Stereochemistry of $\Delta^4$ dehydrogenation catalyzed by an ivy (Hedera helix) $\Delta^9$ desaturase homolog

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The stereochemistry of palmitoyl-ACP $\Delta^\alpha$ desaturase-mediated dehydrogenation has been examined by tracking the fate of deuterium atoms located on stereospecifically monodeuterated substrates-(4S)- and (4R)-[4-$^2$H] palmitoyl-ACP and (5S)- and (5R)-[5-$^2$H] palmitoyl-ACP. It was found that the introduction of the (Z)-double bond between C-4 and C-5 of a palmitoyl substrate occurs with pro-$R$ enantioselectivity—a result which matches that obtained for a closely related homolog-castor stearyl-ACP $\Delta^\alpha$ desaturase. These data show that despite the difference in regioselectivity between the two enzymes, the stereochemistry of hydrogen removal is conserved.

Introduction

Fatty acid desaturases catalyze the highly selective, O$_2$-dependent, 1,2-dehydrogenation of lipidic substrates.\(^1\) The most common example of this important biological reaction features the insertion of a C9–C10 double bond into long-chain fatty acids as shown in Scheme 1. Interestingly, a number of regiochemical variations of a C9–C10 double bond into long-chain fatty acids as shown in Scheme 1.\(^1\) The occurrence of fatty acids with various double bond position or chain lengths is associated with cold acclimation, protection from herbivory and biological signaling.\(^1\) Elucidation of the structural determinants that control desaturase regioselectivity is an intriguing research problem of intrinsic interest in the area of protein engineering.\(^4\) However, to date, only soluble stearoyl-ACP $\Delta^\alpha$ desaturases (ACP = acyl carrier protein) have been purified in sufficient amounts to permit analysis by X-ray crystallography.\(^5\) Recently, a closely related homolog, palmitoyl-ACP $\Delta^\alpha$ desaturase (74% sequence identity), found in ivy (Hedera helix L.) with promising biophysical characteristics has been overexpressed.\(^6\) The availability of two stable, structurally related desaturases with differing regioselectivities offers a unique opportunity to compare active site topographies. A critical element of such an investigation involves determining the enantioselectivity of desaturation for each enzyme in order to validate crystallographic models of substrate–enzyme complexes. In the case of the castor $\Delta^\alpha$ desaturase-mediated dehydrogenation, we have shown that it is the pro-$R$ hydrogens at C-9 and C-10 of substrate that are removed.\(^7\) In this paper, we extend this approach to the stereochemical analysis of the related $\Delta^\alpha$ desaturase.

Results and discussion

The enantioselectivity of $\Delta^4$ desaturase-mediated oxidation was determined by mass spectrometric examination of products derived from C-4,5-dehydrogenation of stereospecifically monodeuterated palmitoyl-ACP derivatives. The methodology for enzymatic preparation of palmitoyl-ACP derivatives from the corresponding carboxylic acid has been developed previously.\(^8\) The synthesis of the required labeled palmitates (4R)-[4-$^2$H]I, (4S)-[4-$^2$H]I, (5R)-[5-$^2$H]I and (5S)-[5-$^2$H]I is shown in Scheme 2.\(^B\) It should be noted that de novo synthesis of these compounds was required because naturally occurring palmitates bearing appropriately situated chiral functionality such as the mid-chain hydroxyl group are not available from natural sources. A number of routes to stereospecifically labelled fatty acids have been reported;\(^9\) we decided to take advantage of the convenient Jacobson epoxide resolution methodology\(^10\) to prepare the four required compounds (Scheme 2A,B). Thus chiral

