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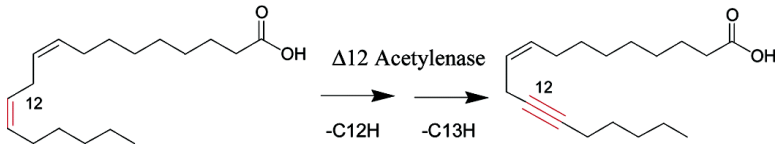

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Mechanistic Study of an Improbable Reaction: Alkene Dehydrogenation by the Δ12 Acetylenase of *Crepis alpina*

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Abstract: The mechanism by which the fatty acid acetylenase of *Crepis alpina* catalyzes crepenynic acid ((9*Z*)-octadeca-9-en-12-ynoic acid) production from linoleic acid has been probed through the use of kinetic isotope effect (KIE) measurements. This was accomplished by incubating appropriate mixtures of regiospecifically deuterated isotopomers with a strain of *Saccharomyces cerevisiae* expressing a functional acetylenase. LC/MS analysis of crepenynic acid obtained in these experiments showed that the oxidation of linoleate occurs in two discrete steps, since the cleavage of the C12−H bond is very sensitive to isotopic substitution ($k_H/k_D = 14.6 \pm 3.0$) while a minimal isotope effect ($k_H/k_D = 1.25 \pm 0.08$) was observed for the C13−H bond breaking step. These data suggest that crepenynic acid is produced via initial H-atom abstraction at C12 of a linoleoyl substrate. The relationship between the mechanism of enzymatic acetylenation and epoxidation is discussed.

Introduction

A wide range of (poly)acetylenic compounds are biosynthesized by fungi, algae, marine sponges, and higher plants.[1,2] Many of these natural products possess antimicrobial, antitumor, and insecticidal properties.[1] While information regarding the biosynthesis of these intriguing compounds remains scarce, results of tracer experiments using plant systems point to the intermediacy of unsaturated fatty acids such as linoleic acid and its 12,13-dehydrogenated product, crepenynic acid ((9*Z*)-octadeca-9-en-12-ynoic acid; Figure 1).[2−5] In the seeds of certain plant species, crepenynic acid is, itself, an end product. Interest in the acetylene bond-forming process has been heightened by the cloning of a cDNA from *Crepis alpina* (*Asteraceae*) which encodes the enzyme responsible for the conversion of linoleate to crepenynate.[3] This rather unique enzyme, termed an acetylenase, bears a striking sequence similarity to a group of membrane-bound, non-heme diiron proteins known as plant oleate Δ12 desaturases (FAD2). In fact, the *Crepis* protein has been shown to be bifunctional, having both oleate Δ12 desaturase and linoleate acetylenase activity (Figure 1). More recently, an enzyme with similar bifunctional (desaturase and acetylenase) activity involved in Δ6 dehydrogenation of fatty acids has also been cloned from the moss *Ceratodon purpureus*.[6] In addition, a combination Δ11 desaturase/acetylenase has been implicated in the biosynthesis of insect pheromones in the genus *Thaumetopoea* (*Lepidoptera*).[7]

The acetylenase-catalyzed reaction is a truly remarkable transformation which has no precedent in the repertoire of biochemical oxidizing systems such as cytochromes P450.[8,9] The process is highly unusual in mechanistic terms since the direct dehydrogenation of alkenes to alkynes is known only to occur when the starting material is activated to attack by strong bases.[10] What is perhaps most puzzling is the fact that the acetylenase-mediated reaction proceeds when an energetically far more facile reaction pathway, namely, epoxidation, would appear to be available. Interestingly, a FAD2-like 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).[3] Dual catalytic behavior of this type (dehydrogenation versus oxo transfer) is also found in the action of FAD2-like enzymes which attack saturated positions along the fatty acid chain. In this case, C−H activation leads to either linoleate or 12-hydroxyoleate (ricino-

