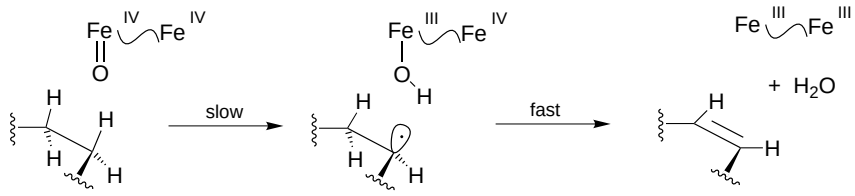
Characterization of a Δ^8-Sphingolipid Desaturase from Higher Plants: A Stereochemical and Mechanistic Study on the Origin of *E*,*Z* Isomers**

Christoph Beckmann, Janine Rattke, Neil J. Oldham, Petra Sperling, Ernst Heinz, and Wilhelm Boland*

Dedicated to Professor Ernst Anders on the occasion of his 60th birthday



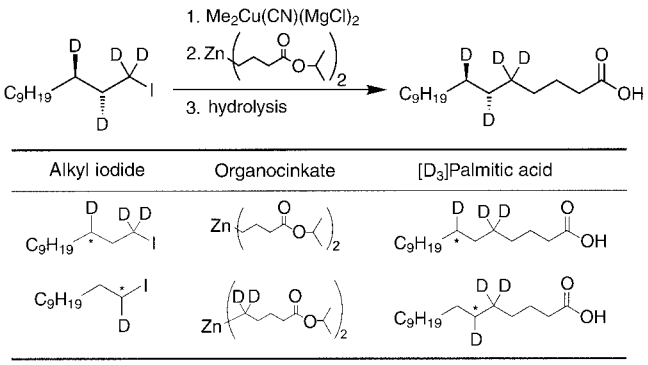
Scheme 1. Schematic representation of a desaturase-catalyzed formation of a double bond in an alkyl chain of a saturated precursor.

Sphingolipids are ubiquitous constituents of animal and plant cell membranes. In addition to their role as membrane components, sphingolipids act as secondary messengers by controlling metabolism and cell growth.[1, 2] Unlike animal cells, in which (4*E*)-sphingenine is the characteristic sphingolipid, plant tissue produces the *E*,*Z* isomers of 4-hydroxy-8-sphingenines (phytosphingenine) and (4*E*)-4,8-sphingadienine.[3]

The properties of unsaturated sphingolipids, acyl lipids, and other fatty acid derived metabolites are determined by the number, position, and configuration of their double bonds. However, the first stereospecifically operating enzymes have only recently been cloned and characterized.[4, 5] All enzymes known to date contained a diiron center with carboxylate and/or histidine ligands as the catalytic unit and allow the oxygen-dependent introduction of double bonds in nonactivated alkyl chains. Both soluble and membrane-bound desaturases are known; these have different consensus motifs, which determine the ligand environment of their diiron centers.[6] As a result of studies with labeled precursors,[7, 8] and from the first crystallographic analysis of a soluble Δ^9-18:0-ACP desaturase from castor seed[9]—together with mechanistic and spectroscopic studies[10, 11]—a uniform mechanistic scenario emerged. Most, if not all, desaturases convert the saturated substrate into an olefin through a highly conserved two-step radical mechanism.[12, 13] In the case of membrane-bound desaturases, removal of the first hydrogen atom by an iron–oxo species at the reactive center of the enzyme (Scheme 1) is rate-limiting and displays a high primary kinetic isotope effect (KIE, $k_{H/D} \approx 5-8$). The second hydrogen atom is lost without a significant KIE.[14–16] In contrast, the soluble stearoyl ACP desaturase exhibits no pronounced KIE for the removal of the first hydrogen atom. Contributions from electron transport, substrate binding, and product release have been discussed as masking factors.[17]

Desaturation proceeds suprafacially[8, 18–21] and removes two *syn*-oriented vicinal hydrogen atoms from an enzyme-controlled conformation of the substrate (Scheme 1). Thus, the products are configurationally pure *E* or *Z* alkenes. With respect to this uniform stereochemical course, enzymes which convert a substrate into a mixture of *E* and *Z* isomers are of special interest.[15, 19] The recently cloned Δ^8-sphingolipid desaturase from higher plants falls into this category.[22, 23] The corresponding gene cloned from sunflowers (*Helianthus annuus*) was heterologously expressed in yeast (*Saccharomyces cerevisiae*), which does not produce unsaturated long-chain sphingobases.[22] The expressed enzyme converts 4-hydroxy-sphinganine (phytosphinganine) into a 7:1 mixture of (8*E*)- and (8*Z*)-4-hydroxy-8-sphingenine (Figure 1). The inhibition of fatty acid biosynthesis in the yeast with cerulenin[24] allows exogenous labeled palmitic acids to be channelled with high efficiency into the sphingolipid metabolism of the cell, thus yielding deuterated 4-hydroxysphinganine (> 95% labeled). Owing to the ease of incorporation, administration of stereospecifically deuterated palmitic acids should give the first stereochemical and mechanistic information (KIEs) on the mode of simultaneous production of *E* and *Z* olefins by a single desaturase.

