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The evolution of desaturases

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1. Introduction

When considering the evolution of desaturases, several different aspects come into focus, the most obvious ones being phylogenetic origins and differentiation of regioselectivities of these enzymes. In this general context the term desaturase includes all enzymes able to activate oxygen and to use this reagent for a subsequent modification of C–H bonds at saturated or monounsaturated carbons in substrates as diverse as alkyl groups, acyl residues in thio-, amide- or oxygen-ester linkage, carotenoids, sphingolipids, aldehydes and sterols [1,2]. The presently known oxygen-dependent modifications do not only include the formation of *cis*- and *trans*-double bonds, they also result in the production of acetylenic bonds, insertion of hydroxy or epoxy groups, and even the postulated decarbonylation of aldehydes or dehydrogenation of ubiquinols [3]. This wide spectrum of reactions is catalysed by proteins which all (as extrapolated from the few examples actually studied in detail) may house a di-iron complex held in place by the side chains of suitable amino acids (histidine, aspartate, glutamate and glutamine), although some similar reactions are catalysed by the heme iron of cytochrome P450 isoforms [4]. It should also be pointed out that the mitochondrial dehydrogenation of ubiquinol by the alternative oxidase [3] does not attack a C–H–, but an O–H bond. If the activity of this enzyme does in fact rely on a di-iron centre, it seems to make use of an overpowered reagent for a reaction which normally involves the completely different di-iron–sulphur cluster of the Rieske protein.

2. Soluble and membrane-bound desaturases are unrelated groups

Based on amino acid sequences, two completely unrelated desaturase groups exist despite some similarities regarding cofactor use and stereochemistry of hydrogen removal [1]. The first group is represented in the present context by the soluble acyl–acyl carrier protein (ACP) desaturases found so far only in plants, where they are localized exclusively in plastids. X-ray analyses of several, functionally different members of this diverse family, including the plant stearoyl-ACP desaturase [5], have provided the prototype model for this type of desaturases. The di-iron complex is kept in a channel formed in the middle along the axis of a four-helix bundle. After docking of the acyl–ACP, the substrate acyl residue has to be inserted with the methyl end going in first. The electrons required for acyl group desaturation are delivered from ferredoxin and have to reach the di-iron centre enclosed by the helix bundle. The docking site for ferredoxin and the actual path of the two successively delivered electrons have not been identified yet. As typical for electron transport within proteins [6], most of the distance may be covered by travelling along the bonding system of the polypeptide (''through bond''), since no cofactors are bound to the enzyme. Similarly, the access pathway of the oxygen is not known, but in contrast to the electrons, the hydrophobic oxygen molecule may slip through transient gaps resulting from conformational protein/helix movements (''breathing''). All these details may require co-evolution following a change in an amino acid in the desaturase or in the electron donor.

The amino acid residues involved in binding the di-iron complex in this type of desaturases form two characteristic D/EXXH motifs [1,2]. In the second group of desaturases they are replaced by three so-called histidine boxes (see below). A functional explanation for this replacement cannot be given, since in many cases both types of enzymes catalyse equivalent reactions. This situation may represent a convergent development of two unrelated proteins which have become desaturases by picking up two iron ions and combining them in slightly different ligand shells resulting in functionally very similar di-iron ensembles required for this type of catalysis.

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The second and larger group of desaturases comprises membrane-bound proteins including the various desaturases involved in the biosynthesis of polyunsaturated fatty acids [1,2]. These enzymes are characterized by the above-mentioned histidine boxes $H(X)_{3-4}H$, $H(X)_{2-3}HH$ and $H/Q(X)_{2-3}HH$. Similar to the first group, they require molecular oxygen and reducing equivalents delivered by ferredoxin (cyanobacterial and plastidial enzymes) or cytochrome b_5 (either in free or fused form). Hydropathy plots reveal 4–6 membrane spanning helices which account for nearly 30% of the amino acid sequence of these proteins. Assuming the formation of four transmembrane helices, the histidine boxes come to lie on the same side of the membrane. Therefore, the scattered sequence parts not involved in the formation of the membrane anchor may build up the catalytically active head. According to hydropathy plots these parts are not integrated into the hydrophobic part of the membrane and rather may represent a membrane-peripheral protein domain. In case that this domain has some structural similarity to the acyl–ACP desaturase as referred to above, a putative substrate channel should open towards the membrane interface to allow access of lipid-bound acyl groups. A similar situation is realized by prostaglandin synthase [7], which extracts arachidonic acid from the membrane bilayer after its release from phospholipids. But so far, no crystal structure is available for any member of the second group of desaturases, and accordingly, considerations regarding the details discussed above lack any structural or experimental basis. On the other hand, this kind of knowledge would be required for an assessment of the relevance of the differences in amino acid sequences observed between the different desaturase families. Our basic assumption on the evolution of paralogous desaturase activities is that after gene duplication [8] the actual active site regarding its size, the chemistry and geometry of oxygen binding as well as the positioning of the ethylene segment to be modified were kept similar within close limits, at least regarding the formation of *cis*-double bonds in acyl groups. According to this picture, the crucial creation of new regioselectivities would not involve the active site, but would mainly affect adjacent sequence parts forming the substrate channel (if it does exist), whereas the membrane-spanning helices may contribute very little to a shift in reaction specificity. We will see whether the alignment of partial sequences from different desaturases and site-directed mutagenesis do confer with these assumptions. A well known, but not understood exception of the rather invariant structure of the active site [1,2] is the replacement of a single histidine by glutamine in the third histidine box of front-end desaturases [$H(X)_{2-3}HH$ by QXXHH].

3. Phylogenetic roots of acyl–ACP desaturases are unknown

A first series of questions refers to the phylogenetic origin of the two desaturase groups and to the timing in the subsequent differentiation resulting in various regioselectivities as well as in other reactivities. Furthermore, it would be particularly interesting to know, which regioselectivities were already present in the last common ancestor of the eukaryotic lineages or whether several regioselectivities had to be developed repeatedly and independently later on? As usual we assume that sequences present in prokaryotes may represent ancestors of proteins found in eukaryotes. The most complex pattern in eukaryotic organisms has evolved in plant cells: in addition to the soluble acyl–ACP desaturases in plastids, they express two sets of membrane-bound desaturases in plastids and in the endoplasmic reticulum (ER). All of them may have different phylogenetic roots as typical for a cell type being the product of sequential, multiple endosymbiosis [9].

Despite the fact that all plants require the constitutive expression of the plastidial stearoyl-ACP-Δ9-desaturase for insertion of the first *cis*-Δ9-double bond present in oleic acid and its polyunsaturated derivatives found in all compartments, it has to be stressed that the origin of this acyl–ACP desaturase is not clear. The obvious phylogenetic sources, the cyanobacteria, lack this soluble protein [10]. For the insertion of the first Δ9-double bond, they make use of a completely different, membrane-bound enzyme with histidine boxes (DES C). It uses the stearoyl ester groups of thylakoid membrane lipids and has a close relationship to the acyl CoA-desaturases (see below). On the other hand, the dating back of plastidial enzymes including desaturases does not necessarily end with cyanobacteria [11]. The oxygenic cyanobacteria are themselves considered as having experienced extensive precyanobacterial gene transfer from different phototrophic prokaryotes. But these organisms, expressing photosystems resembling either PSI (green sulphur bacteria and Gram-positive heliobacteria) or PSII (purple bacteria and green non-sulphur bacteria) of oxygenic photosynthesis, all carry out anoxygenic photosynthesis and, therefore, may produce monounsaturated fatty acids via the anaerobic pathway. The available sequence data from members of these groups (*Rhodobacter*, *Chlorobium*) (see [12]) do not provide evidence for the existence of proteins related to acyl–ACP desaturases.

On the other hand, from a functional point of view, prokaryotes may not at all require a soluble desaturase for introducing the first double bond. In plastids of higher plants the conversion of stearoyl into oleoyl residues is carried out with soluble ACP-substrates before incorporation of acyl groups into membrane lipids. This enables plastids to export oleic acid residues

into the extraplastidial compartment which has no capacity for de novo biosynthesis of fatty acids [13]. The acyl–ACP desaturase and an additionally required acyl–ACP thioesterase are characteristic members of the export machinery by which plastids serve all other cellular compartments. Normally prokaryotes do not continuously export acyl groups, and accordingly, the function of the plastidial acyl–ACP desaturase (and thioesterase) may have necessitated significant reconstruction of a prokaryotic ancestral protein which therefore is difficult to recognize. As mentioned above, the alternative oxidase in mitochondria [3] is a distantly related member of the acyl–ACP desaturase group, but despite its mitochondrial localization, this protein has not necessarily to be derived from those symbiotic prokaryotes of the α-proteobacterial group finally becoming mitochondria [14], where such proteins cannot be found at present.

The only prokaryotic sequences coding for proteins similar to acyl–ACP desaturases, but of unknown function (Fig. 1) are found in different strains of *Mycobacterium* and *Streptomyces* [12]. The relevance of this observation is presently restricted to the fact that such sequences do exist in prokaryotic organisms. Biochemical studies with soluble proteins prepared from *Mycobacterium smegmatis* [15] have demonstrated the existence of an acyl-CoA desaturase which converts C24:0-CoA into Δ15/ω9-24:1. Cofactors were oxygen, NADPH, a reductase and a ferredoxin. This resembles an acyl–ACP desaturase system, but unfortunately, sequence data are not available from *M. smegmatis*. On the other hand, in separate studies with cell-free systems of *Mycobacterium phlei* on aerobic fatty acid desaturation, the introduction of the Δ9-double bond required a 100,000*g*-sediment as enzyme source [16]. Therefore, this desaturase activity was most likely due to a membrane-bound enzyme with histidine boxes and not to a soluble acyl–ACP desaturase. These data point to the interesting possibility that in the genus *Mycobacterium* both soluble and membrane-bound enzymes for desaturation of saturated substrates may be present. In this context it should be mentioned that an efficient *Escherichia coli* system has been developed to select mutants of the plant acyl–ACP desaturase [17]. After appropriate modification, this system could be useful to screen for the presence of functional orthologues of acyl–ACP desaturases in prokaryotes to shed light on the phylogenetic origin of this important desaturase group.

Several plant families express two different acyl–ACP desaturase activities [1], the house-keeping stearoyl-ACP Δ9-desaturase and a paralogous enzyme with deviating regio- and/or chain-length selectivity (Fig. 1). These additional enzymes are involved in the biosynthesis of "exotic" fatty acids for reserve triacylglycerols and share from 60% to 80% amino acid sequence identity with the corresponding house-keeping activity of the same plant. Based on the crystal structure of the stearoyl-ACP Δ9-desaturase [5] and the knowledge of the geometry of the substrate channel in these proteins, the structure of this channel was changed by genetic engineering. In one case two amino acid changes were sufficient to shift the chain-length selectivity of the castor bean enzyme from stearic (18:0) to palmitic acid (16:0) without changing the Δ9-regioselectivity [18]. In an even more impressing experiment [19], the palmitoyl-ACP Δ6-desaturase from *Thunbergia alata* was modified to accept the slightly longer stearoyl substrate group. By exchanging only five amino acids the "exotic" enzyme was completely converted without penalty for specific activity into an enzyme with house-keeping activity, i.e. with changed regioselectivity (from Δ6 to Δ9) as well as chain-length selectivity (from 16:0 to 18:0). This mutant sequence is included in the dendrogram (Fig. 1), but due to the small difference to the parent enzyme (about 0.1%) it does not align with the house-keeping Δ9-desaturase, but stays with the "exotic" precursor Δ6-desaturase. On the other hand, the functionally identical isoforms of the house-keeping Δ9-desaturases (several genes in *Arabidopsis thaliana*) show up as distinct ramifications.

These data on molecular engineering of desaturases support our assumption that actually a very few changes of amino acids at the right place may be sufficient to establish a paralogous activity. These small differences would never result in the deep branchings showing up in Fig. 1 and all the subsequent dendrograms. These branchings are ascribed to sequence drift after gene duplications which have occurred at different time points.