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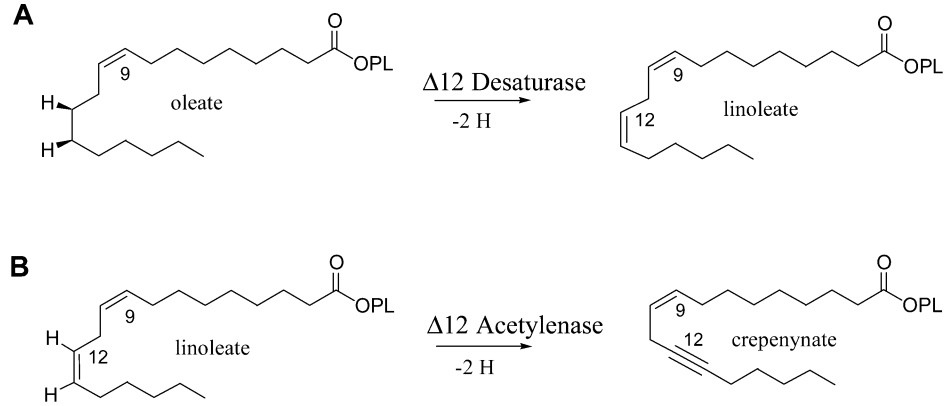

Figure 1. The dehydrogenation reactions catalyzed by oleate desaturase (FAD2)-like enzymes. A, desaturation; B, acetylenation (PL = phospholipid).

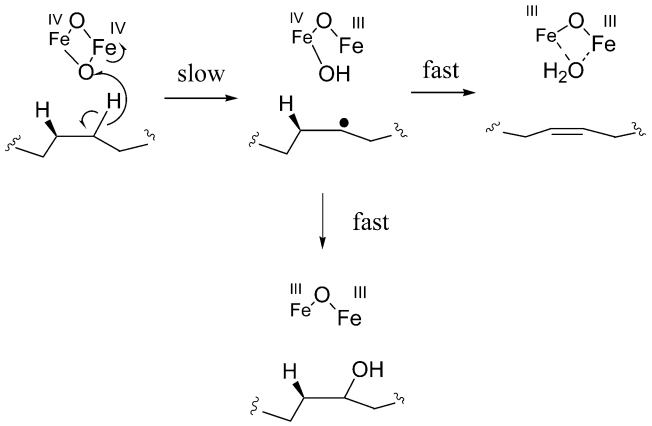

Figure 2. Proposed mechanism of fatty acid desaturases. The structure of the oxidant is speculative.

leate) or mixtures of these products.[11−15] Similar bifunctional behavior has been noted for a number of other desaturases.[16] To account for this dichotomy, a minimal mechanistic model has been constructed which features an initial hydrogen abstraction by the putative high valent, oxygenated diiron center to give a very short-lived carbon-centered radical.[17] This intermediate can collapse via a second rapid hydrogen abstraction to give an olefinic product or by hydroxyl transfer to form an alcohol (see Figure 2). The ratio of rate constants for the two processes appears to depend on subtle differences in the positioning of substrate relative to oxidant in the active site.[13,14,16] It is conceivable that similar considerations may apply to the acetylenase/epoxidase pair, although the barrier to alkene dehydrogenation would, a priori, appear to be very high. A detailed mechanistic analysis of this intriguing system is clearly warranted.

To gain some insight into how the alkene−alkyne transformation proceeds, it is of interest to first determine whether the C−H bonds at C-12 and C-13 are cleaved by the oxidant in a synchronous or stepwise manner. A method for assessing the kinetics of hydrogen abstraction has been developed for desaturase-mediated dehydrogenation through the use of primary kinetic isotope effect measurements.[13,14] In this report, we describe the application of this approach in the first mechanistic study of an enzyme-catalyzed alkene dehydrogenation reaction.

