Fatty acid desaturation: variations on an oxidative theme
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Significant progress in our understanding of the mechanism of fatty acid desaturation has been achieved. The site of initial oxidation has been determined for several membrane-bound desaturases and a common cryptoregiochemical theme has been revealed. The results of several studies, including a detailed analysis of a soluble plant desaturase system, point to a close mechanistic relationship between dehydrogenation and hydroxylation pathways.

Introduction
Fatty acid desaturases catalyze highly stereoselective, regioselective and chemoselective O2-dependent syn-dehydrogenation reactions, as exemplified by the transformation of stearoyl CoA to its oleyl counterpart (Figure 1) [1,2]. Enzymatic reactions of this type play a critical role in adjusting the biophysical properties of membrane lipids and in the biosynthesis of cellular signalling agents such as ceramides, arachidonic acid derivatives and pheromones. The past decade has witnessed significant progress in our understanding of the molecular and structural biology [3–6] as well as the bioinorganic chemistry of fatty acid desaturases [7]. As a result of these efforts, it is now recognized that this family of enzymes can be divided into two classes: first, a large set of integral membrane-bound proteins that have a probable multi-histidine diiron coordination site and act on CoA- or phospholipid-linked substrates; and second, a smaller group of soluble plant desaturases that contain a carboxylate-bridged, diiron cluster similar to that found in methane monooxygenase [8] and which convert the acyl carrier protein (ACP) derivative of substrates to the corresponding product. The mechanistic details of how these remarkable enzymes function are now beginning to emerge. Here, we review recent advances in this area of research.

Mechanistic model
A genetic mechanistic scheme for non-heme, diiron-mediated desaturation, which is based on all of the available experimental evidence, is shown in Figure 2. An initial, energetically difficult C–H activation step, executed by a compound Q-type oxidant [8], produces a carbon-centred radical/FeOH pair that disproportionates to give an olefinic product and iron-bound water either directly or by a one electron oxidation/deprotonation sequence. The reactive intermediate can also collapse by hydroxyl capture to give a secondary alcohol; indeed, several bifunctional desaturase/hydroxylase diiron and monoiron systems are now known [9–12,13••]. The switch controlling the ratio of rate constants associated with each reaction pathway remains an intriguing question. One possibility is that the positioning of the substrate relative to oxidant plays an important role in deciding reaction outcome. That is, if access to the hydrogen β to the putative radical centre is denied (negative catalysis) [14], then oxygenation of substrate would be the only alternative.

Membrane-bound desaturases: cryptoregiochemistry
Recent mechanistic research on this class of desaturases has focussed on pinpointing the site of initial oxidative attack (cryptoregiochemistry [15]) involved in double-bond formation. The most versatile method of achieving this goal has been through the use of a competitive kinetic isotope effect (KIE) method [15]. This approach is based on the premise that initial C–H activation should be energetically more difficult and therefore more sensitive to isotopic substitution than the second C–H bond-breaking step (Figure 2). Typically a 1:1 mixture of regiospecifically deuterated substrate and its non-deuterated parent is incubated with a convenient source of the desaturase and the d1/d0 ratio of the olefinic product evaluated by mass-spectral analysis. In this manner, the intermolecular deuterium isotope effect on C–H cleavages for a large number of membrane-bound desaturases from a variety of aerobic life-forms has been determined (Figure 3). These include Δ9 (rat liver microsomes [16•]), Δ5 (Bacillus subtilis, bacteria [17]), Δ6 (Tetrathymena thermophila, protist [18]), Δ9 (Saccharomyces cerevisiae, yeast [15]; Chlorella vulgaris, green alga [19•]; Spirulina platensis, cyanobacteria [20]), Δ11
(Spodoptera littoralis, insect [21], Δ\(^{12}\) (Arabidopsis thaliana, plant [22]; C. vulgaris, green alga [19•]), and Δ\(^{15}\) (ω-3) desaturases (Caenorhabditis elegans, nematode [23•]; A. thaliana, plant [24]). The study of plant and nematode desaturases was greatly aided by the functional expression of these enzymes in a host yeast system (e.g. [25]).

