Acyl Carriers Used as Substrates by the Desaturases and Elongases Involved in Very Long-chain Polyunsaturated Fatty Acids Biosynthesis Reconstituted in Yeast*

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The health benefits attributed to very long-chain polyunsaturated fatty acids and the long term goal to produce them in transgenic oilseed crops have led to the cloning of all the genes coding for the desaturases and elongases involved in their biosynthesis. The encoded activities have been confirmed in vivo by heterologous expression, but very little is known about the actual acyl substrates involved in these pathways. Using a Δ6-elongase and front-end desaturases from different organisms, we have reconstituted in Saccharomyces cerevisiae the biosynthesis of arachidonic acid from exogenously supplied linoleic acid in order to identify these acyl carriers. Acyl-CoA measurements strongly suggest that the elongation step involved in polyunsaturated fatty acids biosynthesis is taking place within the acyl-CoA pool. In contrast, detailed analyses of lipids revealed that the two desaturation steps (Δ5 and Δ6) occur predominantly at the sn-2 position of phosphatidylcholine when using Δ5- and Δ6-desaturases from lower plants, fungi, worms, and algae. The specificity of these Δ6-desaturases for the fatty acid acylated at this particular position as well as allowing a re-equilibration with the acyl-CoA pool result in the accumulation of γ-linolenic acid at the sn-2 position of phosphatidylcholine and prevent efficient arachidonic acid biosynthesis in yeast. We confirm by using a similar experimental approach that, in contrast, the human Δ6-desaturase uses linoleoyl-CoA as substrate, which results in high efficiency of the subsequent elongation step. In addition, we report that Δ12-desaturases have no specificity toward the lipid polar headgroup or the sn-position.

Very long-chain polyunsaturated fatty acids (VLC-PUFAs) such as arachidonic acid (ARA, 20:4Δ5,8,11,14), eicosapentaenoic acid (20:5Δ5,8,11,14,17), and docosahexaenoic acid (22:6Δ4,7,10,13,16,19) are important constituents of membranes (particularly in the retina and the central nervous system) as well as precursors of several biologically active eicosanoids (1). The presence of VLC-PUFAs in the human diet affects diverse physiological processes involved in cardiovascular, immune, neuronal, and visual functions (2). Many clinical studies have linked PUFAs intake with normal health and development, particularly in the case of newborns and infants (3). VLC-PUFAs are mainly found in fish, in some fungi and lower plants, as well as in a variety of microorganisms of the phytoplankton. With the exception of the anaerobically operating polyketide synthase-like systems found in some marine bacteria and primitive eukaryotes (4), VLC-PUFAs are synthesized by elongation and desaturation of linoleic acid (LA, 18:2Δ9,12) or α-linolenic acid (ALA, 18:3Δ9,12,15) in the endoplasmic reticulum. Most algae, fungi, and lower plants producing VLC-PUFAs possess the entire biosynthetic pathway to synthesize these fatty acids from acetate, whereas mammals, which lack Δ12- and Δ15-desaturases, use as precursors LA and ALA that have to be supplied in their diet and thus are essential fatty acids.

The numerous health benefits attributed to VLC-PUFAs as well as the absence of sustainable and low cost sources has led to the long-term goal of producing such fatty acids in transgenic oilseed crops (5). Using organisms producing VLC-PUFAs such as the fungus Mortierella alpina, the moss Physcomitrella patens, the worm Caenorhabditis elegans, and the diatom Phaeodactylum tricornutum as gene sources, a large collection of sequences coding for elongases and desaturases was created in the last 10 years (reviewed in Ref. 6). Each coding sequence was separately expressed in yeast or plant and the substrate specificity of the encoded enzyme verified so that cDNAs encoding all the enzymatic activities required for DHA synthesis are available.

The fatty acid desaturases involved in VLC-PUFA biosynthesis can be divided into two groups, the ω6-ω3-desaturases and the so-called front-end desaturases (7), which contain a cytochrome b$_5$-domain fused to their N terminus (8). Whereas the latter group of desaturases inserts the new double bond between the fatty acid carboxyl group and a pre-existing double bond, the ω6-ω3-desaturases insert it between a pre-existing double bond and the fatty acid methyl end. Using alkyl/ether glycerolipids and tomato cell cultures, it was unambiguously proven that plant ω6- and ω3-desaturases are acting on lipid-linked substrates (9). In addition, biochemical studies with diacylglycerol; FID, flame-ionization detector; KCS, β-ketocetyl-CoA synthase; FAE, fatty acid elongation; MES, 4-morpholinethanesulfonic acid.

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¶ The abbreviations used are: VLC-PUFAs, very long-chain polyunsaturated fatty acids; LA, linoleic acid (18:2Δ9,12); GLA, γ-linolenic acid (18:3Δ6,9,12); ARA, arachidonic acid (20:4Δ5,8,11,14); PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; DGD, diglycosyl-
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TABLE I

| Source organism                        | Regio-specificity | GenBank Accession No. | Yeast expression vector | Restriction sites used | Source Ref. |
|----------------------------------------|-------------------|-----------------------|-------------------------|------------------------|-------------|
| Phaeodactylum tricornutum              | Δ5                | AF1076892             | pYES2                   | HindIII, XhoI          | (22)        |
| Mortierella alpina                     | Δ5                | AF172755              | pYES2                   | HindIII, XhoI          | (46)        |
| Physcomitrella patens                  | Δ5                | CAD5232               | pYES2                   | HindIII, XhoI          | (47)        |
| Phytophthora megasperma                | Δ6                | AT087296              | pESC-TRP                | HindIII, BamHI         | (48)        |
| Caenorhabditis elegans                 | Δ5                | AF107693              | pVT102-U                | HindIII, XhoI          | (22)        |
| Phaeodactylum tricornutum              | Δ6                | CA11033               | pVT102-U                | HindIII, XhoI          | (49)        |
| Ceratodon purpureus                    | Δ6                | CAB94933              | pYES2                   | HindIII, EcoRI         | (50)        |
| Homo sapiens                           | Δ6                | NP-004256             | pYES2                   | HindIII, XhoI          | (51)        |
| Borago officinalis                     | Δ6                | AAC46970              | pYES2                   | HindIII, XhoI          | (18)        |
| Mortierella alpina                     | Δ5                | AF101510              | pYES2                   | HindIII, EcoRI         | (52)        |
| Helianthus annuus                      | Δ12               | AF251845              | pYES2                   | HindIII, BamHI         | (45)        |
| Phaeodactylum tricornutum              | Δ12               | AF167023              | pYES2                   | HindIII, XhoI          | (31)        |