Materials and Methods

Chemicals. Pentadecanoic acid was obtained from Nu-Chek Prep, Inc. (Elysian, MN). Methyl [11,11-$^2H_2$]-linoleate was synthesized as described previously[18] and saponified to the free acid. [12,12-$^2H_2$]-Stearic acid, [13,13-$^2H_2$]-stearic acid, and [18,18,18-$^2H_3$]-oleic acid were synthesized previously by A. P. Tulloch.[19]

Oligonucleotides. The following deoxyribonucleotides were synthesized (DNA Technologies Group, Plant Biotechnology Institute, Saskatoon, Canada) for use in cloning the *C. elegans fat-2* open reading frame: primer fat2recon, 5′-ATGGCAATCG CTACAAAAGT GAACACAAAT AAAAAGGACC TTGATACAAT CAAGGTACCG GAGCTTCCAT CAGTGG-3′; primer fat2start, 5′-CCGAGCTCAT GGCAATCGCT ACAAAAGT-3′; primer fat2end, 5′-TTTCTAGATT ATTGAGCCTT CTTAGCC-3′.

Primer fat2recon varies from the wild type *fat*-2 sequence[20] in having a G instead of an A at position 4 relative to the translation initiation site. This primer has been designed to conform better to Kozak consensus sequences affecting the efficiency of translation initiation.[21]

DNA Plasmids. The plasmid pSAS050, containing the nematode *Caenorhabditis elegans fat-2* gene, encoding the Δ12 fatty acid desaturase,[20] in a yeast expression vector was prepared as follows. Copy DNA for most of the open reading frame of *fat-2* was derived from the *C. elegans* EST clone yk271h6 (Yuji Kohara, National Institute of Genetics, Japan). DNA sequencing indicated that this clone was lacking 56 bp at the 5′ end of the open reading frame. The open reading frame was reconstructed by PCR amplification with primers fat2recon and fat2start. The resulting PCR product was reamplified with primers fat2start and fat2end and cloned into the topoisomerase-activated vector pYES2.1/V5-His-TOPO using a pYES2.1 TOPO TA Expression Kit (Invitrogen) to create the plasmid pSAS050. This vector provides for galactose-inducible expression mediated by the GAL1 promoter. DNA sequence of pSAS050 was in agreement with the published *fat-2* sequence[20] with the exception of the aforementioned substitution in the second codon.

Yeast Strains. For preparation of isotopically labeled linoleic acids, the yeast strain pSAS050/DTY10-a2 (*MAT*α, *fas2*Δ*::LEU2*, *can1-100*, *ura3-1*, *ade2-1*, *his3-11*, *his3-15*, pSAS050), which is a fatty acid auxotroph capable of Δ9 and Δ12 desaturation of exogenous fatty acids, was constructed by introduction of the pSAS050 plasmid into the

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Table 1. Deuterium Content of Starting Materials and Biosynthetic Linoleic Acids As Determined by LC/MS

position of deuterium label	fatty acid	d_0[a]	d_1	d_2	d_3
12	stearic	0.9 ± 0.2	9.2 ± 0.1	89.9 ± 0.2	
12	linoleic	9.0 ± 0.06	91.0 ± 0.1		
13	stearic	2.5 ± 0.2	6.8 ± 0.1	90.7 ± 0.1	
13	linoleic	9.3 ± 0.3	90.7 ± 0.3		
18	oleic	nd[b]	nd	2.5 ± 0.1	97.5 ± 0.1
18	linoleic	nd	nd	2.1 ± 0.4	97.9 ± 0.4

[a] Percentages are the average of three determinations and standard deviations are shown. [b] nd = not detected.

Saccharomyces cerevisiae strain DTY10-a2 (*MATα, fas2Δ::LEU2, can1-100, ura3-1, ade2-1, his3-11, his3-15*)[22] using a S.c. EasyComp Transformation Kit (Invitrogen). To conduct the isotope effect experiments, a yeast strain (pVT-Crep1/InvSc1) expressing a *C. alpina* desaturase/acetylenase gene (*Crep1*) and a corresponding control strain (pVT-100/Inv*Sc*1) were constructed by transformation of Inv*Sc*1 (*MATa his3D1 leu2 trp1-289 ura3-52 MATα his3D1 leu2 trp1-289 ura3-52*; Invitrogen) with pVT-Crep1 (Sten Stymne, Alnarp, Sweden[3]) and pVT-100,[23] respectively. The pVT-100-derived plasmids in these two strains allow for constitutive expression from the *S. cerevisiae* ADH1 promoter.