4. Two groups of membrane-bound desaturases with histidine boxes

An alignment of nearly 400 desaturase-like sequences belonging to the group of membrane-bound proteins with the above-mentioned histidine boxes results in a separation of two large groups (Fig. 2). They differ in the spacing between the first and third histidine box, whereas the hydrophobic parts (putative membrane anchors) are of similar size in both groups [20]. This difference in length may be ascribed to an insertion/deletion dating far back in evolution as evident from the presence of members from yeast, plants and mammals in both groups. Since the group with the "short" sequence contains only one prokaryotic organism (the carotene hydroxylase of *Paracoccus marcusii*) compared to many prokaryotes in the "long" group, it may be assumed that the "short" sequence was derived by a loss from the "long" sequence. It is interesting that all of the enzymes desaturating acyl groups and sphingolipids are found in

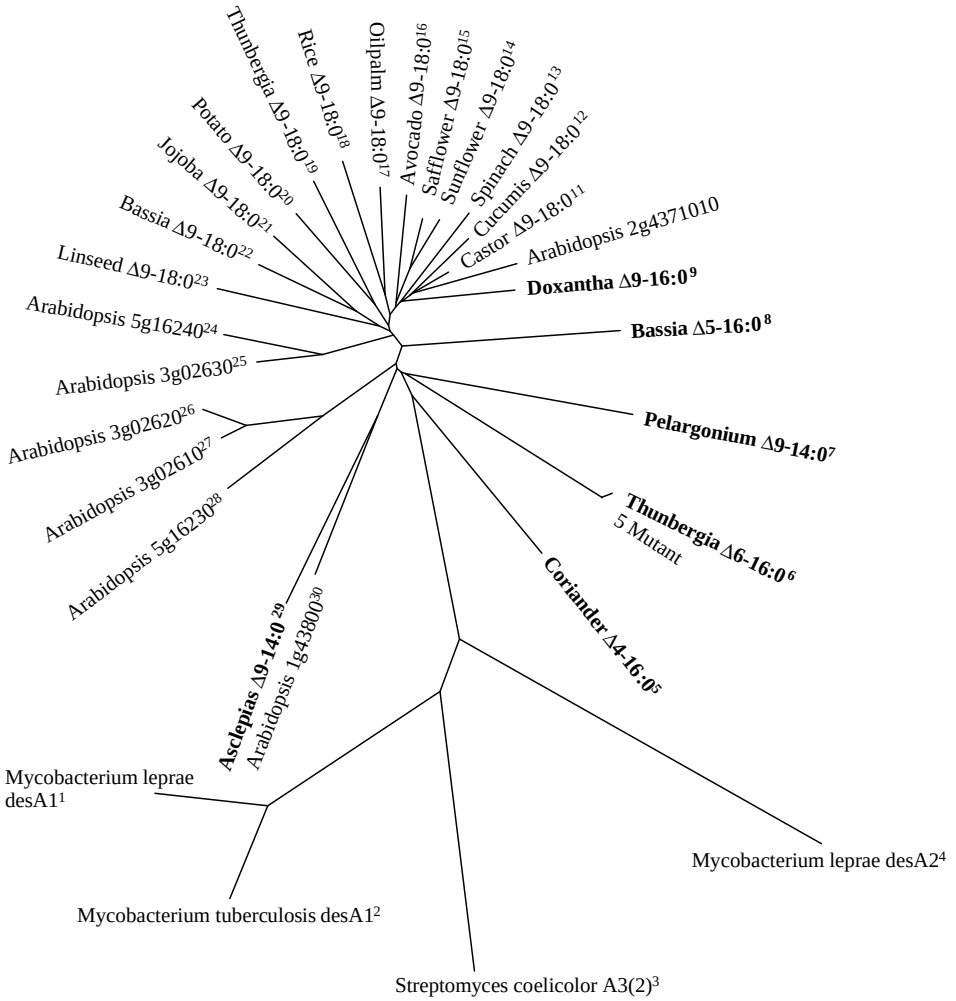


Fig. 1. Dendrogram of soluble acyl–ACP desaturases from plants and related sequences from prokaryotes. The regioselectivity of the introduced *cis*-double bond is indicated by Δ preceding the chain-length selectivity. Enzymes contributing to the synthesis of "exotic" fatty acids are indicated by bold printing. The prototype of the house-keeping stearoyl-ACP Δ9-desaturase is represented by the enzyme from castor bean (11) from which the crystalline structure is known [5]. Isoenzymes from *A. thaliana* are scattered in the upper part of the dendrogram (10, 24–28, 30). The "exotic" Δ6-16:0 desaturase from *Thunbergia alata* (6) has been converted by a change of five amino acids into a house-keeping Δ9-18:0 desaturase [19] which aligns with its "exotic" precursor. The sequences from *Mycobacterium* and *Streptomyces* (even more can be extracted from databases) [12] encode proteins of presently unknown functions. Accession numbers: [1]NP_302431, [2]NP_215339, [3]NP_630790, [4]NP_302317, [5]A47245, [6]AAA82160, [7]AAC49421, [8]AAL26876, [9]AAC05293, [10]NP_181899, [11]AAA74692, [12]AAA33130, [13]CAA44687, [14]CAC80360, [15]P22243, [16]AAF15308, [17]AAD33903, [18]Q40731, [19]AAA61560, [20]P46253, [21]Q01753, [22]AAL26877, [23]P32062, [24]NP_197128, [25]NP_186912, [26]NP_186911, [27]NP_186910, [28]NP_197127, [29]AAC49719, [30]NP_175048.

the group with the larger spacing, whereas the only formally typical desaturase in the other group is the sterol C5-desaturase (Erg3). The other members with a short spacing catalyse hydroxylations at C25 of sterol side chains, at the C4-position of sphingoid bases (Sur2), at the α-position of amide-linked acyl residues of sphingolipids (FAH, Scs7) and the decarbonylation of aldehydes (CER1). With regard to the C4-hydroxylation of sphingoid bases, we would like to point out that the same reaction is catalysed by enzymes from both groups (the monofunctional Sur2 from yeast with a short spacing and the bifunctional DES2 from mouse with a long spacing having desaturase/hydroxylase activity). This situation represents a convergent development between two paralogous proteins.

Furthermore, at this point we would like to recall the above-mentioned overlap in reaction outcome between desaturases and cytochrome P450 enzymes: the C22-*trans*-desaturation of the sterol side chain is catalysed by a cytochrome P450 isoform [21], whereas the hydroxylation at C25 is catalysed by the desaturase-like hydroxylase. As discussed below, hydroxylase functions have also emerged several times in the group with the widely spaced histidine boxes. Since this group comprises the classical acyl group desaturases, we will have a closer look at an enlarged form of this part of Fig. 2. It will be filled up with characteristic members of all presently known desaturase groups with emphasis on functionally expressed sequences. To cover all the different regioselectivities, it will also include two groups

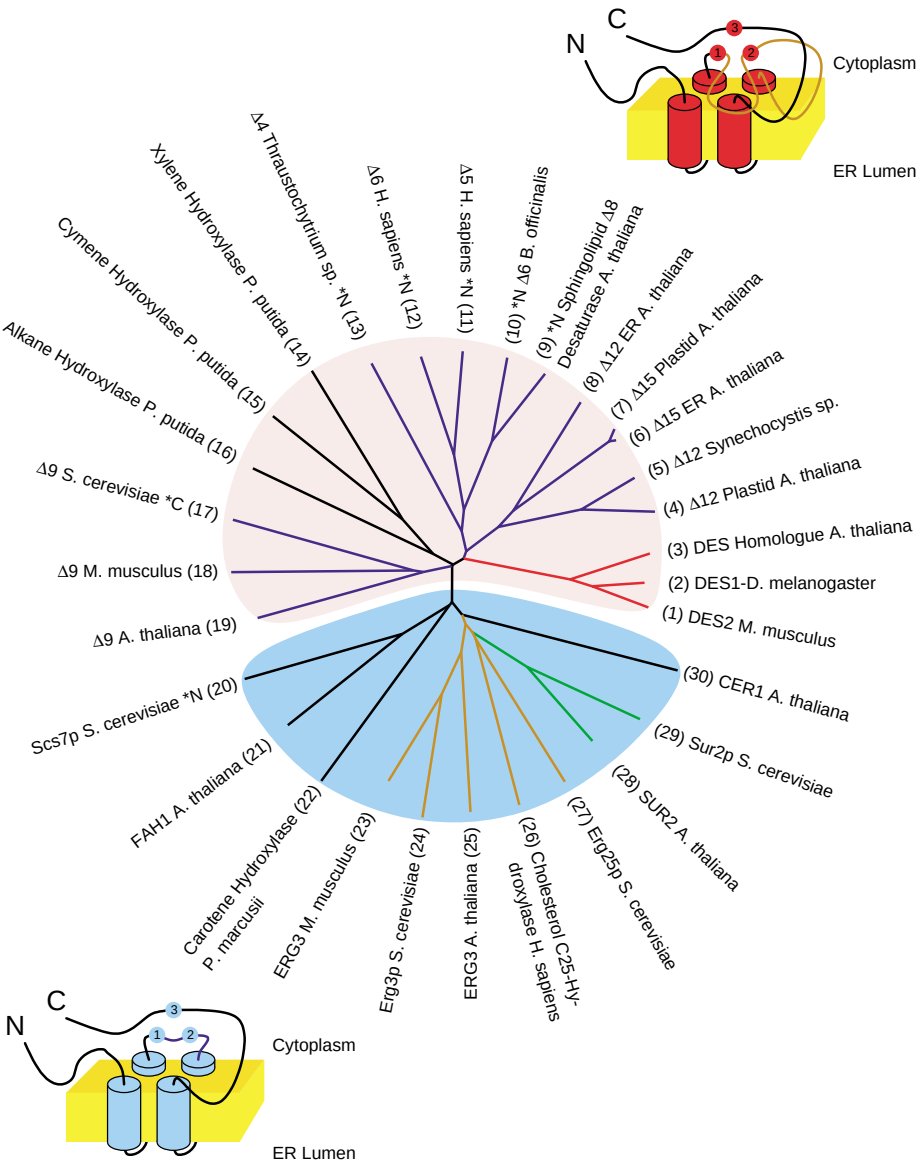


Fig. 2. Dendrogram showing similarities between selected membrane-bound desaturases and hydroxylases. Red, sphingolipid Δ4-desaturases; blue, fatty acid desaturases and sphingolipid Δ8-desaturase (sequence 9); green, sphingolipid C4-hydroxylases; brown, sterol desaturases and hydroxylases. *N, N-terminal cytochrome b_5 fusion protein; *C, C-terminal cytochrome b_5 fusion protein; ER, endoplasmic reticulum. The pink and light blue background indicates two groups of desaturase and hydroxylase sequences distinguished by a different spacing (in primary structure) between the first and second histidine box and between the second histidine box and the third transmembrane helix. The small pictures illustrate the proposed topology of membrane-bound desaturases and hydroxylases, assuming an integration into the ER membrane (yellow) with four transmembrane helices (coloured cylinders) and an active site formed by the three histidine boxes (coloured balls) on the cytoplasmic face of the membrane. In the sequences with the pink background, the first and the second histidine box as well as the second histidine box and the third transmembrane helix are separated by longer sequence segments (highlighted in brown) than in the sequences with the light blue background (corresponding segments highlighted in blue). The parts of the sequence that are missing in the group with light blue background contain a core of hydrophobic amino acids, indicating that they might be incorporated into a possible globular structure on the cytoplasmic face of the membrane or that they might be involved in the formation of a hydrophobic lipid binding site. The dendrogram has been constructed from pairwise similarities of full-length amino acid sequences using T-COFFEE and TreeView. GenBank™ protein accession numbers are: (1) AAM12533, (2) AAM12535, (3) AAD17340, (4) CAA18198, (5) P20388, (6) P48623, (7) P48622, (8) P46313, (9) T47950, (10) AAD01410, (11) AAF70457, (12) AAD31282, (13) AAM09688, (14) P21395, (15) AAB62299, (16) CAB51047, (17) AAA34826, (18) NP 033154, (19) BAA25181, (20) NP 013999, (21) T01359, (22) CAB56060, (23) O88822, (24) NP 013157, (25) AAD38120, (26) NP 003947, (27) NP 011574, (28) AAF43928, (29) NP 010583, (30) AAC24374.

of "short" desaturases. At the present stage, this dendrogram (Fig. 3) may be separated into 6–8 branching systems comprising more or less closely related enzymes.