Scheme 1 Some naturally occurring variations on the prototypical $\Delta^\alpha$ desaturation of hexadecanoate.
epoxides of appropriate chain length were opened with either allyl or 4-but-1-enyl Grignard to give two pairs of regioisomeric, hydroxyheptadec-1-enyl enantiomers. The enantiomeric purity of these intermediates was estimated to be >98% in each case as determined by examination of the $^1$H NMR spectrum of the corresponding (S)-(+)-$O$-acetylmandelate derivatives$^{11}$ ($\Delta \delta$ = 0.21 ppm for the $^1$H NMR resonances assigned to the C-2 vinyl hydrogen). Stereospecific introduction of the monodeuterium label was achieved by a standard tosylation/LiAlD$_4$ displacement sequence. Mass spectrometric evaluation of the resultant monodeuterated terminal alkenes indicated that the isotopic purity of these compounds was very high (>98% d, species, Table 1). The desired monodeuterated palmitates were obtained by oxidative cleavage$^{12}$ of the corresponding heptadec-1-enes and purified by flash chromatography as the methyl esters. The analytical data for these compounds was in accord with the assigned structures; the location of the deuterium label in each case was confirmed by comparison of the $^{13}$C NMR spectral data with that of unlabeled methyl palmitate (See Experimental section).

The ACP derivative of each enantiomer was prepared as previously described and incubated with ivy palmitoyl-ACP $\Delta^4$ desaturase under conditions that maximized olefin production.

![Scheme 2](image)

**Table 1** Isotopic content* of stereospecifically monodeuterated palmitates and $\Delta^4$ desaturated-products

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Products</th>
<th>%Retention of label$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^\text{a}d_0$</td>
<td>$^\text{a}d_1$</td>
</tr>
<tr>
<td>(4R)-[4-2H$_1$]-1</td>
<td>0.9 ± 0.2</td>
<td>99.1 ± 0.2</td>
</tr>
<tr>
<td>(4S)-[4-2H$_1$]-1</td>
<td>1.1 ± 0.2</td>
<td>98.9 ± 0.2</td>
</tr>
<tr>
<td>(5R)-[5-2H$_1$]-1</td>
<td>1.0 ± 0.2</td>
<td>99.0 ± 0.2</td>
</tr>
<tr>
<td>(5S)-[5-2H$_1$]-1</td>
<td>1.3 ± 0.2</td>
<td>98.7 ± 0.2</td>
</tr>
</tbody>
</table>

* Each incubation was repeated two times and the deuterium content is given as an average value ± standard deviation of three independent GC-MS analyses. $^a$% Retention of label = [%(d$_1$(product))/%(d$_1$(substrate)) × 100].

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In each case, the enzymatic ACP product was isolated as the corresponding methyl ester after treatment of the quenched reaction with sodium methoxide solution. GC-MS analysis of the organic extracts allowed determination of the deuterium content of the products. The mass spectrometric data is shown in Table 1 and clearly demonstrate that Δ1 desaturation involves removal of the pro-R hydrogens at C-4 and C-5. That is, essentially complete loss of deuterium was observed upon desaturation of (R)-deuterated substrates while the olefinic product derived from (S)-labelled palmitates retained deuterium label to a very high degree.

The enantioselectivity displayed by the ivy Δ1 enzyme is identical to that elucidated for the homologous castor Δ1 desaturase. Given the high degree of sequence homology, it is tempting to account for the observed conservation of stereochemical preference in terms of a common active site architecture in the region of the putative non-heme diiron dioxo oxidant

\[ \text{In silico analysis of the X-ray structure for castor Δ1 desaturase} \]

has shown that the diiron centre is proximal to the pro-R hydrogens of a docked stearoyl substrate residing in a bent hydrophobic binding pocket. Mechanistic studies using oxygen, sulfur- and fluoride-substituted substrate analogues have suggested that the C-10 pro-R hydrogen of stearoyl-ACP may be removed first in the castor Δ1 desaturase-catalyzed reaction. If the location of oxidant relative to bound substrate is strictly conserved as implied in Scheme 3, then one would predict that the site of initial oxidation would be C-5 for the Δ1 desaturase-catalyzed reaction. Experiments designed to test this prediction are planned. It is hoped that the results of these efforts, together with X-ray crystallographic data of enzyme-substrate complexes can be used to gain more insight into this fascinating set of ultra selective reactions.

