Desaturation, Chain Scission, and Register-Shift of Oxygen-Substituted Fatty Acids during Reaction with Stearoyl-ACP Desaturase†

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Received April 24, 2002; Revised Manuscript Received June 5, 2002

ABSTRACT: Stearoyl acyl carrier protein Δ^9 desaturase catalyzes the NADPH- and O_2-dependent insertion of a cis double bond between the C-9 and C-10 positions of the acyl chain in the kinetically preferred natural substrate 18:0-ACP. In this work, substrate analogues with an oxygen atom singly replacing the methylene groups at the 8, 9, 10, and 11 positions of the stearoyl chain were synthesized, converted to acyloxy-ACPs, and used as probes of desaturase reactivity. Evidence for desaturation, acyloxy chain scission, and register-shift in binding prior to chain scission was obtained. Reactions with acyloxy-ACPs having either O-8 or O-11 substitutions gave a single desaturation product consistent with the insertion of a cis double bond between C-9 and C-10. The k_{cat}/K_M values for the O-8- and O-11-substituted acyloxy-ACPs were comparable to that of the natural substrate, indicating that the presence of an ether group adjacent to the site of reactivity did not significantly interfere either with the desaturation reaction or with the binding of substrate in the proper register for desaturation between C-9 and C-10. For reactions with the O-9 and O-10 acyloxy-ACPs, the k_{cat} values were decreased to ~3% of that observed for 18:0-ACP, and upon reaction, the acyloxy chain was broken to yield an ω-hydroxy fatty alkanoyl-ACP and a volatile long-chain aldehyde. For the O-9 substitution, 8-hydroxyoctanoate and 1-nonanal were obtained, corresponding to the anticipated binding register and subsequent reaction between the O-9 and C-10 positions. In contrast, the O-10 substitution yielded 9-hydroxynonanoyl-ACP and 1-octanal, corresponding to an obligate "register-shift" of acyloxy chain binding prior to reaction between the O-10 and C-11 positions. Register-shift is thus defined as a mechanistically relevant misalignment of acyl chain binding that results in reaction at positions other than between C-9 and C-10. The inability of the O-10 acyloxy probe to undergo reaction between the C-9 and O-10 positions provides evidence that the Δ9D-catalyzed desaturation of stearoyl-ACP may initiate at C-10. Possible mechanisms of the acyl chain scission and implications of these results for the desaturation mechanism are considered.

The soluble acyl-ACP[1] desaturases catalyze the NADPH- and O_2-dependent insertion of cis double bonds into fatty acyl chains that are covalently attached to ACP (*1*). These desaturases are members of a structurally related family of diiron enzymes that also includes the R2 component of ribonucleotide diphosphate reductase (*2*−*4*) and numerous bacterial hydrocarbon monooxygenases (*5*, *6*). All of the above diiron enzymes bind and activate O_2 during catalysis, and a paradigm for reaction involving the generation of a high-valent iron oxidizing state has emerged from extensive studies of biological systems and chemical synthetic analogues (*5*−*11*).

For Δ9D, protein−acyl chain interactions are recognized as important catalytic determinants (*10*, *12*−*14*). This enzyme exhibits remarkably high fidelity for the insertion of a cis double bond between the C-9 and C-10 positions of a long-chain acyl-ACP, including nonnatural acyl-ACP analogues (*14*, *15*). These results imply that an accurate, reproducible generation of the proper binding register for an otherwise nondescript acyl chain is an important aspect of catalysis. An X-ray structure revealed that the diiron center was buried within the protein and alongside a tunnel that might serve as the fatty acyl chain binding site (*16*). It is reasonable that the depth of the diiron center within the protein subunit and the protein−protein interaction surface provided by the ACP portion of the substrate provide important constraints to the positional specificity of double bond formation. Moreover, the exclusive cis stereochemistry observed for the introduced double bonds apparently corresponds to the presence of a prominent bend in this tunnel adjacent to the diiron center (*16*, *17*).

In addition to positional and stereochemical constraints on double bond formation provided by protein structure, the acyl-ACP desaturases exhibit a high degree of selectivity

† This work was supported by National Institutes of Health Grant GM-50853.