Biosynthetic Preparation of Deuterated Fatty Acid Substrates. [12-^{2}H]-, [13-^{2}H]-, and [18,18,18-$^2H_3$]-linoleic acids were prepared by bioconversion as follows: One liter Erlenmeyer flasks containing 200 mL aliquots of minimal medium (SD) lacking uracil and glucose and supplemented with galactose (20 g/L),[24] 4 mg of pentadecanoic acid, 16 mg of [12,12-$^2H_2$]-stearic acid, [13,13-$^2H_2$]-stearic acid, or [18,18,-18-$^2H_3$]-oleic acid, and a final Tergitol (type NP-40) concentration of 1 g/L were inoculated with the DTY10-a2/pSAS050 yeast strain. Cultures were grown for 72 h at 28 °C, followed by 72 h at 15 °C with continuous shaking at 240 rpm. Typically, cultures allowed to reach saturation at relatively low temperatures (15 °C) have been found to give higher yields of desaturation products. The yeast cells were centrifuged and washed with 10 mL of Tergitol solution (type NP-40; 10 g/L). Fatty acid methyl esters were prepared from the yeast pellets according to Reed et al.[18] and the methyl linoleate fractions were purified by HPLC according to Meesapyodsuk et al.[25] A total of 2.4 L of cultures were grown with each dideuterated substrate to produce 2.0 mg of methyl [12-^{2}H]-linoleate (1% yield) and 2.5 mg of methyl [13-^{2}H]-linoleate (1.3% yield) after HPLC purification. From a total of 1 L of yeast culture containing 80 mg of [18,18,18-$^2H_3$]-oleic acid and 20 mg of pentadecanoic acid, 81.4 mg of total fatty acid methyl esters were recovered, from which ~20 mg of HPLC-purified methyl [18,18,18-$^2H_3$]-linoleate (25% yield) was obtained. After purification, labeled methyl esters were saponified to free acids. The position of the deuterium atoms in the monodeuterated linoleic acids was confirmed by ^{1}H NMR (500 MHz) for [12-^{2}H]-linoleate, residual doublet ($^3J_{10,11}$ = 6.3 Hz) for bisallylic hydrogens at C-11; for [13-^{2}H]-linoleate, doublet of doublets ($^3J_{10,11}$ = $^3J_{11,12}$ = 6.8 Hz) for bisallylic hydrogens at C-11.The deuterium content of all deuterated linoleates was determined by LC/MS and is given in Table 1.

Biotransformation of Linoleic Acids by the *C. alpina* Acetylenase. Trial conversions of linoleic acid and [11,11-$^2H_2$]-linoleic acid to the corresponding crepenynic acid were accomplished by adding these substrates (100 μg/mL) to 50 mL aliquots of minimal medium lacking uracil and glucose and supplemented with galactose (20 g/L)[24] and Tergitol (type NP-40; 1 g/L) followed by inoculation with the pVT-Crep1/InvSc1 yeast strain in 250 mL flasks. Cultures were grown for 72 h at 20 °C, followed by 72 h at 15 °C with continuous shaking at 240 rpm. The lower initial temperature (20 versus 28 °C) was found to give higher accumulation of crepenynate. Control cultures using pVT100/InvSc1 were grown similarly with and without linoleic acid added to the cultures.

Isotope Effect Experiments. Competitive KIE experiments were performed in triplicate using 10 mL cultures contained in 50 mL Erlenmeyer flasks. The minimal medium lacking uracil and glucose was supplemented with galactose (20 g/L),[24] Tergitol (type NP-40; 1 g/L) and 1:1 mixtures of either [12-^{2}H]-linoleic acid and [18,18,18-$^2H_3$]-linoleic acid or [13-^{2}H]-linoleic acid and [18,18,18-$^2H_3$]-linoleic acid at 100 μg/mL total. The yeast cells were centrifuged and washed with 10 mL of Tergitol solution (type NP-40; 10 g/L). Fatty acid methyl esters were prepared from the yeast pellets according to Reed et al.[18] and the crepenynate fractions were purified by HPLC according to Meesapyodsuk et al.[25] Prior to LC/MS analysis, all samples were saponified at 80 °C for 1 h with 10% KOH, 10% H_2O in methanol. The mixture was acidified with 50% aqueous acetic acid and extracted into hexane which was then removed under a stream of nitrogen. The samples were dissolved to an appropriate concentration in a mixture of 95% acetonitrile, 5% water, and 0.075% acetic acid before analysis.

