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Catalytic diversity of fatty acid desaturases

Peter H. Buist*

Department of Chemistry, Carleton University, Ottawa, Ontario K1S 5B6, Canada

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Abstract—The highly selective oxidation chemistry carried out by fatty acid desaturases is a potentially important source of novel biocatalytic activity. Recent progress in the mechanistic understanding of this set of reactions will help to guide ongoing protein engineering experiments designed to modify desaturases for specific requirements.

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1. Introduction

The ability to functionalize unactivated hydrocarbons in a selective manner has been an important research objective for many years.[1] Enzymes such as bacterial cytochromes P450 appear to be particularly attractive with a view to engineering robust biocatalysts with useful substrate throughput.[2] Non-heme di-iron-containing enzymes such as methane monooxygenase (MMO),[3] toluene monooxygenase (TMO)[4,5] and alkane ω-hydroxylase (AlkB)[6]are also potentially important in the sense that these systems are capable of oxidizing very strong C–H bonds, and very often in contrathermodynamic fashion (Scheme 1). Fatty acid desaturases such as stearoyl CoA desaturase (SCD) are important members of this group of metalloproteins and feature highly stereo-, regio- and chemoselective dehydrogenation reactions.[7] Traditionally, these enzymes have been regarded as too restrictive in terms of substrate specificity and reaction outcome to be of interest as synthetically useful biocatalysts. However, substantial advances in the structural and mechanistic characterization of desaturases, coupled with the discovery of novel catalytic behaviour, has generated renewed enthusiasm for considering this group of proteins as candidates for optimization through protein engineering. Here, we highlight some of the recent developments in this area with a special focus on the catalytic plasticity of fatty acid desaturases and related enzymes. Earlier reviews have dealt with more general aspects of this topic.[8–11]

2. Structural biology of desaturases

Fatty acid desaturases (>100) have been identified in virtually all aerobic life forms including bacteria, yeasts and fungi, plants and animals, where they play a critical role in lipid biosynthesis. The majority of these enzymes are integral membrane-bound proteins while the structurally distinct soluble desaturase systems are found in

* Tel.: +1 613 520 2600x3643; fax: +1 613 520 3749; e-mail: pbuist@ccs.carleton.ca

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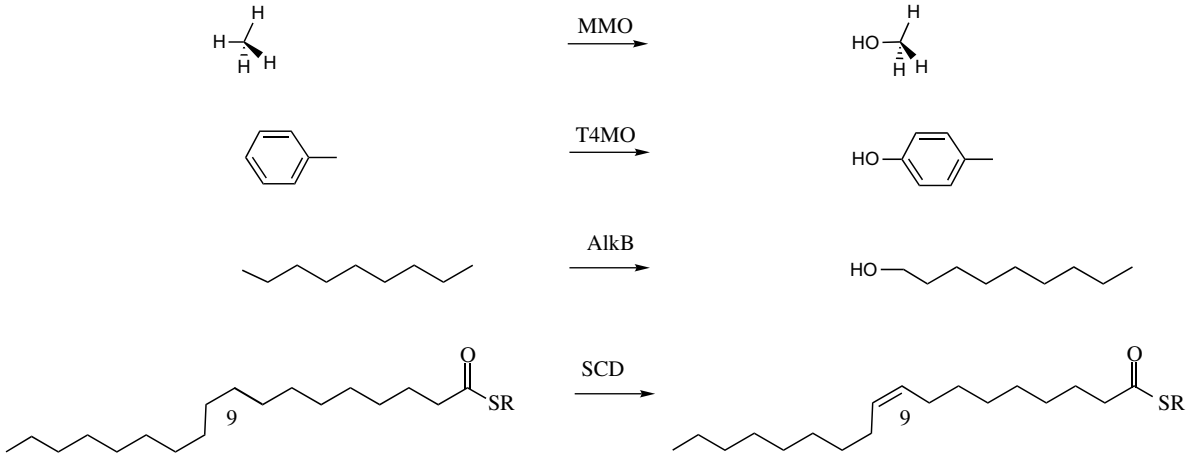


Scheme 1. Some transformations catalyzed by non-heme di-iron-containing enzymes.