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[1] Abbreviations: ACP, holo-acyl carrier protein; *n*:0-ACP, ACP with an *n*-carbon saturated fatty acid covalently attached to ACP through a phosphopantetheine thioester bond; *n*:1-ACP, acyl-ACP with a covalently attached *n*-carbon monounsaturated fatty acid; Δ9D, 18:0-ACP Δ^9 desaturase; GC/EI, gas chromatography/electron ionization; ESI, electron spray ionization; MALDI, matrix-assisted laser desorption; MS, mass spectrometry; MSTFA, *N*-methyl-*N*-trimethylsilyl trifluoroacetamide; O-8, 7-(decyloxy)heptanoic acid; O-9, 8-(nonyloxy)octanoic acid; O-10, 9-(octyloxy)nonanoic acid; O-11, 10-(heptyloxy)decanoic acid; TMS, trimethylsilyl.

10.1021/bi020306d CCC: $22.00
Published on Web 07/12/2002

for acyl chain length (*12, 14, 18*). Steady-state kinetic analysis of the effect of acyl chain length on Δ9D catalysis suggested that each methylene group in an acyl chain of 14−18 carbons in length may contribute approximately −3 kJ/mol of favorable hydrophobic binding energy (*14*). This binding energy was deduced to promote catalysis instead of tighter binding. It is likely that these investigated changes in acyl chain length give rise to altered binding interactions within the tunnel past the diiron center. Correspondingly, mutations that occluded the end of the tunnel shifted the selectivity toward desaturation of shorter acyl chains (*19*).

In this work, fatty acids with an oxygen atom singly replacing the methylene groups at the 8, 9, 10, and 11 positions of a C-18 fatty acyl chain were synthesized, converted to acyloxy-ACPs, and used as probes of Δ9D reactivity. The systematic replacement of carbon with oxygen has provided a metric for assessing the binding register of the acyl chain as well as a mechanistic probe for the subsequent reactivity. The results provide evidence for desaturation, acyloxy chain scission, and "register-shift" in binding prior to chain scission, depending on the position of the ether functional group in the acyl chain. For O-10 ACP, an obligate register-shift of acyloxy chain binding was required prior to an oxidative reaction leading to acyl chain scission between the O-10 and C-11 positions. The unique pattern of reactivity observed with the O-10 ACP analogue provides evidence that Δ9D-catalyzed desaturation reactions may initiate at the C-10 position. As such, the possibility that the Δ9D desaturation reaction may initiate at C-10 represents a distinguishing mechanistic feature when considered in light of the consensus pattern for initial reactivity at the C-H position closer to the carboxyl end of the fatty acid in a wide variety of integral membrane desaturase homologues (*20*−*23*).

MATERIALS AND METHODS

Materials. Ethyl hydrogen pimelate, ethyl hydrogen suberate, and ethyl hydrogen sebacate were purchased from Lancaster (Morecambe, Lancashire, U.K.). Azeleic acid monomethyl ester and all other solvents and reagents were from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Enzymes. Δ9D, ferredoxin, and NADPH:ferredoxin oxidoreductase, and spinach and *E. coli* ACPs were prepared as previously described (*24*−*28*). Glucose-6-phosphate dehydrogenase, superoxide dismutase, and catalase were from Sigma-Aldrich.

Synthesis and Characterization of Acyloxy Fatty Acids. The syntheses were based upon a previous report (*29*) with the modifications indicated below. 7-Hydroxyheptanoic acid, 8-hydroxyoctanoic acid, 9-hydroxynonanoic acid, and 10-hydroxydecanoic acid were prepared by selective reduction of the carboxylic acid group of ethyl hydrogen pimelate, ethyl hydrogen suberate, methyl hydrogen azelate, and ethyl hydrogen sebacate, respectively, by BH_3 in THF (*30*). Heptyl, octyl, nonyl, and decyl *p*-toluenesulfonates were prepared as previously reported (*31*). ^{1}H and ^{13}C NMR spectra were recorded on Bruker 300 and 500 MHz spectrometers, respectively.

