochytrium* sp. to convert DPA into DHA strongly depended on the growth state of the cells since only freshly diluted cultures were able to produce DHA from the radioactive substrate. On the basis of these results, all subsequent labeling studies were focused on *E. gracilis* and *Thraustochytrium* sp. Both were also able to convert [2-^{14}C]22:4$^{\Delta 7,10,13,16}$ (DTA) into the Δ4-desaturated product 22:5$^{\Delta 4,7,10,13,16}$. Moreover, *Thraustochytrium* sp., but not *E. gracilis*, was able to introduce an ω3-double bond into DTA (Figure 3).

Radioactivity could only be detected in the substrates and the Δ4- (and ω3-) desaturated products. Therefore, the operation of the Sprecher pathway, which would give rise to labeled C24 PUFAs as intermediates, seemed unlikely. However, degradation of the radiolabeled substrate via β-oxidation and resynthesis of DHA from the resulting labeled acetyl-CoA by a PKS system could not be excluded. Therefore, we used α-oxidation to investigate the distribution of the radiolabel in DHA isolated from *E. gracilis* and *Thraustochytrium* sp. In the series of fatty acids resulting from this degradation, the radioactivity was restricted to the C22- and C21 fatty acid, in accordance with the labeling pattern of the substrate (Figure 4). These results strongly argued for direct desaturation of the exogenously supplied fatty acid substrate by a Δ4-desaturase operating in both *E. gracilis* and *Thraustochytrium* sp.

Cloning of a Desaturase cDNA from E. gracilis. A partial cDNA clone with high homology to so-called front-end desaturases involved in PUFA biosynthesis was identified by random sequencing of a cDNA library made from *E. gracilis* strain Z. A cDNA clone of 2569 bp was isolated by subsequent library screening (GenBank AY278558). It contained an ORF of 1626 bp, which encodes for a polypeptide of 541 amino acids. The corresponding gene was detected as a single copy gene in the genomic DNA of *E. gracilis* strain Z by Southern blot analysis. A BLAST search

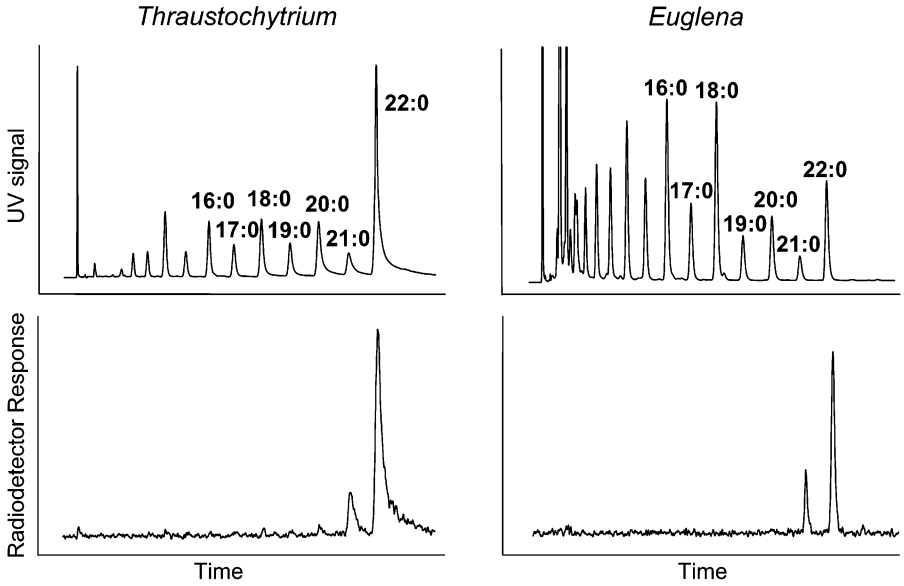

FIGURE 4: α-Oxidation of hydrogenated DHA isolated from *Thraustochytrium* sp. and *E. gracilis*. Labeled DHA was isolated by reversed-phase HPLC from cells incubated with [2-^{14}C]22:5$^{\Delta 7,10,13,16,19}$, hydrogenated, and subsequently α-oxidized as described in Experimental Procedures. The resulting mixture of saturated fatty acids was converted to bromophenacyl ester derivatives and analyzed by reversed-phase radio-HPLC. UV absorption was measured at 254 nm. α-Oxidation of DHA isolated from *Thraustochytrium* sp. was not as effective as that from *E. gracilis* because of the high proportion of endogenous docosahexaenoic acid in the fungus.

