REVIEW

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Fatty acid desaturases: selecting the dehydrogenation channel

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Desaturation (O_2-dependent dehydrogenation) of long chain fatty acyl derivatives is a ubiquitous biotransformation which generates a wide variety of oxidized lipidic natural products with important biological properties. Recent advances in molecular/structural biology coupled with the application of novel mechanistic probes have led to a more sophisticated understanding of how fatty acid desaturases function. In this review, the major milestones along the pathway of desaturase research will be highlighted from a bioorganic perspective. 178 references are cited.

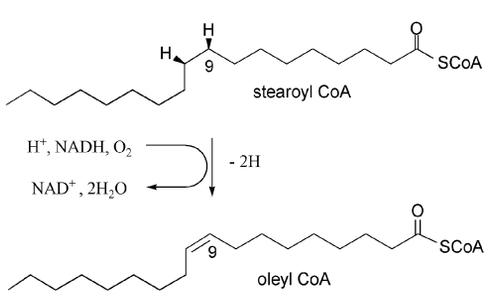
1 Introduction
2 Historical overview
3 Mechanistic models
4 Mechanistic methodology
4.1 Stereochemistry
4.2 Cryptoregiochemistry
4.3 The dehydrogenation/hydroxylation connection
5 Mechanistic features of selected membrane-bound desaturases
5.1 Δ4 sphingolipid desaturase
5.2 Bacterial Δ5 desaturase
5.3 GLA biosynthesis: Δ6 desaturase
5.4 Δ8 sphingolipid desaturase
5.5 Δ9 desaturase
5.6 Δ11 insect desaturases
5.7 Δ12 (FAD2) desaturase
5.8 Δ15 (ω-3) desaturase
5.9 Summary
6 Exotic modifications of plant lipids
6.1 Conjugases
6.2 Acetylenases
6.3 Cyclopropenyl fatty acids
7 Soluble plant Δ9 desaturases
7.1 Stereochemistry
7.2 Cryptoregiochemistry
8 Acknowledgements
9 References

Peter Buist is a Professor of Chemistry at Carleton University, Ottawa, Canada. His progress through the ranks of academia has been greatly aided through the mentorship of Bert Holland, Ian Spenser, David B. McLean (McMaster University), Duilio Arigoni (ETH-Zurich), Gerry Buchanan, Karl Diedrich, Ted Edwards and Don Wigfield (Carleton University). His research interests have centered on the mechanistic study of unusual biological transformations such as methylation of isolated double bonds, sulfur atom insertion into unactivated C–H bonds and O_2-dependent functionalization of hydrocarbons.

Peter Buist

1 Introduction

The O_2-dependent dehydrogenation of lipids is catalyzed by a superfamily of enzymes known as desaturases. A prototypical example of this chemistry is the production of oleyl CoA from stearoyl CoA as shown in Scheme 1. To the organic chemist, this transformation represents a remarkable display of enzymatic regio- and stereocontrol and attempts to duplicate such a feat *via* synthetic catalysts provided an early challenge to biomimetic chemists.[1] Nowadays, understanding the structure, mechanism and regulation of desaturases has become an integral part of research programs in several interdisciplinary areas. For example, studies of cold tolerance in micro-organisms,[2] plants[3,4] and animals[5] have shown how desaturases are recruited to increase the levels of unsaturation in cellular lipids and thus help to maintain the fluidity of cell membranes at low temperatures. Enzymes belonging to the desaturase family have also been implicated in the generation of various exotic, oxidized fatty acids which may be employed by plants to defend against various predators.[6] In the fascinating area of chemical signalling pathways, regio- and stereoselective olefinic bond formation has long been recognized as a critical element of insect pheromone biosynthesis;[7] more recently, this theme has resurfaced in sphingolipid research.[8] At the more applied level, the desire to harness the biosynthetic capacity of plants for the production of designer oils has led biologists to carry out an in depth study of desaturase genes and how they might be used in manipulating plant lipid profiles.[9,10] The use of micro-organisms for this purpose has also been explored.[11] Useful lipid-based products generated in this manner include



Scheme 1 A prototypical fatty acid desaturase-mediated reaction.

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high value industrial feedstocks[12,13] and nutraceuticals.[14] Finally, another important application of desaturase research has become apparent recently in that overexpression of stearoyl CoA Δ9 desaturase (SCD) in animal models has been implicated in a variety of metabolic diseases such as obesity and diabetes.[15,16] The search for specific SCD inhibitors is now underway.

The renaissance of desaturase research in the last decade has resulted in the publication of a number of surveys written by experts in the fields of bioinorganic chemistry,[17,18] biochemistry[19] and molecular biology.[20,21] The reader is also directed to reviews on topics related to desaturases such as methane monooxygenase (MMO),[22–24] non-heme iron co-ordination chemistry[25] and Cytochromes P450.[26–28] This review deals specifically with the bioorganic chemistry of desaturases and represents an expanded version of earlier accounts.[29–31]

2 Historical overview

The modern era of desaturase research was triggered over four decades ago during a lecture by the noted microbiologist, Van Niel who mentioned, in passing, that "certain lipids" can satisfy the oxygen requirement for yeast growth.[32] In the audience that day was the lipid biochemist, Konrad Bloch, who gives an account of his reaction:

"*. . .I rushed to the library and learned that Andreasen and Stier*[33] *had succeeded in culturing yeast anaerobically by adding cholesterol (or ergosterol) and also an unsaturated fatty acid. . .. . . Why oleate formation—presumably by direct H abstraction from stearate—should be an aerobic process seemed intriguing and worth investigating*."[32] Shortly thereafter, Bloomfield and Bloch[34,35] showed that a particulate fraction from yeast could "desaturate" palmitoyl CoA (and by implication stearoyl CoA (Scheme 1)) in the presence of molecular oxygen and NADPH—enzymatic properties which were identical with those of mixed-function oxygenases which inserted oxygen into unactivated C–H bonds (ROH from RH).[36] As has been pointed out recently by Brenner,[37] the term "desaturation" was used to distinguish the O_2-dependent dehydrogenation of unactivated methylene groups from the anaerobic removal of hydrogens at activated positions such as carbons adjacent to the carbonyl function.

Subsequent research on desaturases carried out in the 1960's established the ubiquity of the fatty acid desaturase-mediated pathway in various aerobic life forms including bacteria, yeasts and fungi, plants and animals. During the course of these studies it was found that desaturases could be divided into two main classes—membrane-bound enzymes which utilized fatty acyl CoA or glycerolipid derivatives and soluble systems unique to photosynthetic organisms which operate exclusively on the substrate as the ACP thioester.[38,39] Detailed analysis of the latter system from the phytoflagellate, *Euglena gracialis* showed that electrons flowed from NAD(P)H to the terminal desaturase *via* a flavin-dependent NADPH reductase and the electron transfer protein, ferredoxin.[40] The existence of a similar electron transfer chain consisting of an NADH reductase and cytochrome b5 for the prototypical membranous rat liver Δ^9 desaturase was also established.[41,42] A feature common to both types of systems was the inhibitory effects of cyanide and an insensitivity to CO—both indicators of a non-heme rather than a heme iron catalytic core.

Attempts to prepare stable desaturase preparations for the purposes of structural characterization were met with varying success. In the case of the membranous proteins, enzyme systems which were reconstituted outside the native hydrophobic environment invariably displayed very low activity and were notoriously unstable. Careful fractionation of rat liver microsomes by Strittmatter's group in the 1970's led to the purification of the membrane-associated NADH reductase and cytochrome b5 components as well as the integral membrane-bound desaturase.[43] The presence of functional non-heme iron in the latter protein was confirmed. Hydropathy analysis of its amino acid sequence[44] together with that of a homologous yeast Δ9 desaturase generated the first two-dimensional model[45] featuring two membrane-spanning regions with the bulk of the protein protruding from the cytosolic side of the ER membrane. A more detailed analysis based on a larger sequence set, coupled with site-directed mutagenesis experiments, revealed the presence of eight essential histidines residing in three highly conserved $HX_{(2-4)}H$ boxes.[46] It is now believed that these histidines coordinate to a di-iron centre as documented by Mössbauer data obtained on a closely related non-heme iron-containing ω-hydroxylase.[47,48] It should be noted that a number of membranous desaturases have been shown to contain a fused cytochrome b5 component which presumably facilitates electron transfer from NADH reductase to the catalytic di-iron core.[49,50] To date, no major improvements in experimental protocol have been invented for the purification of membrane-bound desaturases.

In contrast to the situation described above, significant progress in the characterization of the soluble plant desaturases has been achieved in the last decade. Building on the pioneering work of Stumpf and coworkers,[51,52] stearoyl-ACP Δ9 desaturase was purified from castor[53] and safflower embryos[54] and overexpressed in *E. coli* to generate sufficient functional enzyme for characterization by Mössbauer,[55] Resonance Raman,[56] X-ray crystallography[57,58] and Magnetic Circular Dichroism (MCD).[59] These efforts revealed the presence of a non-heme, carboxylate-bridged di-iron core similar to that found in methane monooxygenase (MMO), an enzyme responsible for the hydroxylation of methane.[24] In addition, the crystallographic model of the homodimer revealed a potential substrate binding region in the form of a narrow, hydrophobic pocket transversing the protein. Computer modelling suggested that this space can best accommodate the stearoyl substrate if it adopts a *gauche* conformation at the C9–C10 position in the region proximal to the di-iron core—a state which would facilitate regioselective *syn*-dehydrogenation to produce the desired oleyl product.[57] It should be noted that other soluble plant desaturases differing only in regiochemical preference and substrate chain length specificity share strong similarities in overall sequence and di-iron binding amino acid motifs with those of stearoyl ACP Δ9 desaturase.[19] However, as might be expected, the sequences of these soluble proteins are very different from those of the membranous desaturases.