I. Feussner (unpublished); Patent WO 03012092-A6.

2) Expressed in the yeast strain INVSc1 (Invitrogen).

Δ6-desaturases from P. tricornutum together with the Δ6-elongase from P. patens (22). Despite this success, these reconstitution experiments were rather inefficient compared with the situation in the genuine organisms and regarding the relatively high activities measured with the separately expressed enzymes. A closer look at the activities of the different enzymes expressed to reconstitute VLC-PUFAs biosynthesis in yeast showed that the elongation of endogeneously produced Δ6-fatty acids was less than half that observed with exogenously supplied Δ6-fatty acids. These results suggest a great difference in the availability of exogenously added or in situ produced Δ6-fatty acids for elongation: in contrast to exogenously supplied fatty acids, those produced endogeneously by Δ6-desaturation may remain in a pool that is not available for elongation, which consequently limits VLC-PUFAs biosynthesis.

In the present work we sought to identify which acyl carriers could be used as substrate by the different enzymes involved in the biosynthesis of ARA reconstituted in S. cerevisiae. In view of the data presented above, special attention was paid to phosphatidylcholine and the acyl-CoA pool. Using cDNAs from various organisms, the four activities leading to the synthesis of arachidonic acid from oleic acid (Δ12-desaturase, Δ6-desaturase, Δ6-elongase, and Δ5-desaturase) were expressed in yeast separately or in combination. After short or long incubation times, the fatty acid profiles of various lipid pools were determined in order to evaluate which acyl carriers are preferentially used by the different desaturases and the elongase.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, polymerases, and DNA-modifying enzymes were obtained from New England Biolabs (Frankfurt, Germany) unless indicated otherwise. All other chemicals were from Sigma.

Construction of Vectors—The different fatty acid desaturases and yeast expression constructs used in this study are listed in Table I. Usually, complete open reading frames (ORF) were modified by PCR to create appropriate restriction sites adjacent to the start and stop codons. The amplified DNAs were cloned into the pGEM-T vector (Promega, Madison, WI) before being released and cloned into a yeast expression vector (pVT102-U, pYES2 or pESC-LEU) using the restriction sites inserted by PCR.

Expression in S. cerevisiae—The S. cerevisiae strain C134BYS986 (leu2, ura3, his, pral, prb1, prc1, csp) (22) was used in all the expressions described in this study. Transformation, selection and growth of the transgenic yeast cells have already been described (23). When the cultures had reached an OD600 of about 0.2, expressions were induced by supplementing galactose (2%, w/v) and the appropriate fatty acids to a final concentration of 500 μM. All cultures were then grown for another 24 or 48 h at 20 or 30°C, as indicated, and harvested by centrifugation. For short time pulses, cultures were grown for 24 h at 30°C, reaching an OD600 of about 1.5, before the exogenous fatty acid was added. After 1 min, 2.2-ml aliquots were harvested and the cells...
suspended by short centrifugation (20 s). After removal of the supernatant, the cell pellets were frozen in liquid nitrogen and stored at −80 °C until needed.

Lipid Analysis—Lipid analysis of transgenic yeast cells were made from 150-ml cultures grown for 24 h at 20 °C unless otherwise indicated. Cells were harvested by centrifugation, washed with 30 ml of 0.1 M NaHCO3 and the lipids were extracted on a shaker for 4 h with 15 ml of chloroform (1:1) and then for 1 h with 15 ml of methanol (2:1). The resulting organic phase was extracted with 9 ml of 0.45% NaCl, dried with Na2SO4, and evaporated under vacuum. The residue dissolved in 2 ml of chloroform and corresponded to the total lipid extract. The major lipid classes PC (phosphatidylcholine), PI+PS (phosphatidylinositol and -serine), PE (phosphatidylethanolamine) and NL (neutral lipids) were purified by thin layer chromatography using chloroform/methanol/acetic acid (65:35:8; v/v/v) as solvent mixture. The different lipid fractions were extracted from silica by adding successively 400 μl of water, 2 ml of methanol, and 2 ml of chloroform and vigorously shaken. After adding 2 ml of 0.2 M H3PO4/1 M KCl, the organic phase was extracted and the resulting aqueous phase re-extracted with 2 ml of chloroform. Both organic phases were combined, dried with Na2SO4, and evaporated under argon. The residues dissolved in 2 ml of chloroform corresponded to the different lipid fractions. For quantification, an aliquot of the total lipid extract was resolved by thin layer chromatography using chloroform/methanol/acetic acid (65:35:8; v/v/v) as solvent mixture. After drying, the plate was dipped in 10% CuSO4 in 8% (v/v) phosphoric acid, then developed in chloroform/methanol/acetic acid/ethanol 1:1 (v/v/v/v), and the spots corresponding to free fatty acids and lysophospholipids were scraped and directly transmethylated for gas-liquid chromatography analysis.

Lipid analysis of transgenic yeast cells were made from 150-ml cultures grown for 24 h at 20 °C until needed.