5. Phylogenetic roots of membrane-bound desaturases

In contrast to the situation with the acyl–ACP desaturases, there is more functional evidence for the

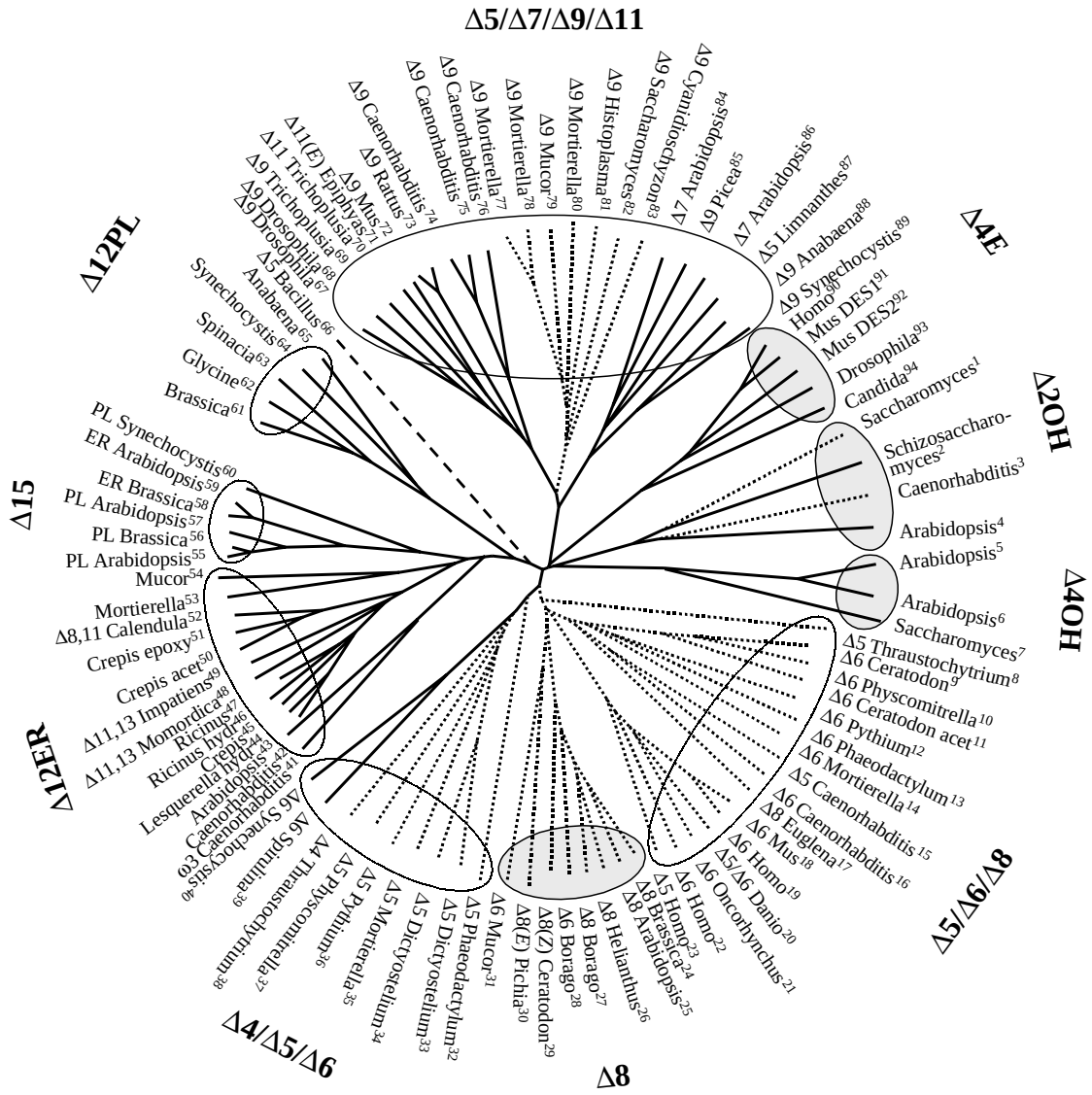


Fig. 3. Regioselectivity phylogram of lipid desaturases and modifying enzymes. Full-length amino acid sequences were aligned and grouped in a radial diagram according to different regioselectivities using the programs CLUSTALX and TreeView. The regioselectivities are marked by numbers (simplified as Δ-desaturases) and subcellular compartments (PL = plastidial, ER = microsomal). The branches designated as Δ2OH and Δ4OH refer to sphingolipid acyl amide α-hydroxylases and sphingolipid sphingoid C4-hydroxylases, whereas Δ4*E* and Δ8 refer to sphingolipid sphingoid desaturases, respectively. All sphingolipid-modifying enzyme groups are marked by grey background. Cytochrome b_5 fusion proteins are indicated by dotted branches, and exotic modifications (hydroxylation, epoxidation, acetylenation and conjugation) are mentioned behind the organism. The prokaryotic desaturase from *B. subtilis* is indicated by a dashed branch. Accession numbers: [1]Z49260, [2]Z97209, [3]Z81038, [4]AF021804, [5]AC012188, [6]AC013289, [7]AAA16608, [8]AF489588, [9]AJ250735, [10]AJ222980, [11]AJ250734, [12]AF419296, [13]AY082393, [14]AF110510, [15]AF078796, [16]Z70271, [17]AF139720, [18]W63753, [19]AF084559, [20]AF309556, [21]AF301910, [22]AF084560, [23]AF199696, [24]AJ224160, [25]AJ224161, [26]X87143, [27]AF133728, [28]U79010, [29]unpublished, [30]unpublished, [31]AF296076 [32]AY082392, [33]AB022097, [34]AB029311, [35]AF054824, [36]AF419297, [37]reference [100], [38]AF489589, [39]X87094, [40]L11421, [41]L41807, [42]AF240777, [43]L26296, [44]AF016103, [45]Y16284, [46]U22378, [47]Patent: WO 96/10075, [48]U86374, [49]AF182520, [50]Y16283, [51]AF182521, [52]Y16285, [53]AJ245958, [54]AF161219, [55]D14007, [56]L22963, [57]D17578, [58]P48624, [59]P48623, [60]D13780, [61]P48627, [62]P48628, [63]X78311, [64]P20388, [65]D14581, [66]AF037430, [67]U73160, [68]AJ271415, [69]AF038050, [70]AF035375, [71]AY049741, [72]M21285, [73]J02585, [74]AF260243, [75]AF260244, [76]AF260242, [77]AF0085500, [78]Y18554, [79]AF026401, [80]AJ278339, [81]X85963, [82]P21147, [83]AB006677, [84]AB017071, [85]AF438199, [86]AB017071, [87]AF247133, [88]D14581, [89]D16547, [90]AF466375, [91]AF466376, [92]AF466377, 93AF466379, [94]C. *albicans* genomic sequence, bases 7499-8611 of contig 6-2340. *Note*: Independent alignments of the cytochrome b_5- and desaturase domains, respectively, as well as of the transmembrane helices result in dendrograms not differing from this figure which is based on full-length desaturase sequences. For clarity, only a selection of available sequences has been used to construct the dendrogram. The inclusion of more of the presently known sequences does not affect the regioselectivity branchings.

existence of prokaryotic members of membrane-bound desaturases with three histidine boxes. A large number of biochemical studies has been carried out on the aerobic introduction of *cis*-double bonds at various positions of acyl substrates using intact bacteria of different systematic groups [22]. Evidence for the involvement of membrane-bound enzymes (most likely desaturases with histidine boxes) and thus excluding soluble acyl–ACP desaturases comes from experiments on the Δ9-regioselectivity of cell-free activities found in the high-speed sediments of *Mycobacterium* [16] and *Pseudomonas* [23]. But so far, only the Δ5-desaturase from *Bacillus subtilis* has been cloned and expressed in functional form in *E.coli* [24]. This clone was also used to complement the *ole1* mutant of *S. cerevisiae* lacking Δ9-desaturase activity (see below) [25].

The Δ5-desaturase from *B. subtilis* may be considered as representing a prototype orthologue of desaturases introducing the first double bond into a saturated acyl chain. In Fig. 3 this Δ5-desaturase forms a single-membered branch. It is deeply separated from all other desaturases including the group containing functionally related enzymes with Δ5-, Δ7-, Δ9- and Δ11-regioselectivities. Therefore, prokaryotic enzymes with other regioselectivities would be very useful to examine possible relationships to eukaryotic enzymes acting on saturated substrates. Several sequences of putative relevance in this context are present in databases which are not included in the alignment because their activity has not been verified experimentally. Similar to the possible use of the above-mentioned *E. coli* system [17] to screen for prokaryotic acyl–ACP desaturases, a system using expression in the *ole1* mutant of *S. cerevisiae* could be established to screen for prokaryotic acyl-CoA desaturases of different regioselectivities [25].

From biophysical studies it is well known that the transition temperature of membrane lipids depends (among other factors) on the location of *cis*-double bonds in the acyl groups. If the evolution of organisms followed a gradient from higher to lower temperatures with the need of appropriate adjustment of biomembrane fluidity, it may well be that the first regioselectivity required was one operating close to the carboxyl or methyl end of the acyl groups to avoid the drastic fluidization resulting from an introduction of the first double bond into the middle of the acyl chains. This is documented by the transition temperatures of phosphatidylcholines (PC) with two stearoyl (58°C), Δ5-*cis*-octadecenoyl (10°C), Δ9-*cis*-octadecenoyl (−20°C) and ω5-*cis*-octadecenoyl (0°C) groups, respectively [26]. In this context it may be mentioned that Archaea make use of a fatty acid- and desaturase-independent way of membrane fluidization by different branching patterns of their ether-linked isoprenoid chains [27].

6. Desaturases introducing the first double bond into saturated substrates

Apart from the phylogenetically singular desaturase from *Bacillus*, the other enzymes known to introduce the first double bond into a saturated acyl group form a common branching system split into three subgroups (Fig. 3). This tripartite structure may be due either to differences in function (form of substrate accepted) or to the phylogenetic separation of the organisms in these groups (phototrophs, animals, and fungi).

The first one comprises enzymes from cyanobacteria and plants. The cyanobacterial desaturases introduce the first Δ9-double bond into (mainly) stearoyl residues at the *sn*-1 position of membrane lipids irrespective of the lipid headgroup [10]. The plant enzyme from chloroplasts of so-called 16:3-plants introduces the first Δ7-double bond exclusively into palmitoyl residues at the *sn*-2 position of monogalactosyl diacylglycerol [28]. As discussed above, the cyanobacterial Δ9-desaturase replaces the plastidial stearoyl-ACP Δ9-desaturase, and the Δ7-desaturase of chloroplasts may actually be a paraloguous activity of this enzyme. But it should be mentioned that the identity of the Δ7-desaturase [29] has not yet been verified by functional expression. The specificity with regard to *sn*-1/2-location of acyl groups will be discussed in more detail below.

Surprisingly, also the Δ9- and Δ5-desaturases from the plants *Picea glauca* [30] and *Limnanthes douglasii* [31] fall into this branch. These enzymes are most likely localized in ER membranes and are believed to use saturated acyl-CoA thioesters. Functionally they would fit better into the next subgroup containing "true" acyl-CoA desaturases. From the clustering with lipid desaturases of these Δ5- and Δ9-desaturases and of other putative acyl-CoA desaturases from plants, another possibility would be that they do not accept saturated acyl-CoAs, but acyl groups of phospholipids as typical for many other desaturases (see below). In this context, it should be mentioned that in plant reserve tissues, other saturated acyl chains are also channelled through PC on their way into triacylglycerols [32]. The fact that acyl-CoA desaturases are membrane-bound enzymes agrees with the partitioning of their substrates from an aqueous solution into hydrophobic surfaces and their immunological detection in ER membranes [33].

The second subgroup comprises acyl-CoA desaturases from insects, nematodes and mammals with Δ9- and Δ11-regioselectivities, with separate enzymes leading to *cis*- or to *trans*-fatty acids. The Δ9- and Δ11-desaturases from the moths (*Trichoplusia ni* [34] and *Epiphyas postvittana* [35]) show a very deep separation and align according to regioselectivity (Δ9 versus Δ11) irrespective of their stereospecificity, since the *trans*-Δ11-desaturase from *Epiphyas* [35] aligns with the *cis*-Δ11-desaturase

from *Trichoplusia* [34]. This may indicate that the Δ11-activity required for hormone biosynthesis is a basic and accordingly old necessity for the life cycle of these organisms. Also *Drosophila melanogaster* contains two acyl-CoA Δ9-desaturases falling into this group [36]. They differ by chain-length specificity with one accepting palmitoyl and stearoyl residues, whereas the other is restricted to myristoyl groups.

The deep separations of these insect sequences contrasts with the high similarity existing between the Δ9-desaturase isoforms found in the nematode *Caenorhabditis elegans* [37] and the fungus *Mortierella alpina* [39] (see below) indicating more recent duplications in those organisms. We would like to point out that all these differences in function are reflected by branchings of different depths, which are in contrast to the engineered differences referred to above (Fig. 1). Therefore, these branchings may not represent the sequence alterations required for a functional change but rather reflect the time elapsed since gene duplication.

The third subgroup contains functionally similar enzymes restricted to the Δ9-regioselectivity, but differing by the fact that they carry a cytochrome b_5-domain fused to the C-terminus of the protein sequence. With the exception of a red alga (*Cyanidioschyzon* [38]), this C-terminal fusion has so far only been observed in fungal Δ9-desaturases, but the clustering of these fusion proteins is not due to their cytochrome b_5-portions, since the same branching does result from an alignment of the desaturase sequences after removal of the cytochrome b_5-domains. The relevance of the fusion with the electron donor will be discussed in connection with the front-end desaturases. The separation of the three genes from *M. alpina* should be pointed out, from which two are closely related, whereas the third one is significantly different regarding sequence and function [39]. For unknown reasons it cannot complement the *ole*1 mutant of *S. cerevisiae*, despite the fact that it shows Δ9-desaturase activity on expression in yeast. The three Δ9-desaturases from *C. elegans* differ by chain-length selectivity, with two closely related enzymes/sequences accepting stearate and palmitate, whereas the third one is limited to palmitate [40].

