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Johnathan A. Napier
IACR-Long Ashton Research Station

Louise V. Michaelson
IACR-Long Ashton Research Station, louise.michaelson@rothamsted.ac.uk

Teresa M. Dunn
Uniformed Services University of the Health Sciences

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Louise Jones
Dept of Biology, University of York, Heslington, York, UK YO10 5YW.
e-mail: alj2@york.ac.uk

A new class of lipid desaturase central to sphingolipid biosynthesis and signalling

Johnathan A. Napier, Louise V. Michaelson and Teresa M. Dunn

Sphingolipids and their phosphorylated metabolites play crucial roles in intracellular signalling in animals, and evidence is emerging for analogous situations in fungi and plants. Central to this signalling pathway is the phosphorylation of the sphingoid long chain base, sphingosine, which yields sphingosine-1-phosphate. Until recently, the enzyme responsible for the biosynthesis of sphingosine was unknown, but the Δ^4-long chain base desaturase that carries out this reaction has now been identified. Orthologues are present in animals, plants and fungi, raising the possibility of using reverse genetics to determine the contribution of sphingosine-1-phosphate to signalling networks.

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Sphingolipids are ubiquitous and essential components of the eukaryotic cell, but until recently these lipids were considered to play a purely biophysical role in cellular homeostasis [1]. The structure of sphingolipids can be considered analogous to phospholipids, comprising two acyl chains linked to a polar headgroup, although the actual composition of the two classes of lipids is different. In the case of sphingolipids, a long chain base (LCB) is amide-linked to a very long chain fatty acid. This LCB is synthesized by the condensation of saturated C_{16-18} fatty acyl CoA with serine, followed by reduction, to yield the sphingoid LCB dihydrosphingosine. This LCB is then acylated to a long chain or very long chain fatty acid esterified to acyl-CoA to yield dihydroceramide, which then, in turn, serves as a substrate for further modifications to generate sphingolipids (primarily by the addition of a polar head group) [1].

Sphingolipid-derived signals

It is now also clear that sphingolipids, and the signalling molecules derived from them, play a dynamic role regulating many biological processes. In particular, work in mammalian systems has identified the sphingolipid-derived molecule sphingosine-1-phosphate (S-1-P) as a potent signal involved in cell proliferation and apoptosis [2]. Analogous phosphorylated LCBs in yeast, such as dihydrosphingosine and phytosphingosine, have also been implicated (by reverse genetics) in stress responses to abiotic shock [3]. More recently, a role for S-1-P has been shown in drought stress and stomatal guard cell closure in plants, linking the phosphorylated lipid-derived signal to this Ca^{2+}-mediated process [4]. Although sphingosine (the precursor of S-1-P) is present in trace amounts in higher plants [5], the levels of S-1-P were shown to increase under drought conditions. Exogenous application of S-1-P resulted in guard cell closure in an EGTA-sensitive manner, and also resulted in diagnostic oscillations of cytosolic free Ca^{2+}. This response was not induced by dihydrosphingosine-1-phosphate [5] (although the activity of phosphorylated metabolites derived from more-prevalent plant LCBs remains to be tested).

Taken together, it is clear that S-1-P is a key signalling molecule in animals, plants and fungi, but the genetic and molecular dissection of its role in cellular processes has been hampered by previous failures to identify key biosynthetic enzymes involved in its synthesis. In particular, the enzyme directly responsible for the synthesis of sphingosine has, until now, been elusive. Sphingosine is synthesized by the Δ^4-desaturation of dihydrosphingosine, introducing a (*trans*) double bond between C4 and C5 of the LCB (Fig. 1) [6]. This reaction is generally believed to occur when the LCB is acylated to the fatty acid, hence this enzyme is also referred to as Δ^4-dihydroceramide desaturase [6]. Identification of the Δ^4-dihydro-(sphingosine/ceramide) desaturase would enable the precise genetic manipulation [via insertional mutagenesis, RNA interference (RNAi) or *trans*-dominant mutants] of sphingosine (and hence, S-1-P) levels *in vivo*, and facilitate the identification of sphingolipid-mediated signalling events.