RESULTS

Exogenously Supplied Fatty Acids Enter the Yeast Lipid Metabolism via the Acyl-CoA Pool—A large body of evidence suggests that exogenously supplied fatty acids enter the yeast cell by concomitant conversion to acyl-CoAs before their use for various metabolic purposes. Nevertheless, to our knowledge the time course of this conversion has not yet been studied experimentally. For a first approximate evaluation of this time scale, a yeast growing culture was pulsed for 1 min with LA before evaluating the fatty acid profile in three different fractions: the total fatty acids, the esterified fatty acids and the acyl-CoA pool (Fig. 1). These patterns were compared with control samples prepared from the same culture just before the addition of LA. Before adding LA (Fig. 1A), the fatty acid composition of total and esterified fatty acids were practically identical, indicating the absence of a significant pool of free fatty acids. The monounsaturated fatty acids (16:1 and 18:1) represented about 76% of the total fatty acids, whereas the saturated 16:0 and 18:0 accounted for about 18 and 66%, respectively. These major fatty acids were also found in the acyl-CoA pool, but in significantly different proportions. Palmitoleoyl-CoA was the major acyl-CoAs species (about 36%), 16:0-CoA, 18:0-CoA, and 18:1-CoA accounted each for about 20%, and myristoyl-CoA represented less than 1%. One minute after adding LA (Fig. 1B), the profiles of the total and esterified fatty acids clearly differed, since LA represented about 50% of the total, but only 4% of the esterified acyl groups. This difference indicates that after 1 min in the presence of LA, only a very small proportion of the exogenously supplied fatty acid has already been channeled into lipids, and that most of the LA, which has been bound to and/or incorporated by the yeast cells, remains in the form of the free fatty acid. In marked contrast, the fatty acid profile of the acyl-CoA pool is dominated by LA, which represents about 60% of the acyl groups, supporting the assumption that exogenously supplied fatty acids are converted to acyl-CoAs when entering the yeast cells. These data also indicated that the acyl-CoA pool could be extensively and specifically flooded with a particular fatty acid within a very short time, enabling the possibility to detect acyl-group modifications taking place in that pool. It should be added that after 24 h, LA was still dominating the acyl-CoA pool, but that the profile of the esterified and total fatty acids were similar (data not shown), indicating that all the LA exogenously supplied had been incorporated and acylated into the yeast lipids.

Elongation of GLA Takes Place in the Acyl-CoA Pool—We then used the same approach with a yeast expressing an ELO-type elongase in order to see whether the elongation of GLA takes place within the acyl-CoA pool. For this experiment, a yeast culture transformed with a construct carrying the gene of the Δ6-elongase from P. patens was grown in the presence of galactose for 24 h before adding GLA so that the elongase was present within the cells before supplying its substrate. The fatty acid composition of the three different fractions defined above were then determined before and 1 min after the addition of GLA. Before the pulse, the fatty acid profiles were similar to those reported in Fig. 1A (data not shown). After 1 min in the presence of GLA (Fig. 1C), GLA represented about 40% of the total, but only 2.5% of the esterified fatty acids, confirming the data obtained with LA. In these two fractions, the elongation product of GLA, i.e. 20:3Δ9,11,14, could not be detected. On the other hand, both GLA and 20:3Δ9,11,14 were present in high proportions in the acyl-CoA pool. Besides the predominant 16:1, each represented more than 25% of the acyl-CoA species only 1 min after the addition of GLA, reflect-
ing an elongation of about 50% in that pool. These data strongly suggest that upon entrance into yeast cells exogenously supplied GLA is converted into GLA-CoA and thus becomes immediately available for ω6-elongation.

Next we analyzed the distribution of GLA and 20:3ω8,11,14 in the different lipids of a yeast culture that had expressed the ω6-elongase in the presence of GLA for 24 h. After extraction and separation of the major lipid classes, the fatty acid pattern of PC, phosphatidylethanolamine (PE), the neutral lipids (NL fraction), and a fraction comprising phosphatidylinositol and phosphatidylserine (PI and PS fraction) were analyzed. In addition, the fatty acid profiles of the sn-1 and sn-2 positions of PE and PC were determined. In the total lipid extract, LA and GLA represented each about 20% of the total fatty acids, indicating that the elongase had converted 50% of GLA (Fig. 2). In the various lipid fractions and sn-positions, GLA and 20:3ω8,11,14 were always found in roughly similar proportions. Both fatty acids were present in equimolar proportions in PC and in the PI + PS and NL fractions, while in PE, GLA was slightly more abundant than 20:3ω8,11,14. Despite a nearly constant GLA to 20:3ω8,11,14 ratio, the content of GLA and 20:3ω8,11,14 in the various lipid fractions and sn-positions clearly differed. Both fatty acids were enriched in PC and the NL fraction (each about 23–24%), but significantly reduced in both PE and the PI and PS fraction (each about 12%). Most importantly, the proportions of GLA and 20:3ω8,11,14 were twice as high at the sn-2 position than at the sn-1 position in both PC and PE. These data suggest that after ω6-elongation in the acyl-CoA pool, various acyltransferases transfer indiscriminately both GLA and 20:3ω8,11,14 into the different lipids, and that the sn-2 positions of PC and PE as well as the NL fraction represent the major acceptors.