Scheme 3 Enantioselectivity of desaturation catalyzed by two structurally related, soluble desaturase homologs.

Conclusions

1. Stereospecifically monodeuterated palmitates can be prepared in high isotopic and enantiomeric purity from readily available chiral epoxides. This synthetic route constitutes a general approach to compounds of this type.

2. The stereochemistry of dehydrogenation mediated by two structurally related, soluble plant desaturases with differing regioselectivity has been compared for the first time. The Δ1 palmityl desaturase isolated from English ivy (Hedera helix L.) removes the vicinal pro-R hydrogens from substrate to generate a (4Z)-palmityl product. Despite the difference in regioselectivity (Δ1 versus Δ2) between the ivy and castor desaturases, the observed enantioselectivity is strictly conserved.

Experimental

General methods

1H and 13C NMR spectra were obtained at 300 and 75.5 MHz respectively on a Bruker Avance 300 spectrometer with the use of dilute CDCl3 solutions. Chemical shifts are expressed in ppm (δ) and are referenced to tetramethylsilane. J-values are reported in Hertz (Hz).

Mass spectra of synthetic intermediates were obtained by GC/MS using a Kratos 1H mass spectrometer coupled to a HP 5980 Series 2 gas chromatograph equipped with a J. & W. DB-5 capillary column (30 m × 0.21 mm), temperature programmed from 120 °C to 320 °C at 10 °C min⁻¹. GC-MS analysis of enzymatic products was carried out using a HP5973 mass spectrometer coupled to a HP6890 GC equipped with a SP2340 capillary column (60 m × 0.25 mm), temperature programmed from 100 °C to 160 °C at 25 °C min⁻¹ and 160 °C to 240 °C at 10 °C min⁻¹. The isotopic content of analytes was estimated by scanning several times per GC peak; the integrated intensities of the individual ions in the pertinent ion cluster were analyzed with the use of HP-ChemStation software and have been corrected for natural isotopic abundances. Care was taken to include the entire GC peak in the integration procedure in order to prevent errors due to fractionation of isotopic species during chromatography.

Flash chromatography with silica gel (230–400 mesh) was used to purify all intermediates and substrates. Visualization of UV-inactive materials on silica gel TLC was accomplished by a combination of water spray or I2 vapor as appropriate.

All reagents and starting materials for organic synthesis were purchased from Sigma-Aldrich and used without purification. Tetrahydrofuran (THF) and diethyl ether (Et2O) were freshly distilled from Na-benzophenone ketyl. All air- and moisture-sensitive reactions were performed under N2. Organic extracts were typically dried by gravity filtration through anhyd. Na2SO4 and solvents were evaporated in vacuo on a Büchi RE 111 Rotavapor.

All buffers and salts, NADH, BSA and other biochemicals were purchased from Sigma-Aldrich. Protein concentrations were measured by the method of Bradford using bovine serum albumin as standard protein. The purification of ivy palmityl-ACP Δ1 desaturase and required cofactors and the synthesis of substrate ACP derivatives has been previously described.

Synthesis of substrates

Preparation of chiral epoxides

1,2-Epoxytetradecene. To 1-tetradecene (3.92 g, 20 mmol) in dichloromethane (15 ml) in a RBF at 0 °C was added m-chloroperbenzoic acid (7.92 g, 55%, 45.4 mmol) dissolved in dichloromethane (300 ml). The solution was left to react for 18 h at 4 °C and then washed with sat. NaHCO3 (3 × 75 ml) and sat. NaCl (3 × 75 ml), dried and evaporated to give the title compound (4.06 g, 96%) as a colourless oil at room temperature. The analytical data for this compound
1,2-Epoxytridecane. From 1-tridecane and MCPBA. A colourless oil at room temperature. Analytical data similar to that of 1,2-epoxytridecane except for $^1$C NMR $\delta$ 52.43, 47.11, 32.47, 31.89, 29.61, 29.60, 29.53, 29.53, 29.42, 29.32, 25.94, 22.66, 14.07; EI MS (rel. intensity) m/z 155 (1), 151 (2), 123 (8), 109 (13), 95 (33), 82 (52), 71 (100), 55 (80), 43 (45).