These studies have revealed two remarkably consistent trends. Firstly, only one of the C–H cleavage steps involved in desaturation is subject to a large deuterium KIE (\(k_H/k_D\) ~5–8); a negligible effect being found at the proximal carbon (\(k_H/k_D\)~1). This clearly indicates a stepwise mechanism and rules out a synchronous removal of hydrogens, as has been suggested previously [26]. Secondly, the isotopically sensitive step uniformly occurs at the carbon closest to C-1, which, according to our mechanistic model (Figure 2), implies that desaturation is initiated at this site. Corroborating evidence for the correctness of these cryptoregiochemical assignments is available in nearly all cases: incubation of a series of thia-substrate analogues with Δ\(^{6}\) and Δ\(^{9}\) desaturases leads to preferential, enantioselective sulfoxidation when the sulfur probe is at the 6- and 9-positions [18,27]. More recently, the formation of low-level quantities of 5-, 9-, 12-, 15-monohydroxy products as byproducts of 5-, 9-, 12-, 15-desaturation, respectively, have been detected [13••]. Finally, enzymes that are closely related to the rat liver Δ\(^{4}\) dihydroceramide desaturase and plant Δ\(^{12}\) oleate desaturase (FAD2) produce exclusively 4- and 12-hydroxylated products [28,29].

The data presented above, coupled with the results of several stereochemical investigations [6], point to a common active-site architecture that has been tuned to give a rich variety of unsaturated fatty acids. This catalytic diversity is also evident in reactions catalyzed by a FAD2 subfamily that lead to the further oxidation of linoleate in plants: 1,4-dehydrogenation producing conjugated dienes, 1,2-dehydrogenation (alkyne formation) and epoxidation [30–32]. A recent KIE investigation on the 1,4-desaturation of linoleate leading to calendate (\((E,E,Z)-8,10,12\)-octadecatrienoate clearly indicates that this reaction is initiated at C-11 (PH Buist and PS Covello, unpublished data).

Insect fatty acid signatures

Variations in desaturase regioselectivity and stereoselectivity are critical to the biosynthesis of species-specific semiochemicals [33] such as insect pheromones. A well-studied case is the S. littoralis desaturating system, which produces a unique blend of monoenoic and dienoic tetradecanoates (Figure 4). Both the stereochemistry and crypto-regiochemistry of these processes have been elucidated through the use of stereospecifically monodeuterated and
regiospecifically dideuterated substrates, respectively. The action of what appears to be a single $\Delta^{11}$ desaturase on two possible substrate conformers via an initial abstraction of the pro-$R$ hydrogen at C-11 leads to a mixture of ($E$/Z)-11-tetradecenoates [21]. Further support for the single enzyme model has been gained recently by the cloning and expression of a related moth desaturase that produces a mixture of ($E$/Z)-11-tetradecenoates from myristoyl CoA [34]. Similarly, a single plant $\Delta^8$ desaturase cloned from *Borago officinalis* catalyzes the formation of a mixture of geometric isomers [35] (see also Update).

Interestingly, the course of the next oxidative step in *Spodoptera* pheromone biosynthesis is dependent on the stereochemistry of the 11-enoic substrate: ($E$)-11-tetradecenoate undergoes C-9-initiated, 1,2-dehydrogenation, whereas the (Z)-isomer is processed to give an ($E,E$)-10,12 tetradecadienoate by 1,4-desaturation (initial site of oxidation at C-10) [36,37]. The stereochemistry of both oxidations has also been determined and found to be closely related, as shown in Figure 4 [38]. The fact that (Z)-9-desaturation is initiated at C-9 and not at the thermodynamically more favourable allylic C-10 position can be attributed to a strict cryptoregiochemical imperative imposed by the enzyme active site.