**Lipid Pools Involved in Arachidonic Acid Biosynthesis**—If the elongation step involved in ARA biosynthesis most proba-
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Fig. 2. Distribution of substrate and product (as % of total fatty acids) within the different lipid fractions isolated from a yeast culture expressing the moss Δ6-elongase. A culture of the yeast strain C13ABYS86, transformed with pESC-LEU-PSE1, was grown for 24 h at 20 °C in the presence of 500 μM GLA. The fatty acid compositions of the lipid extract, the different lipid fractions and the sn-positions of PC and PE were determined as indicated under “Experimental Procedures.” Each value is the mean ± S.D. from three independent experiments.


datably takes place in the acyl-CoA pool, the low elongation yields measured with endogenously produced Δ6-fatty acids suggest that the Δ6-desaturase responsible for their synthesis operates within another lipid pool. To identify this pool, the ARA biosynthetic pathway was reconstituted in yeast by co-expressing the Δ5- and Δ6-desaturases from P. tricornutum together with the Δ6-elongase from P. patens in the presence of exogenously supplied LA. After 48 h of expression, the fatty acid profiles of the total lipid extract, the acyl-CoA pool and the putative desaturase substrate PC were examined (Fig. 3). In the total lipid extract, the acyl-CoA pool, about 50 and 10% of GLA and LA were elongated, whereas the major C20-PUFA was 20:2Δ9,12 (about 3%), resulting from the elongation of LA. Due to the very low elongation of endogenously formed GLA, only traces of ARA were detected (0.4%). In the acyl-CoA pool (Fig. 3B), the endogenous 16:0, 16:1, 18:0, and 18:1 were abundant, but the exogenously supplied LA still represented the major fatty acid species. Its elongation product, 20:2Δ9,12, was present in considerable proportions, whereas both GLA and 20:3Δ6,9,12 were minor components. In the acyl-CoA pool, about 50 and 10% of GLA and LA were elongated, respectively, which is similar to the elongation activities measured with exogenously supplied fatty acids (Fig. 2). In PC (Fig. 3C), LA was predominating, but the content of GLA was more than doubled in comparison to the lipid extract. As the Δ6-desaturation products were also enriched (Fig. 3C, insert), these data suggest that the two desaturation steps involved in the biosynthesis of ARA may take place in PC.

Δ5- and Δ6-Desaturases from P. tricornutum Are Specific for the sn-2 Position of PC—In order to confirm that the Δ6-desaturation of LA occurs in PC, the main lipid fractions of the transgenic yeast culture that had expressed the Δ6-desaturase in the presence of LA were isolated and their fatty acid profiles determined (Table II). In the unfractonated lipid extract, LA and GLA represented 48.0 and 10.5% of the total fatty acids, respectively, indicating that about 18% of LA had been desaturated. The NL fraction had a fatty acid profile similar to that of the lipid extract, but it contained slightly more LA and less GLA. In agreement with the general fatty acid composition of yeast phospholipids (29), the PI + PS fraction was enriched in saturated fatty acids (16:0 and 18:0), whereas PE was enriched in 16:1 and poor in 18:0 (Table II). Although these two fractions had proportions of LA similar to that of the lipid extract, GLA was much less abundant, suggesting that none of these phospholipids was a major site for Δ6-desaturation. In contrast, GLA represented about 22% of the total fatty acids in PC, which is more than two times the proportion in the lipid extract (Table II).

The positional analysis of PE and PC showed that the sn-1 positions of both phospholipids were enriched in 16:0, 16:1, and 18:0, whereas the sn-2 positions were enriched in 18:1, LA and GLA (Table II). In both phospholipids, the LA plus GLA content accounted for about 38 and 78% of the total fatty acids at sn-1 and sn-2 positions, respectively, in line with the sn-2 position of these two phospholipids being a major acyl acceptor. In PE, the GLA level was equally low at both positions (2–6%), whereas in PC GLA represented 3.7% of the total fatty acids at the sn-1 position, but as much as 46% at the sn-2 position. In contrast to the nearly constant educt/product ratio in all fractions reported for the elongase in Fig. 2, the data presented in Table II demonstrate a significant variation of this ratio between the different lipids and the two sn-positions. After 24 h of expression in the presence of LA, the neutral lipid fraction represented about 50% of the total lipids, while PC, PE and the PI + PS fraction accounted for 26, 15 and 8%, respectively (Table II). Assuming that the neutral lipid fraction was equally composed of triglycerides and sterol esters (30), these data indicated that although the sn-2 position of PC was represented only 13% of all acyl groups, it contained about half (56%) of the total GLA. This strongly suggests that the sn-2 position of PC served as the major site for Δ6-desaturation.

To demonstrate that GLA accumulation at the sn-2 position of PC was not resulting from the selectivity of yeast acyltransferase activities but from the Δ6-desaturase activity, a wild type yeast was supplemented with a 3:1 mixture of LA/GLA and grown for 24 h before determining the fatty acid composition of different lipid fractions (data not shown). After this incubation, LA and GLA represented about 46 and 11%, respectively, of the total fatty acids. These percentages mimic the fatty acid profile resulting from the expression of a Δ6-desatu-
Fatty acid profiles of the lipid extract, phosphatidylcholine and the acyl-CoA pool of a yeast culture expressing the algal Δ5- and Δ6-desaturases together with the moss Δ6-elongase. A culture of the yeast strain C13ABY886, transformed with pVT102-U-PtD6 and pESC-LEU-PSE1-PDS, was grown for 48 h at 20 °C in the presence of 500 μM 18:2ω6,ω3. The fatty acid compositions of the lipid extract, phosphatidylcholine, and the acyl-CoA pool were determined as indicated under “Experimental Procedures.”

