Application of KIE and thia approaches in the mechanistic study of a plant stearoyl-ACP Δ^9 desaturase

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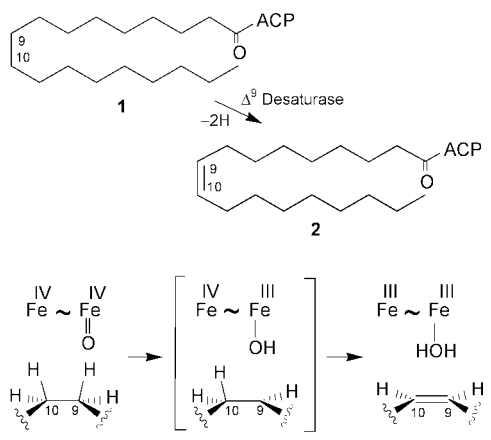
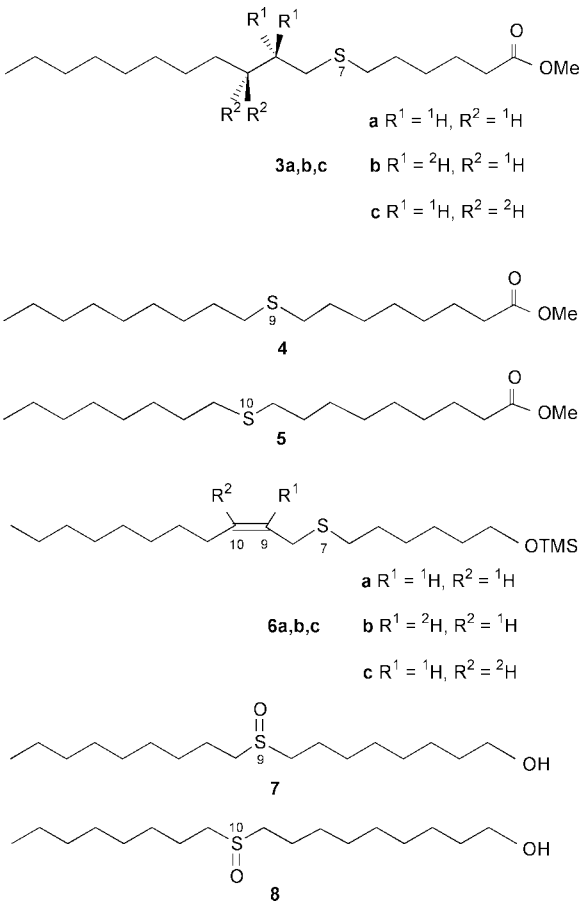
The mechanism of the castor stearoyl-ACP Δ^9 desaturase has been investigated; it has been shown that C–H cleavage at carbons 9 and 10 is not sensitive to deuterium substitution and oxo-trapping experiments using thia analogues revealed that sulfoxidation occurs when the substrate bears sulfur at the 9 and 10 positions.

Fatty acid desaturases[1] constitute an important family of O_2-dependent non-heme diiron-containing enzymes which catalyze the regio- and stereoselective *syn*-dehydrogenation of adjacent unactivated methylene groups. Two structurally unrelated classes[2] of these remarkable catalysts have been identified: a) a large set of membrane-bound proteins which have a probable multi-histidine diiron coordination site[3] and act on CoA- or phospholipid-linked substrates; b) soluble plant desaturases which contain a well-characterized, carboxylate-bridged, diiron cluster[4] similar to that found in methane monooxygenase and which convert the acyl carrier protein (ACP) derivative of substrates to the corresponding product (**1**, **2**, Scheme 1). A generic mechanism for desaturation involves oxidative attack† to give a very short-lived radical intermediate or its iron-bound equivalent which then collapses to give the olefinic product (Scheme 1).‡[5] Recently, a number of substrate-based mechanistic probes[6] have been developed by Buist *et al.*, using a convenient, *in vivo*, yeast Δ^9 desaturase system and some preliminary information has been obtained for the family of membrane-bound desaturases which is consistent with the model presented in Scheme 1.[6] However, very little is known regarding the mechanism of desaturation by the soluble plant desaturases[7] and the issue of whether or not the two classes of desaturases share the same mechanistic features, has yet to be addressed. The availability of homogenous preparations of a soluble plant Δ^9 desaturase from castor seed has prompted us to turn our attention to the bioorganic chemistry of this enzyme system. Here, we describe the application of our kinetic isotope effect (KIE) and 'thia' approaches[6a–c] in the mechanistic study of the soluble castor stearoyl-ACP Δ^9 desaturase.