Identification of the dihydrosphingosine desaturase

The recent paper from Ernst Heinz's group in Hamburg, Germany, reports the identification of the Δ^4-LCB desaturase – this is a major breakthrough in our understanding of sphingolipid metabolism [1,7]. Using a bioinformatic approach, Philipp Ternes *et al.* identified several hundred putative desaturases from several organisms, based on the presence of a catalytically essential motif known as the histidine box [8]. These presumptive desaturases were then further classified on the basis of previously defined substrate- and regio-specificity to identify a novel sub-class of functionally uncharacterized enzymes as potential candidates for sphingosine biosynthesis. This *in silico* analysis was also facilitated by the knowledge that although sphingosine (and therefore the Δ^4-desaturase) is

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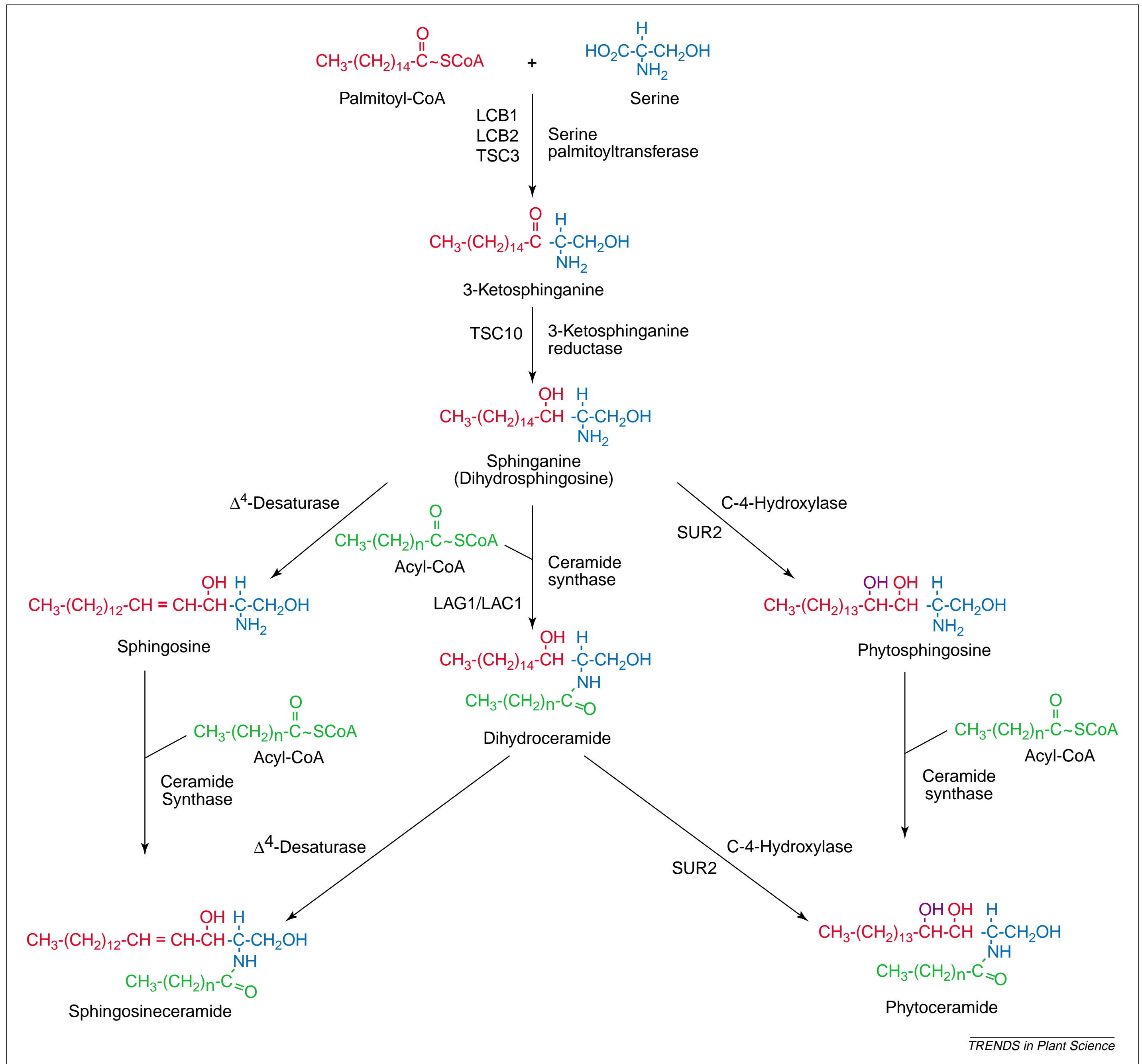