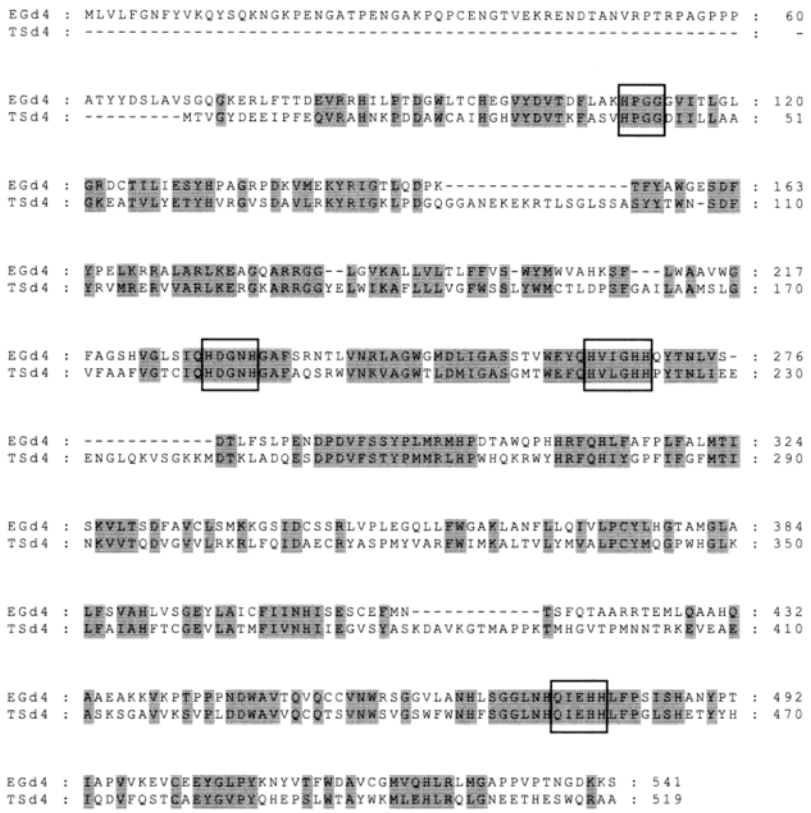

FIGURE 5: Comparison of the amino acid sequences of the Δ4-desaturases from *E. gracilis* (EGd4) and *Thraustochytrium* sp. (TSd4). Frames indicate the conserved histidine boxes and the HPGG motif of the cytochrome b_5 domain. The conserved amino acids are shaded.

revealed highest sequence homology of the amino acid sequence (33% amino acid identity) to the Δ4-fatty acid desaturase from *Thraustochytrium* sp. (*31*) (Figure 5), whereas the identities to Δ5-desaturases from *Dictyostelium discoideum* (*45*) and *Mortierella alpina* (*18*, *19*) are 25% and 24%, respectively. Another property resembling the Δ4-desaturase of *Thraustochytrium* sp. was the larger distance between the last transmembrane helix and the third histidine box. The deduced polypeptide contained the extended HXXXHH motif in the histidine box 2, as compared to the more frequently found HXXHH motif as well as the H/Q exchange in the third histidine box generally present in front-end desaturases. The desaturase contained an N-teminal cytochrome b_5 fusion, including the conserved heme-binding motif (HPGG). The fused cytochrome b_5 also showed a reduction in the number of acidic amino acids, with seven