7. Enzymes modifying sphingolipid components

Sphingolipids are widespread in eukaryotes, but also a few bacteria produce these membrane lipids [41]. Therefore, the enzymes contributing to their biosynthesis may originate from prokaryotic ancestors. The hydrophobic building blocks (ceramides) of these lipids are long-chain bases and long-chain amide-bound fatty acids, which both are substrates for membrane-bound desaturases and hydroxylases. Similar to the group of fatty acyl desaturases just discussed, most of these enzymes have to deal with saturated substrates, but this formal similarity in function regarding the degree of substrate unsaturation is not reflected by sequence similarity. On the other hand, these enzymes provide examples for an evolutionary separation of hydroxylases from desaturases, of enzymes with fused from those with non-fused electron donor and of *trans*-desaturases (i.e. those operating with *trans*-stereochemistry) from *cis*-desaturases.

The four different groups of enzymes (acyl amide α-hydroxylase, sphingoid C4-hydroxylase, sphingoid Δ4-*trans*-desaturase, sphingoid Δ8-*cis*/*trans*-desaturase) represent four independent branches (Fig. 3, grey background). Two of these activities (α-hydroxylase and C4-hydroxylase) are "short" desaturases (Fig. 2), but they are discussed here in a different context. The fact that these four branches originate in the middle of the phylogram indicates a very early separation of these paralogous groups, which may have been present in the oldest eukaryotes. The invariant presence of the N-terminal cytochrome b_5-domain in the Δ8-desaturases, its occasional presence in the α-hydroxylases and its absence in Δ4-desaturases and C4-hydroxylases points to the difficulties in understanding the functional significance or advantage of this fusion [42].

With regard to the discussion of the *sn*-1/2-regioselectivity of cytochrome b_5-fused front-end desaturases (confined in action to the *sn*-2-position, see below), it should be pointed out that the amide acyl residue of sphingolipids is in a configurational position reflecting the *sn*-2-position of *sn*-1,2-diacyl glycerolipids, whereas the alkyl chain of the long-chain sphingoid base corresponds to the *sn*-1 substituent [43]. But in the case of Δ8-sphingolipid desaturases the presence of a cytochrome b_5-fusion is not linked to a preferred *sn*-2-regioselectivity as typical for the fused acyl group desaturases. In this context, another example for the functional overlap between cytochrome P450 and desaturase paralogues becomes evident, since the bacterium *Sphingomonas* makes use of a cytochrome P450 for α-hydroxylation of acyl amide groups in sphingolipids [44].

Furthermore, the sphingoid desaturases and hydroxylases represent examples for the similarity in sequence and initial reaction steps of paralogous di-iron enzymes. Unexpectedly, the Δ4-*trans*-desaturases and the C4-D-hydroxylases have separated long ago as evident from their clustering with long and short enzymes [20], respectively (Fig. 2). On the other hand, in mouse a Δ4-desaturase paralogue acquired additional C4-hydroxylase activity and became a bifunctional enzyme [20]. This may be considered as a recent "reinvention of the wheel". In contrast, desaturase/hydroxylase pairs of closer sequence similarity occur in higher plants (see below).

8. Formation of *trans*-double bonds

Another point of general relevance to be discussed in the context of sphingolipid modification is the occurrence of desaturases with different stereochemistry resulting in *cis*- and *trans*-double bonds. Some members produce either only *trans*-double bonds (Δ4-desaturases from all organisms and Δ8-desaturases from fungi), others yield mixtures containing varying proportions of *cis*- and *trans*-desaturated products (most bifunctional Δ8-desaturases of plant origin) [45] and a minority produces only Δ8-*cis*-desaturated sphingoid bases (some plant enzymes).

Considering fatty acyl group desaturation, the majority of the enzymes produces exclusively *cis*-double bonds, but a few insert *trans*-double bonds. Examples are 3-*trans*-hexadecenoic acid at the *sn*-2 position of plastidial phosphatidylglycerol [28] (not cloned yet and not present in cyanobacteria) and acyl-CoA desaturases from insects converting myristic and palmitic acid into the corresponding 11-*trans*-fatty acids required for pheromone biosynthesis [35]. The stereochemical considerations outlined in the following apply in similar form to the formation of conjugated pairs of *trans*/*trans*- and *cis*/*trans*-double bonds adjacent to a *cis*-double bond in α-eleostearic and punicic acid to be discussed below. The sequence data do not yet allow a recognition of the critical differences in amino acids responsible for the differences in stereochemical outcome of a desaturase reaction. We assume that too many amino acid changes not related to, but compatible with a change in this reaction detail have accumulated and cover the relatively few critical exchanges.

From the mechanism of the reaction at the di-iron site it has been suggested [45,46] that the abstraction of the two protons occurs in a stepwise manner, but stereochemically as a *syn*-elimination (Fig. 4a). The lifetime of the intermediate radical at the first carbon is too short to allow a rotation of the C–C bond with concomitant presentation of the originally *trans*-oriented proton to the di-iron centre. In the case of Δ8-*cis*-double bonds (as in all *cis*-desaturations), two *pro(R)*-protons are lost from the vicinal methylene groups of the long-chain base, whereas in the case of the Δ8-*trans*-double bond the *pro(R)*-C8- and the *pro(S)*-C9-proton are lost [45]. Therefore, if a *trans*-double bond results from a *syn*-elimination, the carbon chain must have adopted a *trans*-conformation before the reaction starts (Fig. 4b). According to this model, the stereochemical outcome of the reaction is not controlled by the actual reactivity of the di-iron centre, but it is a matter of the geometry and size of the hydrophobic cavity in the immediate neighbourhood of the di-iron centre. Actually, the formation of a *trans*-double bond requires less conformational torsion of an extended acyl chain to bring the two protons to be eliminated into a *syn*-position than that required for a *cis*-double bond. Such a *cis*-conformation of a saturated acyl segment can be nicely seen in prostaglandin synthase co-crystallized with linoleic acid, which carries the substituents at the saturated C4–C5 carbon bond in *cis*-conformation [7]. The fact that the bifunctional plant Δ8-desaturases catalyse both *cis*- and *trans*-desaturations may be explained by assuming that their active site cavity is large or flexible enough to bind the substrate in two alternative conformations, each at a time for a single cycle with exclusive *cis*- or *trans*-outcome [45].

In this context experiments on the stereochemistry of the palmitoyl-CoA Δ11-desaturase from insects are most interesting [47]. Depending on the chain length of the substrate, the *trans*-isomer in the *cis*/*trans*-mixture of the Δ11-desaturated product decreases from 85% with tridecanoic acid via 65% with tetradecanoic acid to pure *cis*-products with pentadecanoic-, hexadecanoic- (the normal substrate) and heptadecanoic acids. This change was interpreted with the decreasing chance to accommodate two conformational isomers in the active site, when the length of an overhanging alkyl residue is increased from a methyl to a pentyl group. In view of this discussion, it is not clear whether *cis*-, *trans*- or bifunctional, i.e. stereounspecific desaturases evolved first.

Before ending the discussion of sphingolipid desaturases, the close relationship between the plant sphingolipid Δ8-desaturases and the Δ6-acyl group desaturases from higher plants should be pointed out, which will be discussed in more detail in the context of the front-end desaturases. In fact, the branching system including both activities (sphingolipid Δ8- and acyl group Δ6-desaturases from higher plants) may be considered as part of a larger subfamily (Fig. 3).

9. Towards polyunsaturated fatty acids: additional double bonds

Most eukaryotic organisms, both poikilo- and homoiotherm, accumulate polyunsaturated fatty acids to maintain an appropriate viscosity in the hydrophobic core of biomembranes and to produce various effector molecules. For this purpose separate desaturase groups are required, since the above-mentioned enzymes accept only saturated substrates. We will not discuss the anaerobic polyketide systems producing polyunsaturated fatty acids by an alternative pathway [48].

Based on regioselectivity, two large desaturase groups can be separated which in eukaryotes are also recognized by sequence characteristics. One group introduces the next double bond between the existing one and the methyl end of the fatty acyl chain (''methyl-end'' desaturases). Members of the other group place the next double bond between the existing one and the

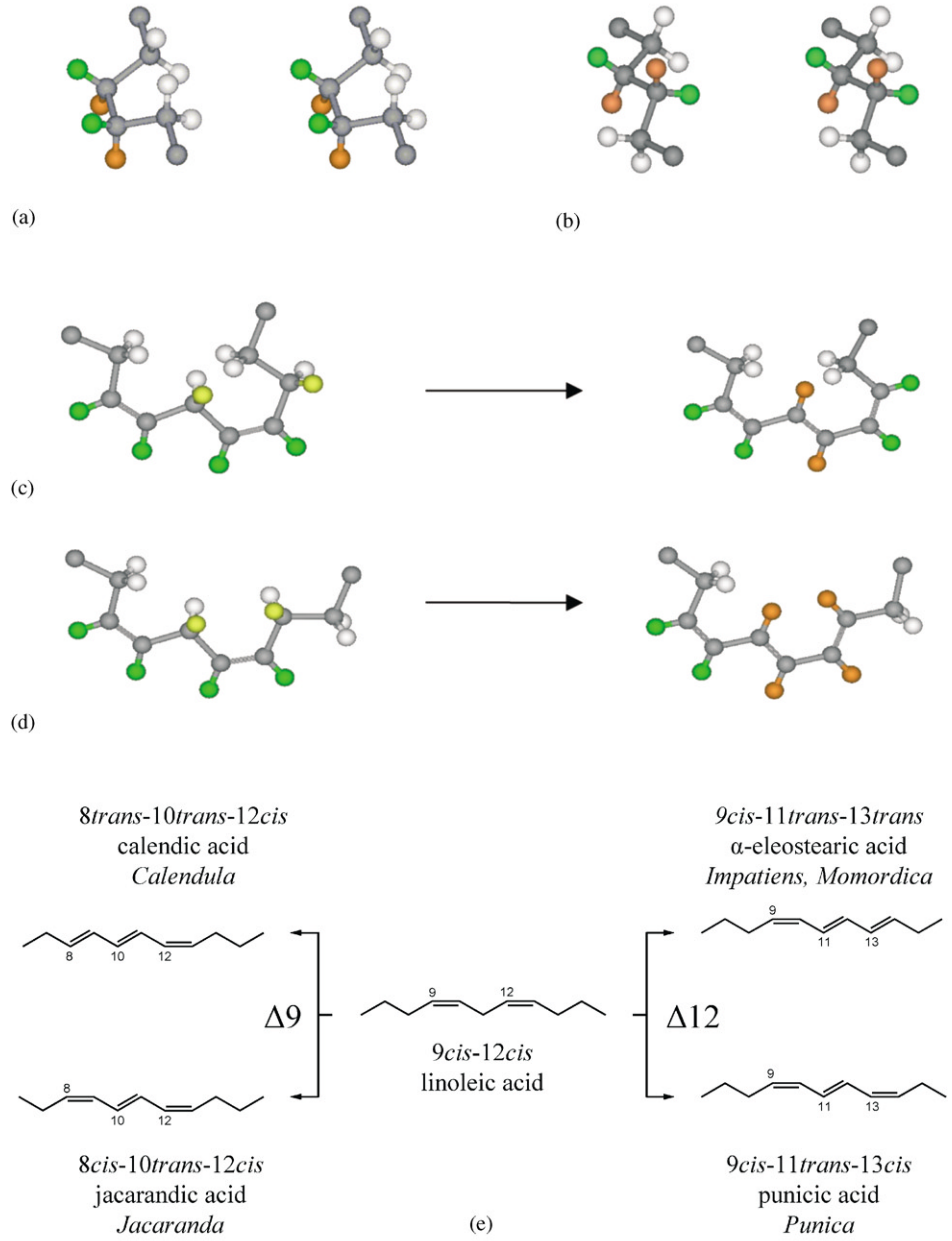


Fig. 4. Possible acyl/alkyl chain conformations enabling a *syn*-elimination of protons as required for the formation of a *cis*- or *trans*-double bond. (a) Stereo pair of an alkyl segment (C1-carbon at the top, methyl end at the bottom) with the two *pro(R)* protons (in green) to be removed for formation of a *cis*-double bond. Prior to proton abstraction the alkyl segment has already adopted a *cis*-conformation. (b) Stereo pair of an alkyl segment (top and bottom as above) with the *pro(R)* proton (green) and the *pro(S)* proton (red) to be removed for formation of a *trans*-double bond. Prior to proton abstraction the chain has already adopted a *trans*-configuration. This model has been proposed from experiments on the *cis*/*trans*-desaturations of long-chain sphingoid bases catalysed by the bifunctional Δ8-desaturase from plants [45]. (c and d) Formation of conjugated trienoic acids from linoleic acid with new *cis*- or *trans*-double bonds (bottom part e of the figure). These reactions are also believed to involve a *syn*-abstraction of two protons (yellow) flanking in allylic 1,4-positions one of the two *cis*-double bonds with olefinic protons in vinylic 1,2-positions (green). The (rate-limiting) [76] removal of one proton from C11 between the two original *cis*-double bonds results always in a new *trans*-double bond (*trans*-olefinic protons red) in conjugation to the *cis*-double bond persisting either at C9 or at C12. (c) A preexisting *cis*-conformation of the saturated substrate segment extending from the double bond to be transformed results in the new *cis*-double bond (*cis*-olefinic protons green) in punicic acid (original 12,13-double bond converted, segment extending towards the methyl end) or jacarandic acid (original 9,10-double bond converted, segment extending towards the carboxyl end). (d) A preexisting *trans*-conformation of the segment extending from the double bond to be transformed results in the new (second) *trans*-double bond (*trans*-olefinic protons red) in α-eleostearic acid (original 12,13-double bond converted, segment extending towards the methyl end) or calendic acid (original 9,10-double bond converted, segment extending towards the carboxyl end). The enzymes involved in the formation of these "exotic" fatty acids are discussed below and included in Fig. 5.