Table II

Fatty acid composition of different lipid fractions from a yeast expressing the Δ6-desaturase from P. tricornutum (PtD6p)

The yeast strain C13ABY886 was transformed with pVT102-U-PtD6, grown 24 h at 20 °C in the presence of 500 μM LA and used to isolate different lipid fractions. Positional analyses were carried out for PC and PE as described under “Experimental Procedures.” Desaturation (%) was calculated as (product × 100)/educt × product) using values corresponding to percent of total fatty acids. Lipid proportions (in %) were determined by densitometric scanning after thin layer chromatography separation. Each value is the mean ± SD from 3–5 independent experiments.

| Fraction | Lipid Extract | NL | PI + PS | PE | sn-1-PE | sn-2-PE | sn-1-PC | sn-2-PC |
|----------|---------------|----|---------|----|---------|---------|---------|---------|
| 16:0     | 15.1 ± 2.1    | 13.2 ± 2.5 | 23.3 ± 5.3 | 13.0 ± 1.9 | 21.6 ± 3.3 | 0.7 ± 0.1 | 15.3 ± 3.1 | 16.9 ± 6.0 |
| 16:1ω9  | 10.5 ± 1.4    | 9.5 ± 1.8 | 6.6 ± 1.8 | 20.7 ± 1.9 | 30.2 ± 1.3 | 3.9 ± 1.3 | 8.4 ± 0.9 | 12.2 ± 1.7 |
| 18:0     | 5.6 ± 0.7     | 6.2 ± 0.7 | 9.8 ± 0.7 | 0.9 ± 0.2 | 2.4 ± 0.3 | 0.5 ± 0.1 | 5.6 ± 0.8 | 11.0 ± 1.3 |
| 18:1ω9  | 9.7 ± 1.1     | 9.4 ± 1.3 | 13.9 ± 3.4 | 11.4 ± 1.7 | 8.8 ± 2.0 | 17.7 ± 2.0 | 9.5 ± 2.2 | 8.7 ± 2.6 |
| LA       | 48.0 ± 1.2    | 53.5 ± 1.0 | 43.2 ± 4.7 | 49.7 ± 2.4 | 35.0 ± 3.0 | 71.0 ± 1.7 | 38.2 ± 2.3 | 35.8 ± 4.1 |
| GLA      | 10.5 ± 0.9    | 8.1 ± 0.8 | 3.1 ± 1.6 | 4.3 ± 1.0 | 2.0 ± 0.9 | 6.2 ± 1.1 | 22.1 ± 0.8 | 3.7 ± 1.1 |

Desaturation (%)

| Fatty acid | % of lipid extract | GLA (% of total) |
|------------|--------------------|------------------|
| 16:0       | 51.0 ± 5.3         | 25.9 ± 1.7 |
| 16:1ω9     | 8.0 ± 0.9           | 2.0 ± 0.9 |
| 18:0       | 13.1 ± 1.5         | 15.0 ± 2.1 |
| 18:1ω9     | 6.7 ± 0.8           | 5.0 ± 0.7 |
| LA         | 3.1 ± 1.6           | 2.3 ± 0.4 |
| GLA        | 3.1 ± 1.6           | 6.0 ± 0.8 |

Retention time

Acyl Substrate Used by Front-end Desaturases from Other Organisms—To see whether the conclusions drawn above can be extended to front-end desaturases from organisms other than algae, we expressed Δ5- and Δ6-desaturases from fungi, mosses, higher plants, worms and mammals in yeast and looked at the desaturation in the different lipid fractions, particularly in PC and at its sn-2 position. The different Δ6-desaturases were expressed in the presence of LA, whereas Δ5-desaturases were co-expressed with the Δ6-elongase from P. patens in the presence of GLA, as it results in high incorporation of 20:3ω5,ω11,ω14 into the sn-2 position of PC (see Fig. 2). As an example, Fig. 5 presents the results obtained with the Δ5-desaturase from the fungus M. alpina. After 24 h, about 50% of GLA had been elongated and 28% of the resulting 20:3ω5,ω11,ω14 had been desaturated to ARA, which represented as much as 5% of the total fatty acids in the lipid extract (Fig. 5A). The proportion of ARA was more than doubled in PC (12% of...
the total fatty acids; Fig. 5B), whereas it was lower in all the other individually analyzed lipid fractions (not shown). On the other hand, ARA accounted for less than 4% of the total fatty acids at the sn-1 position of PC (Fig. 5C), whereas it was lower in all the other individually analyzed lipid fractions (not shown). On the other hand, ARA accounted for less than 4% of the total fatty acids at the sn-1 position of PC (Fig. 5C), but as much as 25% at the sn-2 position (Fig. 5D). The desaturation of 20:3\[^{5,11,14}\] at the sn-2 position of PC was so efficient (82% conversion) that hardly any substrate for the \[^{5}\] desaturase was left at this position after 24 h. As clearly shown in Fig. 5, the enzyme from \textit{M. alpina} resulted in two and three times higher \[^{5}\] desaturations in PC and at its sn-2 position, respectively, than in the lipid extract. The same correlation was found for the \[^{5}\] desaturases from the mosses \textit{P. patens} and \textit{C. purpureus}, the fungus \textit{M. alpina} and the alga \textit{P. tricornutum}, suggesting that the \[^{5}\] and \[^{6}\] desaturases from fungi, lower plants, and diatoms are specific for the sn-2 position of PC. In contrast to these enzymes, the expression of the \[^{5}\] desaturases from man and the higher plant \textit{B. officinalis} led to different patterns. Desaturation was not higher in PC than in the

The results obtained with \[^{5}\] and \[^{6}\] desaturases from all the organisms tested are summarized in Fig. 6. The \[^{5}\] desaturases from the moss \textit{P. patens}, the fungus \textit{Phytophthora megaspora}, the worm \textit{C. elegans}, and the diatom \textit{P. tricornutum} gave results similar to those presented in Fig. 5 for the enzyme from \textit{M. alpina}. Although these desaturases differed in their level of activity in yeast, desaturation was always two and three times higher in PC and at its sn-2 position, respectively, than in the lipid extract. The same correlation was found for the \[^{6}\] desaturases from the mosses \textit{P. patens} and \textit{C. purpureus}, the fungus \textit{M. alpina} and the alga \textit{P. tricornutum}, suggesting that the \[^{5}\] and \[^{6}\] desaturases from fungi, lower plants, and diatoms are specific for the sn-2 position of PC. In contrast to these enzymes, the expression of the \[^{6}\] desaturases from man and the higher plant \textit{B. officinalis} led to different patterns. Desaturation was not higher in PC than in the
lipid extract. The proportions of GLA were about the same in the lipid extract, PC and at its sn-2 position after expression of the human Δ6-desaturase, whereas expression of the Δ6-desaturase from *B. officinalis* resulted in about 50% higher GLA proportions at the sn-2 position of PC as compared with PC or the lipid extract.

