Communications to the Editor

Use of Deuterium Kinetic Isotope Effects To Probe the Cryptoregiochemistry of Δ9 Desaturation

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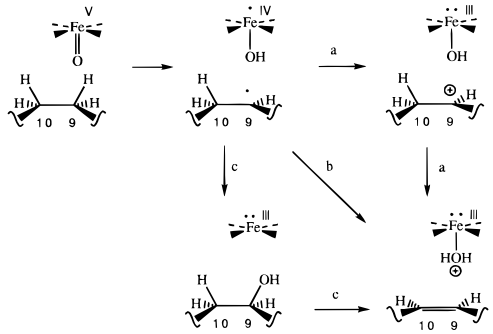
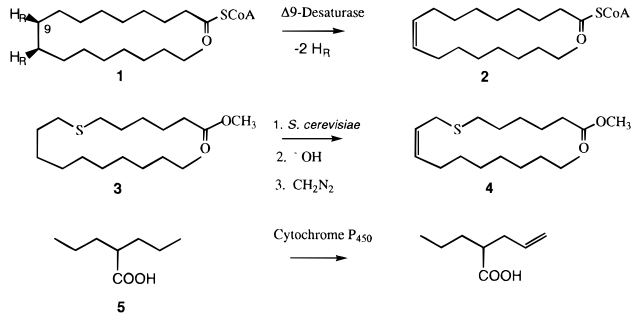
The biological *syn*-dehydrogenation (desaturation) of fatty acids[1] as exemplified by the Δ9 desaturase-mediated transformation of stearoyl CoA (**1**) to give oleyl CoA (**2**) represents one of the more virtuosic displays of enzymatic selectivity. Two classes of desaturases catalyze this intriguing transformation: soluble plant enzymes containing a carboxylate-bridged, nonheme diiron center[2] and less well-characterized, nonheme iron, membrane-bound catalysts as represented by the Δ9 desaturase found in *Saccharomyces cerevisiae*[3] and rat liver.[4] In light of the mounting evidence[5] that desaturases and hydroxylases are structurally related at the protein level, we have adopted the view that desaturations are initiated by a hydrogen abstraction step similar to that proposed for biohydroxylation. Some of the possible subsequent steps to the olefin are outlined in Scheme 1.[6] We have focussed our attention on the Δ9 desaturase of *S. cerevisiae* and have tentatively placed the putative iron−oxo oxidizing species near C-9 of the substrate since this enzyme system consistently oxygenates 9-thia fatty acid analogues more efficiently than the corresponding 10-thia analogues.[12] In this communication, we report the results of a study in which we further investigate the cryptoregiochemistry

Scheme 1



of yeast Δ9 desaturation by measuring the deuterium isotope effect for each individual C−H bond cleavage.[13]

In order to expedite our isotope effect study, we decided to run direct competition experiments involving methyl 7-thiastearate-*9,9-d*$_2$ (**3**-*9,9-d*$_2$) vs methyl 7-thiastearate (**3**) and methyl 7-thiastearate-*10,10-d*$_2$ (**3**-*10,10-d*$_2$) vs methyl 7-thiastearate (**3**). Use of methyl stearate-d_2/d_0 mixtures would have complicated the analysis of the methyl oleate-d_1/d_0 product due to mass spectral interference by endogenous (d_0) oleate. The sulfur atom was placed at position 7 in order to facilitate the synthesis of the deuterated substrates. We have shown previously that methyl 7-thiastearate (**3**) is converted to the corresponding thiaoleate product (**4**).[14]



3-*9,9-d*$_2$ and **3**-*10,10-d*$_2$ were synthesized in 10% and 8% overall yield, respectively, using well-known procedures as shown in Scheme 2.[15] The two deuterated substrates consisted entirely of dideuterated species (within experimental error) as determined by MS; the ^{1}H and ^{13}C NMR spectra were consistent with the location of deuterium label. **3** was available from our previous study. A *ca*. 1:1 mixture of each deuterated substrate and d_0 material (25 mg) was administered as 5% w/v ethanolic solutions to growing cultures (150 mL) of *S. cerevisiae* ATCC 12341 as previously described.[12] Each incubation was carried