the chloroplasts of photosynthetic organisms. Associated with both types of desaturases are typical electron transfer proteins, which serve as a conduit for reducing equivalents from NAD(P)H to molecular oxygen. The overall stoichiometry of the reaction is shown in Eq. 1.

$$\mathrm{NADH + H^+ + O_2 + R\text{-}CH_2{-}CH_2\text{-}R} \rightarrow \mathrm{NAD^+ + 2H_2O + R\text{-}CH{=}CH\text{-}R} \quad (1)$$

Extensive bioinorganic studies[8] of the soluble plant desaturases have indicated the presence of a functional, non-heme, carboxylate-bridged, di-iron centre similar to that found in methane monooxygenase (MMO).[3] X-ray crystallographic analysis of a stearoyl ACP Δ9 desaturase from *Castor* suggests that the substrate binds in an extended, gauche conformation, which would allow stereoselective removal of the pro-*R* hydrogens at C-9 and C-10 (Fig. 1).[12] This model is consistent with the results of a recent stereochemical study[13] and has also been used to guide various protein engineering experiments designed to modify chain length specificity and regiochemistry.[14,15] An interesting feature of this enzyme system is that the binding of the substrate ACP thioester facilitates oxygen-binding to the di-iron centre in preparation for the substrate oxidation event.[16] Substrate binding may be responsible for masking the C–H bond-breaking steps, which have been found to be insensitive to isotopic substitution.[17]

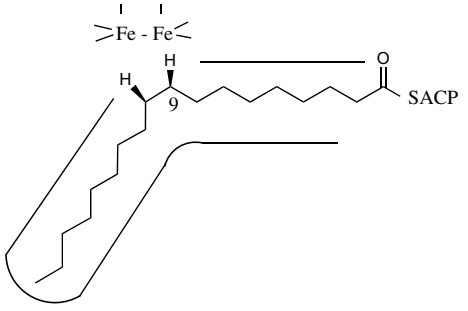
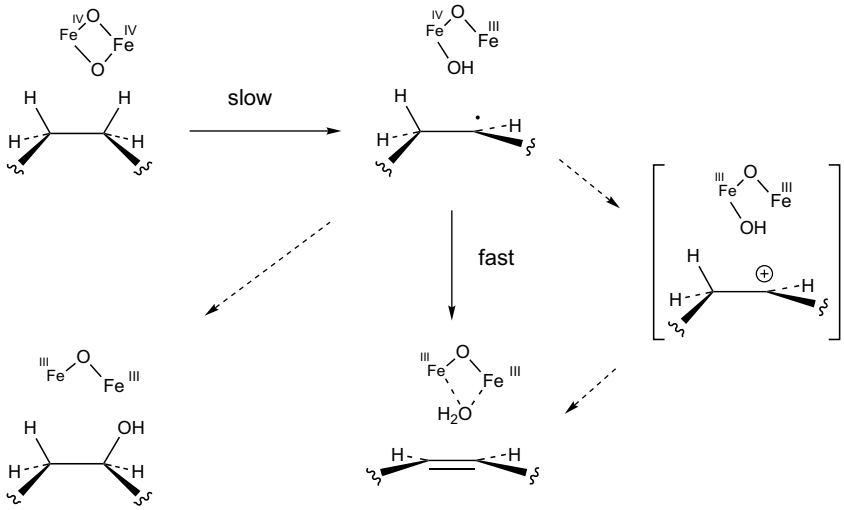


Figure 1. Probable conformation of substrate residing in active site of soluble stearoyl ACP Δ9 desaturase.