Mammalian desaturases like the human Δ6-desaturase have been biochemically characterized as being acyl-CoA desaturases (13, 14). In order to confirm this acyl-substrate specificity, the human Δ6-desaturase (FADS2) and for comparison the Δ6-desaturase of *P. tricornutum* (PtD6p) were expressed in yeast in the presence of LA, and the fatty acids profiles of the lipid extract, the sn-2 position of PC and the acyl-CoA pool were determined (Fig. 7). After expression of the algal Δ6-desaturase, GLA as well as the “side-products” 16:2Δ6,9 and 18:2Δ6,9 were highly enriched at the sn-2 position of PC, but GLA was barely detectable in the acyl-CoA pool. In contrast, after expression of FADS2, GLA was clearly visible in the acyl-CoA pool, but present in similar proportions in both the total fatty acids and at the sn-2 position of PC. This strongly suggests that the human Δ6-desaturase uses CoA-thioesters as acyl substrates. Such acyl substrate specificity could be responsible for the even distribution of both substrate and product of the desaturase shown in Figs. 6 and 7, similar to the situation reported for the elongase in Fig. 2. Further confirmation that the human Δ6-desaturase uses acyl-CoAs as substrates was obtained by its coexpression with the Δ6-elongase from *P. patens* (Fig. 8). When the human Δ6-desaturase was expressed in the presence of LA, 18:2Δ9,12 and 18:3Δ6,9,12 represented 49.5 and 7.5% of the total fatty acids (Fig. 8A). When the Δ6-elongase was expressed in addition (Fig. 8B), nearly all GLA (93%) was elongated to 20:3Δ6,11,14. This result suggests that both enzymes use substrates from the acyl-CoA pool and that the retention of the different intermediates within this pool led to a highly efficient cooperation between the Δ6-CoA-desaturase and elongase.

**Acyl Substrate Used by Δ12/ω6-Fatty Acid Desaturase—** As mentioned above, incubations of tomato cell cultures with alk- enylether glycerolipids resulted in Δ12-desaturated products found at both position of PC and monogalactosyldiacylglycerol (9). Therefore, Δ12/ω6-desaturases may not be specific for the sn-2 position of PC or glycolipids, in contrast to the front-end desaturases from fungi, diatoms, worms and lower plants. When we expressed the Δ12-desaturase from *P. tricornutum* (31) for 24 h in the presence of LA, all the glycerolipid fractions were similarly desaturated (data not shown). In order to get a closer look at the substrate specificity of Δ12/ω6-desaturase, a transgenic yeast producing diglycosyldiacylglycerol (DGD) by expressing the processive glucosyltransferase from *Staphylococcus aureus* (32) was transformed with a vector containing the gene coding for the Δ12-desaturase from sunflower (*Helianthus annuus* L.). After 72 h of expression, the different lipid fractions were isolated to determine the conversion of 16:1 and 18:1 to 16:2 and 18:2 and LA, respectively. As clearly shown in Fig. 9, the Δ12-desaturase from sunflower was not specific for any lipid. In contrast, all the lipids and both positions of PC, PE and DGD displayed similar levels of desaturation for both 16:1 and 18:1. Since similar results were also obtained with the Δ12-desaturase from *A. thaliana* (data not shown), we conclude that plant Δ12/ω6-desaturases use lipid-linked acyl chains as substrates and are most probably not specific for the polar head group of the lipid and the sn-position of the acyl group on the glycerol backbone.

**DISCUSSION**

The goal of this study was to determine which forms of acyl substrate are used by the different enzymes (Δ12-, Δ6-, Δ5-desaturase and Δ6-elongase) involved in VLC-PUFAs biosynthesis. Using the model organism *S. cerevisiae* as heterologous expression system, we have expressed the genes coding for the activities responsible for the conversion of oleic to arachidonic acid and analyzed the fatty acid composition of different lipid fractions and the acyl-CoA pool after short and long incubation times. We made use of the fact that feeding of exogenous fatty
acids results in immediate labeling of the acyl-CoA pool, since uptake is coupled to acyl-thioester formation. These experiments allowed the identification of the acyl carriers used by both the desaturases and the elongase involved in ARA biosynthesis.

Transport of exogenous long-chain fatty acids into S. cerevisiae was shown to rely on the activities of the fatty acid transport protein Fat1p and long-chain fatty acyl-CoA synthetases (primarily Faa1p) (33, 34). Figs. 1 shows that an exogenously supplied fatty acid (LA or GLA) represents the dominating acyl...
group in the acyl-CoA pool already only 1 min after addition to the medium. These data demonstrate for the first time the efficiency of labeling the acyl-CoA pool by exogenous fatty acids, regarding both timing and extent. Wagner and Páltauf (29) have shown that exogenous, radiolabeled 16:0 and 18:1 fatty acids were predominantly incorporated into the phospholipids after 2 min of incubation and that most of the label in PC and PE was found at the sn-2 position. Interestingly, the exogenously supplied fatty acid and its downstream product were still highest at the sn-2 position of PC and PE after 24 h of incubation (Table II and Fig. 2). In addition, the fact that the substrate to product ratio was similar in all the different lipid fractions and positions when expressing enzymes using acyl-CoAs as substrates (the Δ6-elongase in Fig. 2 or the human Δ6-desaturase in Fig. 6) suggests that the fatty acids present within the acyl-CoA pool are transferred indiscriminately into the different lipids by various acyltransferases. The extent to which the different phospholipids are acylated appears to depend mainly on their metabolic involvement in yeast.