7-(Decyloxy)heptanoic Acid (O-8). 7-Hydroxyheptanoic acid (5.00 g, 34 mmol) was added to 60 mL of dry DMF. A 60% dispersion (w/v) of 10 g of NaH in mineral oil was then added with stirring. This mixture was heated at 60 °C for 1 h and then cooled in an ice bath. Decyl *p*-toluenesulfonate (20.0 g, 71 mmol) was added, and the reaction mixture was heated at 80 °C for 60 h. After cooling, the reaction was poured into a mixture of 1 N HCl and ether. The layers were separated, and the water layer was extracted 3 times with hexanes. The organic layers were combined, dried over $MgSO_4$, and stirred with decolorizing carbon for 1 h. The solution was filtered, and the solvent was evaporated under reduced pressure. The resulting product was further purified by silica gel chromatography (80:20, hexanes/ether), yielding 0.6225 g of product (2.2 mmol, 6.4% yield starting from 7-hydroxyheptanoic acid). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.90 (t, 3H, CH_3), 1.08−1.73 (m, 24, methylene envelope), 2.35 (t, 2H, CH_2COO), 3.37 (t, 4H, CH_2OCH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 22.7, 25.7, 26.1, 26.1, 29.0−29.9 (envelope), 31.9, 32.8, 70.8, 70.9, 170.8; ESI-MS m/z = 285.4; GC/EI-MS of the MSTFA derivative m/z = 343, 217, 200, 185, 145, 127, 117, 73.

8-(Nonyloxy)octanoic Acid (O-9) and 9-(Octyloxy)nonanoic Acid (O-10). 8-Hydroxyoctanoic acid (4.64 g, 29 mmol) was reacted with nonyl *p*-toluenesulfonate as above for O-8, and 1.69 g of product was obtained (5.9 mmol, 20.6% yield yield starting from 8-hydroxyheptanoic acid). 9-Hydroxynonanoic acid (5.91 g, 34 mmol) was reacted with octyl *p*-toluenesulfonate as described above for O-8, and 0.303 g of product was obtained (1.06 mmol, 3.1% yield starting from 9-hydroxyheptanoic acid). The ^{1}H NMR and MS spectra of the products matched literature values (*29*).

10-(Heptyloxy)decanoic Acid (O-11). 10-Hydroxydecanoic acid (4.51 g, 24 mmol) was reacted with heptyl *p*-toluenesulfonate as above for O-8, and 0.276 g of product was obtained (0.97 mmol, 4.1% yield starting from 10-hydroxyheptanoic acid). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.90 (t, 3H, CH_3), 1.08−1.73 (m, 24, methylene envelope), 2.35 (t, 2H, CH_2COO), 3.37 (t, 4H, CH_2OCH_2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 22.6, 24.6, 26.1, 26.2, 29.0−29.29.9 (envelope), 31.6, 31.9, 70.9, 71.0, 179.5; ESI-MS m/z = 285.4; GC/EI-MS of the MSTFA derivative m/z = 343, 245, 213, 155, 145, 129, 117, 73.

Enzyme Steady-State Kinetics and Product Formation. Enzyme reactions were performed in 50 mM HEPES buffer, pH 7.8, containing 50 mM NaCl. A typical assay contained 5−120 μM of the acyloxy derivatives of spinach ACP, 2 μM ferredoxin reductase, 9.5 μM ferredoxin, 40 units/mL of superoxide dismutase, 800 units/mL of catalase, 10 units/mL of G6PD, 3.5 mM glucose 6-phosphate, and 800 μM NADPH in a total volume of 1 mL. The reactions were incubated in a rotary shaker at 150 rpm at 25 °C for 2 min before initiation by the addition of 0.02−1.7 nmol of Δ9D. Aliquots were taken at timed intervals and quenched by rapid mixing with 150 μL of THF. For reactions with either O-9 or O-10 ACPs, an internal standard of heptadecanol was present in the THF. Initial reaction velocities were determined using linear least-squares fitting from the time-dependence for appearance of the *n*:1 products in reactions with O-8 and O-11, or from the time-dependence for disappearance of the *n*:0 substrate peak in reactions with O-9 and O-10 as determined by GC/EI-MS. The steady-state parameters k_{cat} and K_M were determined by nonlinear least-squares fitting of the initial desaturation velocity and substrate concentration

data to the Michaelis−Menten equation: $v = k_{cat}[S]/(K_M + [S])$.

Acyl Chain Extraction and Derivatization. THF-quenched samples were diluted with 300 μL of deionized water. To prepare a sample for GC/EI-MS analysis, 5 mg of $NaBH_4$ was added, and the sample was then incubated at 37 °C for 15 min (*14*). Excess $NaBH_4$ was quenched by the addition of 100 μL of 1 N HCl saturated with NaCl. A 30 μL aliquot of 10 N NaOH was then added, and the sample was extracted twice with 750 μL of $CHCl_3$. The combined $CHCl_3$ extracts were dried under N_2 at 37 °C and resuspended in 60 μL of hexanes. The fatty acids were converted to the silyl ether derivatives by the addition of 5−10 μL of MSTFA. Excess MSTFA was quenched by the addition of 2−3 μL of CH_3-OH.