For many years, bioorganic chemists have been reluctant to study the mechanism of desaturation. There were good reasons for this: the difficulty of obtaining purified enzyme desaturase preparations, the need to activate substrate as complex thioesters or glycerolipids, the lack of good spectroscopic techniques to study non-heme iron-containing enzymes and the ambiguity introduced by the conformational mobility of substrates. However, the development of novel probes, coupled with dramatic improvements in molecular biological and bioinorganic approaches has allowed the detailed examination of long-standing mechanistic problems as we document in the remainder of this review.

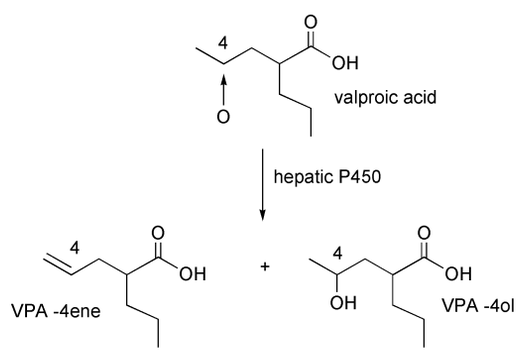
3 Mechanistic models

Mechanistic models for desaturase-mediated dehydrogenation have been formulated on the basis of the paradigms generated for the closely related bio-hydroxylation reaction. Indeed, to the uninitiated, desaturation appears to be a well-disguised hydroxylation/dehydration sequence. However, in the first definitive review on fatty acid desaturation,[60] Bloch noted that the putative hydroxylated intermediates, when incubated with desaturases, were not converted to the corresponding olefinic products. What had been established already at this early date

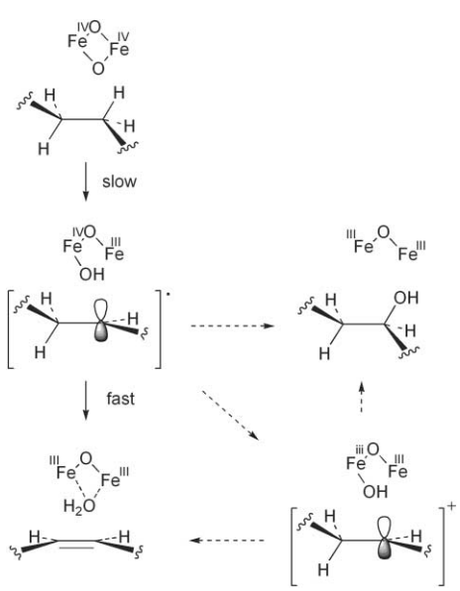
using *in vivo* microbial Δ9 desaturating systems and appropriately labelled substrates was that the removal of hydrogens in stearoyl CoA was a pro*R*-selective *syn*-dehydrogenation process.[61,62] However, the question as to which hydrogen was removed first was not settled with certainty until relatively recently[63] (See Section 5.5).

The nature of the intermediate(s) involved in desaturase-mediated reactions remained unclear for many years; indeed a mechanistic proposal for desaturation is absent from Walsh's authoritative book on enzyme mechanisms.[64] A fresh perspective on the problem was provided in 1987 with the demonstration that hepatic cytochrome P450, a well-studied hydroxylating system, could also act as a dehydrogenase when oxidizing the common epileptic drug valproic acid (Scheme 2).[65] Further examples of cytochrome P450-mediated dehydrogenation were documented by Ortiz de Montellano.[66] The mechanistic proposal presented in the latter work was adopted as a generic mechanistic model for fatty acid desaturation in 1992[67] and an updated version is shown in Scheme 3. (A similar scheme was also presented at this time by Akhtar and Wright in their wide-ranging Natural Product Report entitled "A unified mechanistic view of oxidative reactions catalyzed by P-450 and related Fe-containing enzymes").[68] The underlying assumption was that both heme iron- and non-heme iron-based oxidations of unactivated methylene groups were likely to involve high-valent iron oxo species which operate by some sort of hydrogen abstraction mechanism.[69]

The current consensus mechanism for desaturation (Scheme 3) is consistent with all of the available experimental evidence and is thought to be equally valid for both soluble and membrane-bound desaturase systems. The structure of the



Scheme 2 C-4-initiated bimodal oxidation of valproic acid.

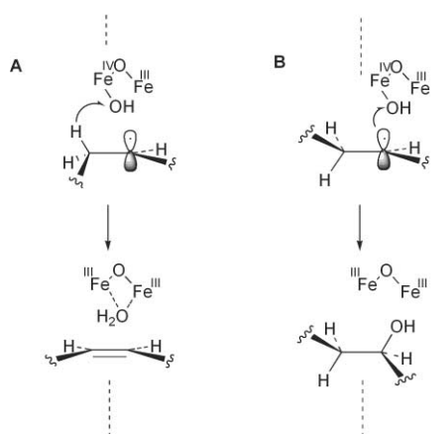


Scheme 3 Generic mechanism for fatty acid desaturation showing its relationship to hydroxylation. The structures of the di-iron oxidant and the reactive intermediates are speculative.

Compound Q-type iron oxidant[70] is speculative and is based on parallel mechanistic work involving sMMO.[24] Desaturation is initiated by an energetically difficult C–H activation step to produce a carbon-centred radical/FeOH pair which disproportionates to give an olefinic product and iron-bound water either directly or *via* a one electron oxidation/deprotonation sequence. Many membrane-bound (but not soluble) desaturases apparently allow a competing "hydroxyl rebound"[71] (better termed as SH_2[72]) pathway to give variable amounts of a secondary alcohol byproduct.[73]

The nature of the switch controlling reaction outcome (dehydrogenation *vs.* oxygenation) is a critically important issue which has not been fully resolved to date. An early suggestion, borrowed from the Cytochrome P-450 literature[74] was that hydroxylation proceeds *via* a radical intermediate but if rapid oxidation of this species to a carbocationic intermediate is promoted in some way then subsequent deprotonation rather than hydroxide transfer to carbon would ensue.[75] In other words, a possible factor controlling reaction pathway is the redox potential of the putative radical intermediate. This idea has been revived recently in connection with the demonstration that methane monooxygenase, sMMO, which is a "pure" ethane hydroxylator, generates dehydrogenated products from substrates such ethylbenzene, potentially *via* the stabilized benzyl cation.[76] Interestingly, a complementary result was obtained using the soluble stearoyl ACP Δ9 desaturase which normally does not produce detectable levels of alcohol.[77] This enzyme, when presented with a 9-fluoro-substituted substrate produces a small amount of a β-hydroxylated product (Section 7.2, Scheme 15) possibly *via* a radical intermediate which is reluctant to undergo one electron oxidation to give a strongly destabilized β-fluorinated carbocation.[77]

However, the observations discussed above can also be explained in terms of simple steric effects and indeed the results of recent site directed mutagenesis experiments on membrane-bound desaturases point in this direction.[78] That is, relatively conservative changes in hydrophobic residues of a membranous bifunctional 12-hydroxylase/12-desaturase were able to affect the relative amounts of oxygenated *vs.* dehydrogenated products. Precise interpretation of these results is difficult in the absence of structural data but it is possible to imagine at least two parameters which might be affected by modest changes in active site architecture: 1) alignment of the carbon–hydrogen bond β to the half filled orbital of the carbon-centred radical and 2) positioning of the iron hydroxyl relative to the two carbons of the incipient double bond (Scheme 4). For desaturation to proceed, hydrogen or proton abstraction must occur *via* the appropriate *syn*-periplanar alignment of orbitals and the iron hydroxyl reagent must be in the correct position to complete the desaturation pathway. Violation of either of these two conditions would lead to hydroxylation by default. The



Scheme 4 Active site scenarios leading to **A** desaturation and **B** hydroxylation.

rather stringent requirements for desaturation would also explain why relatively non-substrate specific oxidants such as hepatic cytochrome P450s rarely act as dehydrogenases due to an inability to exert sufficient control over the conformation of substrates.

A third factor which might influence reaction outcome is the ligand environment of the iron centre and the thermochemistry associated with the desaturase-derived formation of the water–iron complex. This issue was raised in connection with detailed studies involving the non-heme, monoiron-containing enzyme clavaminate synthase which generates either hydroxylated or desaturated product depending on the nature of a pendant amino substituent gamma to the site of initial oxidation.[79] The relevance of this analysis to the fatty acid systems remains to be established.

It should be noted that the above argumentation is predicated on the notion that hydroxylation and desaturation proceed *via* a common radical intermediate. This idea was originally postulated on the basis of a near identity in the magnitude of the *intramolecular* primary deuterium kinetic isotope effects for the hydroxylation and desaturation of valproic acid by the model P450 reaction alluded to above (Scheme 2).[80] This issue has not been explicitly addressed for a fatty acid desaturase system. The possibility also remains that hydroxylation proceeds *via* the nonsynchronous, concerted pathway originally proposed by Newcomb for Cytochrome P450-mediated oxidations[81] and that desaturated product is formed *via* a separate mechanistic pathway involving a discrete radical (or carbocationic) intermediate.

4 Mechanistic methodology

The bioorganic investigation of desaturase-mediated reactions relies heavily on the use of sterically unobtrusive probes which can function as good substrate analogues. As was discovered already by James and coworkers, substrates bearing methyl substituents close to the site of oxidation were not processed by desaturases.[82] Thus researchers in the desaturase area have focused on the use of isotopic labels and the isosteric replacement of methylene groups with oxygen or sulfur and hydrogen with fluorine. An additional complication is the fact that substrate analogues must be compatible with the enzymatic preparation of the requisite thioester or glycerolipid derivative. Finally, it should be emphasized that with the exception of experiments using the soluble plant Δ9 desaturase,[77] a hepatic Δ4 sphingolipid desaturase[83] and a sterol Δ5 desaturase,[84,85] all recent mechanistic work has involved the use of intact organisms or yeast expression systems. These considerations have placed limitations on the types of mechanistic questions which can be asked and the approaches used to supply the answers.