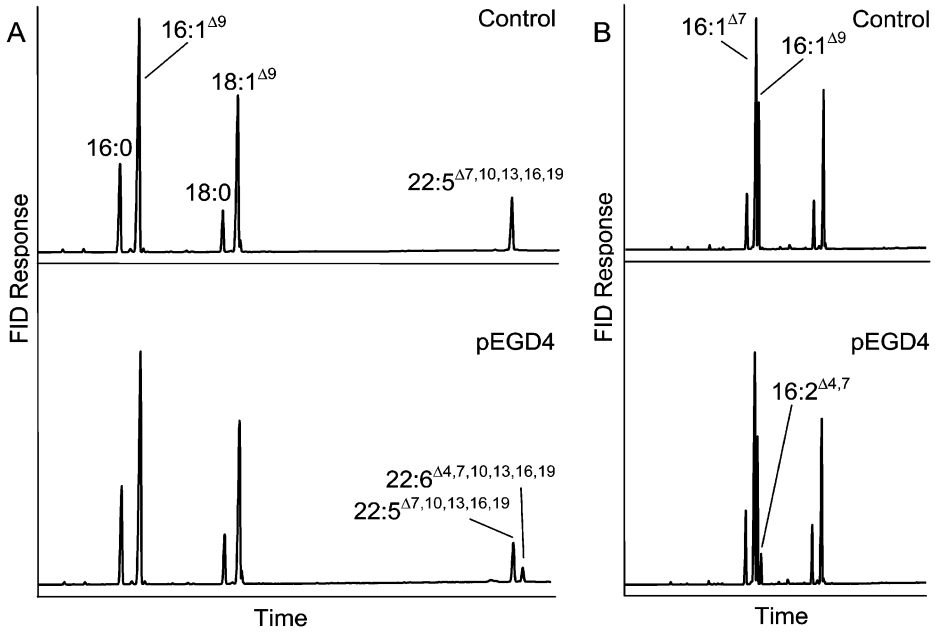

FIGURE 6: Fatty acid profiles of transgenic *Saccharomyces cerevisiae* expressing the Δ4-desaturase (pEGD4) or the empty vector (Control). The yeast cells were incubated for 4 days with exogenously supplied $22{:}5^{\Delta7,10,13,16,19}$ (A) or $16{:}1^{\Delta7}$ (B). FAMEs were prepared from whole cells and analyzed by GLC.

acidic amino acids in the heme-binding domain compared to the average 12 acidic amino acids generally found in free cytochrome b_5 (*46*). Interestingly, the *E. gracilis* cytochrome b_5 domain had a long N-terminal extension, which is also present in the Δ6-desaturases of *Physcomitrella patens* (*16*) and *Ceratodon purpureus* (*44*), but the cytochrome b_5 domain of the *E. gracilis* polypeptide did not show significant similarity to the cytochrome b_5 domains of the moss desaturases. The *E. gracilis* ORF was annotated EgD4, coding for EgD4p.

Expression of EgD4 in S. cerevisiae. The ORF coding for EgD4p was cloned into the pYES2 yeast expression vector, resulting in pEGD4. This recombinant vector and the empty control vector were used for the transformation of *S. cerevisiae* 334 (*41*). Expression was carried out for 4 days at 15 °C in the presence of galactose and $22{:}5^{\Delta7,10,13,16,19}$ as a potential fatty acid substrate. A comparison of the total fatty acids obtained from yeast expressing the ORF coding for EgD4p with the control revealed the presence of a new peak emerging after $22{:}5^{\Delta7,10,13,16,19}$ (Figure 6). This peak could not be attributed to endogenous fatty acids or to the exogenously supplied substrate. The new fatty acid had the retention time of $22{:}6^{\Delta4,7,10,13,16,19}$, demonstrating that EgD4p is a Δ4-fatty acid desaturase involved in the production of DHA. Under the conditions used, 29.7 ± 2.8% of $22{:}5^{\Delta7,10,13,16,19}$ was converted to DHA by EgD4p. To determine the substrate specificity of EgD4p, expressions in the presence of other potential fatty acid substrates were carried out. Although highest activity of EgD4p was observed with $22{:}5^{\Delta7,10,13,16,19}$, some other fatty acid substrates were also accepted. Although none of the other substrates tested (16:0, $16{:}1^{\Delta9}$, 18:0, $18{:}1^{\Delta9}$, $18{:}2^{\Delta9,12}$, $18{:}3^{\Delta6,9,12}$, $20{:}3^{\Delta8,11,14}$) were accepted, 28.7 ± 2.5% of $22{:}4^{\Delta7,10,13,16}$, 21.4 ± 3.3% of $16{:}3^{\Delta7,10,13}$, and 7.4 ± 0.8% of $16{:}1^{\Delta7}$ were converted to their Δ4-desaturated products. The structures of $22{:}4^{\Delta4,7,10,13,16}$ and $16{:}2^{\Delta4,7}$ were confirmed by GLC/MS of the DMOX derivatives. Neither the absence of an ω3-double bond nor a reduction in chain length abolished EgD4p activity, whereas the presence of a Δ7-double bond was required.