The data presented in Fig. 1C show that only 1 min after addition of GLA to the culture medium, this fatty acid as well as its elongation product 20:3ω6,11,14 account in nearly equal proportions for more than 50% of the acyl species of the acyl-CoA pool. In contrast, GLA represents only a minor peak in the esterified fatty acid profile, whereas 20:3ω6,11,14 is not detected at all. In view of the size of the different pools of acyl groups, i.e. acyl-CoA thioesters versus lipid-bound oxygen esters, this result is not surprising. Quantitative acyl-CoA measurements with yeast have shown that lipid-bound acyl groups exceed acyl-CoA thioesters by a factor of about 2000 (35). With the method used in the present study and in the presence of 500 μM exogenous fatty acid, we found even larger factors (data not shown). Throughout the present study, the activity of the elongase deduced from educt/product ratios in the acyl-CoA pool always reflected the total activity measured with exogenously supplied fatty acids (about 10 and 50% conversion of LA and GLA, respectively). These data cannot prove that the elongation of GLA takes place within the acyl-CoA pool. It seems accordingly that similar to the situation encountered in the elongation of monounsaturated fatty acids catalyzed by KCS/FAE condensing enzymes (21), the elongation steps involved in VLC-PUFAs biosynthesis most probably utilize acyl-CoAs as substrates and produce acyl-CoAs as products.

Our experimental procedures also allowed the identification and/or confirmation of the different acyl carriers used as substrate by the various desaturases involved in VLC-PUFAs biosynthesis. Biochemical studies have shown that the first desaturase, the Δ12-desaturase responsible for the synthesis of LA from oleic acid, acts on both sn-positions of PC (10, 11, 36). The data presented in Fig. 9 confirm these results, but also show that the activity of Δ12-desaturases is not restricted to PC. Since it was unambiguously proven that such enzymes can act on lipid-linked substrates (9), and because of the low level of lipid remodeling in yeast observed in this study, we are forced to conclude that Δ12-desaturases are not specific for any polar head group and display activity on both sn-positions of all glycerolipids. In contrast to this very wide acyl carrier specificity, the Δ6- and Δ5-desaturases involved in VLC-PUFAs biosynthesis were shown to be very specific for the acyl chain esterified at the sn-2 position of PC in most cases (Fig. 6). Front-end desaturases from algae, fungi, lower plants, and worms were mainly active on this particular position, which resulted in the accumulation of the desaturation products at the sn-2 position of PC (Table II). The results obtained with the human Δ6-desaturase FADS2 confirm that the front-end desaturases from mammalia in contrast most probably use CoA-thioesters as acyl carriers. Whereas the desaturases discussed above resulted in significant variation of the educt/product ratio between the different lipids and the sn-positions of PC (Table II and Fig. 6), the educt/product ratio resulting from the expression of the human Δ6-desaturase was roughly constant in the different lipids and sn-positions. This situation reflected the pattern resulting from the expression of the acyl-CoA elongase (Fig. 2). Although we carried out several experiments with the human Δ6-desaturase, we could not detect desaturation products in the acyl-CoA pool 1 min after adding the fatty acid.

Fig. 9. Desaturation of 16:1 and 18:1 in the different lipid fractions of a culture expressing a Δ12-desaturase from a higher plant together with a processive glucosyltransferase from a bacterium. A culture of the yeast strain C13ABYS86, transformed with pYES2-HaD12 (H. annuus Δ12-desaturase) and pESC-LEU-SaGT (S. aureus glucosyltransferase), was grown for 72 h at 20 °C. The fatty acid compositions of the lipid extract, the different lipid fractions and the two sn-positions of PC, PE and diglucosyldiacylglycerol (DGD) were determined as indicated under “Experimental Procedures.” Desaturation (%) was calculated as (product × 100)/(educt + product) using values corresponding to percent of total fatty acids. Each value is the mean ± S.D. from three independent experiments.
acyl-CoA:sterol-acyltransferases (encoded by exons) are exclusively made from acyl-CoAs and sterols by a set of two transferases (encoded by genes). TAG are mainly synthesized in yeast (37). LRO1-mediated deacylation followed by resynthesis of acyl-CoA as the reverse activity of the acyl-CoA:lysophosphatidylcholine acyltransferase. This latter enzyme has been described in microsomes prepared from B. officinalis seeds, where GLA was found in both PC and PE, but in vitro assays with [14C]-18:1 have shown that the sn-2 position of PC was the preferred site for desaturation (10, 11). When we expressed the B. officinalis Δ6-desaturase in yeast in the presence of LA, this desaturase was not specific for PC, since significant proportions of GLA were also found in PE and in the PI + PS and neutral lipid fractions, but in both PC and PE, desaturated fatty acids were nearly exclusively found at the sn-2 position (data not shown). The B. officinalis Δ6-desaturase appears to be highly specific for the sn-2 position, but its specificity toward the polar head of lipids differs from the other front-end desaturases.