4.1 Stereochemistry

The two stereochemical issues relevant to desaturation reactions are first, the determination of enantioselectivity of hydrogen removal at the prochiral centres and secondly, the relative stereochemistry of C–H bond cleavage at the adjacent carbons (*syn vs. anti*).

The first problem was initially solved using tritium-labelled substrates derived from naturally occurring or chemically resolved alcohols of known absolute configuration and monitoring the loss of label with reference to ^{14}C-labelled substrate.[61,62,86] Fatty acids bearing enantiomerically enriched, monodeuterated methylene groups can also be generated *in situ via* the biosynthetic machinery of the fatty acid synthase using [2-^{13}C, 2-$^{2}H_3$]-acetate as a precursor. ^{13}C NMR could then be used to monitor the loss of deuterium at even-numbered carbons.[87,88] More recently, the use of synthetic methods to generate alcohols in high enantiomeric excess has facilitated the preparation of chirally, monodeuterated substrates.[89] These materials must also bear a remote mass label for investigations conducted *in vivo* in order to avoid interference with endogenous d_0 product in the subsequent mass spectral analysis of products.

The relative stereochemistry of hydrogen removal at the two adjacent methylene groups is most easily determined through use of *erythro*- and *threo*-dideuterated substrates which are in turn, readily available by stereospecific reduction of the appropriate olefinic precursor. An elegant way of analyzing both the absolute and relative stereochemistry of desaturase-mediated reactions is through the use of enantiomerically enriched dideuterated substrates. These can be obtained *via* a highly enantioselective, *anti*-hydrogenation procedure using the enoate reductase from a *Clostridium tyrobutricum*.[90]

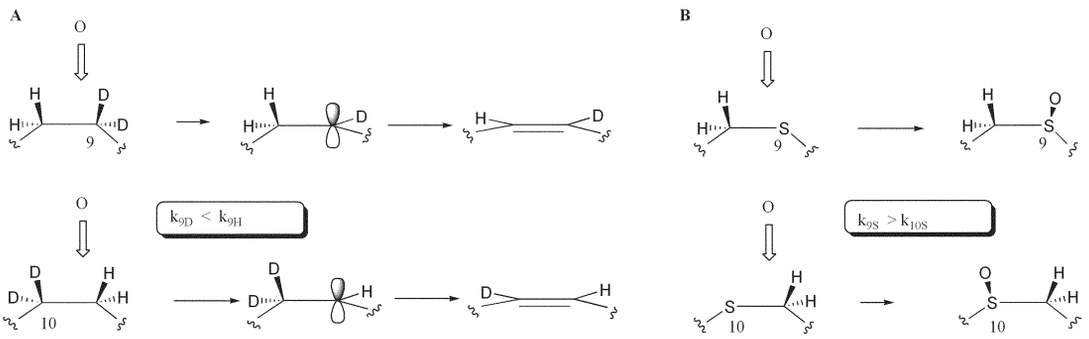
An indirect method for assessing desaturase stereochemistry is through the use of the appropriate thia analogues as oxo traps and a determination of the absolute configuration of the product sulfoxides *via* a suitable chiral NMR shift reagent.[75] In addition, the stereochemistry of hydroxyl byproduct formation during desaturation (Scheme 3) should match the enantioselectivity of hydrogen removal at that position.

4.2 Cryptoregiochemistry

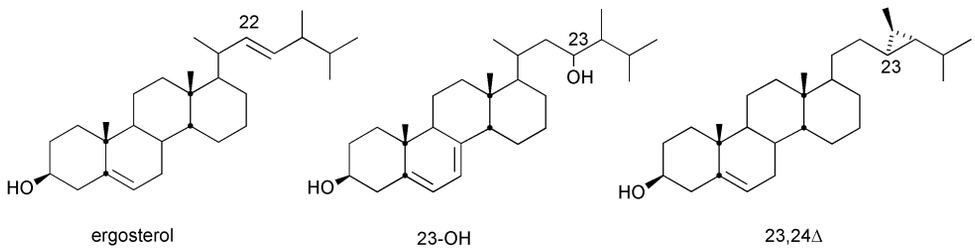
The site of initial oxidative attack (cryptoregiochemistry[91]) involved in double bond formation is a fundamental mechanistic parameter which is most easily determined through the use of KIE (kinetic isotope effect) methodology. This approach is based on the reasonable premise that the initial C–H bond cleavage step should be energetically more difficult and therefore more sensitive to isotopic substitution than the second C–H bond breaking step (Scheme 3). This is precisely what is found experimentally. In two early investigations, competitive KIE information was obtained as a bonus during investigations on the stereochemistry of desaturation.[61,86] Thus, the $^{3}H/^{14}C$ ratio of each residual, stereospecifically-tritiated substrate obtained after incubation was compared with the starting ratio. Significant enrichment of tritium in only one of the starting materials signalled a large primary ^{3}H isotope effect attached to the loss of that tritium atom in the first step of desaturation. More recently, the most frequently used method for the measurement of the primary deuterium KIE on each C–H cleavage has involved incubation of a ~ 1 : 1 mixture of regiospecifically dideuterated substrate and its non-deuterated parent with a convenient source of the desaturase; the d_1/d_0 ratio of the olefinic product (Scheme 5A) is then compared to the starting d_2/d_0 ratio of the substrate by mass spectrometric analysis.[91] The use of racemic, monodeuterated substrates can also give essentially the same information.[92] In both methods, secondary deuterium KIEs are embedded in the value for the primary kinetic isotope effects but this perturbation is expected to be small.[93] Because these experiments yield competitive KIEs, reliable results can be obtained using either *in vivo* or *in vitro* desaturating systems. These data correlate well with a direct measurement of primary deuterium kinetic isotope effects on Vmax and Vmax/Km.[85,94]

Corroborating evidence for the correctness of the cryptoregiochemical assignments obtained from KIE studies has been obtained in two cases through the use of thia probes.[75,95] The relative amounts of sulfoxide obtained by oxo transfer from each thia isomer reflect the positioning of the putative di-iron oxidant relative to substrate (Scheme 5B). Sulfoxide levels can be quantitated through mass spectrometric or NMR analysis of product mixtures. The sensitivity of the latter method can be greatly enhanced through the use of ω-fluoro-tagged thia substrates followed by ^{19}F NMR analysis.[96,97]

To date, explicit attempts to trap out the putative radical intermediate through the use of cyclopropyl radical clocks have not been successful.[98,99] However, a number of cases of what appears to be a hydroxyl trapping process (Scheme 3) have



Scheme 5 Complementary probes for determining the site of initial reaction in desaturase-mediated reactions: **A** KIE test and **B** thia test.



Scheme 6 Products of Δ22 desaturation of sterol side chain found in yeast (ergosterol), a mutant yeast (23OH) and a marine organism (23,24Δ).

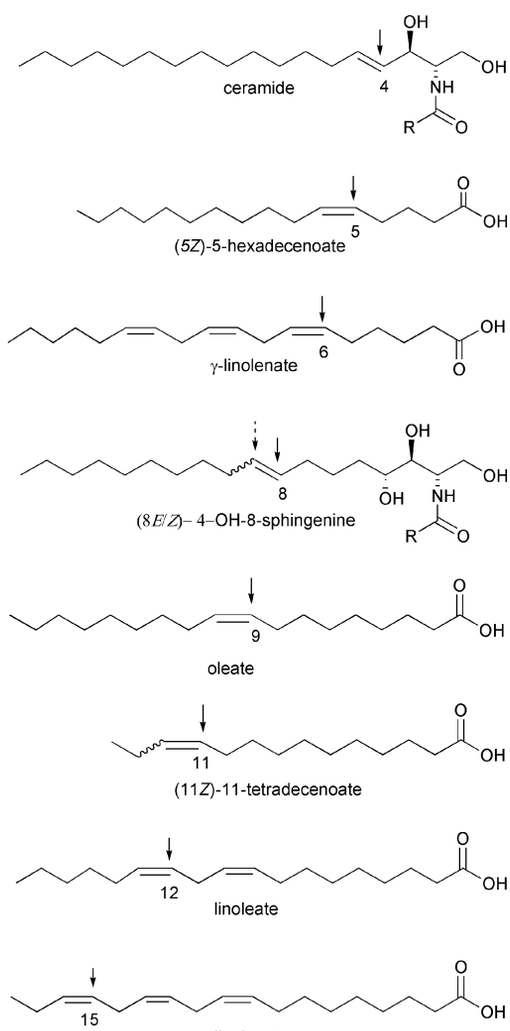
come to light recently.[73] In all cases, the position of oxygenation matches that predicted from the results of a KIE or thia test and constitutes a serendipitous confirmation of the cryto-regiochemical assignment. An interesting example of what may be a similar phenomenon is provided by the isolation of a hydroxylated C23 product in lieu of the normal 22–23-desaturated material in the sterol fraction of a yeast mutant.[100] The site of initial oxidation is currently not known for Δ22 desaturation but this observation along with the discovery of an unusual cyclopropyl product in a marine organism may be providing an important clue (Scheme 6).[101]

4.3 The dehydrogenation/hydroxylation connection

As mentioned in Section 3, a complete mechanistic description of desaturase function must include an answer to the question: what is the switch which controls hydroxylation *vs.* desaturation pathways (Scheme 3)? Development of new approaches designed to shed some light on this important problem is an area of active research and the discovery of several bifunctional desaturase/hydroxylase systems provides new opportunities.[73,102] The expectation is that advances in the structural biology of desaturases will provide sufficiently detailed active site topologies which can be used to assess the consequences of on-going site directed mutagenesis experiments.[73] In this manner, a detailed analysis of what distinguishes a desaturase from a hydroxylase can be carried out in conjunction with possible, high level computational experiments of the type currently being conducted on MMO systems.[103] In addition, design and synthesis of sophisticated biomimetic inorganic models may also provide useful information.