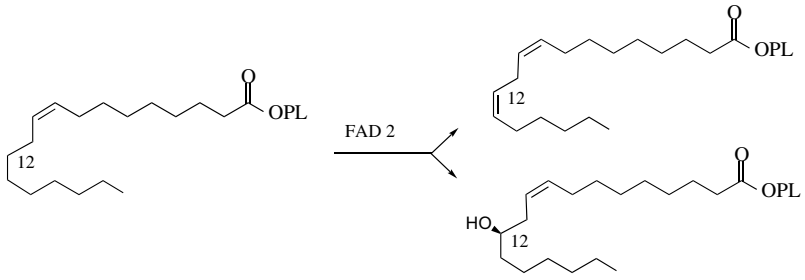
While detailed structural characterization of membranous desaturases has not been achieved to date, the available evidence points to the presence of a multi histidine-coordinated, di-iron catalytic core, also thought to be present in AlkB.[18,19] Both hepatic and yeast stearoyl CoA Δ9 desaturases (SCD) require fatty acyl substrates to be in the CoA thioester form. In most other cases, the substrates possess a glycerolipid headgroup and presumably enter the catalytic cavity via the supporting phospholipid membrane. It appears that membrane-bound desaturases possess a somewhat more capacious binding site than that found in the soluble proteins (vide infra). For the former class, it has also been shown through numerous KIE studies that the initial C–H cleavage step leading to olefinic product is kinetically significant.[11]

3. Mechanistic considerations—the hydroxylase/desaturase connection

The consensus mechanistic model for desaturase-mediated dehydrogenation (Scheme 2) is based on an early proposal of Ortiz de Montellano for the corresponding cytochrome P450 enzymes.[20] It is assumed that such a scheme applies to all O_2-dependent, iron-mediated, dehydrogenations requiring an energetically difficult, initial C–H activating step. In the case of fatty acid desaturases (both soluble and membrane-bound), the active oxidant is postulated to be a compound Q-type (Fe_2O_2) species similar to that postulated for MMO-catalyzed biohydroxylation.[3] The most direct route to olefin involves a slow hydrogen abstraction step to give a short-lived carbon-centred radical, which collapses rapidly to give an olefinic product by a second carbon-hydrogen cleavage. (The involvement of a carbocationic intermediate, which undergoes deprotonation is also possible.) This mechanistic model is consistent with the frequent observation of a large primary deuterium kinetic isotope effect on the initial C–H cleavage while the KIE on the following step is typically close to unity as would be expected for collapse of a highly unstable radical or carbocationic intermediate. These data[21] rule



Scheme 2. Generic mechanism for fatty acid desaturation showing its relationship to hydroxylation. The structure of the di-iron oxidant and the reactive intermediates are speculative.



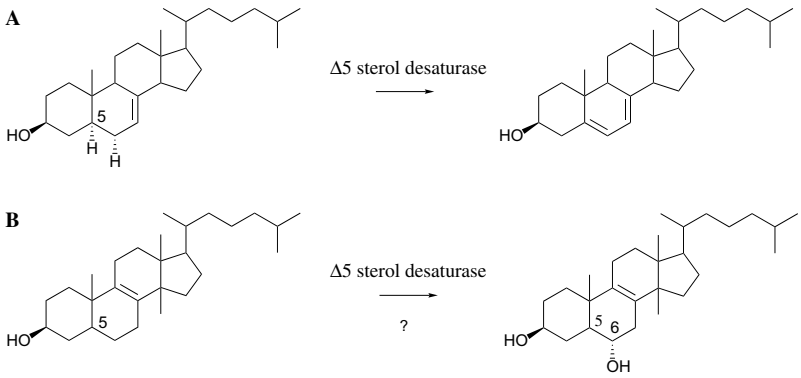
Scheme 3. Biotransformation of oleate to give linoleate and ricinoleate by a bifunctional Δ12 desaturase.

out a synchronous removal of hydrogens as has previously been suggested.[22] The notion that it is the intermediate iron hydroxy species that is responsible for removal of the second hydrogen is consistent with the fact that all desaturations studied to date involve a *syn*-removal of neighbouring hydrogens.[11]