Mass Spectrometry. In this study, fatty acid products were analyzed by LC/MS using electrospray ionization. Using this technique, a mass spectrum consisting almost entirely of a $(M-H)^-$ ion cluster is generated with concomitant enhancement of sensitivity relative to GC/MS operating with electron impact ionization. Fatty acids from incubation experiments were analyzed using a Quattro LC instrument (Micromass, Manchester, UK) controlled by Masslynx version 3.5 software and coupled to an Agilent 1100 HPLC system fitted with a 12.5 × 2.1 mm Zorbax XDB-C8 precolumn in series with a 100 × 2.1 mm Genesis C18 analytical column. The mobile phase consisted of a gradient mixture (pH ~3.3) beginning with 85% acetonitrile, 15% water, and 0.075% acetic acid (held 2 min) increasing to 95% acetonitrile, 5% water, and 0.075% acetic acid over 8 min. MS analysis was performed using negative electrospray ionization (ES^-) with a capillary voltage of 2.6 kV and a cone voltage of 35 V. Deuterium content was obtained for linoleic and crepenynic acids over a scanning range of *m*/*z* 275 to 285 and was acquired at a rate of 0.08 s per scan with a cycle time of 0.10 s. Integrated chromatograms were constructed with a 0.5 *m*/*z* range centered around the ions of interest. The integrated intensities of the individual ions were corrected for natural isotopic abundance and the isotopic ratios were determined using corrected intensities for each compound: *m*/*z* 279, $(M-H)^-$ (linoleic acid); *m*/*z* 280, $(M-H)^-$([12-^{2}H]-linoleic acid and [13-^{2}H]-linoleic acid); *m*/*z* 281, $(M-H)^-$ ([11-$^2H_2$]-linoleic acid); *m*/*z* 282, $(M-H)^-$ ([18,-18,18-$^2H_3$]-linoleic acid); *m*/*z* 277, $(M-H)^-$ (crepenynic acid); *m*/*z* 279, $(M-H)^-$ ([11-$^2H_2$]-crepenynic acid); *m*/*z* 280, $(M-H)^-$ ([18,18,18-$^2H_3$]-crepenynic acid).

Results

To determine the relative kinetic importance of C12−H and C13−H bond rupture in the conversion of linoleate to crepenynate, competitive isotope effect experiments were undertaken in a manner similar to that employed by ourselves and others in the study of related desaturase reactions.[13,14] The methodology for determining the intermolecular primary deuterium KIE on each individual C−H cleavage step in the acetylenase-mediated reaction involved mass spectral analysis of the crepenynate product **2** derived from direct competition between the appropriate regiospecifically monodeuterated linoleates [12-^{2}H]-**1**, [13-^{2}H]-**1**, and its mass-labeled (ω-trideutero) analogue [18,18,18-$^2H_3$]-**1** (see Figure 3). The use of the latter substrate, which is deuterated at a position remote from the site of dehydrogenation, provides a convenient means of tracking the

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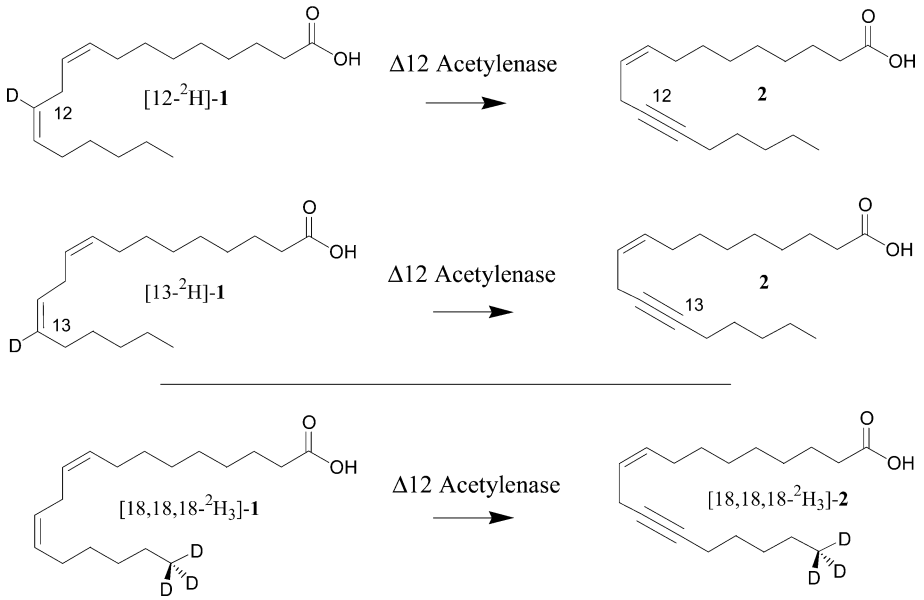