Herein we report that the Δ^8-sphingolipid desaturase from *H. annuus* produces both the (8*E*)- and (8*Z*)-4-hydroxy-8-sphingenine in a stereospecific manner by *syn* elimination of two vicinal hydrogen atoms. Interestingly, both transformations display different KIEs. Stereospecifically deuterated palmitic acids, which were required for analysis of the desaturation process, were synthesized following a modified protocol of Thum et al.[25] [5,5,6,7-D_4]-(6*R*,7*R*)-palmitic acid (> 98% *ee* per center) and racemic [5,5,6- and 5,5,7-D_3]-palmitic acids were synthesized; the latter were used for the determination of KIEs. The fatty acids were obtained by alkylation of chiral alkyl iodides (Scheme 2) with functionalized organocuprates.[26] The alkylation proceeded without loss



Scheme 2. Synthesis of labeled palmitic acids from deuterium-labeled alkyl iodides and functionalized organozincates.

[*] Prof. Dr. W. Boland, Dipl.-Chem. C. Beckmann, J. Rattke, Dr. N. J. Oldham
Max-Planck-Institut für Chemische Ökologie
Winzerlaer Str. 10, 07745 Jena (Germany)
Fax: (+49) 3641-571-202
E-mail: Boland@ice.mpg.de
Dr. P. Sperling, Prof. Dr. E. Heinz
Institut für Allgemeine Botanik, Universität Hamburg
Ohnhorststr. 18, 22609 Hamburg (Germany)

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or scrambling of deuterium (>98% D substitution per center). The protocol is highly flexible and the combination of appropriate starting materials allows almost any position to be labeled with hydrogen isotopes. The two additional deuterium atoms at C5 were required to secure the origin of metabolites in the event that both deuterium atoms from C6 and C7 of the precursor acids were removed.

Cells of the transgenic yeast, which express the gene of the Δ^8-sphingolipid desaturase from sunflower, were grown in the presence of cerulenin and labeled palmitic acids. After alkaline hydrolysis, the released sphingobases were converted by Sanger's reagent into their dinitrophenyl (DNP) derivatives and preseparated by TLC.[23] The derivatives could be analyzed as negative ions by ESI MS with very high sensitivity and essentially free from background. By passing through a reversed-phase silica HPLC column (RP-18), the *E* and *Z* isomers of the DNP-modified 4-hydroxy-8-sphingenine were separated and analyzed individually (Figure 1). Both isomers