5 Mechanistic features of selected membrane-bound desaturases

The primary sequences of a large number of membrane-bound desaturases have been organized into distinct clusters corresponding to the different regiochemistries of double bond introduction.[21,50] However, as pointed out in the introduction, only crude 2-D models based on hydropathy analysis are currently available for this class of desaturase and thus the long sought after correlation between protein structure and positional specificity remains elusive. Indirect information on active site architecture has been gained through the stereochemical and cryptoregiochemical study of several fatty acid desaturases (Scheme 7). Progress in this area has been greatly



Scheme 7 Cryptoregiochemistry of various fatty acid dehydrogenations catalyzed by membrane-bound desaturases. Arrows indicate the site of initial oxidation as determined by KIE effects.

promoted through the close collaboration between molecular biologists and organic chemists and the details of these investigations are listed in the following sections. It should be noted

that the membrane-bound, non-heme mono-oxygenase of *Pseudomonas oleovorans* (*alk B*), which converts medium chain n-alkanes to 1-alkanols serves as a mechanistic and structural model for this set of desaturases.[48,104] Two mechanistic studies involving *alk B* point to the intermediacy of radical but not carbocationic species along the reaction pathway.[105,106]

5.1 Δ4 sphingolipid desaturase

Sphingolipid derivatives containing an (*E*)-4-ene or 4-hydroxy functionality have been implicated as critical signalling agents in many biological processes such as cell proliferation and apoptosis in mammals,[107] gamete-specific cell-cycle progression in *Drosphila*[108] and stress responses in plants[109] and micro-organisms.[110] A large Δ4-sphingolipid desaturase family has been identified using an bioinformatic approach and the previously suspected close relationship between dihydroceramide 4(*E*)- dehydrogenation and 4(*R*)-hydroxylation was firmly established.[102] This conclusion is also supported at the mechanistic level. A pioneering *in vivo* rat study by Stoffel *et al.*[86] showed that Δ4-desaturation involves *syn*-removal of the C(4)-H_R and C(5)-H_S hydrogens and that this process was likely initiated at C-4 on the basis of a large primary ^{3}H isotope effect at C-4 and not at C-5 (the latter result constitutes one of the first pieces of evidence for the stepwise nature of desaturase-mediated oxidations and has only very recently[111] been given the attention it deserves). The stereochemical analysis of a bifunctional Δ4-desaturase/4-hydroxylase from *Candida albicans*[111]and an *in vitro* cryptoregiochemical study of a rat liver microsomal Δ4-desaturase[83] confirm the results of Stoffel *et al.* and clearly show that 4-hydroxylation and Δ4-desaturation are both initiated by removal of the pro*R* C-4 hydrogen. As stated by the authors,[111] the availability of the bifunctional *Candida* system would appear to offer a particularly convenient opportunity to determine more precisely the point at which desaturation and hydroxylation pathways bifurcate. Another important development in the sphingolipid area is the discovery of a potent synthetic dihydroceramide Δ4 desaturase inhibitor which features a novel cyclopropene moiety at the C-4,5 position.[112] A more detailed study of the mechanism of inhibition has been carried out recently.[113]

5.2 Bacterial Δ5 desaturase

As pointed out in the introduction, one way of maintaining lipids of biological membranes in the fluid, liquid crystalline state at suboptimal temperatures is through the (*Z*)-dehydrogenation of their fatty acyl hydrocarbon chains. An early example of this phenomenon was uncovered by Fulco who showed that the (*Z*)-Δ5 desaturation of cell membrane phospholipids in various species of *Bacillus* could be induced when the temperatures of bacterial cultures are lowered from 37 to 20 °C.[114] The control mechanisms involved in the signal transduction process have been worked out by Mendoza and coworkers.[115] Analysis of the Δ^5 desaturase gene[116] found in *Bacillus subtilis* revealed that this protein occupied its own unique position in the sequence space membrane-bound desaturases.[21] Protein fusion expression using alkaline phosphatase, combined with site-directed mutagenesis experiments and hydropathy analysis have yielded a novel 2-D model of the *Bacillus* Δ5 desaturase.[117] The availability of a well-characterized *Bacillus subtilis* system which processes exogenously supplied palmitate to *cis*-5-hexadecenoate upon cold shock treatment expedited a cryptoregiochemical analysis of this system. The intermolecular, competitive, primary deuterium KIE on each C–H cleavage step of Δ5 desaturation was then determined to be 3.9 ± 0.4 at C-5 while the C_6–H bond breaking step was shown to be insensitive to deuterium substitution (KIE = 1.17 ± 0.02).[118] According to our mechanism (Scheme 3), these results suggest that the site of initial oxidation for Δ5 desaturation is at C-5. In accordance with this result, Shanklin and coworkers have recently detected low levels of 5-hydroxy-palmitate derived from the *Bacillus* Δ5 desaturase expressed in *E. coli*.[73] The stereochemistry of Δ5 desaturation has not been elucidated.

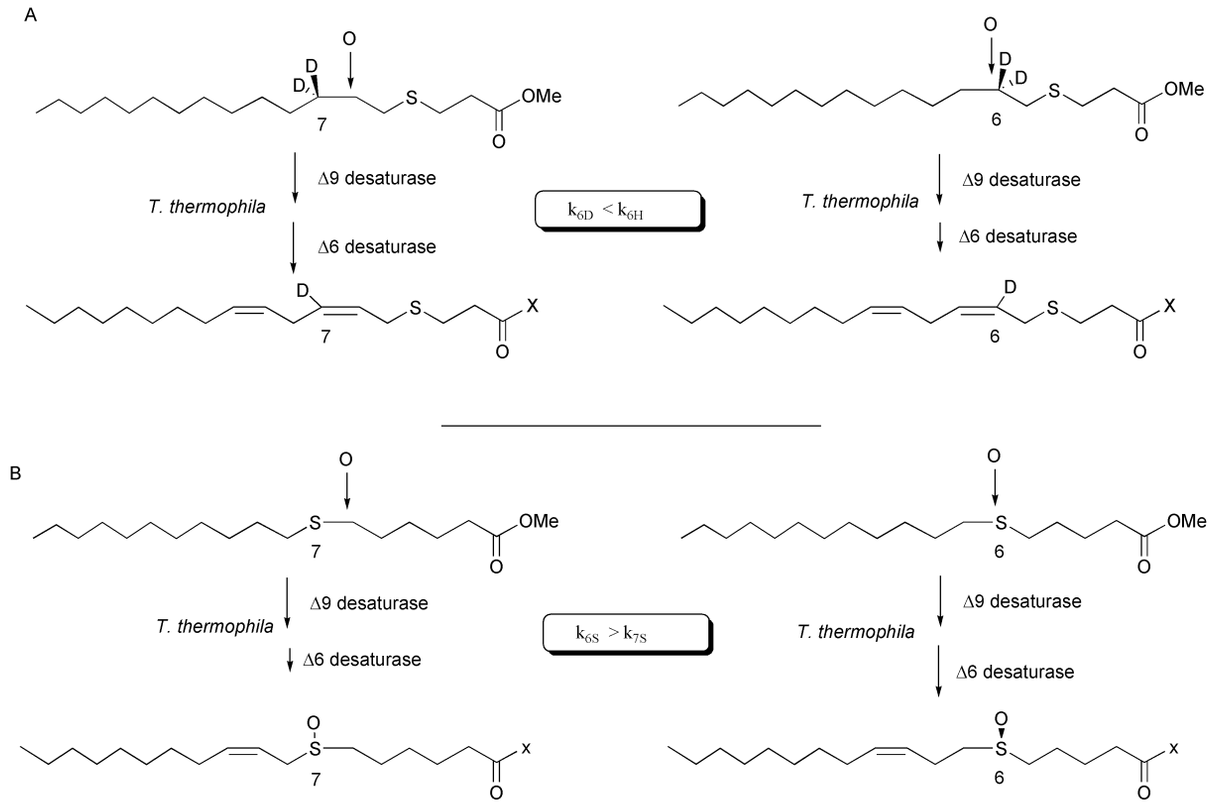
5.3 GLA biosynthesis: Δ6 desaturase

The Δ^6 desaturation of a linoleyl substrate to produce γ-linolenic acid (GLA) is an important step in the biosynthetic pathway leading to arachidonic acid and other polyunsaturated fatty acids (PUFAs) in animals.[119] Surprisingly, a related process also occurs in some plants and the value of GLA-containing dietary supplements such as evening primrose oil (King's cure-all) in managing various disease states has been well-documented.[120] Thus considerable effort has been expended by plant biotechnologists in the characterization of Δ6 desaturase genes from various sources. It was as a consequence of this activity that the first example of a desaturase fused to an N-terminal cytochrome b5 domain, the Δ6 desaturase from borage, was discovered.[49] Another interesting finding was the existence of a bifunctional Δ6-desaturase/acetylenase in *Ceratodon purpureus*.[121] To date, no animal or plant Δ6 desaturase has been studied mechanistically; however the methodology for the cryptoregiochemical and partial stereochemical analysis has been worked out using the protist *Tetrahymena thermophilia* as a convenient source of a Δ6 desaturase.[95] As shown in Scheme 8, application of both the competitive KIE test and the thia test strongly suggests that Δ6 desaturation in this system is initiated at C-6: large primary deuterium KIE at C-6 ($k_H/k_D = 7.1 \pm 0.5$), negligible KIE at C-7 ($k_H/k_D = 1.04 \pm 0.05$) and preferential (> 10 fold) sulfoxidation at S-6 *vs.* S-7. Interestingly, both results are consistent with an earlier prediction based on the differing levels of incorporation of 6,6-d_2- *vs.* 7,7-d_2-oleates into Δ6-desaturated lipids of *Tetrahymena*.[122] In addition, the 6-sulfoxide produced by Δ6 desaturase-mediated oxo transfer was shown to be formed in high enantiomeric excess (> 95% ee) and its absolute configuration (*S*) suggested that initial H-abstraction at C-6 of the parent substrate occurs with the same stereochemical preference as that previously determined for oleate and linoleate biosynthesis.[61,62]