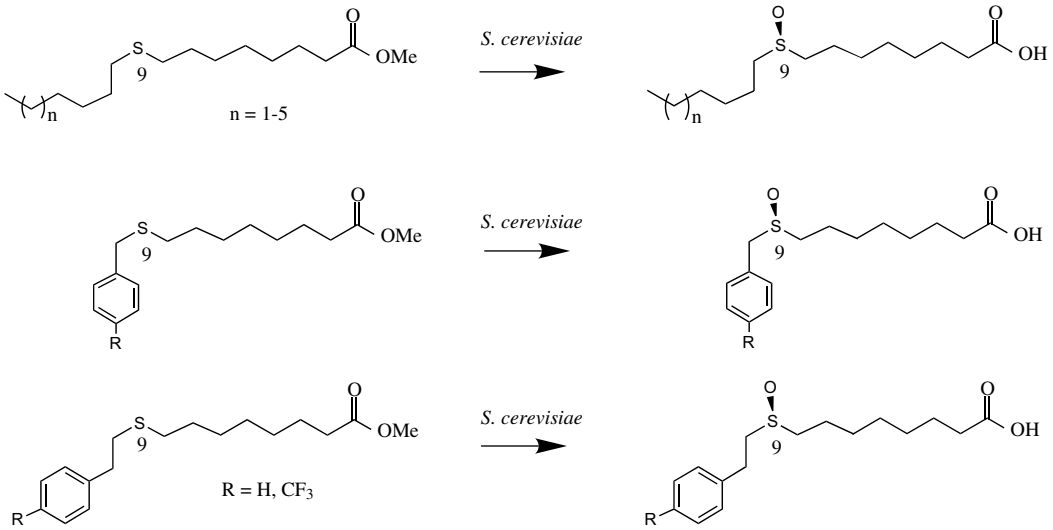
As is shown in Scheme 2, all desaturases are latent hydroxylators and indeed many membrane-bound desaturases apparently allow a competing 'hydroxyl rebound' pathway to give variable amounts of a secondary alcohol byproduct at the putative site of initial oxidation.[23] This bimodal behaviour appears to be relatively common and suggests that these proteins can, in principle, be engineered to be enantioselective hydroxylators—a valuable biocatalytic function. Indeed, site directed mutagenesis experiments involving changes in hydrophobic residues of a membranous, bifunctional 12-hydroxylase/12-desaturase were able to affect the relative amounts of oxygenated versus dehydrogenated products (Scheme 3).[23,24] Understanding the switch, which controls the two pathways is critical to the interpretation of such experiments. Shaik and co-workers have recently addressed this issue computationally for cytochrome P450s–enzymes, which normally give hydroxylated products:[25] a desaturation pathway is apparently favoured by steric hindrance to hydroxyl rebound and by substrate features, which promote a carbocationic intermediate. The relevance of this work to non-heme di-iron systems remains to be determined but substituent effects appear to affect the dehydrogenation/hydroxylation ratio for reactions catalyzed by soluble methane monooxygenase, sMMO[26] and soluble stearoyl ACP Δ9 desaturase.[13]Another apparent case of substrate-induced pathway switching has come to light recently during an investigation of antifungal drug resistance in yeast strains. It is postulated that a sterol Δ5 desaturase acts as an α-6-hydroxylase when presented with an unnatural substrate bearing an 8,9-double bond and a C14-methyl group (Scheme 4).[27,28] Interestingly, Rahier has found that Δ5 desaturation of the normal substrate is initiated by removal of the α-C6 hydrogen.[29] This system would appear to offer a unique opportunity to study the hydroxylase/desaturase switch using substrates of known conformation.

4. Desaturases as enantioselective sulfoxidases

The first explicit attempt to demonstrate that desaturases could act as oxygenases was accomplished by screening for sulfoxide formation using a series of thia-analogues and an in vivo baker's yeast Δ9 desaturating



Scheme 4. Δ5 Sterol desaturase-mediated dehydrogenation (**A**) and a possible example of substrate-induced hydroxylation (**B**).



Scheme 5. Typical examples of enantioselective biosulfoxidation as carried out by a yeast Δ9 desaturase.