GC/EI-MS Analyses. The instrument was a Hewlett-Packard 6890 GC equipped with a Hewlett-Packard 7683 auto injector and an HP-5MS column (30 m × 0.25 mm, 0.25 μm film thickness) connected to either a flame ionization detector or a Hewlett-Packard 5973 electron ionization mass sensitive detector. The injector was maintained at 250 °C. During the GC/EI-MS analyses, the column temperature was maintained at 50 °C for 2 min, then increased at 35 °C/min to a temperature of 175 °C, held for 2 min at 175 °C, increased at 2 °C/min to 220 °C, increased at 45 °C/min to 250 °C, and then held at 250 °C for 7 min. Under the conditions utilized, the silylated products and substrates eluted at ∼18 and ∼19 min, respectively. Products were identified by comparison of the retention times and time-dependent appearance of parent ions and fragments with correct *m*/*z* values. The *m*/*z* values determined by GC/EI-MS after derivatization were confirmed using ESI-MS on underivatized samples. Positions of double bonds introduced by the Δ9D reaction with O-8 and O-11 ACP were determined by GC/EI-MS analysis of the MSTFA- and bis-(methylthio)-derivatized fatty alcohol products obtained from $NaBH_4$ treatment of acyl-ACPs (*14*, *32*). Fractional desaturation of the O-8 and O-11 fatty acids was calculated by dividing the *n*:1 peak area by the sum of the *n*:0 and *n*:1 peak areas. The total nanomoles of *n*:1 formed during the reaction was calculated by multiplying the total nanomoles of *n*:0-ACP introduced into the reaction by the fractional conversion. For O-9 and O-10 fatty acids, the fractional substrate conversion was calculated by comparison with an internal standard of heptadecanol.

Identification of Product Aldehydes. Reactions containing 100 nmol of either O-9 or O-10 ACP in 250 μL of the mixture described above in *Enzyme Steady-State Kinetics and Product Formation* were performed for 30 min in sealed vials. The reactions were quenched by the addition of 62 μL of hexanes to the sealed vial and vigorously mixed using a vortex mixer. The organic and aqueous phases were separated by centrifugation, and the hexane layer was removed for GC/EI-MS analysis. The products were identified by comparison of retention times and fragmentation patterns with authentic samples.

ESI-MS Analyses. To prepare a sample for an ESI-MS analysis, 15 μL of 10 N NaOH was added, and the sample was incubated at 40 °C for 10 min in order to release the acyl chain from phosphopantetheine. Subsequently, 250 μL of 1 N HCl saturated with NaCl was added, and the resulting mixture was extracted twice with 750 μL of $CHCl_3$. The combined $CHCl_3$ extracts were dried under N_2 at 37 °C and dissolved in 75 μL of CH_3OH. The instrument was an Applied Biosystems MDS Sciex API 365 LC/MS/MS triple quadrupole spectrometer equipped with a Perkin-Elmer ABI 140D HPLC inlet system and operated in the negative ionization mode.

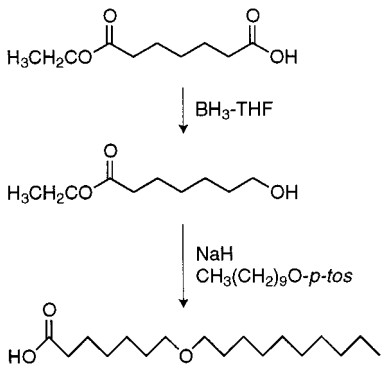

FIGURE 1: Synthesis of 7-(decyloxy)heptanoic acid (O-8) as a representative example of the protocols used to synthesize O-8, O-9, O-10, and O-11 acyloxy fatty acids.