**Soluble plant desaturases**

The most important member of this class of proteins is the castor stearoyl–ACP $\Delta^9$ desaturase — a dimer consisting of two identical subunits. This enzyme has been characterized by X-ray crystallography and a narrow, hydrophobic binding pocket that can accommodate a stearoyl substrate has been identified [39]. Some of the fundamental mechanistic parameters of the stearoyl–ACP $\Delta^9$ desaturase have been determined recently. Hydrogen removal was shown to occur in *syn* fashion with pro-$R$ enantioselectivity [40]. Using regiospecifically deuterated substrates, it was demonstrated that the intermolecular deuterium KIE on C–H cleavage at both the 9- and 10-positions was ~1 [41]. It is likely that the intrinsic KIE values associated with C–H cleavage were masked by another kinetically more important step in the catalytic cycle, such as substrate binding — an event that is known to induce O$_2$ binding [42]. The prediction that the diiron oxidant is not located symmetrically between C-9 and C-10 of the substrate was confirmed through the use of thia-substituted analogues. It was found that 10-thiastearoyl ACP was converted cleanly to the corresponding sulfoxide, whereas oxidation of the 9-thia isomer gave a mixture of the sulfoxide as a minor product along with a novel, as yet uncharacterized, product [41,43,44].

In an effort to link desaturase-mediated dehydrogenation with the hydroxylation chemistry executed by methane monoxygenase (MMO) [6], the oxidations of ($R$)- and ($S$)-9-fluorostearoyl ACP were compared (Figure 5) [40]. Under conditions of partial and complete conversion, the latter fluoroanalogue was processed to give the fluoroolefin.
with the anticipated stereochemistry along with a small amount of a threo-fluorohydrin. By 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 hydroxylated compounds. The observed shift in desaturase regioselectivity (Δ9 to Δ10) through the use of unnatural substrates had been noted previously [45] but the reversal of stereochemical outcome (‘cis’- to ‘trans’-olefin) was completely unexpected. A detailed mechanistic analysis of the (R)-9 fluorosubstrate reaction led to the conclusion that dehydrogenation and hydroxylation were initiated at the same carbon in accord with the mechanistic scheme depicted in Figure 2. The elevation of Δ9 desaturase-mediated hydroxylation activity from undetectable (<0.1% of total products) for the parent substrate to ~10% for both 9-fluorosubstrates is noteworthy. Further insight into the origin of this substituent effect might be obtained by the application of high-level computational studies of the type used to analysis the mechanism of MMO [46].

Conclusions
The invention and application of novel mechanistic probes has led to a more sophisticated understanding of how fatty acid desaturases and related enzymes [47•,48] carry out their ultra-selective oxidation chemistry. This information can now serve as a useful platform for the design of novel desaturase inhibitors—an activity of growing importance given the key role that desaturases play in the life cycle of the cell [49]. In addition, the results of cryptoregiochemical and stereochemical analyses can be used to validate the structures of substrate/desaturase complexes as these become available. Currently, the barrier to obtaining structural data of this sort remains very high in the case of membrane-bound desaturases, as these proteins have been notoriously difficult to isolate in sufficient quantity [50]. Nevertheless, with the help of new microbial expression systems [51], it is hoped that detailed structural information on this important class of enzymes will become available in the near future.

Update
Very recently, the Δ8 sphingolipid desaturase alluded to in our discussion on insect fatty acid signatures has been examined using stereospecifically and regiospecifically deuterated substrates [52]. It was found that both $E$ and $Z$ olefinic isomers were formed in a stereospecific manner by syn-elimination of two vicinal hydrogens. Further mechanistic studies on this fascinating system are ongoing.
Acknowledgements
This review is dedicated to the outstanding co-workers at Carleton University who have worked on desaturase-related projects over the past 15 years. Our understanding of desaturases has also been broadened considerably through fruitful collaborations with John Shanklin (IRNL, Upton, NY, USA); Caroline Nugier and Henri Parin (ENSCR, Rennes, France); Brian Dawson (HPFB-HC, Ottawa, Canada); Gemma Fabriàs (IQAB-CSIC, Barcelona, Spain); and Pat Covello (PBI, Saskatoon, Canada).

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tetradecanoic acids and their use in investigating the enzymatic reaction of a 1,4-desaturation reaction. The strong similarity to related 1,2-desaturation reactions is noted. These papers (see also [38]) constitute the first stereochemical and cryptoregiochemical analysis of a 1,4-desaturation reaction. The strong similarity to related 1,2-desaturation reactions is noted. See annotation to [37].


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