carboxyl terminus of the acyl group (''front-end'' desaturases [49]). The alignments in Fig. 3 suggest that ancient separations led to three desaturase groups which comprise the Δ12/Δ15 (ω6/ω3)-members, the Δ4-, Δ5- and the cyanobacterial Δ6-desaturases as well as the third group with the Δ6/Δ5-members. Before discussing some details of this separation, particularly the clear delineation of two groups of front-end desaturases and the combined clustering of the methyl-end desaturases, a short comment on desaturase nomenclature should be made.

Based on the mode of locking in on the ethylene segment to be desaturated, desaturases may be separated into more than the two groups just mentioned [50]: those measuring their distance from the carboxyl end of the acyl group (Δ-mode); those measuring from the methyl end (ω-mode); those recognizing an existing double bond and inserting the next methylene-interrupted double bond towards the methyl end and the ''front end''-desaturases inserting the additional double bond towards the carboxyl end. In most cases, but actually for unknown reasons, the additional double bond forms or extends a methylene-interrupted pattern. Well-known exceptions to this rule are found in gymnosperm plants which produce series of Δ5,9-non-methylene-interrupted fatty acids of different chain length in seed oils [51].

Throughout this text, we will not pay specific attention to these differences and for simplicity use mainly the Δ- or ω-assignments. The structural basis for these specificities is not known and, apart from the third histidine box of the front-end desaturases (see below), may not involve the active site. The conservative structure of the active site may also include an invariant positioning of the di-iron centre with regard to the ethylene segment to be desaturated, since in all *cis*-desaturases investigated so far including the Δ5-desaturase from *Bacillus* [52], irrespective of the front- or methyl-end type, a large deuterium/proton kinetic isotope effect was observed with the carboxyl-closer and not with the distal methylene group [53].

For recognizing the phylogenetic roots of polyunsaturated fatty acids, we first will have a look at bacterial sources. Prokaryotic enzymes inserting in an oxygen-dependent reaction a second *cis*-double bond towards the methyl end of a Δ9-unsaturated acyl chain are unknown, and only occasionally 5,10-hexadecadienoate has been observed in *Bacillus licheniformis* [22]. Recently, reservations have been expressed on the reproducibility of these observations [25]. Therefore, immediate phylogenetic roots of these groups of desaturases in non-phototrophic prokaryotes are not obvious. The single, but significant exceptions are oxygenic cyanobacteria. Some members produce 6,9,12,15-*all cis*-octadecatetraenoic (stearidonic) acid and, therefore, have four different desaturases which all have been cloned [10]. On the other hand, a precyanobacterial source or origin is unknown, since all anoxygenic phototrophic prokaryotes produce only monounsaturated fatty acids [54]. We are left with a diversity of cyanobacterial regioselectivities not paralleled in any other type of prokaryote. Whether it is correlated with the fact that cyanobacteria were the first organisms producing, withstanding and finally making use of oxygen cannot be answered.

The cyanobacterial desaturases catalysing the synthesis of C18-polyunsaturated fatty acids up to stearidonic acid, i.e. the Δ6-, Δ9- and Δ12/Δ15-desaturases are located in completely different branching systems (Fig. 3). This suggests that they have been developed very long ago from a common ancestor. Multiple series of gene duplications and subsequent separation into paralogous regioselectivities may have led to the four groups of present-day cyanobacteria [10] with Δ9, Δ9 + Δ12, Δ9 + Δ12 + Δ6 and Δ9 + Δ12 + Δ6 + Δ15 regioselectivities, respectively. On the other hand, this fact and the corresponding alignment (Fig. 3) cannot be taken as evidence that it was a cyanobacterial gene pool which served as source to recruit all desaturases required for the biosynthesis of polyunsaturated fatty acids in eukaryotes.

10. The group of Δ12/ω6- and Δ15/ω3-desaturases

The alignments shown in Fig. 3 suggest that enzymes with both Δ12- and Δ15-regioselectivities have been developed from a common ancestor. The question is: of which regioselectivity and origin was this precursor? For the following reasons, it is more likely that the enzyme at the basis of this branching may have had Δ12-regioselectivity. Present-day Δ12-desaturases require substrates with a preexisting Δ9-double bond, which are converted in high yield into 9,12-dienoic acids [55]. On the other hand, present-day enzymes with Δ15-regioselectivity prefer 9,12-dienoic fatty acids and hardly accept Δ9-monoenoic substrates to produce 9,15-dienoic acids [50,56,57].

In Fig. 3 the cyanobacterial Δ12- and Δ15-desaturases are part of a single-rooted branching system which does not only comprise the corresponding members of higher plants, but also of fungi and nematodes, irrespective of their localization in membranes of plastids or ER. Despite some hesitation, we have to consider this as indicating a common and direct heritage of all these enzymes, particularly including fungal and animal ER enzymes. In this context it should be mentioned that primitive fungi belonging to the group of Oomycota (comprising genera with C20-polyunsaturated fatty acids such as *Pythium* and *Phytophthora*) are considered to have biochemical and cytological similarities to some members of the large and heterogeneous group

of chrysophytic algae, many of which produce polyunsaturated very long-chain fatty acids [58]. In particular, the dinoflagellates are known for their complicated cytology. Primary, secondary and even tertiary symbiotic events including acquisition of plastids by "symbiogenesis" with an eukaryotic alga, loss of this plastid and final reacquisition of another type of plastid, always paralleled by a partial transfer of genes into the nucleus, resulted in a mosaic structure of the genetic system [59]. Such events may have contributed to the evolution of eukaryotic cell types equipped with different sets of organelles [60].

As mentioned above, plants have two sets of independently operating desaturases, including those involved in the biosynthesis of polyunsaturated fatty acids [13,28]. Therefore, an obvious question is: where do the plastidial and ER desaturases come from? For a closer consideration of this question, we constructed a separate phylogram enlarging the corresponding branching system of Fig. 3 after inclusion of additional sequences (Fig. 5). These alignments suggest a common origin of plastidial and cyanobacterial $\Delta12/\omega6$-desaturases (lines 20/21 at the bottom of Fig. 5) in line with the origin of plastids from cyanobacterial symbionts [61]. An even closer similarity between the plastidial and cyanobacterial $\Delta15/\omega3$-desaturases points to the same phylogenetic relationship, and according to this alignment also the corresponding $\Delta15$-desaturases in the plant ER are derived from a cyanobacterial $\Delta15$-source, most likely from a symbiont (top three lines 1/2/3 in Fig. 5). It should be pointed out that a similarly close relationship does not show up between the plastidial (line 21) and the ER $\Delta12$-desaturases. In contrast, the $\Delta12$-desaturases and the related paralogues of the plant ER (lines 8-19) branch off at a different point in this system together with fungal $\Delta12$-desaturases (lines 6/7, *Mucor rouxii* [62] and *M. alpina* [62]) and with close similarity to the $\Delta12/\Delta15$-group (lines 4/5) from the nematode *C. elegans* [64,65], which forms a parallel ramification from this point. Nevertheless, in plants the $\Delta12$- and $\Delta15$-desaturase regioselectivities, irrespective of plastidial or ER localization, seem to have been developed long ago as cyanobacterial paralogues, which have been transferred (in separate events?) to the eukaryotic host to become a green plant cell with $\Delta12$-desaturases in plastids, $\Delta15$-desaturases in plastids and ER and $\Delta12$-desaturases in the ER.

A different situation seems to be realized in the nematode *C. elegans* (Fig. 5). The formation of a separate branch and its ramification into a $\Delta12$- and a $\Delta15/\omega3$-desaturase [64,65] suggest that this organism may have duplicated its own $\Delta12$-desaturase (of unknown descendence) to develop one copy into a nematode-specific $\Delta15/\omega3$-paralogue [37]. A similar event may have led to the $\Delta6/\Delta5$-desaturase pair of the nematode (see below). Fungal $\Delta12$-desaturases (*M. alpina* [63] *Mucor rouxii* [62]) are also found deeply separated in this branching, but sequence information of fungal $\Delta12/\Delta15$-pairs are not available, since no fungal $\Delta15/\omega3$-desaturase has been cloned yet.

To conclude this chapter, we want to point out that there are many questions which cannot be answered at present: where do the prokaryotic orthologues of eukaryotic desaturases come from, are they all of cyanobacterial origin, to what extent have some eukaryotes developed their own paralogues, or has occasional horizontal/symbiogenetic gene transfer [59,66,67] contributed to the full complement of regioselectivities in different organisms, as observed for example in the nematode *Caenorhabditis* which in its natural diet may swallow more cyanobacteria than *E. coli* cells?

11. "Exotic" paralogues derived from Δ12-desaturases

The $\Delta12$-desaturase group contains members which catalyse reactions other than the normal *cis*- or *trans*-desaturations, although all are believed to involve the activation of oxygen by a di-iron centre [1,46]. These reactions give ricinoleic acid due to C12-D-hydroxylation of oleic acid (*Ricinus communis* [68], *Lesquerella fendleri* [69]), vernolic acid by C12–C13-*cis*-epoxidation of linoleic acid (*Crepis palaestina* [70]) and crepenynic acid by desaturation/acetylenation of linoleic acid with concomitant creation of a C12–C13 triple bond (*Crepis alpina* [70]). In other plants vernolic acid can also be formed by a cytochrome P450-catalysed epoxidation of linoleoyl groups [71].

The paralogous activities just mentioned are realized in several unrelated plant families (Fig. 5) and show up as branches separated from the corresponding housekeeping $\Delta12$-desaturases. All these enzymes are believed to use as substrate the *sn*-2-bound fatty acyl residues of PC similar to the normal $\Delta12$-desaturase [72]. A similar evolution may have occurred with a $\Delta6$-desaturase from the moss *Ceratodon purpureus*, from which in addition to a normal $\Delta6$-*cis*-desaturase a bifunctional desaturase/acetylenase enzyme was cloned, which converts the $\Delta6$-double bond by a further desaturation into a C6–C7 triple bond [73].

In contrast to the development of different regioselectivities of acyl group-*cis*-desaturases, the establishment of the exotic reactivities may have required some modifications of the active site to result in some change in the interaction between oxygen, the di-iron cluster and the substrate. But it is not clear, which proportion of the separation between sequences of normal and exotic desaturases is due to the need for this reconstruction of the active centre and which may reflect the time point of evolutionary separation.