Fig. 1. Generalized scheme of ceramide synthesis and modification. Sphingolipid long chain bases (LCBs) are synthesized via the condensation of saturated C_{16} or C_{18} fatty acyl CoAs with serine, with subsequent reduction to yield dihydroxysphingosine. This LCB is then the subject of modification, either by the C4 hydroxylase SUR2 (to yield phytosphinganine) or by the Δ^4-dihydrosphingosine desaturase (to yield sphingosine). The precise substrate for these modifications (dihydroceramide versus unacylated LCB) remains to be definitively proven. Ceramide synthesis requires the LAG1 and LAC1 gene products. In the case of higher plants and some fungi, a second double bond at the Δ^8 position is prevalent in LCBs (not shown). For clarity, no stereochemistry is represented. However, the naturally occurring sphingoid bases are D-*erythro*-sphinganine, D-*erythro*-sphingosine and D-*ribo*-phytosphingosine.

present in animals, plants and most fungi, it is lacking in the yeast *Saccharomyces cerevisiae*. Thus, candidate genes must be present across a wide range of species, but absent in *S. cerevisiae*. This led to the identification of a new cluster of open reading frames (ORFs) that are present in humans, mouse, *C. elegans*, *Arabidopsis* and *Candida albicans*, which were then subjected to functional characterization. Expression of these desaturase ORFs in *S. cerevisiae sur2Δ* mutants (which only accumulate the substrate LCB dihydrosphingosine) led to the identification of the Δ^4-LCB desaturase orthologues from human, mouse, *Drosophila* and *C. albicans* [7].

New class of lipid desaturase

Final identification of the Δ^4-dihydrosphingosine desaturase facilitated comparisons with previously characterized desaturases. First, the new sphingolipid desaturase did not contain the characteristic cytochrome b_5 domain that had previously been observed in 'front-end' polyunsaturated fatty acid desaturases and the sphingolipid Δ^8-LCB desaturase of higher plants [9]. Similarly, the Δ^4-dihydrosphingosine desaturase specifically introduces *trans* double bonds, whereas the Δ^8-LCB desaturase is stereo-unselective. This implies that the Δ^4- and the Δ^8-LCB desaturases are

evolutionarily diverged (if related at all). This is also true for the cytochrome b_5 fusion Δ^4-fatty acid desaturase recently identified from *Thaustochytrium*, which although apparently sharing the same regio-specificity as the Δ^4-LCB desaturase, does not share any significant homology [10]. Second, the Δ^4-LCB desaturase also shows no sequence similarity to the *S. cerevisiae* Sur2p, which synthesizes phytosphingosine by the C4 hydroxylation of dihydrosphingosine (Fig. 1) [11]. Because these two enzymes share the same regiospecificity and substrate, they might be predicted to display a paralogous relationship; but this is not the case. Ternes *et al.* identified one ORF from mouse as being bifunctional, carrying out C4 hydroxylation and desaturation, explaining the absence of Sur2p orthologues in (mammalian) organisms that synthesize phytosphingosine [7]. As noted by the authors, this provides good evidence of the convergent evolution of enzymes responsible for C4 hydroxylation, and serves as an important corollary to the multiple examples of desaturase evolution by divergence [12].