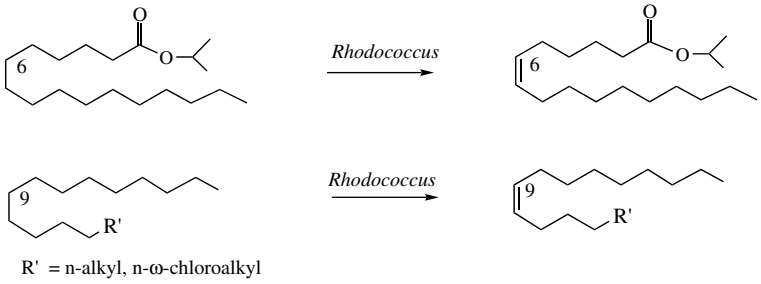
system (Scheme 5).[30] It was assumed that thia substrates, added to the medium of actively growing microbial cultures, would enter the cellular fatty acyl CoA pool[31]and be sulfoxidized by the Δ9 desaturase in a regioselective manner. This is precisely what was observed. An unexpected bonus was that the polar sulfoxy products were not incorporated into the lipids but were transported into the medium as the free acids—a phenomenon that greatly facilitated product isolation. It was found that maximal yields of sulfoxide were achieved when the sulfur atom was in the C-9 position and when the effective chain lengths of substrate ranged from C15–C19.[32] Somewhat surprisingly, substrates bearing pendant benzyl and phenethyl substituents were also oxygenated.[33,34] In all cases, the enantioselectivity of oxo transfer was very high (>95% ee) and matched the pro *R* stereochemistry of hydrogen removal for the parent desaturase-mediated reaction.[35] The ratio of in vivo S-9 to S-10 sulfoxidation was in the range of 2–3:1,[30,33,35,36] an observation, which was consistent with the results of subsequent KIE studies, which showed that Δ9 desaturation was initiated at C-9.[37] The opposite trend in *S*-oxidation regioselectivity was observed when a soluble plant Δ9 desaturase was used as the catalytst: 10-sulfoxides were produced in much higher yield than 9-sulfoxides.[17,38,39] The latter result correlates well with the regiochemistry of induced hydroxylation when a series of oxo-substituted[40] or monofluorinated[13] substrate analogues are processed by this enzyme.

The scope of regioselective desaturase-mediated sulfoxidation was tested using an active Δ6 desaturase system found in *Tetrahymena thermophila* cultures. Again, oxo transfer was found to be highly enantioselective and most efficient when the substrate sulfur atom replaced the C-6 methylene group—known from KIE studies to be the site of initial oxidative attack.[41]

It should be noted that the use of baker's yeast as a sulfoxidizing reagent stimulated other researchers to explore this capability with non-fatty acyl aromatic sulfides.[42] Excellent enantioselectivities were obtained; however it appears that an endogenous cytochrome P450 may have been responsible for the catalytic activity observed in these cases.

5. Tunable regioselectivity

One of the attractive features of desaturases is the wide range of regioselectivities, which occur in biological systems.[9] This variability is reminiscent of that exhibited by



Scheme 6. Headgroup-dependent regioselectivities exhibited by a *Rhodococcal* desaturating system.

mammalian lipoxygenases (5-, 8-, 11-, 12-, 15-LOs).[43] A major goal is to elucidate the structural determinants for positional specificity and some modest progress has been made in this direction.[15,44] The primary sequences of a large number of desaturases have been sorted into the two major classes (soluble and membrane-bound) and further organized into subfamilies corresponding to the different regiochemistries of double bond introduction.[9] For the soluble class of desaturases, Δ4 and Δ6 desaturases are known along with the ubiquitous plant Δ9 enzyme. The set of membranous desaturases exhibit a much greater range of regioselectivity-from Δ3 to Δ15. Most commonly, the position of the incipient double bond (Δ*n*) is determined by the number of methylene units (*n*) from the substrate acyl group and some variation in substrate chain length (within certain limits) as well as the presence of mid-chain heteroatom substitution[45] is permitted. An alternative mode of regiochemical control, (ω-*n*), features the methyl terminus as the recognition point, although examples of this type of selectivity are relatively limited. A notable example of bimodal regioselectivity is illustrated in Scheme 6: a *Rhodococcus* mutant is capable of inserting a double bond at the C-6,7 position when presented with a palmitoyl substrate and at the C-9,10 position of simple hydrocarbons or ω-halocarbons.[46] This has been exploited in the multigram preparation of these unsaturated materials using a repeat–batch membrane bioreactor with a phase inversion design.[47] More recently, a subtle influence of headgroup on regiochemistry has been uncovered for a plant desaturase, which Δ9 desaturates palmitoyl phosphotidyl choline (PC) but dehydrogenates with Δ7 regioselectivity if the palmitoyl substrate bears a monogalactosyl-diacylglycerol (MGDG) headgroup.[48]