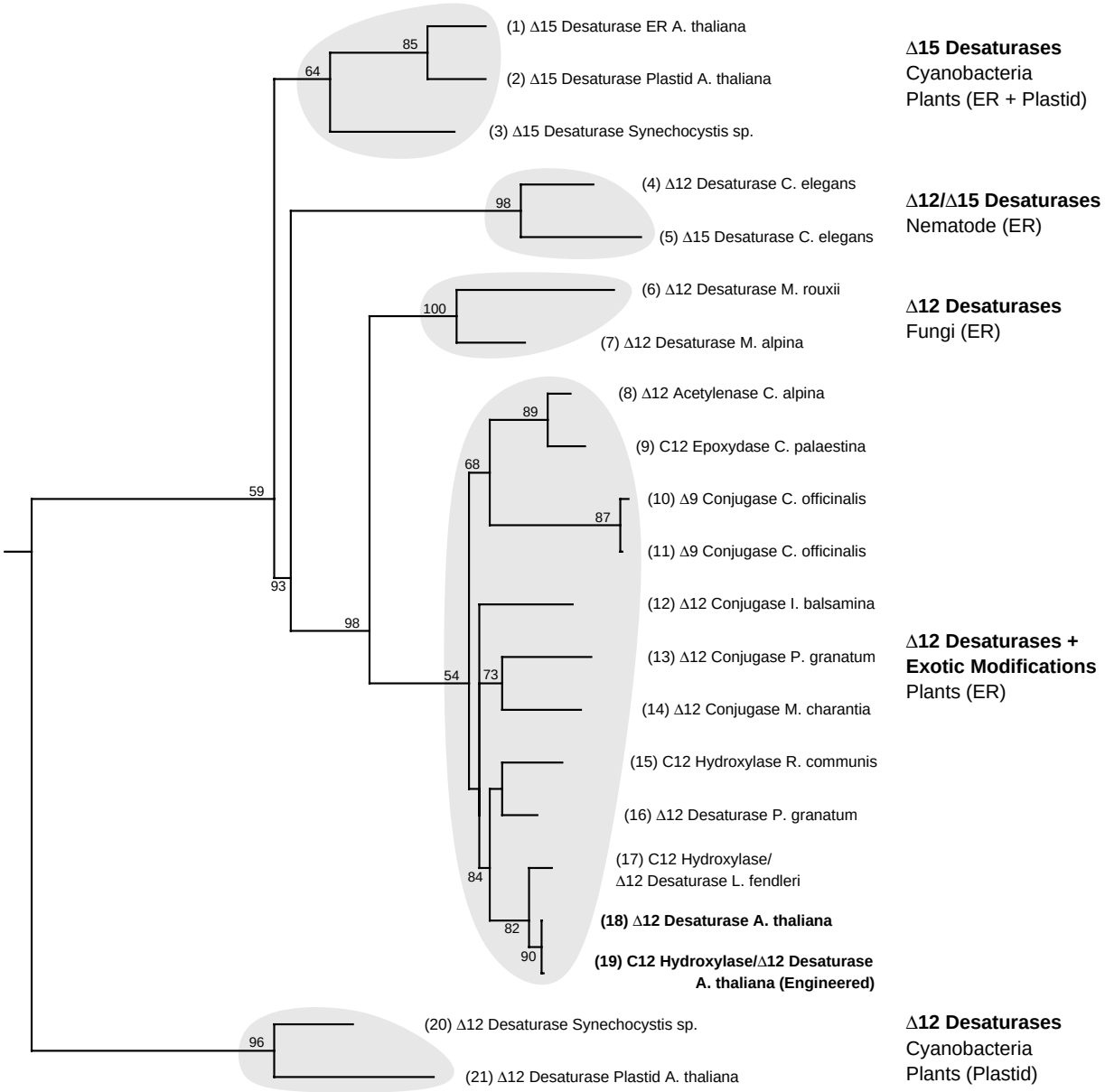


Fig. 5. Phylogram of the group of Δ12/ω6- and Δ15/ω3-desaturases. All enzymes may require acyl lipids as substrates. Five subgroups are highlighted with a shaded background. These are the Δ12/ω6-desaturases from cyanobacteria and plastids, the Δ15/ω3-desaturases from cyanobacteria, plastids and the plant ER, Δ12/ω6- and Δ15/ω3-desaturases from the nematode *C. elegans*, Δ12/ω6-desaturases from fungi, and Δ12/ω6-desaturases from the plant ER. The latter group also includes enzymes responsible for the "exotic" modifications primarily found in plant storage lipids (conjugases, epoxydases, acetylenases, and hydroxylases). The maximum likelihood tree has been constructed with TREE PUZZLE and ATV from an alignment of full-length amino acid sequences created with T-COFFEE. The numbers on the internal branches are quartet puzzling support values which indicate the reliability of the branching pattern on a scale from 0 to 100. The root of the tree has been inferred from the dendrogram in Fig. 3. SWISS-PROT/TrEMBL or GenBank™ (*) protein accession numbers are: (1) P48623, (2) P46310, (3) Q55240, (4) Q9XUB8, (5) Q21056, (6) Q9UVR3, (7) Q9Y8H5, (8) O81931, (9) O65771, (10) Q9FPP7, (11) Q9FPP8, (12) Q9SP62, (13) AJ437140*, (14) Q9SP61, (15) Q41131, (16) AJ437139*, (17) O81094, (18) P46313, (19) reference [75], (20) P20388, (21) P46312.

The actual number of changes required for converting a desaturase into a hydroxylase may be very low as shown by site-directed mutagenesis. A comparison between Δ12-desaturases and C-12-hydroxylases (two enzymes from *Ricinus communis* [68] and *Lesquerella fendleri* [69]) revealed seven amino acids always being different between desaturases and hydroxylases, four of which where within a distance of five residues from the histidine boxes. These four amino acids in the wild-type Δ12-desaturase (FAD2) of *A. thaliana* were replaced by the corresponding residues of the castor bean hydroxylase, and the recombinant gene transformed into the *fad2*-mutant of *A. thaliana* [74]. Some transformed plants accumulated significant proportions of both

linoleic and ricinoleic acid (and its derivatives) indicating the creation of a bifunctional desaturase/hydroxylase. When the wild-type hydroxylase of *L. fendleri* was expressed in the same *fad2*-mutant, it also turned out to be a bifunctional enzyme producing linoleic and ricinoleic acid [69]. A similar bifunctionality is displayed by the mouse DES2 (Δ4-*trans*-sphingolipid desaturase/C4-hydroxylase) as discussed above [20].

A change of four amino acids, sufficient to convert a desaturase (line 18 in Fig. 5) into a bifunctional desaturase/hydroxylase [74] (line 19), cannot be depicted by two separate branches in the dendrogram (Fig. 5) and demonstrates that most of the differences contributing to the separation of the various branches are functionally silent. In the course of similar experiments it was also shown that many desaturases have low hydroxylase activity (Δ12-, Δ9-, Δ5- and Δ15-desaturases from *Arabidopsis*, *Saccharomyces*, *Bacillus* and *Linum*, respectively) [75]. This points to the similarities between desaturation and hydroxylation reactions, which was also confirmed by showing that the two hydroxylases operated with a high kinetic isotope effect in the substitution of the *pro(R)* proton by a hydroxyl group at C12 of the acyl group. The same proton is removed by the Δ12-desaturase [28,76].

Another interesting group of paralogous Δ12-desaturases comprises the so-called "conjugases" [77]. Similar to the triple bond-forming acetylenases, they require *cis*-double bonds, but instead of removing the pair of residual olefinic (vinylic) protons, they may abstract two allylic protons from the two methylene groups adjacent to a *cis*-double bond of a 1,4-diene system [53] (Fig. 4e). The enzymes removing allylic protons adjacent to the Δ12-double bond of linoleic acid (9,12-18:2) may be considered as having retained or slightly expanded the regioselectivity of their precursor desaturase, since their products contain an unaltered *cis*-Δ9-double bond. They produce punicic (9-*cis*, 11-*trans*, 13-*cis*-18:3, in *Punica granatum*) [78] and α-eleostearic acid (9-*cis*, 11-*trans*, 13-*trans*-18:3, in *Momordica charantia* and *Impatiens balsamina*) [77]. Both enzymes have been cloned (from the plants given in brackets) and expressed in yeast and soybean.

Based on the structure of two other conjugated fatty acids of plant origin, there may exist another pair of paralogous conjugases, also related to the normal Δ12-desaturases (Fig. 4e). But so far only one group has been cloned from *Calendula officinalis* [79,80]. They show a seemingly larger shift in regioselectivity, since they do not alter the *cis*-12-double bond, but remove two allylic protons from the methylene groups adjacent to the Δ9-double bond of linoleic acid producing jacarandic (8-*cis*, 10-*trans*, 12-*cis*-18:3) and calendic acid (8-*trans*, 10-*trans*, 12-*cis*-18:3). In *C. officinalis* two slightly different isoenzymes with identical functions are expressed (lines 10/11 in Fig. 5). Studies with deuterium-labelled substrates showed that the enzyme forming calendic acid showed a kinetic isotope effect on removal of deuterium from C11, but not from C8. This represents a shift of the rate-limiting attack by one carbon atom from C12 as observed for the normal desaturase towards C11 of the conjugase. Therefore, the regioselectivity with regard to the critical first attack is not changed very much, and the same may be true for the formation of punicic and jacarandic acid. This is in line with the fact that the cloned members of these two pairs converting linoleic to conjugated trienoic acids stay clearly within the Δ12-branching system (lines 10–14 in Fig. 5) pointing to their phylogenetic root.

Both the 8, 10- and the 11, 13-*trans,trans*-isomers may be formed by a *syn*-elimination of either two allylic *pro(R)* or two *pro(S)* hydrogen atoms, which can be brought into a position very similar to the two *pro(R)* protons removed by the normal *cis*-desaturases (Fig. 4d). When the corresponding *cis,trans*-pairs are produced, the *trans*-double bond is always formed next to the persisting *cis*-double bond and thus separates two *cis*-double bonds in the final products. The formation of the proximal *trans*- and the distal *cis*-double bond (Fig. 4c) may involve more conformational changes in the substrate linoleic acid than required for formation of the *trans*/*trans*-pair (Fig. 4d).

12. Front-end desaturases carry a cytochrome b_5-domain

The acyl group desaturases forming the remaining large branching systems in Fig. 3 operate with front-end regioselectivities and cover C4–C8 of the carbon chain. We will first discuss some common characteristics of these enzymes before dealing with details of this branching system.

A general characteristic of these proteins is the fact that they carry their electron donor as a fused cytochrome b_5-domain at their N-terminus [42,81]. In some members an additional N-terminal stretch precedes this cytochrome b_5-domain displacing it into a middle position. The only exceptions are the non-fused Δ6-desaturases from cyanobacteria which in addition do not use cytochrome b_5 but ferredoxin as electron donor [10]. To understand their evolution, we will again first look for putative prokaryotic orthologues, since the enzymes shown are most likely ER enzymes with two exceptions: the cyanobacterial Δ6-desaturase just mentioned (from *Synechocystis* and *Spirulina*) [10] and an annotated, but not verified desaturase from *Mycobacterium tuberculosis* [82]. This is the only prokaryotic desaturase carrying a cytochrome b_5-fusion. Because of the parasitic life style of *Mycobacterium* and a possible horizontal gene transfer [67], the relevance of this occurrence is unclear.

Another and common characteristic of these desaturases is the structure of the third histidine box, in which for unknown reasons the first histidine is replaced by glutamine [1]. Site-directed conversion of this glutamine to histidine and thus creating a box as found in the other desaturases, resulted in complete loss of activity [83].

A further peculiarity of the fused desaturases, at least from plants [28,84] and fungi [85], is the fact that they are limited in activity to the *sn*-2-bound acyl groups of phospholipids, in particular of PC. But such a fusion is not required for *sn*-2-selectivity: as mentioned above, two well known enzymes from plants desaturate *sn*-2-bound palmitoyl groups forming *cis*-7- or *trans*-3-hexadecenoic acid in two different and specific plastidial lipids [28]. These plastidial enzymes do not carry a cytochrome b_5-domain. In contrast, the sphingolipid Δ8-desaturase which carries an N-terminal fusion [86], introduces the double bond into a substrate representing the equivalent position of an *sn*-1-bound acyl group as outlined above [43]. In this context it should be mentioned that the unfused plant Δ12- and Δ15-desaturases, both from plastids and the ER, are not limited in their activity to a specific *sn*-position of their substrate and accept acyl groups in both positions and irrespective of lipid headgroup [10,28]. Furthermore, the above-mentioned cyanobacterial Δ6-desaturases do not contain a fused electron donor and are confined in their action to the *sn*-1-bound acyl group [10].