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(6) The putative radical intermediate[7] could follow at least three different pathways: one-electron oxidation/H$^+$ elimination (pathway a),[8a,b] disproportionation (pathway b),[9] or a hydroxyl rebound[10a] (SH_2)[10b]/fast Fe^{3+}-promoted dehydration sequence (pathway c). In addition, the possibility that organoiron intermediates[11] (not shown) are involved in these reactions cannot be excluded at the present time.
(7) Very recent results from the Newcomb laboratory have suggested that this species is not a true intermediate but, at least in the case of certain cyclopropylcarbinyl radicals, behaves as "a component of a reacting ensemble" with a very short lifetime of *ca.* 70 fs: Newcomb, M.; Letadicbiadetti, F. H.; Chestney, D. L.; Roberts, E. S.; Hollenberg, P. F. *J. Am. Chem. Soc.* **1995**, *117*, 12085.
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(13) An overall, intermolecular deuterium isotope effect of 6.0 was obtained using the closely related rat liver Δ9 desaturase and noncompetitive comparison of oxidation rates for stearoyl CoA-*9,9,10,10-d*$_4$ vs stearoyl CoA .[4]
(14) Buist, P. H.; Dallmann, H. G.; Rymerson, R. R.; Seigel, P. M.; Skala, P. *Tetrahedron Lett.* **1988**, *29*, 435. Our whole cell system allows us to use thiastearate methyl esters which are presumably converted intracellularly to the corresponding CoA thioesters prior to desaturation. The thiaoleate products can then be isolated by a cell hydrolysis/methylation sequence. The use of *S. cerevisiae* under conditions of adequate glucose supply prevents extensive metabolism of the thiastearate substrates and/or the thiaoleate products which in turn might lead to nondesaturase-related isotopic fractionation.
(15) The tosylate of decan-1-ol-*1,1-d*$_2$ was prepared by reduction of decanoic acid using $LiAlD_4$, followed by treatment with TsCl. 1-bromononane-*1,1,-d*$_2$ was similarly available by $LiAlD_4$ reduction of nonanoic acid followed by bromination of the resultant alcohol using PBr_3.

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Table 1. Intermolecular Isotopic Discrimination in Δ9 Desaturation of Methyl 7-Thiastearate-*9,9-d_2* and Methyl 7-Thiastearate-*10,10-d_2*

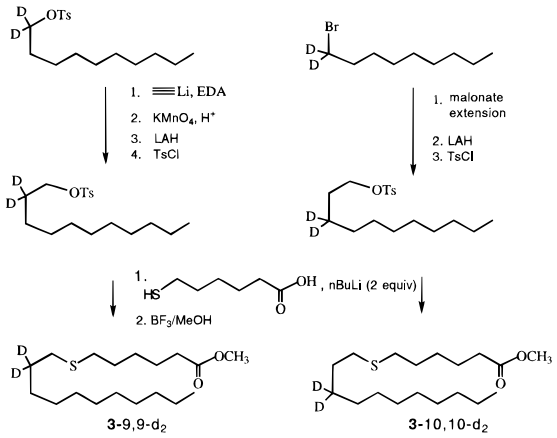
	isotopic composition (mol %)[a]						
	substrates[b]			products			
expt	d_0	*9-d_2*	*10-d_2*	d_0	*9-d_1*	*10-d_1*	KIE
1	47.2 ± 0.7	52.8 ± 0.7		86.8 ± 0. 4	13.2 ± 0.4		7.4 ± 0.3
2	47.0 ± 0.7	53.0 ± 0.8		86.1 ± 0. 8	13.9 ± 0.8		7.0 ± 0.4
3	46.8 ± 0.3	53.2 ± 0.3		86.0 ± 0.4	14.0 ± 0.4		7.0 ± 0.2
							(7.1 ± 0.2)[c]
4	49.2 ± 0.4		50.8 ± 0.4	52.3 ± 0.8		47.7 ± 0.8	1.13 ± 0.03
5	50.9 ± 0.3		49.1 ± 0.3	50.3 ± 0.1		49.7 ± 0.1	0.98 ± 0.01
6	49.7 ± 0.4		50.3 ± 0.4	49.2 ± 0.6		50.8 ± 0.6	0.98 ± 0.02
							(1.03 ± 0.09)[d]