Scheme 1

Our first experiments involved an attempt to decipher the cryptoregiochemistry (site of initial oxidation) of desaturation by determining the individual primary deuterium isotope effects for each individual C–H cleavage: according to Scheme 1, the first, energetically difficult, hydrogen abstraction should be much more sensitive to isotopic substitution than the second C–H bond cleavage. The required regiospecifically deuterium labelled substrates **3b**,**c** along with the non-labelled parent **3a** were available from previous studies.§[6b] 1 : 1 Mixtures of **3b** : **3a** and **3c** : **3a** as their ACP thioesters (14 nmol) were incubated separately with freshly prepared desaturase (0.02 nmol) and required cofactors[7,8] (total volume of 250 μL) under conditions adjusted to allow *ca*. 15% conversion of substrate.[9] The reactions were quenched *via* addition of THF and after reductive workup ($NaBH_4$), extraction with hexane and derivatization with trimethylchlorosilane (TMCS), the product mixtures **6b** : **6a** and **6c** : **6a** were analyzed by GC-MS (60 m SP-23 capillary column, MS scan rate: 2.14 scans s^{-1}). The competitive primary deuterium KIE was computed by comparing the isotopic content of substrates and products[6b,c] and found to be $k_H/k_D = 1.03 \pm 0.02$ at C-9 and $k_H/k_D = 0.95 \pm 0.02$ at C-10



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(average of two incubations). Similar results were obtained when the incubations of **3a**–**c** were run[7,8] in the noncompetitive mode: k_H/k_D = 1.10 ± 0.10 at C-9 and k_H/k_D = 1.13 ± 0.18 at C-10 (average of two incubations). These findings are in marked contrast with those reported for all membrane-bound desaturases studied to date.[6b,c,10] These include bacterial Δ^5, protozoan Δ^6, yeast, algal and bacterial Δ^9, insect Δ^9 and Δ^{11}, plant and algal Δ^{12} and animal and plant ω-3. All of these systems show a similar pattern of isotope effects (one large (k_H/k_D = 4–7), one negligible (k_H/k_D ~ 1)), clearly indicating the stepwise nature of the reaction for this class of desaturase and the kinetic importance of the initial C–H activation step. The absence of a measurable KIE for both C–H cleavages in the case of the soluble stearoyl-ACP Δ^9 desaturase is almost certainly due to the fact that these steps are masked by other enzymatic events such as electron transfer, substrate binding, product release, *etc*.[11] Thus it is clear that the substantial differences in protein structure, enzyme location, and substrate head group for the two classes of desaturases result in very different overall kinetic mechanisms.

We then turned to our 'thia' approach which probes the relative distance between the putative iron oxidant and substrate methylene groups by comparing the efficiency of oxo transfer to 9- and 10-thia analogues. In a preliminary set of experiments, the required pair of thiastearates **4** and **5** along with **3a** as control were incubated separately as their ACP thioesters with soluble stearoyl-ACP Δ^9 desaturase under conditions similar to those used for KIE experiments but adjusted for higher conversion (0.04 nmole enzyme, 10 min incubation). Products of these incubations were isolated and analyzed by GC-MS as the TMS derivatives. Under these conditions, the thia substrate bearing sulfur at the 7-position **3a** was cleanly converted to the corresponding *cis*-olefinic product **6a**; only traces of unreacted starting material were observed for incubations using **4**- and **5**-ACP.