Purification of Acyl-ACPs for MALDI-MS Analyses. HPLC purifications of acyloxy-ACPs and ω-hydroxy fatty acyl-ACPs were performed on a Hewlett-Packard series 1100 HPLC equipped with a Varian Microsorb-MV 300 Å C-18 column. In these experiments, *E. coli* ACP was used to permit optical detection at 260 nm. Reactions containing 200 nmol of O-9 or O-10 ACP in 500 μL of the reaction mixture described above in *Enzyme Steady-State Kinetics and Product Formation* were run for 30 min and then quenched by the addition of 100 μL of THF. Aliquots of the quenched reaction (100 μL) were loaded onto the HPLC column equilibrated in a solvent of 35% (v/v) CH_3CN and 65% (v/v) 90:10 H_2O with 0.1% TFA/CH_3CN (buffer A). After 2 min, the solvent was changed to 65% (v/v) CH_3CN and 35% buffer A over a 13 min period. This solvent was maintained for 8 min, and then the solvent was changed back to 35% (v/v) CH_3-CN and 65% buffer A over a 2 min period. The acyl-ACPs eluted at ∼14 min, and fractions were collected, pooled, and concentrated by evaporation of the solvent under N_2 at 37 °C. The MALDI-MS analyses were obtained from either an α-cyano or a sinapinic acid matrix using a Bruker BIFLEX III spectrometer equipped with a 337 nm nitrogen laser.

RESULTS

Preparation of Acyloxy-ACPs. Figure 1 shows the synthetic steps used to produce the O-8 fatty acid. Analogous procedures were used to produce O-9, O-10, and O-11 fatty acids. The ω-hydroxy carboxylic acid intermediates were prepared by selective reduction of the carboxylic acid group of the corresponding carboxylic acid ester. The ω-hydroxy carboxylic acids were then reacted with the appropriate *p*-toluenesulfonic acid alcohol derivative to yield a long-chain acyloxy fatty acid in 3−20% yield relative to the starting carboxylic acid ester. The resulting acyloxy fatty acids were coupled to either spinach or *E. coli* holo-ACP using purified holo-ACP synthase, providing the desired acyloxy-ACPs in ∼10−50 mg quantities for use as reactivity probes.

Steady-State Kinetic Analyses. Initial studies revealed that both the O-8 and the O-11 ACPs were readily desaturated

Table 1: Kinetic Parameters for the Reaction of Δ9D with Acyloxy-ACPs

substrate	k_{cat}[a] (min^{-1})	K_M (μM)	k_{cat}/K_M ($\mu M^{-1}\cdot min^{-1}$)	R[b]
18:0[c]	49 (3.8)	3.9 (1.4)	13	0.969
O-8	12 (1.0)	6.6 (2.8)	1.8	0.956
O-11	30 (2.6)	7.4 (2.9)	4.0	0.961
O-9	2.4	nd[d]	nd	
O-10	1.2	nd	nd	

[a] k_{cat} values reported per diiron site. Errors (in parentheses) were derived from nonlinear least-squares fitting. [b] Correlation coefficients provided from nonlinear least-squares fitting. [c] Data from (*14*). [d] Not determined.

by Δ9D. Table 1 shows the k_{cat}/K_M, k_{cat}, and K_M values determined from steady-state kinetic analyses for both O-8 and O-11 ACPs. These kinetic parameters were only marginally different from those of 18:0-ACP, indicating an overall similarity in the reactivity of the acyloxy-ACPs and the natural substrate. The relatively weaker binding of the acyloxy-ACPs indicated by the small increases in the apparent K_M values (Table 1) may be due to a number of effects, such as unfavorable interactions of the more polar substrate with hydrophobic portions of the substrate binding tunnel, or an alteration in bond angles, distances, or rotational energies introduced by the ether group, forcing the acyloxy chain to adopt a less favorable conformation. Once bound, however, the favorable k_{cat} values observed for both O-8 and O-11 ACPs (12 and 30 min^{-1}, respectively) relative to 18:0-ACP (49 min^{-1}) imply that these acyloxy-ACPs may react in a manner closely mimicking the natural substrate.

Measurement of the time-dependent disappearance of either O-9 or O-10 ACPs revealed that the k_{cat} values for these substrates were less than 5% of that observed for 18:0-ACP when the acyloxy-ACP concentration was ~120 μM (Table 1). Due to these low k_{cat} values, K_M values were not determined for either O-9 or O-10 ACP. For comparison, however, a previous study of the dependence of steady-state kinetic parameters on acyl-chain length showed that increasing the acyl chain length between C-14 and C-19 resulted in an ~100-fold increase in k_{cat} and k_{cat}/K_M, but only a ~3-fold nonsystematic variation in the K_M values (*14*). Our interpretation of these results was that hydrophobic binding interactions provided an important energetic contribution to catalysis. For O-9 and O-10 ACPs, the k_{cat} values were most similar to those observed from 15:0-ACP, suggesting the decreased reactivity may arise in part from alterations in hydrophobic binding interactions. Alternatively, the positioning of the ether O-atoms in the active site may interfer with certain aspects of the reaction mechanism, such as a requirement for C−H bond breakage as a part of the catalytic cycle.