More roles for sphingolipid signals?

Another interesting aspect to the functional identification of the Δ^4-dihydrosphingosine desaturase was that the human and *Drosophila* orthologues had previously been isolated in unrelated genetic and molecular studies, but those experiments had failed to identify the true function of the enzyme. The human orthologue (termed MLD for membrane lipid desaturase) was identified in a two-hybrid screen for proteins that interacted with the epidermal growth factor receptor, and ectopic expression of MLD was shown to modulate expression of the EGF receptor [13]. In addition, EGF stimulates the production of S-1-P, therefore the identification of MLD as the enzyme responsible for sphingosine synthesis adds weight to the implication that sphingolipid signalling occurs in EGF receptor signalling. A second, perhaps more exciting, example of emerging roles for sphingosine (and metabolites) is highlighted in *Drosophila* [7,14]. A mutant blocked in cell-cycle progression at the G2/M transition had been serendipitously designated *des* (for degenerative spermatogenesis) [14]. However, this phenotype was only present in male flies, with females carrying the same allele displaying full fertility. The identification of *des* as the *Drosophila* orthologue of the Δ^4-sphingosine desaturase implicates sphingosine and therefore, S-1-P, in gamete-specific cell-cycle progression [7]. Given the importance of regulating the cell cycle, particularly in terms of human health, this hitherto unsuspected role for sphingolipids is likely to prove highly significant. Importantly, the authors have used their expression system to solve conundrums that had proved intractable to genetic or molecular dissection, highlighting the need for functional analysis even in this post-genomic era.

Sphingolipid signalling in plants

Almost predictably, higher plants have proved less keen to give up their secrets. It is interesting to note that although Ternes *et al.* succeeded in demonstrating the activity of Δ^4-LCB desaturases from human, mouse, *Drosophila* and the fungus *C. albicans*, a presumptive higher plant orthologue from tomato (*Lysopersicum esculentum*) failed to demonstrate any such activity [7]. Whether this indicates the need for some additional requirements (such as specific substrate or co-factors) for the reconstitution of the plant enzyme activity has yet to be resolved. Examination of the *Arabidopsis* genome sequence for Δ^4-LCB desaturase orthologues reveals the presence of only one clear candidate ORF (At4 g04930). Although it has yet to be demonstrated that this ORF encodes the Δ^4-LCB desaturase, the apparent lack of redundant homologues make this gene a clear target for mutagenesis studies.

Metabolic pathways as effectors of developmental processes

Evidence for sphingolipids and derived signals in plants continues to emerge. Not only has S-1-P been demonstrated to play a role in Ca^{2+}-mediated guard cell closure, but more recent work has identified a sphingosine transfer protein as being involved in programmed cell death. Mutant alleles of the *Arabidopsis* gene ACD11 (accelerated cell death) result in constitutive expression of defense-related genes and apoptotic hypersensitive responses [15]. Acd11p encodes a protein related to mammalian glycolipid transfer protein, although the plant orthologue specifically binds sphingosine but not ceramide [15]. It has also been shown that a tomato orthologue of the LAG1 component of the ceramide synthase mediates the plant's resistance to fungal toxins [16].

Thus, sphingolipids and their metabolites are now clearly implicated in plant signalling and development and further examples are likely to emerge via functional characterization of the many *Arabidopsis* genes implicated by homology in sphingolipid metabolism. When compared to animals, higher plants display a far greater heterogeneity in their LCB composition (and hence potential phosphorylated metabolites). This enhanced potential for novel signalling molecules could reflect their sessile nature and the need to deal with a range of biotic and abiotic stresses. It seems likely that sphingolipids play a central role in multiple aspects of plant biology, and both functional characterizations and analytical methods described by Ternes *et al.* represent further advances towards dissecting these contributions. The prospective combination of insertional mutagenesis with biochemical analysis should help to elucidate the role(s) of sphingolipid LCB desaturation in *Arabidopsis* signalling networks.