Figure 3. Transformation of deuterated linoleate substrates to the corresponding crepenynates.

cleavage of substrate bearing hydrogen at both C12 and C13. The competitive design of these KIE experiments is highly suited to the study of membrane-bound enzymes such as CREP1, since these systems are notoriously unstable outside their native cellular environment and are conveniently studied in vivo. This approach was first used in the study of a yeast Δ9 desaturase[26] and has now been successfully applied to a number of desaturases.[13,14] Baker's yeast (*S. cerevisiae*) has been found to act as a suitable host for the expression of a wide range of membranous desaturating systems by providing the necessary cofactors, electron-transfer proteins, a suitable hydrophobic matrix, and a facile means of introducing substrate in the correct acylated form.[27] The availability of a *S. cerevisiae* strain bearing a functionally expressed *C. alpina* Δ12 acetylenase[3] greatly expedited our KIE study.

Incubation conditions for our KIE measurements were optimized in a preliminary experiment: linoleic acid was administered to cultures of the pVT-Crep1/DTY10-a2 strain of *S. cerevisiae*. These were incubated at 20 °C for 3 days to permit relatively rapid growth and then at 15 °C for a further 3 days to reach saturation at a temperature that has been found to give better substrate conversion rates. The cells were harvested by centrifugation and the lipids were isolated. Analysis of the fatty acids as methyl esters by GC revealed that exogenous linoleic acid had been converted to crepenynic acid to the extent of 0.15% of total cellular fatty acids, a result similar to that observed previously.[3] Control experiments with pVT-100/Inv*Sc*1 indicated that the production of crepenynate was dependent on the presence and expression of the CREP1 enzyme. Given the bifunctional nature of CREP1 (see Introduction), the contribution of low level production of d_0-linoleate from endogenous oleate was assessed. This was accomplished via an incubation using [11,11-$^2H_2$]-linoleic acid which was available from a previous study.[18] The deuterium content of crepenynate product was determined by LC/MS analysis of the free acid (see Materials and Methods). It was found that the isotopic content of the acetylenic product (0.4 ± 0.2% d_0, 5.2 ± 0.3% d_1, 94.5 ± 0.3% d_2) was essentially identical to that of recovered substrate (0.6 ± 0.2% d_0, 0.7 ± 0.2% d_1, 98.7 ± 0.2% d_2) with respect to d_0 content. This result demonstrated the suitability of this in vivo system for the determination of deuterium kinetic isotope effects. The apparent formation of < 5% d_1-crepenynate may be due to a minor amount of deuterium exchange at the bis-activated C11-position.

The deuterated linoleic acids required for our KIE study were prepared by incubating regiospecifically deuterated stearic and oleic acids[19,28] with yeast mutants having both endogenous Δ9 desaturase and heterologous Δ12 fatty acid desaturation activity.[27] For this purpose, *S. cerevisiae* cultures containing the *C. elegans* Δ12 desaturase were found to be particularly efficient at desaturation of oleic acid under the culture conditions used. Using the yeast strain pSAS050/DTY10-a2, it was possible to produce the desired deuterium-labeled linoleic acids from the corresponding deuterated stearic and oleic acids in ~1% and ~25% yields, respectively (see Materials and Methods). As expected, the yield of linoleate from oleic acid, which required only a single desaturation step, was much higher than that realized in the cases which required the concerted action of both the endogenous Δ9 desaturase and the *C. elegans* Δ12 desaturase. The deuterium content of the starting materials and linoleate products was determined by LC/MS and is listed in Table 1.