A putative Δ6 desaturase has been found in a *Rhodococcus* mutant which converts isopropyl palmitate into its (*Z*)-6-ene derivative. Interestingly, the same system also Δ9-desaturates mid- to long-chain n-alkanes and 1-chloroalkanes. This report constitutes a rare example of hydrocarbon desaturation and, as such, is worthy of closer examination.[123]

5.4 Δ8 sphingolipid desaturase

In 1998, a novel sphingolipid Δ8 desaturase was characterized which, at the level of its primary sequence, bears a strong resemblance to the plant Δ6 desaturases discussed above.[124,125] A unique feature of this protein is that it catalyzes the introduction of a double bond at the 8,9 position of phytosphinganine with less than 100% stereoselectivity (7 : 1 (*E*) : (*Z*)). While the production of a stereoisomeric mixture of olefins has good precedent in insect pheromone biosynthesis (see Section 5.6), the generation of such isomers at positions remote from the potentially more mobile methyl terminus is highly unusual. Apart from the broader biological significance of such apparently "sloppy" biochemistry, it was of interest to probe this lack of precision in the dehydrogenation event more closely. The sphingolipid Δ^8 desaturase gene cloned from sunflower (*Helianthus annuus*) was expressed in yeast and the stereochemistry of hydrogen removal assessed by incubation with an appropriate enantiomerically enriched precursor bearing deuterium at the vicinal pro*R* positions.[92] ESI-MS analysis of the sphingolipid olefinic products showed that desaturation had proceeded with *syn*-stereochemistry for both isomers. The cryptoregiochemical analysis of the same system carried



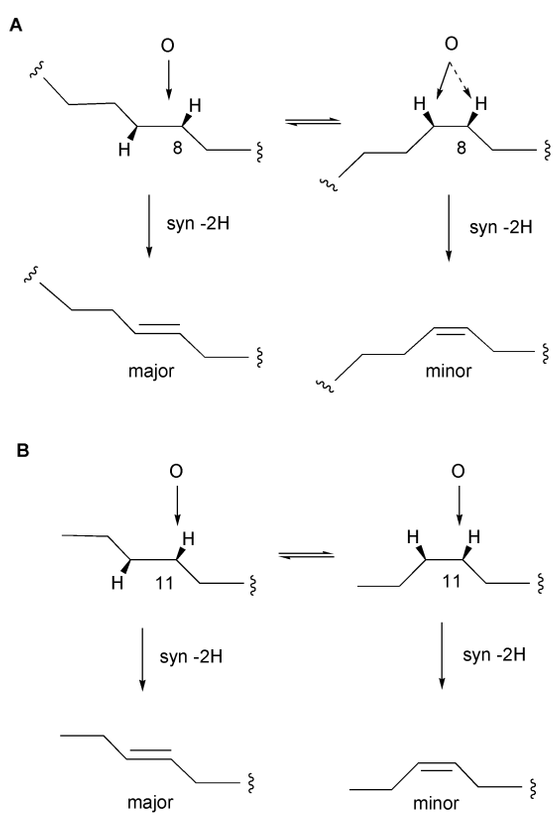
Scheme 8 Determination of cryptoregiochemistry of Δ6 desaturation *via* **A** KIE test and **B** thia test. Reactions are initiated at C6 (S6).

out using a pair of mass-labelled racemic monodeuterated substrates yielded unexpected results. While the site of initial oxidation for the production of the major (*E*)-isomer appears to be at C-8 (primary KIE at C-8 ~2; at C-9 ~ 1), the KIE signature found for the (*Z*)-isomer appeared to be reversed (KIE at C-8 ~2, C-9 ~ 4).[92] In the latter case, neither of the KIE values is close to unity; this may be interpreted in terms of a less than absolute preference for initial attack at C-9 (Scheme 9A). A more extreme example of unselective oxidation of this type has been documented in Cytochrome P450-catalyzed formation of Δ3-desaturated valproic acid on the basis of a KIE study and a mixture of regioisomeric radical intermediates has also been postulated in this case.[126]

5.5 Δ9 desaturase

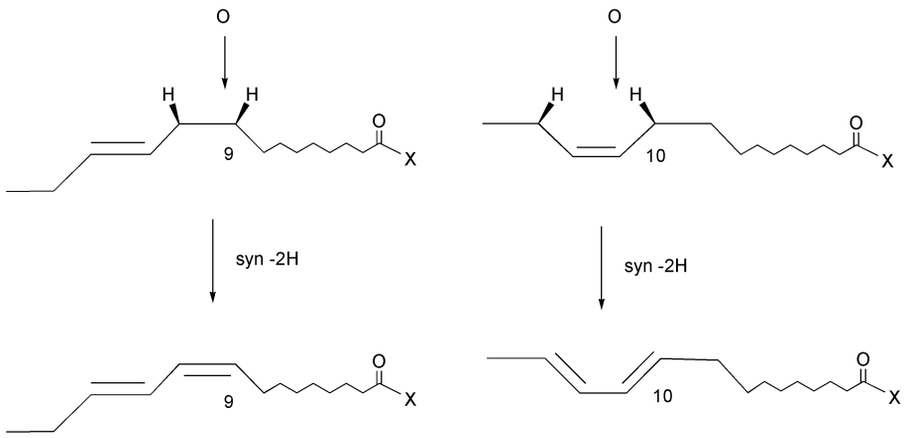
Δ9 desaturation has long been regarded as the prototypical reaction for this class of biological oxidation because of the ubiquitous presence of this enzymatic activity in virtually every aerobic organism from bacteria to humans. Sperling and Heinz have analyzed the various subgroups found within the Δ9 desaturase envelope.[21] The yeast stearoyl CoA desaturase has played a prominent role in mechanistic investigations because its high *in vivo* activity in actively growing cultures has allowed various new mechanistic probes to be tested in convenient fashion.[67,91,127] As a result of this research, the early conclusions of Bloch on desaturase mechanism were upheld, namely that the Δ9 dehydrogenation of stearoyl derivatives by membranous desaturases was initiated by abstraction of the C-9 (H_R) hydrogen in an isotopically sensitive step and that the reaction was completed by a very rapid second removal of the C-10 (H_R) hydrogen to give oleyl CoA. Similar KIE results have been obtained in a cyanobacterial system.[128]

Detection of trace quantities of 9-hydroxystearate[73] in the yeast lipid fraction also supports the cryptoregiochemical assignment. A long standing apparent contradiction between KIE data obtained for two different microbial Δ^9 desaturases has led to much confusion in the literature and has only recently been resolved. Thus competitive $^3H/^{14}C$ KIEs measured using



Scheme 9 Two examples of desaturation reactions which lead to a mixture of stereoisomeric products: **A** Δ8 desaturation of sphingolipids in *Helianthus annuus* and **B** Δ11 desaturation of myristoyl CoA in *Spodoptera littoralis*. Putative site of initial oxidative attack is indicated by the arrows.

an *in vivo Corynebacterium diptheriae* system clearly pointed to the operation of a KIE at C-9 but not at C-10 (*vide supra*).[61] However, KIE measurements employing labelled substrates originally designed to test the stereochemistry of Δ9 desatura-



Scheme 10 Comparison of 1,2 and 1,4 dehydrogenation of (*E*)-11-tetradecenoate and (*Z*)-11-tetradecenoate as it occurs in *Spodoptera littoralis*. The oxidant is positioned at C-9 and C-10 respectively as determined by KIE studies.

tion in *Chlorella vulgaris* led to the inference that C–H cleavage reactions at C-9 and C-10 are both sensitive to deuterium or tritium substitution.[62] The latter result raised the possibility that a fully synchronous rather than a stepwise dehydrogenation process may be operating in some cases. A re-examination of *Chlorella* Δ^9 desaturation using a more direct approach clearly showed that cleavage of the C9–H bond was very sensitive to deuterium substitution ($k_H/k_D = 6.6 \pm 0.3$) while a negligible isotope effect ($k_H/k_D = 1.05 \pm 0.05$) was observed for the C10–H bond-breaking step.[63] These data confirmed that Δ^9 desaturation in this organism occurs in two discrete steps and that the site of initial oxidation is at C-9. A corollary of these findings is that membranous Δ9 desaturases can coexist with or replace the soluble equivalent in photosynthetic organisms. The existence of a membrane-bound Δ9 desaturase in white spruce has now also been confirmed.[129]

An unexpected spin off from this mechanistic work was the demonstration that the yeast Δ9 desaturase could be used to carry out highly enantioselective sulfoxidations of 9-thia fatty acid analogues of various chain lengths and bearing a variety of aromatic groups.[130] The polar sulfoxy products are excreted into the media as the carboxylic acids from which they can be conveniently extracted. This cellular clearing mechanism may serve to prevent a toxic accumulation of 9-thia-derived lipids which might inhibit the Δ9 desaturase as has been seen *in vitro* using rat-liver microsomes.[131]