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Johnathan A. Napier*
Louise V. Michaelson
IACR-Long Ashton Research Station, Long Ashton, Bristol, UK BS41 9AF.
*e-mail: jon.napier@bbsrc.ac.uk

Teresa M. Dunn
Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA.

Function of the alternative oxidase: is it still a scavenger?

Anthony L. Moore, Mary S. Albury, Paul G. Crichton and Charles Affourtit

The alternative oxidase is a respiratory chain protein found in all higher plants, fungi, non-fermentative yeasts and trypanosomes. Its primary structure suggests that it is a new member of the di-iron carboxylate protein family. Recent sequence analysis indicates an evolutionary relationship between primitive members of this protein family and the alternative oxidase, suggesting that its early function was to scavenge di-oxygen. However, modelling of plant growth kinetics suggests a different function.

The alternative oxidase has been an enigma with respect to its structure and function. An insight into the role of this enzyme is now emerging from the recent analysis by Cláudio Gomes *et al.* [1] who propose that alternative oxidases are evolutionarily related to other di-iron proteins and that an early function of these proteins was to scavenge di-oxygen. However, Lee Hansen *et al.* [2] suggest that the present-day function of this enzyme is to allow flexible control of ATP synthesis to maintain growth rate homeostasis.

Plant alternative oxidase

The respiratory chain of plant mitochondria is similar to that found in mammalian systems in that it contains the four respiratory chain complexes: Complex I (the NADH dehydrogenase), Complex II (succinate dehydrogenase), Complex III (the bc_1 complex), and Complex IV (cytochrome c oxidase) along with the ATP synthase. On an evolutionary time-scale, this basic respiratory chain was obviously formed early, and has remained highly conserved throughout the development and divergence of the plant and animal kingdom. In addition to being able to oxidize internally generated NADH by Complex I, most plant mitochondria possess a complex series of dehydrogenases located on the outer and inner surface of the inner membrane that can oxidize NAD(P)H in a rotenone-insensitive and non-energy-conserving manner [3]. However, one of the most characteristic features of plant mitochondria is the presence of an additional terminal oxidase, the alternative oxidase, the activity of which is insensitive to inhibitors of cytochrome c oxidase and the bc_1 complex. The plant alternative oxidase is a 32 kDa homodimer that branches from the main respiratory chain at the level of the ubiquinone pool and catalyses the four-electron reduction of oxygen to water. The resultant free energy is lost as heat and oxygen reduction is not coupled to proton pumping [4–8]. Apart from its role in thermogenesis, the biological function of alternative oxidase is not fully understood. Its role is generally considered to allow a turnover of the Krebs cycle under high cytosolic energy charge or to protect the cell against oxidative stress.

Structure of the alternative oxidase

Until recently, little was known about the overall structure or nature of the active site of the alternative oxidase. However, in 1995, James Siedow and colleagues proposed a structural model [9] on the basis of sequence comparisons, in which the active site of the alternative oxidase contains a non-haem di-iron centre. In this model, the alternative oxidase is considered to be an integral membrane protein possessing two membrane-spanning helices connected by a α-helix located in the inter-membrane space. In 1999, Martin Andersson and Pär Nordlund [10] pointed out that this model did not agree with the structure of other well-characterized di-iron proteins. This led them to propose a revised model that not only had a greater degree of resemblance to other di-iron enzymes but also, importantly, considered the oxidase to be an interfacial rather than a transmembrane protein [5,10]. Recent site-directed mutagenesis experiments on plant [11] and trypanosomal alternative oxidases [12] are generally supportive of such a structure, although they do question the extent to which the primary coordination sphere of the di-iron centre is universally conserved and hence raise the possibility of species-specific variations [8].

Although sequence identity between di-iron proteins is generally too low to use conventional phylogenetic analysis, Gomes *et al.* [1] used structure-orientated alignments of 56 di-iron protein sequences, including the various alternative oxidases, to derive a phylogenetic relation (Fig. 1). It is evident from this dendrogram that the alternative oxidases from all sources examined form a distinct cluster and that all 56 di-iron proteins are structurally

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