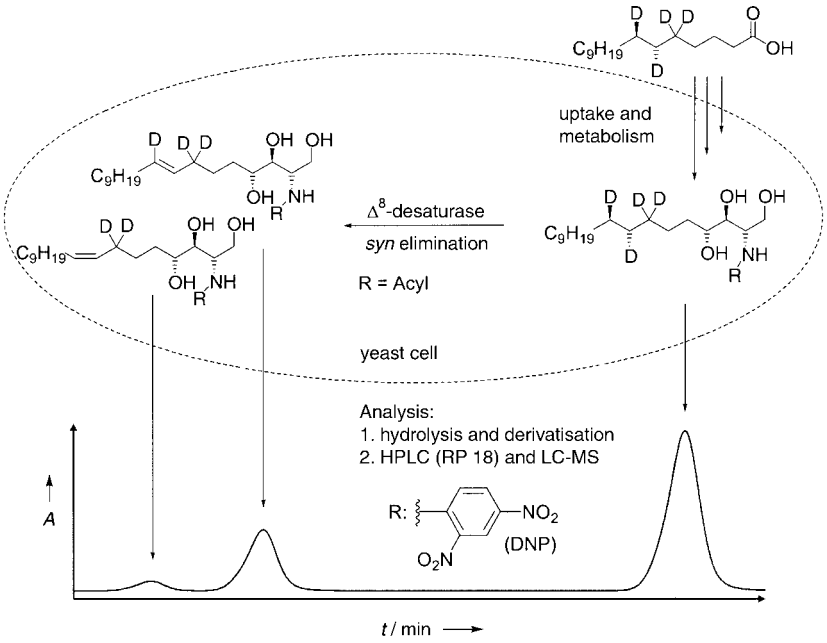

Figure 1. Biosynthesis and desaturation of 4-hydroxysphinganine. Deuterium-labeled palmitic acid from the culture medium enters the transgenic yeast cell and is metabolized via *N*-acyl-4-hydroxysphinganine into acylated (8*E*)- and (8*Z*)-4-hydroxy-8-sphingenine. The *E*/*Z* isomers of their DNP derivatives were separated by reversed-phase HPLC (RP 18) with UV detection (350 nm).

from the transformation of [5,5,6,7-D_4]-(6*R*,7*R*)-palmitic acid displayed spectra with an intense $[M-H]^-$ pseudomolecular ion, with no further fragmentation. The major isomer, (8*E*)-4-hydroxy-8-sphingenine gave rise to a single $[M-H]^-$ ion ($m/z = 483$); the 8*Z* isomer also gave rise to a single ion ($m/z = 482$). In addition, both isomers displayed a weak signal at $m/z = 480$, which corresponded to the $[M-H]^-$ ion of unlabeled, natural 4-hydroxy-8-sphingenine. The *E* isomer was formed stereospecifically by the loss of a single deuterium atom along with a single hydrogen atom from C8 and C9, respectively, of the saturated 4-hydroxysphinganine precursor. In contrast, the 8*Z* isomer was generated with simultaneous removal of two deuterium atoms (C8-D_R and C9-D_R) of the saturated precursor.

To determine the position of the remaining deuterium atoms in (8*E*)-4-hydroxy-8-sphingenine, the double bond was oxidized with OsO_4[27] and the resulting diol cleaved with $NaIO_4$ under phase-transfer conditions.[28] The aliphatic cleavage product, decanal, was then analyzed by GC/MS (chemical ionization, *i*-butane). Decanal from the oxidative degradation of (8*E*)-4-hydroxy-8-sphingenine was found to possess one deuterium atom at the carbonyl group,[29] which demonstrates the simultaneous removal of the C8-D_R and the C9-H_S atoms. Decanal from the oxidative degradation of (8*Z*)-4-hydroxy-8-sphingenine possessed no deuterium atom, which confirmed that the two remaining deuterium isotopes on the metabolite ($m/z = 482$) were located in the polar head of the 4-hydroxy-8-sphingenine at C7 (marker isotopes at C5 of the administered palmitic acid). These findings suggest that both desaturation steps leading to either the 8*E* or the 8*Z* isomer of 4-hydroxy-8-sphingenine involved the *syn* elimination of two vicinal hydrogen atoms. Any influence from unspecific isomerases of the yeast cells on the *E*/*Z* ratio of the products could be excluded, since the expression of desaturases from other plants in the same system resulted in different and characteristic *E*/*Z* mixtures of the unsaturated products.[22, 23] Moreover, the resulting *E*/*Z* ratio proved to be sensitive to isotopic substitution at C8 and/or C9 on the saturated precursor and hence is intrinsically linked to the mechanism of desaturation.[30]

The KIEs of the removal of the hydrogen atoms from C8 and C9 of the sphingolipid precursor were determined by mass spectrometric analysis of the metabolites from racemic [5,5,6- and 5,5,7-D_3]palmitic acids (Scheme 2) fed to the transgenic yeast. All experiments followed the above protocol and were carried out at least in triplicate. Average values of the resulting KIEs are compiled in Table 1.

In contrast to previously studied systems, two distinct and different KIEs were found for the removal of the two hydrogen atoms from C8 and C9 of the saturated precursor en route to the 8*Z* isomer. The KIEs associated with the production of the (8*E*)-isomer follow the usual trend: a large KIE for the removal of the hydrogen atom attacked initially and a low KIE for the subsequent loss of the second hydrogen atom, which is in agreement with the two-step radical mechanism of previously studied desaturases.[31, 12] Since both isomers are generated by the same enzyme, a uniform mechanism that involves a

Table 1. KIEs of the desaturation of specifically deuterated sphingolipids.

Δ^8-Phytosphingenine	Atom	$k_{H/D}$
E isomer	C8	1.91 ± 0.14
	C9	1.16 ± 0.04
Z isomer	C8	2.07 ± 0.16
	C9	3.79 ± 0.59

KIEs were calculated from the ratio of the intensities of the pseudomolecular ions of [D_3]- and [D_2]phytosphingenine after correction for the abundance of their ^{13}C satellite peaks.