To assess the intermolecular primary deuterium KIE's at C-12 and C-13 for the Δ12 acetylenase reaction, a ~1:1 mixture of each vinyl-labeled, monodeuterated linoleate with the methyl-labeled, trideuterated compound (~ 0.5 mg each) was administered to growing cultures (10 mL) of the *S. cerevisiae* strain pVT-Crep1/Inv*Sc*1 using conditions identical to that of the trial experiments. The deuterium content of the recovered linoleic acid and crepenynic acid products isolated from the cellular lipid extract was assessed by LC/MS (see Table 2).

Mass spectral analysis of the crepenynate fraction revealed that in both incubations this material consisted essentially entirely of a d_3/d_0 mixture indicating a loss of one deuterium

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Table 2. Competitive Isotopic Discrimination in the *C. Alpina* Acetylenase-Mediated Formation of Crepenynic Acid from [12-^{2}H]-Linoleic Acid or [13-^{2}H]-Linoleic Acid versus [18,18,18-$^2H_3$]-Crepenynic Acid from [18,18,18-$^2H_3$]-Linoleic Acid

position of isotope in d_1 species	trial	linoleic acid[a] substrate			crepenynic acid product		KIE[c]
		d_0[b]	d_1	d_3	d_0	d_3	
C-12	1	4.8	48.3	46.9	16.3	83.7	11.1
	2	5.0	48.8	46.2	14.9	85.1	15.8
	3	5.1	49.2	45.7	14.9	85.1	17.0
	average						14.6 ± 3.0
C-13	1	4.2	45.2	50.6	44.8	55.3	1.23
	2	4.4	44.9	50.7	43.0	57.0	1.33
	3	4.4	45.0	50.6	45.7	54.3	1.18
	average						1.25 ± 0.08

[a] Residual substrate isolated from yeast cells. The isotopic composition of cellular linoleate was found to be similar (average deviation = ± 4%) to that measured prior to incubation. Thus we find no enrichment in nondeuterated substrate, indicating that the condition for low substrate conversion has been met, as is required for competitive KIE measurements.[36] [b] The deuterium content (%) of each species is given as an average value based on three LC/MS runs. d_0, d_1, and d_3 refer to undeuterated, monodeuterated, and trideuterated species, respectively. [c] The average KIE (three incubations) ± standard deviation is shown.

from the d_1-substrate as expected (see Table 2). Product kinetic isotope effects (k_H/k_D) for C−H cleavage at C12 or C13 were calculated using the ratio

$$\frac{[\%d_3P + \%d_3P\ (\%d_0S/\%d_3S)]/[\%d_0P - \%d_3P\ (\%d_0S/\%d_3S)]}{[\%d_3S + \%d_0S]/[\%d_1S]}$$

where P = product and S = substrate. This expression is based on a standard equation[29] which has been modified to include a correction for the amount of d_0 substrate (d_0S) contributing to d_0 product (d_0P) in addition to that derived by deuterium loss from d_1 substrate (d_1S). This correction is given by the ratio $\%d_3P$ ($\%d_0S/\%d_3S$) and assumes, as is reasonable, that d_0- and d_3-substrates behave identically with respect to Δ12 acetylenation.

Use of the equation shown above indicated the operation of a large primary deuterium isotope effect (14.6 ± 3.0 average of three experiments) 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, average of three experiments) (Table 2).