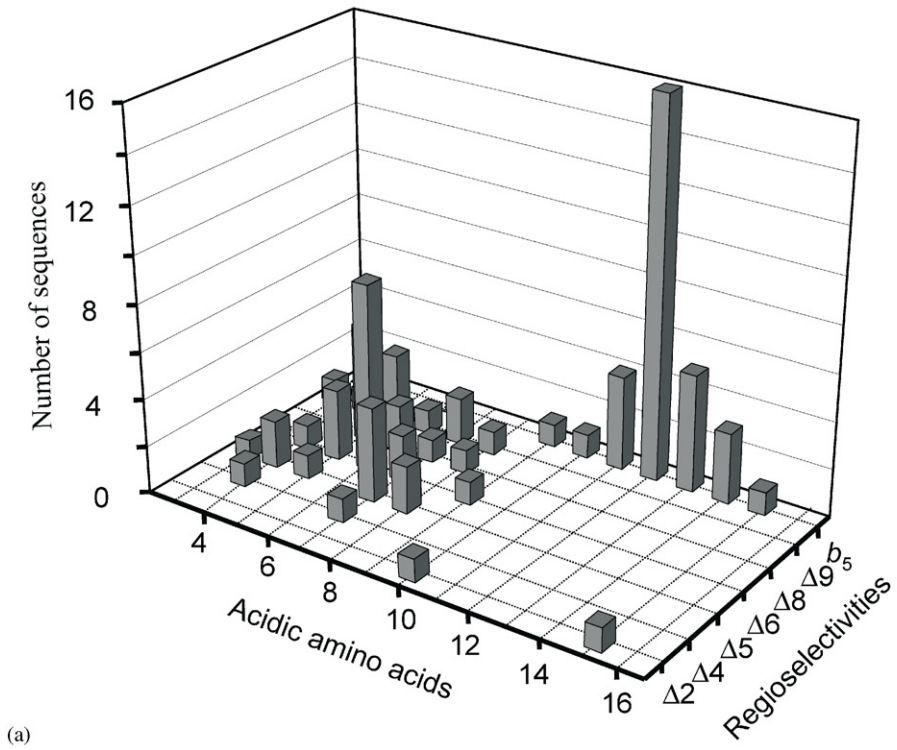
Despite the recognition of these useful details, the most interesting questions of both functional and phylogenetic relevance are the following: which cytochrome b_5-source was used for fusion, when was it realized and what was the reason for this fusion in view of the fact that many other desaturases work perfectly without this fusion? The following considerations may contribute to a partial answer, at least in the case of plant cells, since it may be assumed that they have acquired their desaturases from cyanobacterial ancestors by symbiosis [61].

The cyanobacterial Δ6-desaturase receives the electrons required for desaturation from ferredoxin [10], which is not available for ER enzymes. A transfer of this gene into an eukaryote and the targeting of the protein into the ER [87] would have resulted in very low activity as is in fact observed after expression of the cyanobacterial Δ6-desaturase in the nucleocytoplasmic compartment of tobacco cells [88]. Therefore, an increase in activity would require a more efficient transfer of electrons from cytochrome b_5, which is the most abundant electron donor available in the ER.

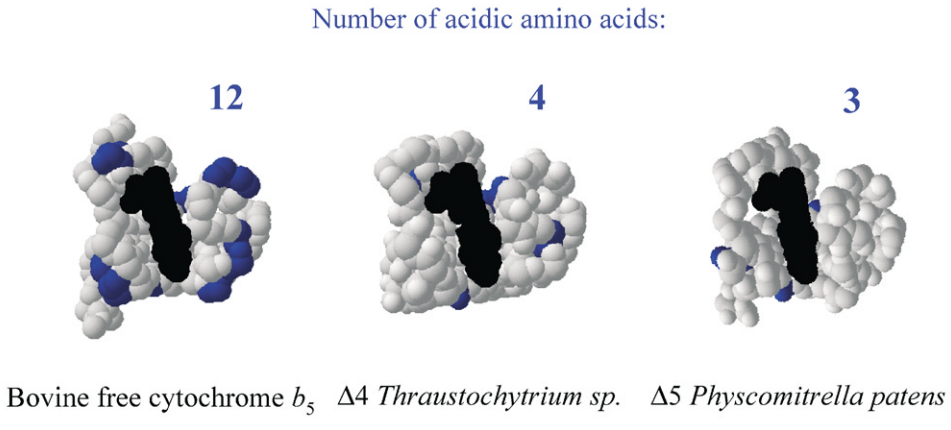
One consequent way to improve this situation would have been a fusion of a duplicated copy of the cytochrome b_5-head to one end of the desaturase sequence, which in this case was the N-terminus. Following its fusion, the cytochrome b_5-head was modified by replacing several acidic amino acid residues in the vicinity of the heme slot [42]. This decrease in acidic amino acids can be seen in the cytochrome domains of all fusion desaturases (Fig. 6), including the recently identified acyl-CoA dehydrogenase [89]. It has been speculated that this change may stabilize a permanent and more hydrophobic interaction between the cytochrome b_5-domain and the actual desaturase part [42]. A similar strategy may have resulted in the C-terminal fusion of cytochrome b_5 to an acyl-CoA desaturase originating from another prokaryotic, but not necessarily cyanobacterial cell. The most ancient fusion seems to be realized in the above-mentioned desaturase from *Mycobacterium* [82] which also shows a significant reduction of acidic amino acids in the cytochrome b_5-domain (only 5 left, not included in Fig. 6). But it would be interesting to find additional fusion desaturases in free-living prokaryotes to be sure of the prokaryotic origin of these enzymes.

But what would have been the source of cytochrome b_5 for such a fusion in prokaryotes? It should be mentioned that most present-day bacteria [12], including cyanobacteria and *Mycobacterium* [82], do not contain free cytochrome b_5. Even the cyanobacterial nitrate reductase, which receives electrons from ferredoxin, does not contain the cytochrome b_5-domain as typical for the cytoplasmic enzyme from higher plants [90]. Similar to other assimilatory nitrate reductases of prokaryotes [91], this domain of the cyanobacterial enzyme is replaced by a sequence binding iron–sulphur centres [92]. Interestingly, a free prokaryotic cytochrome b_5 has so far only been detected in the anaerobic photosynthetic bacterium *Ectothiorhodospira vacuolata*, where it is a periplasmic protein [93]. Other ER desaturases such as the Δ12- and Δ15-desaturases not carrying a cytochrome b_5-domain may have evolved to interact efficiently with the free cytochrome b_5, the phylogenetic origin of which is not clear yet.

For an additional, but independent alignment we used the cytochrome b_5-domains of all fusion proteins depicted in Fig. 3. The resulting dendrogram does not differ from Fig. 3 which is based on the full-length sequences. Furthermore, we carried out a separate alignment by using only the transmembrane helices of the proteins shown in Fig. 3. Also this alignment resulted in the same branching as that obtained with the full-length sequences. This is further evidence for the priorities governing the branching of these dendrograms. With regard to the fusion proteins it indicates that these fusions are very ancient events. But the question is: how ancient? If the N-terminal fusion is a phylogenetically rare and singular event, then one could conclude that all fused front-end desaturases, irrespective of regioselectivity and eukaryotic phylum, may have had a common fused ancestor. Evidently, all these



(a)



(b)

Fig. 6. Reduction in the number of acidic amino acid residues in the cytochrome b_5-domain of fusion desaturases. (a) Only those aspartate and glutamate residues were counted, which contribute to the negatively charged heme-binding domain represented by the sequence between the highly conserved W22 and G77 (numbering refers to the bovine cytochrome b_5 sequence; P00171). The acidic residues of this domain are considered to be involved in docking to the electron donor and acceptor. Sequences of free cytochrome b_5 from plants (X71441; X80008, X75670, L22209, M87514, U79011, AB007802, AB007801, AF503284, CAA04703, CAA04702, AAM61330), from fungi (P40321, AB022443, O94391, CAB53082, CAB91687, AAG23835) and animals (L38464, P04166, P00168, P00167, P00170, P00172, P00171, P00174, P00169, D13205, P56395, AF003141, NP_510335) were analysed as well as cytochrome b_5-fusion desaturases with different regioselectivities as follows: Δ2 (α-acyl amide hydroxylase): Z49260, Z81038; Δ4: AF489589, unpublished; Δ5: AF078796, AF054824, AB029311, AB022097, AF199596, AY082392, AF489588, AF419297, AF309556, *P. patens* [100]; Δ6: Z70271, U79010, AB021980, W53753, AF084559, AJ250734, AJ250735, AJ222980, AF110510, AF309557, AF301910, AY082393, AF084560, AF419296, AF309556; Δ8: AF005096, X87143, AJ224160, AJ224161, AC005397, AF133728, AF031194, CAB61031, AF139720; Δ9: X85963, Y10421, J05676, AB006677, AF026401, D83185, Y18553, Y18554, CAC81988. The recently identified Δ6-acyl lipid desaturase from *Mucor rouxii* (AF296076) not shown in this figure has 5 acidic amino acid residues in its heme-binding domain. (b) Number and location of acidic amino acid residues in the heme-binding domain of free cytochrome b_5 and the homologous domains of fusion desaturases. These models are based on three-dimensional fold prediction of cytochrome b_5 domains of fusion desaturases with bovine cytochrome b_5 as modelling template. C-α trace of bovine microsomal cytochrome b_5 (Protein Data Bank code 1_CYO) including the heme group, and of the fused cytochrome b_5 domain located at the N-terminus of the Δ4-acyl lipid desaturase from the marine single-cell eukaryote *Thraustochytrium* sp. (amino acid residues 6–84 of accession number AF489589) [101] and of the Δ5-acyl lipid desaturase from the moss *Physcomitrella patens* (amino acid residues 27–113) [100]. This figure was generated using the SWISS-MODEL Protein Modelling Server and the Swiss-Pdb Viewer [119–121]. They are turned into a position, which allows to view the heme disk (in black) from the top with the two propionate residues (one with the characteristic bending) facing the viewer. According to this presentation, the acidic residues (in blue) are preferentially lost from the left and right flanks which therefore may contribute to non-polar interaction with electron donor and acceptor as discussed [42].

considerations do not answer the question, but accentuate the problem.

13. Desaturases with Δ4-, Δ5-, Δ6- and Δ8-regioselectivities

For a closer look into the branching system comprising the Δ4- to Δ8-desaturases we constructed a separate unrooted dendrogram to show the complexity of this group (Fig. 7) and to substantiate some obvious conclusions to be drawn.

One group in this branching system comprises front-end desaturases with Δ4-, Δ5- and Δ6-regioselectivity. From all acyl group desaturases presently known, those assembled in this branching system operate closest to the carboxyl end. The deep bifurcations do not allow the recognition of the ancestral regioselectivity. The Δ6-regioselectivity is exceptional in this group and represented by the only prokaryotic members, the two desaturases from cyanobacteria (*Spirulina platensis* and *Synechocystis* sp., sequences 28 and 29 in Fig. 7) [10]. Not taking into account these cyanobacterial enzymes, a remarkable and highly significant separation is seen between the Δ6- and the Δ5-desaturases in fungi, algae and mosses (*Pythium irregulare* [94,95], sequences 20 and 25; *M. alpina* [63,96,97], sequences 18 and 24; *Phaeodactylum tricornutum* [98], sequences 19 and 21; *Physcomitrella patens* [99,100], sequences 16 and 22, Fig. 7), since the Δ5- and Δ6-desaturases of these organisms show up in different branching systems. This indicates that these two activities were separated a very long time ago in these organisms compared to the relatively recent invention of one of these activities in animals. Similarly wide gaps separate the Δ5- and Δ4-desaturase from *Thraustochytrium* [101] (a primitive eukaryote, sequences 1 and 23 in Fig. 7). Since this organism produces 4,7,10,13,16,19-docosahexaenoic acid, it should express a full complement of front-end and Δ12/ω6- as well as Δ15/ω3-desaturases in parallel to the polyketide-like system which produces this highly unsaturated fatty acid in an anaerobic sequence not requiring desaturases [102].

The alignment also places the two Δ5-desaturases from the single-cell eukaryotic slime mould *Dictyostelium discoideum* [103] (sequences 26 and 27 in Fig. 7) into this group. Similar to the enzyme from *Limnanthes* [31], one of these enzymes accepts saturated acyl groups, preferentially 16:0, but both are more active with Δ9- and Δ11-monounsaturated acyl groups and produce dienoic acids. Based on sequence similarities, the N-terminal location of the cytochrome b_5-domain and the histidine/glutamine exchange in the third histidine box, these two enzymes are clearly related to the other Δ5-front-end desaturases of this group normally involved in the biosynthesis of polyunsaturated fatty acids. Therefore, these two enzymes from *Dictyostelium* may represent primitive members of front-end desaturases working with monounsaturated substrates and producing non-methylene-interrupted 5,9-fatty acids, as also typical for some gymnosperms [51]. But so far no experiments on the use of polyunsaturated fatty acids as possible substrates by the expressed Δ5-desaturases from *Dictyostelium* were carried out, and the actual form of the substrate is also not known [103]. The capacity for introducing the Δ5-double bond into a saturated acyl group may be considered as a reconversion of a paralogous polyenoic fatty acid desaturase.

The sphingolipid Δ8-desaturases from plants [86] and fungi form two separate parts of a common branching system (sequences 6–11 in Fig. 7) in line with function and taxonomy. The fungal sphingolipid desaturases are pure *trans*-desaturases, whereas the plant enzymes operate with different stereochemistry (*cis*, *trans* and bifunctional) as outlined above.