Positional Analysis of Phosphatidylcholine. In contrast to the acyl-CoA desaturases of animals, which act on fatty acid CoA thioesters (*47*), the substrates of Δ4-, Δ5-, and Δ6-desaturases from plants and fungi are thought to be fatty acyl residues that are linked to the *sn*-2 position of phosphatidylcholine (PC) (*48*−*50*). To investigate if this might also prove true for the Δ4-desaturase from *E. gracilis*, we used $16{:}1^{\Delta7}$ and $22{:}4^{\Delta7,10,13,16}$ as substrates and carried out a positional analysis of the fatty acids in PC as a dominating and representative component of the phospholipid mixture isolated from transgenic yeasts expressing pEGD4. Both substrates were incorporated into PC. The recovered polyunsaturated C22 fatty acids from PC (the sum of $22{:}4^{\Delta7,10,13,16}$ and $22{:}5^{\Delta4,7,10,13,16}$) were similarly distributed between the *sn*-1- and *sn*-2 position, accounting for 2.3% and 2.9%, respectively (Figure 7). A comparable observation was made with the synthetic $16{:}1^{\Delta7}$ and its desaturation product (31.9% in the *sn*-1 position and 27% in the *sn*-2 position). However, there was a significant difference in the positional distribution of the Δ4-desaturated products, which in contrast to both Δ7-unsaturated educts were highly enriched in the *sn*-2 position. In the *sn*-2 position, 2.4% of $22{:}5^{\Delta4,7,10,13,16}$ was detected, that is, 20 times more than the 0.1% in the *sn*-1 position. Similarly, the proportion of $16{:}2^{\Delta4,7}$ in the *sn*-2 position was about 10 times higher as compared to that in the *sn*-1 position of PC, despite the fact that the substrate was equally available in both *sn*-positions.

DISCUSSION

In this study, we report the application of in vivo radiolabeling studies in combination with α-oxidation to gain biochemical evidence for the involvement of a Δ4-desaturase in the synthesis of DHA from DPA in *E. gracilis* and *Thraustochytrium* sp. The use of DTA as an additional