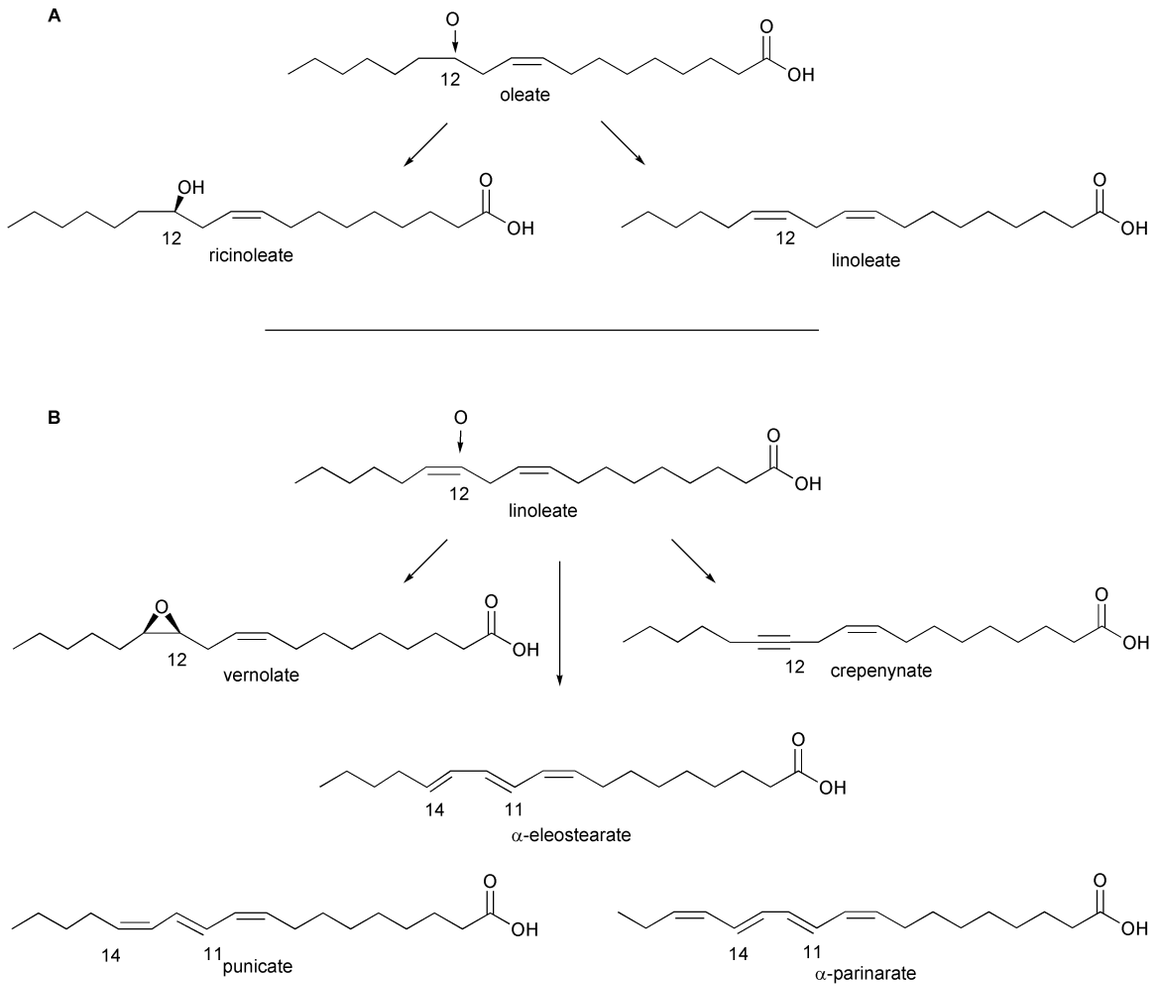
5.6 Δ11 insect desaturases

The involvement of desaturases in the production of volatile insect pheromones was first recognized some two decades ago. Variations in desaturase regio- and stereoselectivity are logical mechanisms for generating species-specific chemical signals.[132] The first stereochemical analysis of a Δ11 insect desaturase was carried out by Boland *et al.* who showed that the reaction in *Mamestra brassicae* proceeded with *syn* removal of pro*R* hydrogens.[133] Similar results were obtained for pheromone biosynthesis found in *Manduca sexta* and *Bombyx mori*.[134] The Fabrias laboratory has been very active in sorting out the stereochemical and cryptoregiochemical details of a *Spodoptera littoralis* desaturating system which produces a unique blend of mono- and dienoic tetradecanoates. It is hypothesized that the monoenes are formed by the action of a single Δ^{11} desaturase[135] which can accommodate two rapidly equilibrating substrate conformers (Scheme 9B). The initial abstraction of the pro*R* hydrogen of the stationary C-11 methylene group generates a short-lived radical intermediate which undergoes a second hydrogen abstraction to generate a mixture of (*E*/*Z*)-11-tetradecenoates.[136] The relative stereochemistry of hydrogen removal is "*syn*" in both cases.[89] Use of an 11-cyclopropylundecanoyl probe failed to intercept the putative radical intermediate through a ring opening rearrangement or hydroxyl quenching; instead a highly strained cyclopropylidene product was formed, indicating the very high commitment to the dehydrogenation pathway displayed by these systems.[99]

The remaining components of the *Spodoptera* pheromone blend are biosynthesized by the action of a Δ9 desaturase which processes the (*E*)-11-tetradecenoate *via* C-9-initiated dehydrogenation to give a 9(*Z*),11(*E*)-diene. This process is characterized by an extremely high primary deuterium KIE ($k_H/k_D = 22.9 \pm 2.8$) at C-9 which was attributed to hydrogen tunneling.[137] The cryptoregiochemistry matches that of all membranous Δ9 desaturases studied to date. Thus, the presence of a neighbouring (*E*)-double bond in the substrate does not influence the choice of initial oxidative attack which is obviously determined by a strict, active-site imposed regiochemical imperative. Interestingly, 11(*Z*)-tetradecenoate is oxidized, possibly by the same enzyme, to give (*E*,*E*)-10,12-tetradecadienoate by 1,4-desaturation (initial site of oxidation at C-10).[138] The stereochemistry of both transformations has also been determined and shown to be "*syn*" and they are related to each other in a formal sense as depicted in Scheme 10.[139,140]

5.7 Δ12 (FAD2) desaturase

The "essential fatty acid", linoleic acid, is biosynthesized in the plant by one of two enzyme systems: 1) a plastidial enzyme (*FAD6*) which uses the methyl terminus as a reference point and has been classified as an ω-6 desaturase because it introduces the double bond six carbons from the ω-carbon; 2) an extraplastidial system known as oleate Δ12 desaturase (FAD2) which is selective for C-12,13 oxidation independent of chain length and acts on 9(*Z*)-monoenoic substrates.[141] When presented with a 10(*Z*)C19:1 substrate, this enzyme will use the existing double bond as a reference point and produce a methylene interrupted diene: 10(*Z*)13(*Z*)C19:2.[142] FAD2 desaturase has attracted a great deal of attention since a closely related enzyme found in *Ricinus communis* converts oleate to (*R*)-12-hydroxystearate and a *Lesquerella* bifunctional system produces a mixture of 12-hydroxylated and 12-monoenoic product (Scheme 11A).[143] Indeed, wild type FAD2 found in the model plant *Arabidopsis thaliana* possesses inherent, low level hydroxylation activity as do a number of other membrane-bound enzymes (*vide supra*).[73] It has also been shown that the ratio of 12-ol/12-ene can be adjusted through site-directed mutagenesis experiments involving as few as four amino acid residues.[78] More recently, it has been shown that changes in amino acid at positions 148 and 324 of *Arabidopsis* FAD2 play an important role in determining the ratio of dehydrogenation *versus* hydroxylation.[73] The molecular biological and stereochemical data implied that both linoleate and ricinoleate formation were initiated by abstraction of the pro*R* C-12 hydrogen.[62,73] Support for this hypothesis was obtained by comparing the primary deuterium KIE on C–H cleavage



Scheme 11 **A** C-12-initiated bimodal oxidation of oleate by FAD2. **B** Species-specific oxidation of linoleate by FAD2 variants. The site of initial oxidation for crepenynate formation is at C12.

at C-12 and C-13 using a strain of *S. cerevisiae* containing a functionally expressed plant oleate Δ^{12} desaturase from *Arabidopsis thaliana.*[144] As anticipated, a large primary deuterium KIE was observed at C-12 (k_H/k_D = 7.3 ± 0.4) and essentially no effect at C-13 (k_H/k_D = 1.05 ± 0.04).[145] Similar results have been obtained for the oleate Δ^{12} desaturase in *Chlorella.*[63]

5.8 Δ15 (ω-3) desaturase

Cryptoregiochemical analyses of the type described in the previous section have also been carried out in mechanistic studies on the formation of α-linolenic acid (ALA) in plants (FAD3)[146] and in the nematode worm *C. elegans* (FAT1).[147] The latter system normally produces eicosapentaenoic acid (EPA) from arachidonic acid.[148] These enzymes are more accurately termed ω-3 desaturases rather than Δ15 since, when presented with a series of fatty acyl substrates of varying chain length, a double bond is always introduced three carbons from the methyl terminus. Using strains of *S. cerevisiae* containing *fad-3*[149]or *fat-1*[148] genes and the appropriate, regiospecifically deuterated substrates, it was found that ω-3 desaturation proceeds with large primary deuterium kinetic isotope effects only at the ω-3 position in both cases.[146] A similar trend in KIEs (one large, one negligible) was obtained for this reaction as it occurs in *Chlorella vulgaris*.[150] These results clearly point to ω-3 (18 − 3 = 15) as the site of initial H-abstraction for ω-3 desaturases; importantly, small amounts of 15-hydroxylinoleate have been found in flax seed oil—a rich source of α-linolenate.[73] Surprisingly, the stereochemistry of hydrogen removal for either FAD3 or FAT1 catalyzed transformations has not been determined to date.