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transient C-centered radical should hold for both isomers (as shown in Scheme 1). Thus, the C8-H_R atom of a staggered alkyl segment should be exposed directly to the active center of the desaturase if an *E* isomer is to be formed. Following removal of this hydrogen atom, the radical intermediate at C8 could suffer a second electron transfer or undergo a direct β-cleavage with simultaneous transfer of the C9-H_S atom to the reactive center without a significant KIE (Table 1, $k_{H/D} = 1.16 \pm 0.04$). The production of the 8*Z* isomer requires a *gauche* conformation of the substrate in the relevant area and proceeds with initial hydrogen abstraction from C9 (Figure 1). Since the initial attack on the hydrogen atoms en route to the *E* or *Z* isomers occurred at different methylene groups, a common reactive intermediate (radical) is ruled out. Our data support a mechanism by which the Δ^8-sphingolipid desaturase directly and independently converts two differently populated conformations of the same substrate with either *anti* or *gauche* orientation about the C8–C9 axis into *E* or *Z* alkenes.

Owing to the simultaneous production of 8*E* and 8*Z* double bonds and to the different KIEs for the production of the two isomers (Table 1), the Δ^8-sphingolipid desaturase is different from the hitherto studied and stereospecifically operating fatty acid desaturases. Moreover, in the case of the 8*Z* isomer, initial attack is directed onto a hydrogen atom distal to the polar head (C9), while all previously studied desaturases,[31, 12] including the recently described Δ^4-*trans*-dihydroceramide desaturase from rat liver,[32] attack a hydrogen atom of the proximal C atom. Despite these differences, the Δ^8-sphingolipid desaturase removes, en route to the *E* and *Z* isomers, the hydrogen atoms from exactly the same spatial positions as all other previously studied *E*- or *Z*-selective fatty acid desaturases.[18, 19] It remains to be seen whether or not *E*/*Z* mixtures of other desaturases, for example, those from pheromone glands of insects,[15, 19] follow similar principles and also rely on single enzymes, or whether in these organisms two stereospecifically operating enzymes generate the isomeric mixtures. More work on the mechanistic basis of the Δ^8-sphingolipid desaturase is required and will be reported soon.

Experimental Section

The open reading frame of a Δ^8-Sphingolipid desaturase cDNA from *Helianthus annuus* was cloned behind the constitutive ADH1 promotor of the yeast expression vector pVT-U-102 and transformed in *Saccharomyces cerevisiae* INVSc1.[22] Transgenic yeast cells were cultured for 4 d at 30 °C at $OD_{600} \sim 1.0$ in a medium containing Cerulenin (25 μM; Sigma) and [D]palmitic acid (0.25 mM) in complete minimal medium-dropout-uracil (100 mL) with 2% raffinose and 1% tergitol-NP40 (Sigma). Cells were harvested by centrifugation, washed, and directly hydrolyzed (10% $Ba(OH)_2$ (w/v) in H_2O/Dioxan (1:1), 24 h, 110 °C). The released long-chain sphingobases (LCB) were converted into the DNP derivatives and prepurified by TLC (silica gel 60, $CHCl_3$/MeOH 9:1 v/v). The configurational isomers of the DNP derivatives were separated by HPLC on reversed-phase silica (RP 18, GROM-SIL 120 ODS-5, 3 μm, 125 × 2 mm, ST, Grom, Herrenberg) with a gradient of 0.2 mL min^{-1} from 60% MeOH/CH_3CN/2-propanol (10:3:1 v/v/v) and 40% water (10 min) to 20% water and, finally 0% water (40 min). The DNP derivatives were analyzed by ESI MS on a Micromass Quattro II mass spectrometer (Micromass, Manchester, UK). Spectra were recorded in the negative-ion mode (source temperature: 100 °C, desolvation temperature: 250 °C, cone voltage: 35 Volt).

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[29] Oxidative degradation of [7,7,9-D_3]-(8*E*)-phytosphingenine yields a mixture of decanal and [1-D]decanal. The ratio of labeled and unlabeled decanal corresponds to the ratio of labeled and natural (*E*)-4-hydroxy-8-sphingenine prior to the cleavage.
[30] C6-labeled palmitic acid yields the *E*/*Z* isomers of 4-hydroxy-8-sphingenine in a ratio of about 6:1, whereas the C7-labeled equivalent gives a lower ratio of the *Z* isomer (ca. 11:1) as a result of high KIE.
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