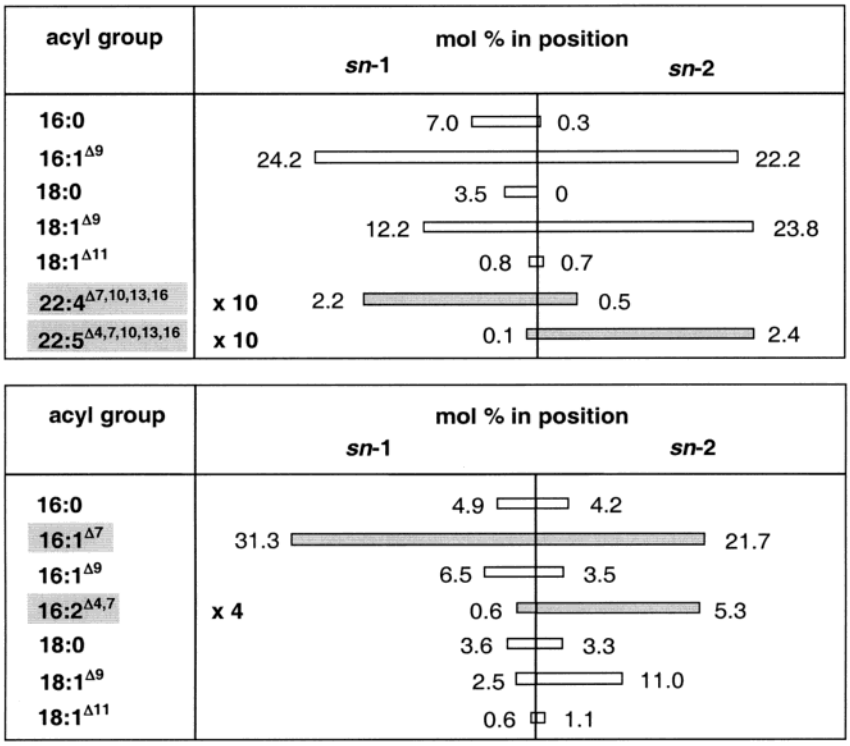

FIGURE 7: Positional distribution of fatty acids in phosphatidylcholine. Yeasts expressing pEGD4 were supplemented with 22:4$^{\Delta 7,10,13,16}$ (top) or 16:1$^{\Delta 7}$ (bottom) and grown for 5 days before PC was purified from total lipids and subjected to a positional analysis of the fatty acids. Δ4-Desaturation substrates and products are marked with a gray background. When indicated, the lengths of the bars (but not the percentages) were multiplied by factors of 4 or 10 for better visualization. The experiment was repeated twice. Results are from one representative experiment.

substrate revealed the existence of an ω3-desaturase acting on C22 fatty acid substrates in *Thraustochytrium* sp. and the absence in *E. gracilis*. This alga is probably also missing an ω3-desaturase activity acting on C20 fatty acyl residues since Nichols and Appleby (*51*) observed the synthesis of arachidonic acid but not of eicosapentaenoic acid or DHA from radiolabeled C18 and C20 fatty acid substrates. The availability of the ω3-desaturase gene from *Thraustochytrium* sp. may allow to merge the ω6-pathway with the ω3-pathway at different stages to increase the proportion of ω3-PUFAs in transgenic oilseed plants, such as rapeseed, with high proportions of linoleic acid. The desaturase might also be involved in the introduction of the ω3-double bond in C18 and C20 ω6-fatty acids, reflecting the activity of the ω3-desaturase of *Caenorhabditis elegans* (*52*). Although *Thraustochytrium* sp. and *Schizochytrium* sp. are both members of the Thraustochytriaceae, they showed a very different behavior in the labeling studies. While *Thraustochytrium* sp. efficiently desaturated the exogenously supplied DPA and DTA, *Schizochytrium* sp. did not use any of these substrates (data not shown for DTA), which is similar to observations made by Metz et al. (*13*) with different C18 fatty acid substrates. Furthermore, Metz et al. showed that DPA and DHA synthesis from malonyl-CoA was restricted to the membrane-free supernatant fraction which, therefore, did not seem to involve membrane-bound enzymes such as acyl-lipid desaturases and elongases. The isolation of a gene cluster coding for a PKS system with high homology to the *Shewanella* PUFA PKS system supported the hypothesis that DHA synthesis in *Schizochytrium* sp. is achieved by a PKS system. Whether DHA synthesis in *Thraustochytrium* sp. might also rely to some extent on a PKS system in addition to the desaturase/elongase pathway has yet to be determined. Since only freshly diluted cultures of *Thraustochytrium* sp. with a high proportion of flagellated zoospores were able to desaturate DPA to DHA, the desaturase/elongase pathway and the PKS system might operate in different stages of the fungal life cycle.