Surprisingly, the linoleic/linolenic acid Δ6-desaturases from higher plants [104] (sequences 10 and 11 in Fig. 7) are part of this group of plant enzymes [105] and do not align with the other Δ6-desaturases, including those from algae [98] and mosses [99] (sequences 15–20 in Fig. 7). In all higher plants, desaturated sphingolipids are ubiquitous membrane components, whereas Δ6-desaturated polyunsaturated fatty acids are rare, but nevertheless occur in widely varying proportions in nearly 50 different families [106]. These families are spread all over the taxonomic system of angiosperm plants including mono- and dicotyledoneous groups. If the occurrence of Δ6-polyunsaturated fatty acids is paralleled by the expression of Δ6-desaturases with sequence similarities to the sphingolipid-Δ8-desaturase, then one could conclude that both represent paralogous activities originally found in all angiosperms and not limited to a few families such as *Boraginaceae*, *Onagraceae*, *Ranunculaceae* and *Asteliaceae* [49]. From both activities only the Δ8-sphingolipid desaturase is constitutively expressed and, therefore, may represent the ancestral form. A similar origin may have the Δ6-desaturase from *Mucor rouxii* [107] (sequence 6 in Fig. 7) which shows up in this branch and not together with other fungal Δ6-desaturases (sequences 18 and 20 in Fig. 7, see below). Unfortunately, the sphingolipid Δ8-desaturase from this fungus has not been cloned yet.

The Δ6-desaturases involved in polyenoic fatty acid synthesis of fungi [63,94], algae [98] and mosses [99] form another branching (sequences 15–20 in Fig. 7), which also comprises the fatty acid Δ8-desaturase from the unicellular alga *Euglena* [108] (sequence 12 in Fig. 7) and the Δ6/Δ5-desaturase pair of *C. elegans* [109–111] (sequences 13 and 14 in Fig. 7). In view of the existence of the separate branch of ancient Δ5-desaturases not comprising the nematode Δ5-desaturase, we conclude

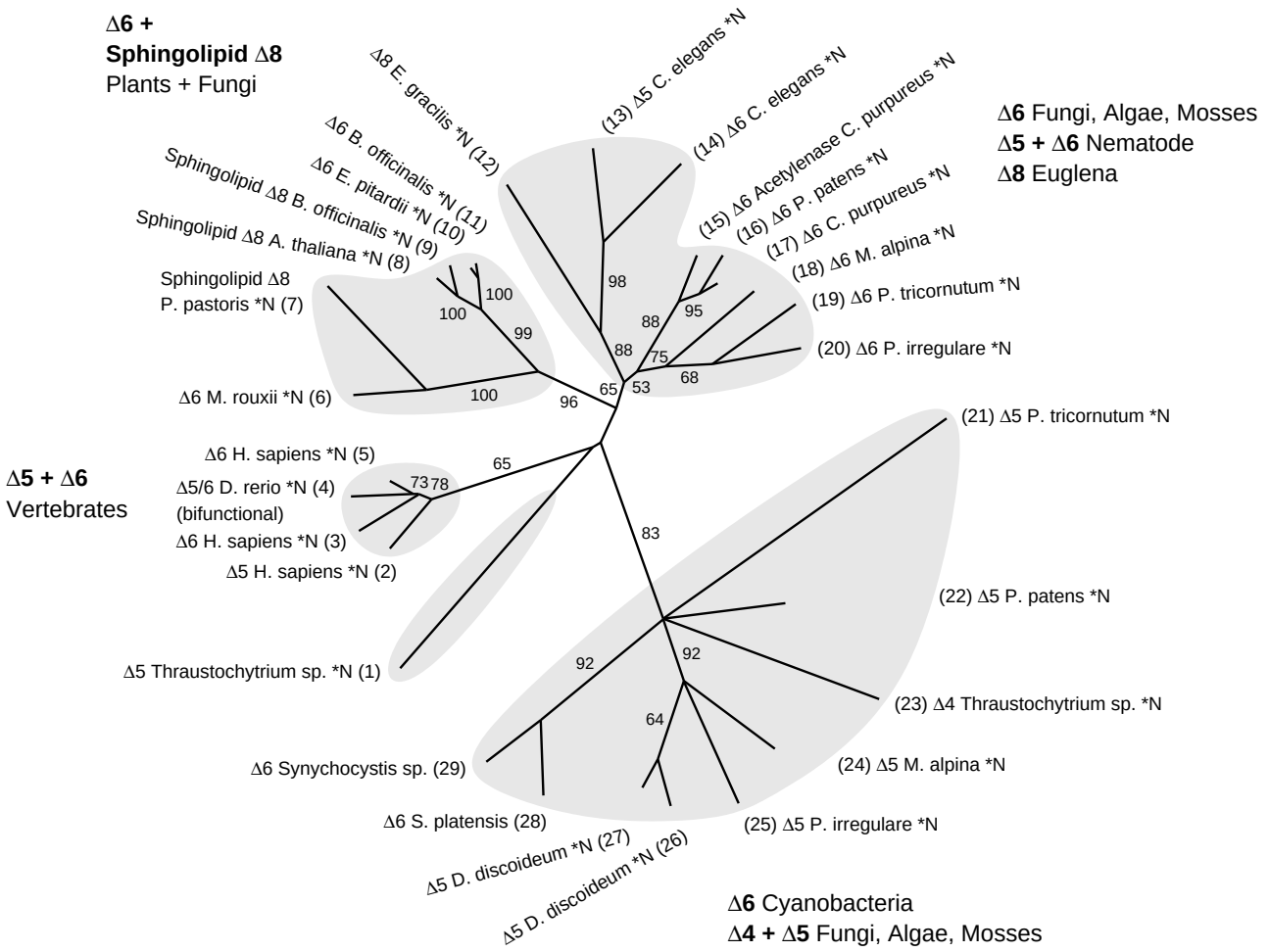


Fig. 7. Unrooted phylogram of the group front-end desaturases. The enzymes are acyl group desaturases unless a different substrate (sphingolipid) or activity (acetylenase) is indicated. Five subgroups are highlighted with a shaded background. These are the sphingolipid Δ8- and acyl lipid Δ6-desaturases from plants and fungi, a second group of Δ6-desaturases from fungi, algae, mosses and the nematode *C. elegans* (including a Δ5-desaturase from *C. elegans*, a Δ8-desaturase from *E. gracilis*, and a Δ6-acetylenase from *C. purpureus*), the Δ5- and Δ6-desaturases from vertebrates, and a heterogeneous group consisting of Δ6-desaturases from cyanobacteria and Δ4- and Δ5-desaturases from fungi, algae, and mosses. Finally, the Δ5-desaturase from *Thraustochytrium* sp. has been assigned into a separate group since no association to any of the other groups can be inferred. The maximum likelihood tree has been constructed with TREE PUZZLE and TreeView from an alignment of full-length amino acid sequences created with T-COFFEE. The numbers on the internal branches are quartet puzzling support values which indicate the reliability of the branching pattern on a scale from 0 to 100. SWISS-PROT/TrEMBL protein accession numbers are: (1) Q8S3C1, (2) Q9NRP8, (3) Q9Y5Q0, (4) Q9DEX7, (5) O95864, (6) Q9HDG8, (7) unpublished, (8) Q9ZRP7, (9) Q9FR82, (10) Q8VZZ1, (11) O04353, (12) Q9SWQ9, (13) Q9XTB7, (14) O61388, (15) Q9LEN0, (16) Q9ZNW2, (17) Q9LEM9, (18) Q8X174, (19) Q8RXB0, (20) Q944W4, (21) Q8RXB1, (22) reference [100], (23) Q8S3C0, (24) O74212, (25) Q944W3, (26) Q9Y1W0, (27) O96099, (28) Q54795, (29) Q08871.

that the nematode Δ5-desaturase was developed later by gene duplication from the Δ6-desaturase [37]. It should be recalled that also the pair of Δ12/Δ15-desaturases [64,65] of this nematode was developed from a late duplication [37]. Therefore, an ancestral form of this nematode may have had just three desaturases (Δ9, Δ12 and Δ6), and the capacity to produce arachidonic acid was acquired by two subsequent duplications to develop the Δ5- and the ω3-desaturase within the nematode lineage.

An even closer correlation between the two Δ5/Δ6-desaturases is evident in vertebrate animals [112–115], which form a branching system of their own (sequences 2–5 in Fig. 7). This may indicate that also these organisms converted one front-end desaturase of presently unknown regioselectivity (Δ5 or Δ6) after gene duplication into a pair with members responsible for either Δ6- or Δ5-desaturation. In the zebrafish *Danio rerio* [116] there is even a single bifunctional enzyme which catalyses both reactions with similar efficiency (sequence 4 in Fig. 7). Despite the seemingly small differences in distance between the carboxyl group and a double bond at Δ4 (C22), Δ5 (C20) or Δ6 (C18), it should be kept in mind that the corresponding

substrates differ with regard to their chain length (given in brackets).

This points to the general possibility that evolution of paralogous activities may have occurred stepwise with an intermediate form of a bifunctional enzyme. The examples discussed so far include the sphingolipid Δ8-*cis*/*trans*-desaturase [86], oleic acid Δ12-desaturase/C12-hydroxylase [69], sphingolipid C4-hydroxylase/Δ4-desaturase [20], linoleic acid Δ6-desaturase/acetylenase [73] and the C18/C20-polyenoic acid-Δ6/Δ5-desaturase just mentioned [116]. Regarding the number of different desaturases required for the biosynthesis of 4,7,10,13,16,19-docosahexaenoic acid, another form of bifunctionality was detected after expressing a Δ6-desaturase of human origin (sequence 3 in Fig. 7) in yeast. This enzyme accepted two substrates of significantly different chain length [117], since it introduced the Δ6-double bond into linoleic acid (9,12-18:2) as well as into 9,12,15,18-tetracosatetraenoic acid (24:4). This may answer the long-lasting question regarding the number of different enzymes catalyzing these reactions [118].

14. Conclusions

What have we learned about the evolution of desaturases from these alignments? The existing multiplicity of desaturase enzymes may have originated in a single ancestral form. The regioselectivity of this ancient form cannot be deduced from our alignments. The only presently known prokaryotic cells producing polyunsaturated fatty acids by oxygen-dependent desaturases are cyanobacteria. Is it justified to conclude that it was this gene pool of ancient oxygenic phototrophs from which sequences were recruited to provide a starter set for eukaryotic cells developing into different directions? On the other hand, even the cyanobacteria do not provide a clue regarding the phylogenetic origin of the plant acyl–ACP-desaturases.

The alignments suggest that an ancient eukaryotic desaturase set has already included separate enzymes with Δ5-, Δ6-, Δ9- and Δ12-regioselectivities as well as the enzymes for sphingolipid modification. All front-end desaturases carrying a cytochrome b_5-domain at their N-terminus may have arisen from a common and ancient fusion desaturase with Δ5- or Δ6-regioselectivity. In some eukaryotes, comparatively late gene duplications gave rise to "local", i.e. parallel developments of some of the basic regioselectivities [Δ6 from sphingolipid-Δ8 (plants), Δ5 from Δ6 (nematode), Δ5/Δ6 from a common precursor (vertebrate animals)]. These developments may have included bifunctional intermediates. The few presently available sequence pairs of Δ12- and Δ15-desaturases from different phyla seem to indicate that the Δ15-regioselectivity is a descendant of an ancient Δ12-desaturase.

In higher plants, more recent and local duplications of Δ12-desaturases resulted in various paralogous activities regarding reaction outcome, in some cases again leading to bifunctional enzymes. Similarly, the acyl-CoA-desaturases have been modified separately within the various eukaryotic phyla regarding chain-length selectivity, regiospecificity and stereochemistry. Depending on the time elapsed since the gene duplications, more or less deep separations are seen irrespective of the extent of functional difference. Similar considerations apply to the house-keeping and exotic acyl–ACP desaturases of plants.

From these data, the experiments on paralogous activities created by genetic engineering and the identical dendrograms resulting from alignments of full length or partial sequences (cytochrome b_5-domains or transmembrane helices) we conclude that most of the sequence differences leading to the branching systems shown in the various figures are not required to separate the biochemical functions. The differences in amino acid sequence are related to the time point of gene duplications and represent accidental and functionally neutral genetic drift. The phylogenetic resolution ends with a basic eukaryotic cell, but does not differentiate between phototrophic (algae, mosses and higher plants) and heterotrophic cell types (fungi).

Nevertheless, these alignments allow a very useful prediction of the putative function of a newly cloned desaturase available either in full length or only as partial clone. On the other hand, a functional expression for verification of the reactivity is always recommended.

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