To probe for products of oxo transfer, a second set of incubations was carried out using the ACP-derivative of each thiastearate (**3a**, **4**, **5**, 60 nmol) along with enzyme (4 nmol) and required cofactors[7,8] (in a total volume of 1.5 ml for 30 min). The CH_2Cl_2 extracts of reduced ($NaBH_4$) reaction mixtures were examined by silica gel TLC (90% EtOAc–hexane, H_2O spray detection, detection limit = 1–2 nmole). A search for the anticipated sulfoxide product alcohols was conducted with the use of authentic standards.¶ Significantly, only in the case of 9- and 10-thia substrates (**4**-, **5**-ACP), were the corresponding sulfoxides (**7**, **8**) detected (R_f = 0.20 and 0.16, respectively). Sulfoxidation of **4**- and **5**-ACP was not observed in the absence of enzyme or enzyme lacking a supply of NADPH. No sulfones were detectable. The structural assignments of the sulfoxide products were confirmed by electrospray MS analysis of material isolated from the TLC by CH_2Cl_2 extraction.‖ This constitutes the first report of an *in vitro* desaturase-mediated sulfoxidation and substantiates earlier reports of similar results for an *in vivo* yeast Δ^9 desaturase system.[12] However, it is interesting to note that in the latter case, sulfoxidation of a series of 9-thia-analogues was always more efficient (2–3-fold) than the production of the corresponding 10-sulfoxides,[6a,13] which led to the conclusion that the site of initial oxidation was at C-9 in good agreement with the observance of a large KIE effect at C-9 but not at C-10 (see above).[6b] In contrast, it appears that for the soluble Δ^9 desaturase, the trend is the reverse: we consistently see a greater amount of sulfoxide (7–12-fold)†† from the 10-thia substrate (**5**-ACP) than from the corresponding 9-isomer under conditions of partial and complete substrate consumption. This clearly implies that the 9-thia substrate is being converted to additional products possibly arising from oxidative attack α to the sulfur; experiments designed to address this issue are currently in progress. In addition, the determination of the absolute configuration of the sulfoxide products presents an impressive challenge to the methodology we have developed previously.[6a]

In summary, we have demonstrated fundamental differences in the behaviour of the two major classes of desaturase enzymes and have taken our first steps towards defining the crypto-regiochemistry of a structurally well-defined plant stearoyl-ACP Δ^9 desaturase. It is hoped that these efforts will complement ongoing active-site X-ray crystallographic work[4a] and other mechanistic studies[7] on this fascinating catalytic system.

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Notes and references

† We have chosen to use a generic iron oxo representation of the active oxidant although other structures are possible. For an in depth analysis of the inorganic chemistry involved in diiron-mediated oxidations, see Ref. 4*c*.

‡ The intermediate could collapse rapidly to give alkene *via* a one electron oxidation–deprotonation sequence, simple disproportionation or Lewis acid (Fe^{3+})-catalyzed dehydration of a hydroxy intermediate. Newcomb *et al.*, have recently raised the possibility that these sorts of C–H activations may proceed, in part, *via* cationic intermediates produced by insertion of OH^+ into the unactivated C–H bond as the first step, followed by rapid loss of water from a protonated alcohol intermediate.[5a,b]

§ A trial incubation using **3a**-ACP revealed that this material was an excellent substrate and the structure of the 7-thia alkene **6a** was confirmed by comparison with an authentic standard on the basis of MS and GC retention time. The presence of the thia-substituent does not affect the regiochemistry of desaturation.[6b,c,10] The standard was prepared by LAH reduction of the carbomethoxy group of the methyl 7-thiaoleate available from previous experiments[12] followed by derivatization *via* TMCS.

¶ Authentic standards were prepared from available synthetic methyl thiastearates[12] by LAH reduction of the terminal methyl ester group followed by sulfoxidation using *m*-chloroperbenzoic acid.

‖ **7**: *m*/*z* 305 ($M + H^+$), 287 [$(M - H_2O) + H^+$], 269 [$(M - 2H_2O) + H^+$], 177 [$(M - HOCH_2(CH_2)_5CH{=}CH_2) + H^+$], 161 [$((M - H_2O) - CH_3(CH_2)_6CH{=}CH_2) + H^+$], 111 ($C_8H_{14} + H^+$); **8**: *m*/*z* 305 ($M + H^+$), 287 [$(M - H_2O) + H^+$], 269 [$(M - 2H_2O) + H^+$], 175 [$((M - H_2O) - CH_3(CH_2)_5CH{=}CH_2) + H^+$], 163 [$(M - HOCH_2(CH_2)_6CH{=}CH_2) + H^+$], 125 ($C_9H_{16} + H^+$).

†† The amount of sulfoxide products was estimated using known amounts of authentic standards on the same TLC plate.

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