5.9 Summary

The mechanistic study of various membrane-bound desaturases described in the preceding sections has revealed three remarkably consistent trends. First, only one of the C–H cleavage steps involved in desaturation is subject to a large deuterium KIE (typical k_H/k_D ~ 4–8), a negligible effect being found at the proximal carbon (k_H/k_D ~ 1). These results clearly indicate a stepwise mechanism. Secondly, the isotopically sensitive step normally occurs at the carbon closest to C-1 which, according to our mechanistic model (Scheme 3), implies that desaturation is initiated at this site. Thirdly, hydrogen removal is pro*R* selective and occurs with *syn* stereochemistry. Taken together, these data point to a highly conserved active-site architecture which is depicted in Fig. 1. Modulation of the distance between the methyl (ω-*n*) or C-1 (Δ*n*) terminal recognition sites through natural protein engineering yields the various regioselectivities found in biology. The maintenance of a constant cryptoregiochemical and stereochemical motif over such a wide range of regiochemical preferences is somewhat surprising and was not anticipated at the outset.

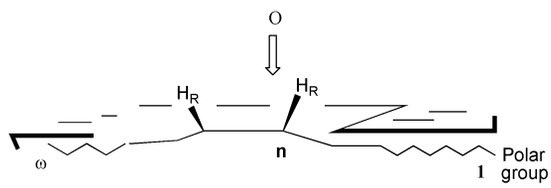


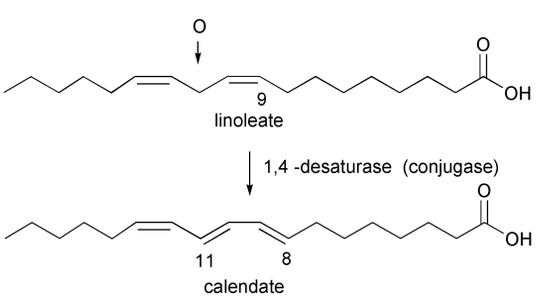
Fig. 1 Active site model of membrane-bound desaturases showing that reaction is normally initiated by removal of the pro*R* hydrogen closest to C1. Reactions proceed uniformly with *syn* stereochemistry.

6 Exotic modifications of plant lipids

A number of enzymatic systems belonging to the plant oleate Δ12 desaturase (FAD2) subfamily have been identified recently which gives rise to a collection of lipids bearing a rich variety of functional groups including conjugated dienes,[151–157] alkyne[158] and epoxy moieties[158](Scheme 11B). These plant products are thought to participate in a stress response strategy.[159] The bioorganic characterization of these fascinating systems is only just beginning.

6.1 Conjugases

Sequence analysis of plant genes responsible for the production of conjugated trienoic acids of the type displayed in Schemes 11B, 12 revealed the involvement of FAD2 variants.[151] Thus it appeared that these highly unsaturated materials are biosynthesized by an apparent (1,4)-dehydrogenation process analogous to that proposed for the more common (1,2)-dehydrogenation reactions of fatty acid desaturases. Support for this hypothesis had already been obtained by Crombie *et al.* some years ago: the results of labelling experiments using marigold seed homogenates and labelled linoleate precursors demonstrated that calendic acid (Scheme 12) is produced by loss of hydrogen from C-8 and C-11 and no oxygenated intermediates could be detected.[160] Further evidence for the close relationship between 1,2- and 1,4-dehydrogenation was obtained for a *Spodoptera littoralis* desaturating system as discussed previously in Section 5.6 (Scheme 10).[140] The availability of a convenient yeast expression system for *Fac2*—a *Calendula officinalis* gene encoding the (1,4)-desaturase involved in calendic acid production—allowed a KIE study to be carried out in convenient fashion.[161] It was found that the site of initial hydrogen abstraction is at C-11 as would be expected for a FAD2 variant; recall that the parent enzyme initiates oxidation at C-12 (Scheme 11A). Thus only a slight shift in the position of the iron oxidant relative to substrate is required to perform the 1,4-dehydrogenation reaction. The path is now open to examine the topology of oxidation for the other FAD2-like enzymes involved in the formation of α-eleostearic, punicic and α-parinaric acid from linoleic acid.



Scheme 12 C-11-initiated 1,4-dehydrogenation of linoleate in *Calendula officinalis*.

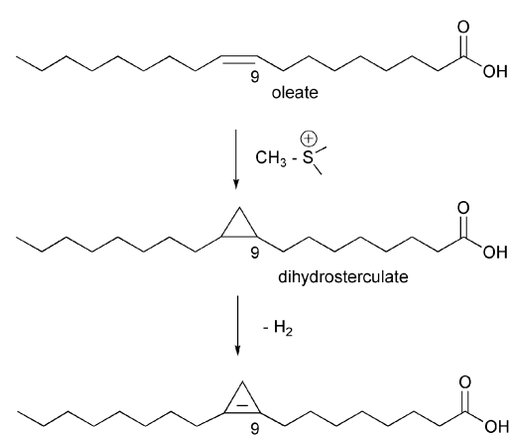
6.2 Acetylenases

The dehydrogenation of linoleate to give crepenynate (Scheme 11B) represents an excellent example of enzymatic behaviour which appears to break the "rules" of classical organic chemistry. The critical question arises: what is the key factor which prevents the energetically far more facile epoxidation reaction from taking place? A major advance in this area was achieved by the cloning of a cDNA from *Crepis alpina* (*Asteraceae*) which encodes the enzyme responsible for the conversion of linoleate to crepenynate.[158] A closely related enzyme found within the genus *Crepis*, bearing a high degree of sequence similarity to the *C. alpina* acetylenase, converts a linoleoyl substrate exclusively to vernolate (12,13-epoxyoleate) (Scheme 11B). Other examples of acetylenase-catalyzed chemistry have been found to accompany Δ6 and Δ11 desaturation of fatty acids in the moss *Ceratodon purpureus*[121] and insect *Thaumetopoea* (*Lepidoptera*) respectively.[162]

Given the fact that non-heme di-iron centres are capable of cleaving the strong C–H bonds found in methane, and methyl groups of n-alkanes, it is not inconceivable that the alkene/alkyne transformation proceeds along a mechanistic pathway similar to that shown for the desaturation of alkanes (Scheme 3). In addition, the requirement for an eclipsed arrangement between vicinal orbitals (Scheme 4) is clearly met in (*Z*)-alkenes. Recently experimental support for a conventional two step mechanism was obtained by a KIE study using the *Crepis* acetylenase expressed in a yeast expression system.[163] The operation of a large primary deuterium isotope effect (KIE = 14.6 ± 3.0) was demonstrated for the carbon–hydrogen bond cleavage at C12 while the C13–H bond breaking step was found to be relatively insensitive to deuterium substitution (KIE = 1.25 ± 0.08). Interestingly, the site of initial oxidation for this acetylenase matches that found for FAD2-mediated desaturation of oleate. Further insight into the factor(s) controlling acetylenation *vs.* epoxidation will require the purification and structural characterization of the two enzymes from *Crepis* which perform these reactions. Some progress towards achieving these goals has been made recently.[164]

6.3 Cyclopropenyl fatty acids

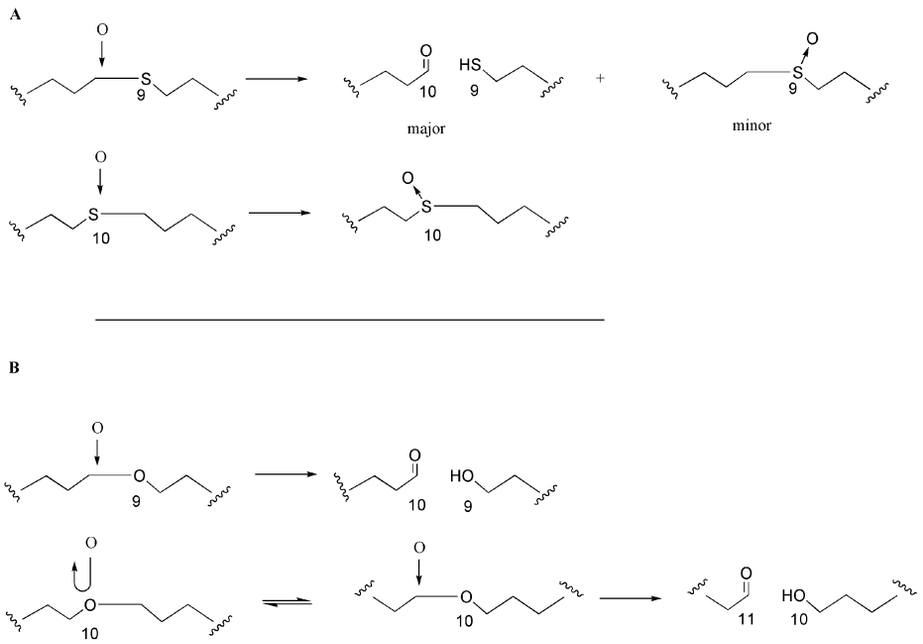
The highly strained cyclopropene-containing fatty acids are produced from unsaturated precursors in several tropical plant species (*Sterculia*, *Malva*) by a cyclopropanation/desaturation sequence (Scheme 13). The cyclopropane synthase from *Sterculia foetida* has recently been expressed in tobacco cells; efforts to clone the desaturase responsible for sterculate formation can be anticipated.[165,166] Several lines of evidence point to the specific inhibition of membranous desaturases such as SCD by the highly strained cyclopropene-containing fatty acids.[167,168] Further advances in our understanding of the biosynthesis and biological activity of sterculate can be anticipated in the near future given the current interest in SCD (See Introduction).



Scheme 13 Biosynthetic route to sterculate in *Sterculia foetida*.