Products from O-8 and O-11 ACPs. Figure 2 shows the structures of the TMS-derivatized fatty alcohols obtained from reactions with O-8 and O-11 ACPs. Figure 2 also shows mass data obtained from these derivatives that define the double bond position. In the TMS derivatives, an M· peak of $m/z = 327$ was observed for both compounds, while the m/z values of 203 for O-8 ACP and 243 for O-11 ACP correspond to fragments that have lost the acyl chain past the ether group (Figure 2A,B, left panels). For O-11 ACP, the fragment with $m/z = 243$ also indicates the presence of

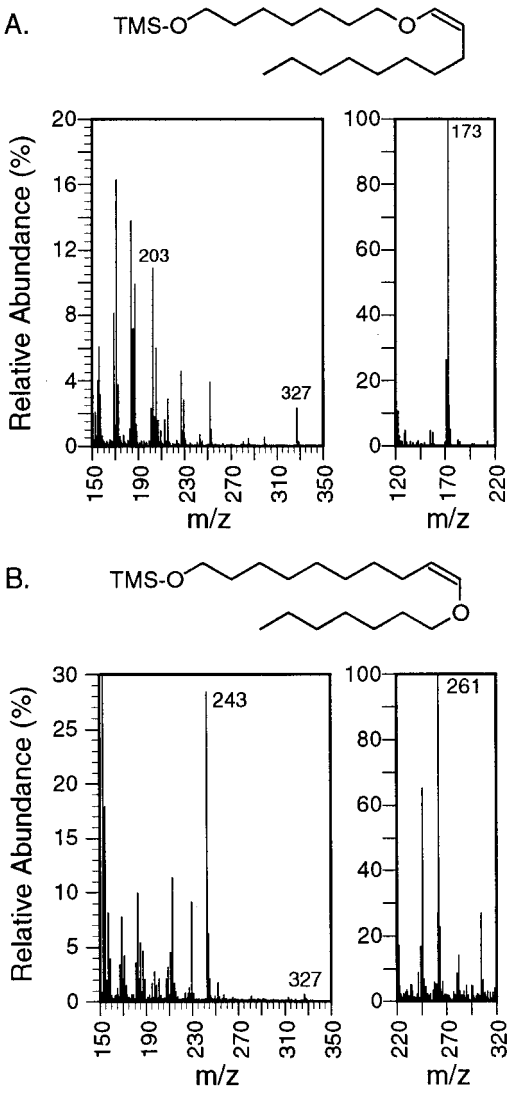

FIGURE 2: Δ9D-catalyzed desaturation of either (A) O-8 or (B) O-11 ACP. The left panels show mass spectra of the TMS-derivatized products, while the right panels show mass spectra of the TMS- and bis(methylthiol)-derivatized products. Diagnostic mass peaks described under Results are indicated.

a double bond introduced by the enzyme. Furthermore, the TMS and bis(methylthiol) fragmentation products with $m/z = 173$ obtained for O-8 ACP (Figure 2A, right panel) and $m/z = 261$ obtained for O-11 ACP (Figure 2B, right panel) indicate that a double bond was introduced between the C-9 and C-10 positions. These results correspond to the known positional selectivity for Δ9D-catalyzed desaturations, i.e., exclusive reaction between the C-9 and C-10 positions. Furthermore, and with respect to the minor changes in K_M values measured for these substrates, the presence of the ether substitutions did not alter substrate binding sufficiently to yield any detectable alternative products. We therefore conclude that upon reaction, the O-8 and O-11 ACP substrates had achieved a binding register corresponding to that of the natural substrate. Thus, for the example of O-11 ACP, the C-9, C-10, and O-11 atoms occupy positions within the active site equivalent to those occupied by the C-9, C-10, and C-11 atoms of 18:0-ACP. Upon reaction, O-11 ACP thus gives exclusive double bond formation between C-9 and C-10.