Despite the lack of detailed structural information, some features of active site architecture have been elucidated for numerous membrane-bound desaturases of varying regioselectivities. Thus, we now know that it is the pro-*R* hydrogen (or its topological equivalent) at the carbon closest to C-1, which is removed first, followed by the second hydrogen proximal to the methyl terminus, in overall *syn* fashion.[11] This indicates that the local desaturase active-site topology is highly conserved and should allow for the accurate prediction of the regiochemistry and stereochemistry of any latent membrane-bound desaturase-mediated oxygenation reaction (cf. Section 3).

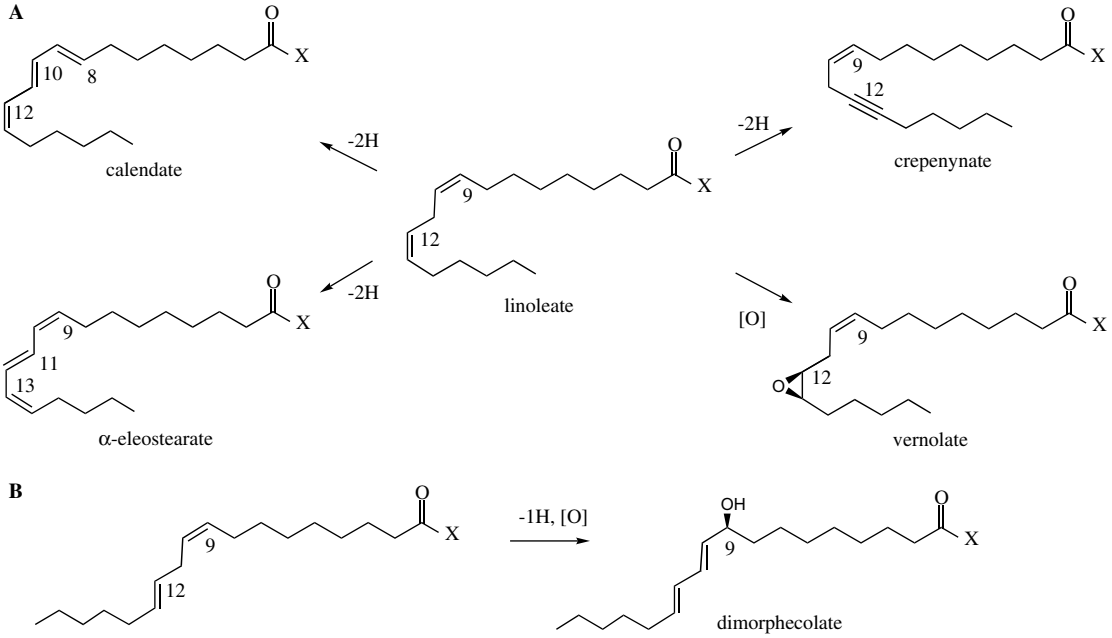
6. Exotic transformations catalyzed by desaturase-related enzymes

Nowhere is the catalytic diversity of desaturases exhibited more spectacularly than in the species-specific production of unusual plant fatty acids derived from linoleic acid (Scheme 7). Significant advances in the ability to functionally express the enzymes (FAD2 family) responsible for these transformations in a microbial host has greatly facilitated their mechanistic characterization and function-based classification. The picture that is beginning to emerge is that through subtle changes in FAD2 active site architecture, the course of linoleate oxidation can be altered to give a unique product.