Because of the high proportion of DHA found in the Thraustochytriaceae, this group has always been of great interest for the industrial production of this fatty acid, either by large-scale cultivation of the organism or by the eventual transfer of the biosynthetic genes to oilseed crops. The same applies to DHA-producing dinoflagellates such as *Crypthecodinium cohnii*. *E. gracilis*, however, produces only minor amounts of this LCPUFA. In this report, we demonstrate that *E. gracilis* is as efficient at desaturating exogenously supplied fatty acid substrates at the Δ4-position as *Thraustochytrium* sp., and we succeeded in the cloning of a Δ4-desaturase cDNA from this organism, whose gene product is involved in the biosynthesis of DHA. The amino acid sequence contains the N-terminal fusion of cytochrome b_5 and the H/Q exchange in the third histidine box typically found in front-end desaturases. The cytochrome b_5 domain harbors an N-terminal extension found in the moss desaturases (*16*, *44*), a property not observed with the Δ8-desaturase from *E. gracilis* (*14*), the Δ4-desaturase from *Thraustochytrium* sp. (*31*), or front-end desaturases from other organisms. The Δ4-desaturase and the Δ8-desaturase from the alga share only 14% identity, which suggests an early evolutionary separation of the enzymes. The Δ4-desaturase from *E. gracilis* seems to be more closely related to the corresponding desaturase from *Thraustochystrium* sp. and also contains a prolonged amino acid stretch between the

transmembrane helix closest to the C terminus and the third histidine box. The enlarged distance between this transmembrane region and the last histidine box may contribute to the Δ4-regioselectivity of the enzymes from *E. gracilis* and *Thraustochytrium* sp. As with the *Thraustochytrium* protein, the *E. gracilis* Δ4-desaturase did not strongly differentiate between ω3- and ω6-substrates, as evident from the acceptance of both DPA and DTA. This nonselectivity toward the methyl end has been observed with several other desaturases and elongases operating with the carboxyl-close segment of the acyl substrate (*14*, *16*, *31*). Another property of the *E. gracilis* desaturase is its broad substrate specificity. Fatty acids differing by six carbon atoms in chain length were desaturated to a high proportion. To date, only mammalian Δ6-desaturases were shown to possess similarly broad chain-length specificities (*27*, *53*). These desaturases may be involved in the Sprecher pathway since they desaturated both Δ9-C18 and Δ9-C24 polyunsaturated fatty acids when expressed in yeast. It may well be that the Δ4-desaturase that introduces the double bonds into the Δ7-desaturated C22 and C16 fatty acids tested here might also be responsible for the Δ4-double bonds in some unusual fatty acids present in *E. gracilis*, such as pentadecadienoic acid ($15{:}2^{\Delta 4,7}$), pentadecatetraenoic acid ($15{:}4^{\Delta 4,7,10,13}$), and $21{:}5^{\Delta 4,7,10,13,16}$ (*34*). However, this broad substrate specificity should not restrict the potential use of the desaturase in DHA production by transgenic plants since its activity is limited to fatty acid substrates containing a Δ7-double bond, which are not present in seed oils of higher plants. A positional analysis of phosphatidylcholine from transgenic yeast expressing the Δ4-desaturase from *E. gracilis* showed that the Δ4-desaturated products were highly enriched in the *sn*-2 position. This might indicate a positional specificity of the desaturase for lipid-bound acyl substrates. However, the present data do not exclude the possibility that acyl-CoAs represent the actual substrates for the enzyme and that the Δ4-desaturated acyl residues are preferentially transferred to the *sn*-2 position of the phospholipid.

To summarize, we provided biochemical evidence for the presence of a Δ4-fatty acid desaturase involved in DHA biosynthesis in *E. gracilis*. Mass sequencing of a cDNA library from *E. gracilis* resulted in the isolation of a Δ4-desaturase cDNA clone. The desaturase was able to convert DPA to its Δ4-desaturated product DHA when expressed in yeast and, consequently, represents a second candidate for the production of the nutritionally relevant DHA in transgenic plants.