Although our experimental approach could differentiate all these desaturases according to the acyl carriers used as substrates, we could not demonstrate that the activity of any of these enzymes was absolutely restricted to an unique acyl substrate. For example, the data presented in Fig. 9 could be compatible, if considered at their own, with the operation of the Δ12-desaturase with acyl-CoA substrates. Similarly, the presence of minor proportions of desaturated fatty acids at the sn-1 position of PC and in most of the other lipids resulting from the expression of front-end desaturases specific for the sn-2 position of PC raises the question as to whether this was due to lipid remodeling or to substrate unspecificity. On the other hand, the distribution of the desaturated products varied considerably between the different desaturases assayed, whereas lipid remodeling should be considered as being always the same in the various expressions carried out with the same yeast strain. Therefore, it seems more probable that these desaturases are preferentially, but not absolutely specific for the sn-2 position of PC. As shown in Table II for the Δ6-desaturase from P. tricornutum, about 96% of the total GLA was found at the sn-2 position of PC and in the neutral lipids, which represented half of all lipids. These data indicate that after 24 h in the presence of 500 μM LA, all the different pathways involved in storage lipid biosynthesis in yeast were highly active and that lipid remodeling mainly transferred GLA from PC into the neutral lipids. Three main pathways responsible for storage lipid synthesis have been described in yeast (37). TAG are mainly synthesized from DAG and acyl-CoAs by the acyl-CoA:DAG-acyltransferase (encoded by DGA1) (38), whereas sterols ester are exclusively made from acyl-CoAs and sterols by a set of two acyl-CoA:sterol-acyltransferases (encoded by ARE1 and ARE2) (39). The third route involves the phospholipid:DAG-acyltransferase activity (encoded by LRO1), which in yeast specifically transfers the acyl chain from the sn-2 position of PC to DAG, yielding TAG and sn-2-lyso-PC (40). If we consider that the low elongation of endogenously produced GLA indicates that GLA is present in very low level in the acyl-CoA pool (see next), then most of the GLA made in PC is transferred to the NL fraction without passing through the acyl-CoA pool. Accordingly, the phospholipid:DAG-acyltransferase as well as activities synthesizing DAG from PC (phospholipase C or D, cholinephosphotransferase) (41) may account for most of the GLA synthesized in PC, but found in the NL fraction.

Despite the fact that we could not demonstrate an absolute acyl carrier specificity of any desaturase, our results clearly show the existence of different groups of desaturases regarding the acyl carriers used as substrate. Many indirect approaches have already led to a recognition and assignment of these specificities, but our data represent an independent and more direct approach since all the different enzymes, i.e. lipid- or CoA-linked, have been expressed in the same system for direct comparison and the analyses included both putative lipid substrates and acyl-CoAs. In a phylogenetic tree with various desaturases (42), the different regiospecificities (Δ5, Δ6, Δ9, or Δ12) define separate branches, although front-end desaturases with Δ4-, Δ6-, or Δ8-regiospecificity do not form clear-cut groups so that the exact regiospecificity of an unknown front-end desaturase must always be confirmed by heterologous expression. In such phylogenetic trees, the B. officinalis Δ6-desaturase groups together with the sphingolipid long-chain base Δ8-desaturases, rather than with the other front-end desaturases. This was interpreted as indicating that the Δ6-desaturases from higher plants have arisen by gene duplication from sphingolipid Δ5-desaturases (42). This particular phylogenetic origin may in turn explain that the Δ6-desaturases from higher plants have an acyl carrier specificity different from that of the other front-end desaturases, as clearly shown in the Fig. 6. In addition, we would like to point out that in phylogenetic alignments the front-end Δ5- and Δ6-desaturases from mammalia and fish always form a deeply separated branch (42). On the basis of the few presently known sequences, this grouping was attributed to the general separation of vertebrates from the other organisms. On the other hand, a criterion never considered as influencing these alignments is desaturase similarity based on substrate preference, i.e. using acyl-CoAs or lipid-linked acyl groups. A high priority of this difference could also result in the separation of these desaturases, from which at least the mammalian ones use acyl-CoA substrates. Furthermore, there is desaturase bifunctionality with regard to reaction outcome (for example desaturation and hydroxylation), stereochemistry (cis and trans double bonds) and regiochemistry (Δ5- and Δ6-desaturation), there may also be bifunctionality with regard to the acyl group position (sn-1 or sn-2), the lipid headgroup or CoA- versus lipid-linked substrate. At present it is not possible to recognize these alternatives, which could interfere with the interpretation of our data, from the amino acid sequences of the various desaturases.

The involvement of different acyl carriers as demonstrated in this study explains the poor yields obtained when reconstituting ARA biosynthesis in yeast (18, 22). The Δ6-desaturase from P. tricornutum mainly uses the LA in the sn-2 position of PC, while the Δ6-longelangase from P. patens requires GLA in the acyl-CoA pool. Therefore, an inefficient transfer of the Δ6-desaturated products from PC into the acyl-CoA pool most probably represents a bottleneck. As shown in Fig. 3B, S. cerevisiae appears to possess some enzyme(s) capable of releasing GLA from the sn-2 position of PC. Several activities can be responsible for this transfer in yeast, such as phospholipase A2-mediated deacylation followed by resynthesis of acyl-CoA as well as the reverse activity of the acyl-CoA:lysophosphatidylcholine acyltransferase. This latter enzyme has been described in yeast, but not studied in the presence of PUFAs (43). Using microsomal preparations from developing safflower cotyledons and rat liver, it was shown that the reverse reaction catalyzed by the acyl-CoA:lysophosphatidylcholine acyltransferase represented less than 5% of the forward reaction (44). In our study, fatty acids exogenously supplied or endogenously produced in the acyl-CoA pool were found enriched in PC and in particular at its sn-2 position. Wagner and Paltapuf (29) also found most of the exogenously supplied, labeled fatty acids at this particular position.
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position even after 2 min, suggesting that transfer from the acyl-CoA pool into the sn-2 position of PC is very efficient in yeast. As is clearly shown in Fig. 7 the reverse reaction is significantly lower and results in the accumulation of GLA at the sn-2 position of PC, which leads to low elongation activity. These data suggest that a bottleneck, which limits VLC-PUFAs production in yeast, may be an insufficient supply of acyl-CoA substrates to the elongase.

Recently, we obtained the first transgenic linseed producing ARA or EPA by expressing the 5- and 6-elongases from P. tricornutum together with the 5-desaturase from the seeds of higher plants.2 Similar to the results obtained in yeast, 6-desaturated fatty acids were present in high proportion in total fatty acids, but nearly absent from the acyl-CoA pool, suggesting that the same problem exists in the seeds of higher plants. In order to produce high levels of VLC-PUFAs in oil seed crops, particularly docosahexaenoic acid (22:6n-3), the synthesis requires another elongation step after the lipid-linked 5-desaturation, this bottleneck has to be circumvented.

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