7 Soluble plant Δ9 desaturases

A major milestone in desaturase research was achieved in the mid-1990's with the publication of the Lindqvist X-ray structure of the castor stearoyl ACP Δ9 desaturase.[57] This work provided the first clue as to how active site topology might influence the conformational preference of a mobile alkane moiety such that a thermodynamically less stable (*Z*)-olefinic bond is generated upon dehydrogenation. A description of some of the work leading up to this event is given here (personal communication, John Shanklin, BNL): "*Previous attempts at*



Scheme 14 Mechanistic probes of a soluble plant Δ9 desaturase. **A** Regioselective oxidative cleavage and sulfoxidation chemistry. **B** Oxidative cleavage of oxo-substrate analogues residing in normal and abnormal conformations.

purification had stalled because the desaturase was labile until fairly well enriched. We overcame this problem by extracting several kilos of avocados at a time, making a delipidated crude extract and passed ~5 liters of this through a 1 ml substrate analogue affinity column. Once eluted from the column, the enriched protein was subjected to cation affinity chromatography that yielded several milligrams of a fairly pure desaturase enzyme. This was used for antibody production and subsequently for cDNA isolation from a castor library we obtained from Dave Dennis. Functional confirmation of the gene's identity was achieved by expression of the cDNA in yeast and assaying extracts. A desaturase gene from which the transit-peptide-encoding portion had been excised, was expressed in an E. coli T7 expression system. To produce sufficient quantity of functional enzyme for crystallography we employed a 100 liter fermenter for culture production, and for purification, switched our microbore analytical columns for preparative columns. The desaturase proved very difficult for obtaining good crystallographic data. I estimate we consumed 5–10 grams of protein (representing ~1000 liters of E. coli culture) before achieving the 1996 crystal structure with Ylva Lindqvist."

Two further X-ray structures of this protein with bound azide and acetate have been obtained recently and give important information as to some of the changes in iron co-ordination that take place as substrate and oxygen are processed.[58] These results correlate well with what is known from a detailed MCD study—namely that substrate binding to reduced enzyme promotes oxygen binding *en route* to a Compound Q-like oxidant which is capable of attacking unactivated C–H bonds.[59] The overall kinetics of these events are complicated by the fact that the desaturase is a functional homodimer but the details of substrate binding and diiron reduction have been worked out in some detail by Fox and coworkers.[169–173] It should be emphasized that the structure of the active oxidizing species is not known with certainty and the use of soluble methane mono-oxygenase (sMMO) as a model in this respect may well be an oversimplification.

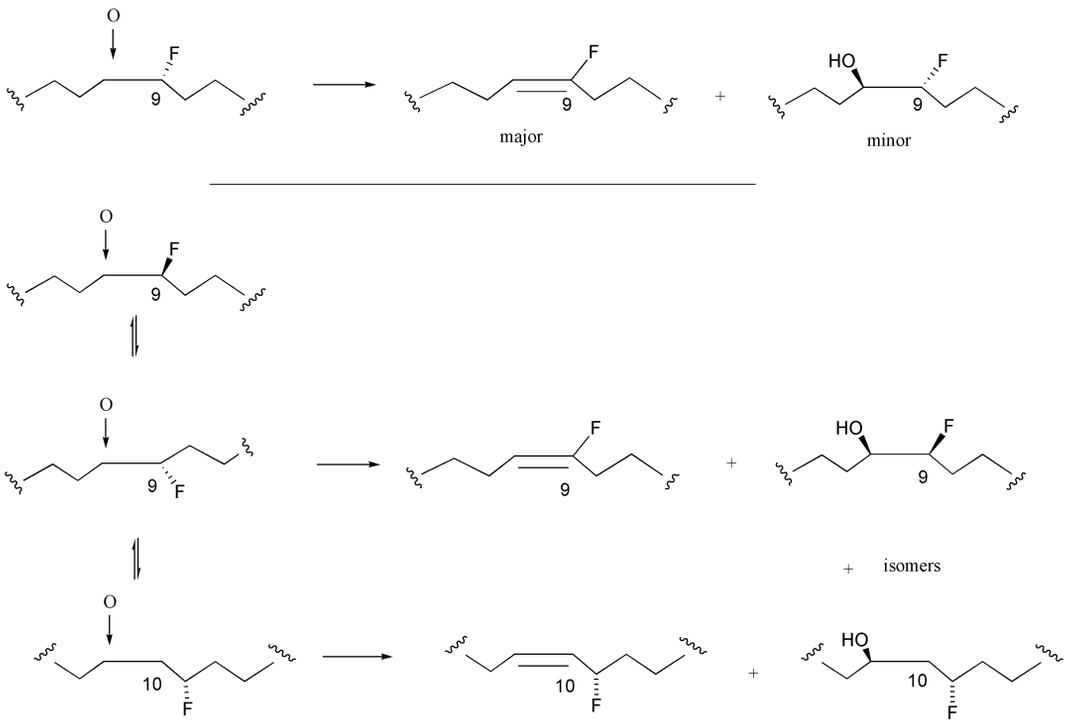
From the point of view of deducing the bioorganic mechanism, it is unfortunate that the C–H bond breaking steps are obscured kinetically in this system by other enzymatic events. This became apparent when the intermolecular deuterium KIEs on C–H cleavage at both the 9- and 10-positions were measured to be ~ 1 through the use of the standard KIE test (Scheme 5A). This fact has prevented the direct determination of the site of initial oxidation—a parameter which was so readily acccessible for membrane-bound desaturases (*vide supra*). The use of natural abundance deuterium measurements may be helpful in this regard but this approach remains to be validated.[174] Other mechanistic probes have yielded valuable information as to how the substrate might reside in the active site of stearoyl ACP desaturase.

7.1 Stereochemistry

Using stereospecifically deuterated substrates, prepared in a manner similar to that employed by Bloch,[61] it was shown that the substrate hydrogens are removed in *syn*-fashion with a pro*R* enantioselectivity at C-9 and C-10.[175] Interestingly, this stereochemical preference is the same as that determined for the structurally unrelated membrane bound Δ9 desaturases (Fig. 1). The current computer model of the substrate in the active site of the Lindqvist X-ray structure is consistent with the experimental data in that the pro*R* hydrogens are pointing towards the catalytic iron centres. However it is important to keep in mind that this structure was obtained without bound substrate and it is known that substantial changes in di-iron co-ordination do take place upon substrate binding.[59] On the other hand, the results of protein engineering experiments on soluble desaturases have, in part, been rationalized using the current substrate binding model.[176]

7.2 Cryptoregiochemistry

The site of initial oxidation for stearoyl ACP Δ9 desaturase-mediated dehydrogenation cannot be ascertained from the current X-ray crystallographic model.[57] However application of the thia test (Scheme 5B) gave interesting results.[176] It was found that 10-thiastearoyl ACP was converted cleanly to the corresponding sulfoxide while oxidation of the 9-thia isomer gave the corresponding sulfoxide in much lower yield (~10%). When substrates bearing sulfur at other carbon sites were incubated with the desaturase, normal dehydrogenation products were detected. This set of experiments was repeated using ω-fluorinated thia probes, followed by ^{19}F NMR analysis with similar results.[96] In the case of the 9-thia experiment, a novel product was observed which was thought to arise by a chain cleavage process (Scheme 14A). These data have recently been confirmed



Scheme 15 Comparison of (*R*)- and (*S*)-9-fluorostearate oxidation by a soluble plant Δ9 desaturase leading to novel oxygenated and olefinic products.

by Fox and coworkers and in addition, it has been shown that oxygenation at sulfur was an aerobic process by ^{18}O labeling.[177]

The results of the thia test are diametrically opposed to those obtained for desaturation by the membrane-bound yeast Δ9 desaturase (Scheme 5B)—a reaction which almost certainly is initiated at C-9. Thus one can conclude that the soluble enzyme may in fact operate by initial attack at the C-10 position. Some support for this hypothesis is available from work using probes containing other heteroatoms. Incubation of a series of oxo-substrates generated the expected chain cleavage product from a 9-substituted analogue but when the 10-oxo-substrate was processed, a regiochemical error was induced (Scheme 14B) rather than attack at C-9.[178] A similar loss of regiochemical precision has been observed with racemic 10-monofluoro-stearate; no 9-hydroxylated products were detected with these substrates as well (Behrouzian, Shanklin and Buist, unpublished results). In addition, the oxidation of (*R*)- and (*S*)-9-fluorostearoyl ACP was compared (Scheme 15).[77] The latter fluoroanalogue was processed to give the fluoroolefin with the anticipated stereochemistry along with a small amount of a *threo*-fluorohydrin, derived aerobically as shown by ^{18}O-labelling. In contrast, the (*R*)-fluorostearate—a substrate designed to block the dehydrogenation pathway—was oxidized to give mainly "error" fluoroolefinic products along with a mixture of minor 10- and 11-hydroxylated compounds. The observed shift in desaturase regioselectivity (Δ9 to Δ10) mimics the behaviour of the 10-oxo-substrate (*vide supra*) and other unnatural substrates[98] but the reversal of stereochemical outcome ("*cis*"- to "*trans*"-olefin) was completely unexpected. Observation of deuterium-induced branching using regio-specifically labelled (*R*)-9-fluorosubstrates led to the conclusion that the two pairs of olefinic/hydroxylated products were obtained from two rapidly equilibrating conformers which suffer initial oxidative attack at C-10 and C-11 as shown in Scheme 15. While the weight of the evidence certainly points to C-10 as the site of initial oxidation for the soluble Δ9 desaturase, this cryptoregiochemical assignment must be regarded as tentative. In this context, it may be possible to dissect the small deuterium KIE on C–H cleavage observed recently in rapid-mixing experiments into contributions from the C-9 and C-10 positions *via* regiospecifically-deuterated substrates.[173]

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