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REFERENCES

1. Poulos, A. (1995) *Lipids 30*, 1−14.
2. Horrocks, L. A., and Yeo, Y. K. (1999) *Pharmacol. Res. 40*, 211−225.
3. Abbadi, A., Domergue, F., Meyer, A., Riedel, K., Sperling, P., Zank, T. K., and Heinz, E. (2001) *Eur. J. Lipid Sci. Technol. 103*, 45−55.
4. Yongmanitchai, W., and Ward, O. P. (1989) *Process Biochem. 24*, 117−125.
5. Jiang, Y., and Chen, F. (2000) *Process Biochem. 35*, 1205−1209.
6. Russell, N. J., and Nichols, D. S. (1999) *Microbiology 145*, 767−779.
7. Kendrick, A., and Ratledge, C. (1992) *Lipids 27*, 15−20.
8. Yazawa, K. (1996) *Lipids 31* (Suppl.), S297−300.
9. Tanaka, M., Ueno, A., Kawasaki, K., Yumoto, I., Ohgiya, S., Hoshino, T., Ishizaki, K., Okuyama, H., and Morita, N. (1999) *Biotechnol. Lett. 21*, 939−945.
10. Yu, R., Yamada, A., Watanabe, K., Yazawa, K., Takeyama, H., Matsunaga, T., and Kurane, R. (2000) *Lipids 35*, 1061−1064.
11. Takeyama, H., Takeda, D., Yazawa, K., Yamada, A., and Matsunaga, T. (1997) *Microbiology 143*, 2725−2731.
12. Allen, E. E., and Bartlett, D. H. (2002) *Microbiology 148*, 1903−1913.
13. Metz, J. G., Roessler, P., Facciotti, D., Levering, C., Dittrich, F., Lassner, M., Valentine, R., Lardizabal, K., Domergue, F., Yamada, A., Yazawa, K., Knauf, V., and Browse, J. (2001) *Science 293*, 290−293.
14. Wallis, J. G., and Browse, J. (1999) *Arch. Biochem. Biophys. 365*, 307−316.
15. Qi, B., Beaudoin, F., Fraser, T., Stobart, A. K., Napier, J. A., and Lazarus, C. M. (2002) *FEBS Lett. 510*, 159−165.
16. Girke, T., Schmidt, H., Zähringer, U., Reski, R., and Heinz, E. (1998) *Plant J. 15*, 39−48.
17. Zank, T. K., Zähringer, U., Beckmann, C., Pohnert, G., Boland, W., Holtorf, H., Reski, R., Lerchl, J., and Heinz, E. (2002) *Plant J. 31*, 255−268.
18. Michaelson, L. V., Lazarus, C. M., Griffiths, G., Napier, J. A., and Stobart, A. K. (1998) *J. Biol. Chem. 273*, 19055−19059.
19. Knutzon, D. S., Thurmond, J. M., Huang, Y. S., Chaudhary, S., Bobik, E. G., Jr., Chan, G. M., Kirchner, S. J., and Mukerji, P. (1998) *J. Biol. Chem. 273*, 29360−29366.
20. Huang, Y. S., Chaudhary, S., Thurmond, J. M., Bobik, E. G., Jr., Yuan, L., Chan, G. M., Kirchner, S. J., Mukerji, P., and Knutzon, D. S. (1999) *Lipids 34*, 649−659.
21. Sakuradani, E., Kobayashi, M., and Shimizu, S. (1999) *Gene 238*, 445−453.
22. Michaelson, L. V., Napier, J. A., Lewis, M., Griffiths, G., Lazarus, C. M., and Stobart, A. K. (1998) *FEBS Lett. 439*, 215−218.
23. Watts, J. L., and Browse, J. (1999) *Arch. Biochem. Biophys. 362*, 175−182.
24. Napier, J. A., Hey, S. J., Lacey, D. J., and Shewry, P. R. (1998) *Biochem. J. 330*, 611−614.
25. Domergue, F., Lerchl, J., Zähringer, U., and Heinz, E. (2002) *Eur. J. Biochem. 269*, 4105−4113.
26. Cho, H. P., Nakamura, M., and Clarke, S. D. (1999) *J. Biol. Chem. 274*, 37335−37339.
27. de Antueno, R. J., Knickle, L. C., Smith, H., Elliot, M. L., Allen, S. J., Nwaka, S., and Winther, M. D. (2001) *FEBS Lett. 509*, 77−80.
28. Leonard, A. E., Kelder, B., Bobik, E. G., Chuang, L. T., Parker-Barnes, J. M., Thurmond, J. M., Kroeger, P. E., Kopchick, J. J., Huang, Y. S., and Mukerji, P. (2000) *Biochem. J. 347*, 719−724.
29. Beaudoin, F., Michaelson, L. V., Hey, S. J., Lewis, M. J., Shewry, P. R., Sayanova, O., and Napier, J. A. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 6421−6426.
30. Voss, A., Reinhart, M., Sankarappa, S., and Sprecher, H. (1991) *J. Biol. Chem. 266*, 19995−20000.
31. Qiu, X., Hong, H., and MacKenzie, S. L. (2001) *J. Biol. Chem. 276*, 31561−31566.
32. Bajpai, P. K., Bajpai, P., and Ward, O. P. (1991) *J. Am. Oil Chem. Soc. 68*, 509−514.
33. Yokochi, T., Honda, D., Higashihara, T., and Nakahara, T. (1998) *Appl. Microbiol. Biotechnol. 49*, 72−76.
34. Korn, E. D. (1964) *J. Lipid Res. 5*, 352−364.
35. Domergue, F., Spiekermann, P., Lerchl, J., Beckmann, C., Kilian, O., Kroth, P., Boland, W., Zähringer, U., and Heinz, E. (2003) *Plant Physiol. 131*, 1648−1660.
36. Calvayrac, R., and Douce, R. (1970) *FEBS Lett. 7*, 259−262.
37. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911−917.
38. Siebertz, H. P., Heinz, E., Joyard, J., and Douce, R. (1980) *Eur. J. Biochem. 108*, 177−185.
39. Halgunset, J., Lund, E. W., and Sunde, A. (1982) *J. Chromatogr. 237*, 496−499.
40. Donahue, T. F., and Cigan, A. M. (1990) *Methods Enzymol. 185*, 366−372.
41. Hovland, P., Flick, J., Johnston, M., and Sclafani, R. A. (1989) *Gene 83*, 57−64.