[a] The mol % of each isotopic species is given as an average value based on two to three GC/MS runs. [b] The six starting mixtures were individually prepared. [c] The average KIE at C-9 ± standard deviation. [d] The average KIE at C-10 ± standard deviation.

out three times. After 24 h of growth, the cellular fatty acids (ca. 30 mg) were isolated from the centrifuged cells by a standard hydrolysis (1 M 50% ethanolic KOH)/methylation (CH_2N_2) sequence, and the deuterium content of thiaoleate products (<10% of the total fatty acid fraction) was assessed by GC/MS (30 m DB-5 capillary column, temperature programmed from 120 °C, (held, 2 min) to 320 °C at 10 °C/min; MS scan rate: 0.5 s/decade; 5−8 scans per GC peak; the integrated intensities of the individual ions in the molecular ion cluster were recorded using a SUN SPARC I workstation equipped with Kratos Mach 3 software and have been corrected for natural isotopic abundances.) Product kinetic isotope effects (KIEs) (k_H/k_D) were calculated using the ratio [% d_0 (product)/ % d_1 (product)] / [% d_0 (substrate) / % d_2 (substrate)]. In this manner, a large primary deuterium isotope (7.1 ± 0.2, average of 3 experiments)[16] was determined for the carbon−hydrogen bond cleavage at C-9, while the C-10− H bond-breaking step was shown to be essentially insensitive to deuterium substitution (KIE = 1.03 ± 0.09, average of 3 experiments). The analytical data is displayed in Table 1.

These results represent the first direct determination of the KIE[17] on each of the two C−H bond cleavage steps involved in fatty acid desaturation and strongly suggest that the yeast Δ9 desaturase initiates oxidation at carbon 9 in a relatively slow, isotopically sensitive step in accordance with our mechanistic model. The second C−H cleavage appears to be fast as might be expected for any of the three pathways shown in Scheme 1. A similar pattern of isotope effects (one large (5.58, C-4) and one small (1.62, C-5)) was observed for the cytochrome P_{450}-mediated 4,5-dehydrogenation of valproic acid (**5**)—a reaction

Scheme 2



thought to be initiated by hydrogen abstraction at C-4.[18] It is also interesting to note that in very early experiments involving 9- and 10-monotritiated stearates and a bacterial Δ9 desaturase, a kinetic isotope effect at C-9 but not at C-10 was observed.[19] Our results are not consistent with a desaturase mechanism involving a synchronous removal of the 9,10-hydrogens—a possibility which has been raised previously.[20,21]

In conclusion, we believe that the methodology described in this paper will prove very useful in the study of other desaturases such as the recently discovered soluble, diiron plant Δ9 desaturases for which no mechanistic information is currently available.[22]

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(16) A small proportion (<10%) of the observed isotope effect may be due to an α-secondary isotope effect. Cf.: Jones, J. P.; Trager, W. F. *J. Am. Chem. Soc*. **1987**, *109*, 2171.

(17) The magnitude of the primary deuterium isotope effect at C-9 must be regarded as a lower limit for the "intrinsic" kinetic isotope effect since our value is derived via an intermolecular competition experiment and is therefore subject to partial masking by other enzymic steps. Large "intrinsic" primary deuterium isotope effects are not uncommon for biological hydrocarbon activations thought to involve iron−oxo species. Cf. ref 16.

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