(S)-(−)-1,2-Epoxytetradecane. Glacial acetic acid was added (62.5 µL, 1.10 mmol) to a solution of [(S,S)-N,N′-bis(3,5-di-tert-butylsalicylidene)-1,2-cyclohexanediaminato(2−)] cobalt(II) (63 mg, 0.10 mmol) in dichloromethane (2 mL) and the solution was stirred 30 minutes and then concentrated in vacuo to give a crude brown solid. This residue was cooled to 0 °C and a solution of 1,2-epoxytetradecane (4.40 g, 20.7 mmol) in isopropanol (1 mL) was added. H$_2$O (375 µL, 21 mmol) was added dropwise with stirring. The reaction mixture was allowed to warm to room temperature; the reaction was allowed to proceed for 48 h. Hexanes (40 mL) were added to the product mixture and the turbid solution filtered to remove a solid precipitate. The filtrate was concentrated (40 mL) were added to the product mixture and the turbid solution filtered to remove a solid precipitate. The filtrate was concentrated in vacuo to yield the title compound (1.21 g, 85% yield) as a low melting solid.

(R)-(−)-1,2-Epoxytetradecane. From [(R,R)-N,N′-bis(3,5-di-tert-butylsalicylidene)-1,2-cyclohexanediaminato(2−)] cobalt(II) and 1,2-epoxytetradecane. Colourless oil at room temperature. R$_f$ 0.34 (5% EtOAc in hexanes); All spectral data were similar to that of (R,S)-1,2-epoxytetradecane. [α]$^\text{D}_2$ = + 5.8° (c 1.48, CHCl$_3$) lit.* + 4.31° (c 1.42, CHCl$_3$).

(R)+(−)-1,2-Epoxyoctadecane. From [(R,R,R)-N,N′,N″-tris(3,5-di-tert-butylsalicylidene)-1,2-cyclohexanediaminato(2−)] cobalt(II) and 1,2-epoxytetradecane. Colourless oil at room temperature. R$_f$ 0.34 (EtOAc/hexanes 1 : 20). All spectral data were similar to that of (R,S)-1,2-epoxytetradecane. [α]$^\text{D}_2$ = + 5.8° (c 1.48, CHCl$_3$) lit.* + 4.31° (c 1.42, CHCl$_3$).

Preparation of chiral alcohols

(S)-5-Hydroxy-1-heptadecene. To allyl magnesium bromide (1 M in THF, 6.6 mL, 6.6 mmol) at 0 °C was added, slowly with stirring, (S)-(−)-1,2-epoxytetradecane (906 mg, 4.27 mmol) dissolved in THF (1.5 mL), followed by dilithium tetrachlorocuprate solution (50 mL, 24 mg LiCl and 34 mg CuCl$_2$ in 2.5 mL THF). The mixture was allowed to warm to rt and stirred for 2 h. The reaction was quenched by the addition of crushed ice (5 g). The mixture was transferred to a separatory funnel, and the reaction vessel rinsed with Et$_2$O (10 mL) and 3 M HCl (3 mL). The aqueous layer was extracted with Et$_2$O (3 × 6 mL) and the combined organic layers were washed with 10% NH$_2$OH (3 × 6 mL), 10% Na$_2$SO$_4$ (1 × 4 mL), H$_2$O (2 × 4 mL) and sat. NaCl (1 × 5 mL). The solution was dried and concentrated in vacuo to yield the title compound (1.05 g, 4.14 mmol, 97%) as a low melting solid.