42. Fischer, W., Heinz, E., and Zeus, M. (1973) *Hoppe-Seyler's Z. Physiol. Chem. 354*, 1115−1123.
43. Christie, W. W. (1998) *Lipids 33*, 343−353.
44. Sperling, P., Lee, M., Girke, T., Zähringer, U., Stymne, S., and Heinz, E. (2000) *Eur. J. Biochem. 267*, 3801−3811.
45. Saito, T., Morio, T., and Ochiai, H. (2000) *Eur. J. Biochem. 267*, 1813−1818.
46. Sperling, P., and Heinz, E. (2001) *Eur. J. Lipid Sci. Technol. 103*, 158−180.
47. Okayasu, T., Nagao, M., Ishibashi, T., and Imai, Y. (1981) *Arch. Biochem. Biophys. 206*, 21−28.
48. Galle-Le Bastard, A. M., Demandre, C., Oursel, A., Joseph, M., Mazliak, P., and Kader, J. C. (2000) *Physiol. Plant. 108*, 118−124.
49. Jackson, F. M., Fraser, T. C., Smith, M. A., Lazarus, C., Stobart, A. K., and Griffiths, G. (1998) *Eur. J. Biochem. 252*, 513−519.
50. Griffiths, G., Stobart, A. K., and Stymne, S. (1988) *Biochem. J. 252*, 641−647.
51. Nichols, B. W., and Appleby, R. S. (1969) *Phytochemistry 8*, 1907−1915.
52. Meesapyodsuk, D., Reed, D. W., Savile, C. K., Buist, P. H., Ambrose, S. J., and Covello, P. S. (2000) *Biochemistry 39*, 11948−11954.
53. D'Andrea, S., Guillou, H., Jan, S., Catheline, D., Thibault, J. N., Bouriel, M., Rioux, V., and Legrand, P. (2002) *Biochem. J. 364*, 49−55.

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