Discussion

The pattern of KIE's obtained in these experiments (one large (C12), one small (C13)) clearly supports a stepwise mechanism for the acetylenase-mediated dehydrogenation of linoleate. The very strong secondary, vinyl C−H bond (BDE = 107 kcal/mol[30]) at C-12 would appear to be broken first in an energetically difficult and therefore isotopically sensitive step. This event is followed by a rapid cleavage of the weakened C13−hydrogen bond. (The estimated bond dissociation energy of a vinyl CH bond adjacent to a vinyl radical is ∼35 kcal/mol.[30]) The fact that the value for the primary KIE at the C12 position (14.6 ± 3.0) exceeds the theoretical limit calculated using classical transition state theory (k_H/k_D = 6.9 (301 K) − 7.5 (288 K), ν = 3030 cm^{-1} (vinyl CH)) may reflect the contribution of quantum-mechanical tunneling[31] to this process—a phenomenon which is not unknown in the enzymatic activation of strong C−H bonds.[32]

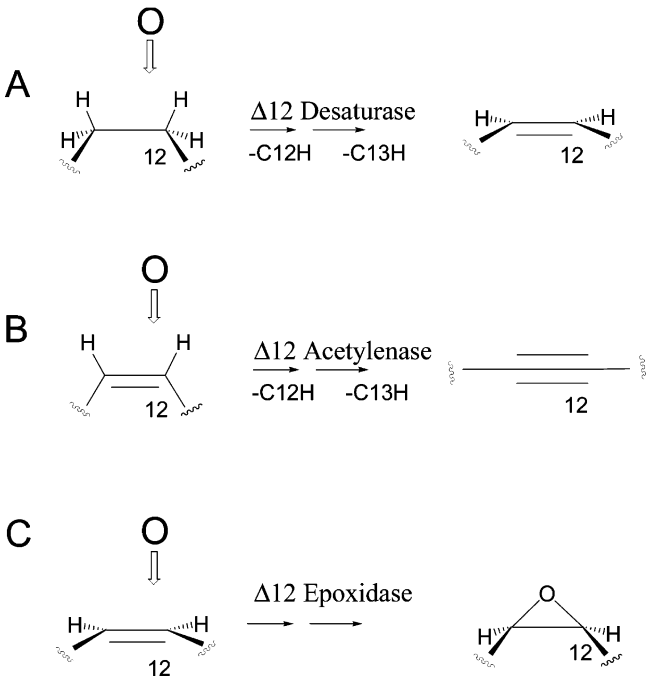

Figure 4. Comparison of possible substrate orientations with respect to oxidant for desaturation (A), acetylenation (B), and epoxidation (C).

Our finding that the Δ12 acetylenase initiates oxidation at C12 correlates with KIE data obtained for Δ12 desaturase-mediated desaturation of oleate.[17,33] The latter reaction also exhibits a large primary deuterium KIE at C12 (7.3 ± 0.4) and a negligible KIE at C13 (1.05 ± 0.04)—data which are consistent with an initial, kinetically important hydrogen abstraction step at C12 followed by facile formation of unsaturated product (see mechanistic model, Figure 2). The similarity in "cryptoregiochemistry" for the two enzymes is consistent with the inclusion of this acetylenase in the FAD2-like family of enzymes on the basis of sequence similarity. The desaturase−acetylenase connection is further emphasized by the fact that the *Crepis* Δ12 acetylenase also possesses weak oleate Δ12 desaturase activity.[3] The most probable relationship between substrate conformations for the two catalytic activities is portrayed in a generic manner in Figure 4 (A and B) taking into account the known pro-*R* enantioselectivity at C12 and C13 for Δ12 desaturation.[34]

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Crepenynate would be produced via a conformation that allows the substrate to approach the oxidant in the plane formed by the adjacent C12−C13 sp^2 centers (Figure 4B). Such an angle of attack would prevent a competing, and energetically more favorable, epoxidation pathway, which requires the oxidant to be positioned above the olefinic plane (Figure 4C). The latter conformation would presumably be optimized in the epoxidation of linoleate by the related FAD2-type Δ12 epoxidase.[3] Further insight into the factor(s) controlling acetylenation versus epoxidation will require the purification and structural characterization of the two enzymes from *Crepis* which perform these reactions. Some progress toward these goals has been made recently.[35]

In summary, we have elucidated, for the first time, some of the fundamental mechanistic characteristics of a unique enzymatic dehydrogenation process—the conversion of an unactivated alkene to the corresponding alkyne. The success of our approach opens the door to a closer examination of the mechanisms underlying the formation of acetylenic natural products—a rich cache of bioactive compounds.

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