(R)-5-Hydroxy-1-heptadecene. From (R)(+)-1,2-epoxytetradecane and allyl magnesium bromide. Low melting solid at rt. R$_f$ 0.08 (5% EtOAc in hexanes). Analytical data similar to that reported for corresponding racemic compound, >98% ee (1H NMR of (S)-(−)-O-acetylamidomale derivative).
Preparation of stereospecifically deuterated palmitates

Methyl (4R)-[4-2H1]-palmitate (4SR)-[4-2H1]-1. A solution of (S)-5-hydroxy-1-heptadecane (906 mg, 3.57 mmol) in dry pyridine (7.5 mL) was treated with TsCl (1.41 g, 7.14 mmol) at 0 °C. The solution was stirred for 1 h at 0 °C and left at 4 °C (3 days). The precipitated pyridinium hydrochloride salt was dissolved by adding H2O (25 mL) to the reaction mixture and the aqueous layer extracted with Et2O (3 × 30 mL). The combined organics were washed with sat. CuSO4 (4 × 15 mL), H2O (2 × 15 mL), 5% NaHCO3 (2 × 15 mL), sat NaCl (1 × 15 mL), dried and concentrated in vacuo to give the tosylate (1.29 g) as a viscous oil. 1H NMR δ 7.79 (d, J 9.0 Hz, 2H), 7.33 (d, J 9.0 Hz, 2H), 5.70 (m, 1H), 4.98 (dm, J 5.1 Hz, 1H), 4.92 (m, 1H), 4.57 (p, J 6.0 Hz, 1H), 2.44 (s, 3H), 1.94–2.06 (m, 2H), 1.61–1.73 (m, 2H), 1.15–1.33 (m, 22H), 0.88 (t, J 6.6 Hz, 3H). The tosylate intermediate (1.14 g, ca. 2.8 mmol) was dissolved in dry ether (5 mL) and lithium aluminium deuteride (372 mg, 8.8 mmol) was added; the reaction mixture was stirred for 5 h at rt under N2. The reaction was quenched with H2O (12 mL) and 6 M HCl (21 mL) and the mixture was stirred for 30 min. The reaction was terminated with the addition of NaOMe at 55 °C for 30 min. The residue was acidified with acetic acid (100 µL) and extracted with hexane (2 × 2 mL). The phases were separated by centrifugation and the combined organics were evaporated under a steady stream of N2 and the residue was diluted with 100 µL of hexane for analysis by GC-MS.

Desaturase assay. Reactions of acyl-ACP derivatives with Δ4 desaturase were carried out at room temperature. Each reaction mixture consisted of ivy Δ4 desaturase dimer (2.4 nmol), dithiothreitol (400 nmol), bovine liver catalase (16 nmol), Anabaena vegetative ferredoxin (4.7 nmol), maize root NADPH:ferredoxin reductase (0.3 nmol), acyl-ACP (10.6 nmol) in a total volume of 660 µL of buffer. The reaction was initiated by the addition of NADPH (0.7 µmol) in buffer (32 µL) and allowed to continue for 30 min. The reaction was terminated with the addition of toluene (1 mL) and the thioster linkage was transesterified to give the corresponding methyl ester with freshly prepared 0.5 M NaOMe at 55 °C for 30 min. The residue was acidified with acetic acid (100 µL) and extracted with hexane (2 × 2 mL). The phases were separated by centrifugation and the combined organics were evaporated under a steady stream of N2 and the residue was diluted with 100 µL of hexane for analysis by GC-MS.

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Methyl (5SR)-[5-2H1]-palmitate (5SR)-[5-2H1]-1. From (S)-6-hydroxy-1-heptadecane. Obtained as a white solid. The spectral data of the title compound was identical to that of the corresponding (S)-enantiomer.

Methyl (5R)-[5-2H1]-palmitate (5RS)-[5-2H1]-1. From (R)-6-hydroxy-1-heptadecane. Obtained as a white solid. The spectral data of the title compound was identical to that of the corresponding (R)-enantiomer.
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