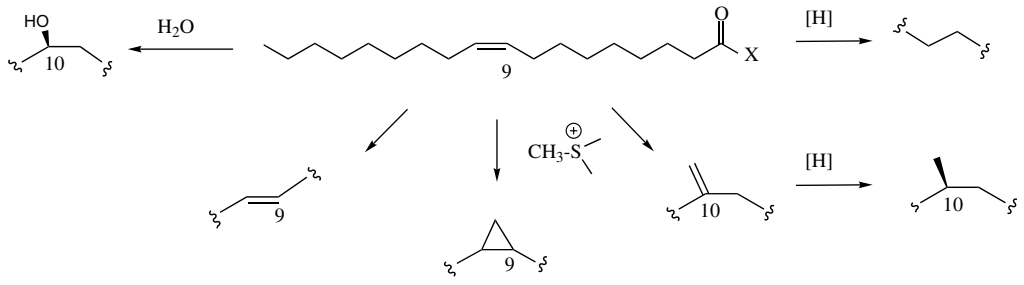
Probably the most intriguing case of 'natural' protein engineering in this context is the dehydrogenation of linoleate to give crepenynate,[49] a reaction that is without any laboratory precedent and that must surely qualify as a prime example of 'extreme enzymology' (Scheme 7A). This process proceeds by a stepwise mechanism as indicated by a large primary deuterium isotope effect on C–H cleavage at C-12 while the C13–H bond breaking step is insensitive to deuterium substitution.[50] Even more remarkable is the fact that a closely related FAD2 variant generates vernolate (12,13-epoxyoleate) by a standard epoxidation reaction. The nature of the switch governing these two pathways is a fascinating mechanistic problem.

Another interesting pair of reactions, which uses linoleate as a common substrate is 1,4-dehydrogenation to give calendate or α-eleostearate (Scheme 7A). The former reaction proceeds by sequential hydrogen atom abstraction at C11 followed by C8 as determined by KIE studies.[51] The cryptoregiochemistry of α-eleostearate formation[52] is unknown as is the enantioselectivity of either reaction. Recently, it has been shown that a geometric isomer of linoleate is converted to a C-9 hydroxylated product (dimorphecolate) presumably by regioselective hydroxyl trapping of an allylic radical (or carbocationic) intermediate derived by hydrogen abstraction at C-11 (Scheme 7B).[53] Methyl dimorphecolate can be converted to highly enantiomerically enriched (*R*)-9-hydroxystearate via hydrogenation.

An impressive display of oxidative chemistry similar to that depicted in Scheme 7 is also found in the biosynthesis



Scheme 7. Species-specific oxidation of linoleate by FAD2 variants (A) and a unique FAD2-catalyzed transformation of linoleate in *Dimorpheteca* involving oxygenation at C-9 (B).



Scheme 8. Various microbial transformations of oleate.

of insect pheromones. Early indications[54] are that the mechanisms of these reactions follow the same crypto-regiochemical and stereochemical rules as those being elucidated for the plant systems. Cloning of insect-derived desaturases into a yeast host has also been achieved, thus opening the door to the development of novel microbial biocatalysts.[55]

7. Other oleochemical functional group transformations

Selective, non-oxidative transformations of unactivated double bonds are intriguing mechanistically and potentially useful in synthesis in that they can be conducted under mild conditions. A number of such reactions are used by microorganisms to modify oleic acid and its isomers. These include hydration,[56] hydrogenation,[57] isomerization,[58,59] cyclopropanation[60] and methylenation[61] (Scheme 8). All of these processes could potentially be modified to allow the use of alternate substrates. A driving force for synthesizing novel fatty acid analogues is the recent discovery that mammalian stearoyl CoA Δ9 desaturase (SCD) may qualify as a therapeutic target in the treatment of obesity and diabetes.[62] The search for efficacious inhibitors of SCD based on a fatty acid structural template is now of current interest. The use of the biocatalytic reactions outlined in this section, and elsewhere in this review, may play an important role in attaining this scientific objective.

8. Summary

In this report, we have highlighted some of the regio- and stereoselective transformations that are catalyzed by fatty acid desaturases. In addition to the parent dehydrogenation reactions, highly selective hydroxylation, sulfoxidation and epoxidation is also observed through the use of alternate substrates or protein engineering. As we gain a more sophisticated understanding of the structural basis for desaturase function, one can envisage extending this exquisite chemistry to a wider range of acylic and cyclic substrates through the use of suitably modified enzymes.

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