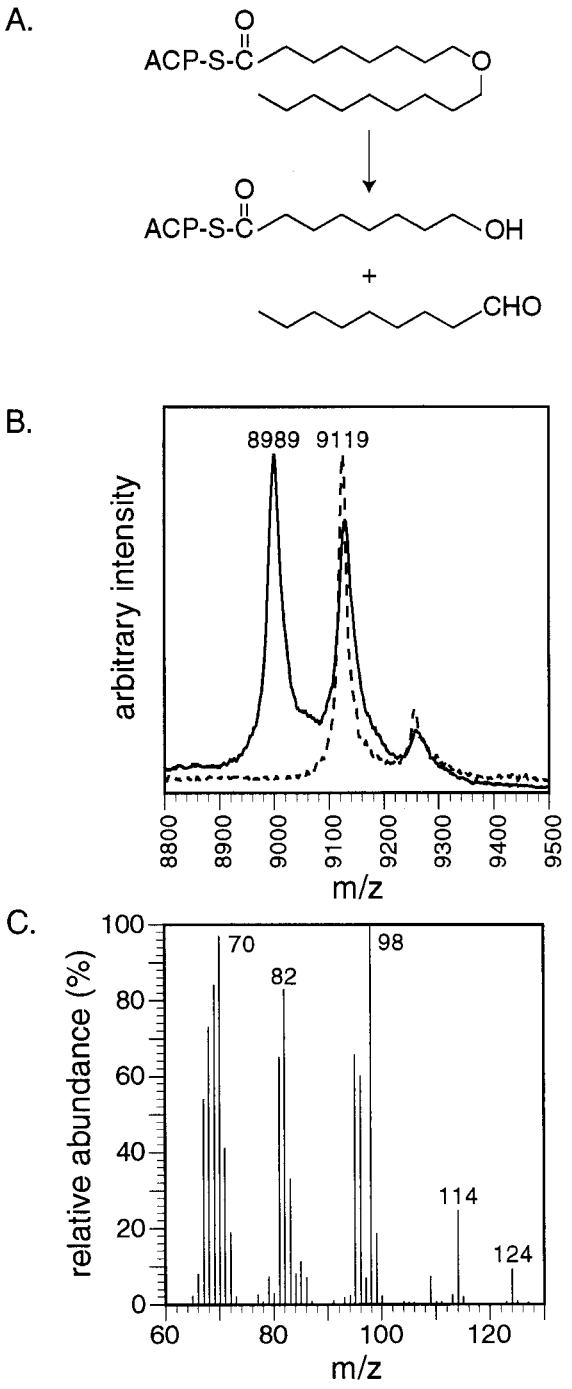

FIGURE 3: Mass spectral data obtained on the products of the reaction of O-9 ACP with Δ9D. (A) Reaction scheme showing structures of the substrate and products. (B) MALDI-MS analysis of the HPLC-purified acyl-ACP product. Dashed line, substrate O-9 ACP, observed *m*/*z* is 9119, expected *m*/*z* is 9116. Solid line, 8-hydroxyoctanoyl-ACP, observed *m*/*z* is 8990, expected *m*/*z* is 8989. The peak with *m*/*z* of 9260 arises from a fraction of the O-9 ACP preparation that retained the *N*-terminal Met residue on ACP. (C) GC/EI-MS spectrum of 1-nonanal produced from chain cleavage of O-9 ACP.

Reaction of O-9 ACP. Figure 3 shows the structures and mass spectra of the products obtained from the reaction with O-9 ACP. Initial GC/EI-MS analyses revealed the absence of long-chain acyloxy product. An HPLC separation of the acyl-ACP product followed by MALDI-MS analysis suggested that only 8-hydroxyoctanoate remained bound to ACP after the reaction of O-9 ACP (Figure 3B, where O-9 ACP has an expected *m*/*z* = 9116 and 8-hydroxyoctanoyl-ACP has an expected *m*/*z* = 8989). The assignment of the ACP-bound fragment as 8-hydroxyoctanoate was further corroborated by a base-catalyzed cleavage of the acyl ester and ESI-MS analysis of the released acyl group, which gave the predicted *m*/*z* = 159 (not shown). The presence of the 8-hydroxyoctanoyl fragment of the initial O-9 fatty acid demanded that scission of the acyloxy chain had occurred in preference to a desaturation reaction. Figure 3C shows the results of a GC/EI-MS analysis of the contents extracted from a sealed vial (required to retain volative products during sample workup) containing the O-9 ACP reaction. This mass spectral pattern is indistinguishable from that obtained from an authentic sample of 1-nonanal and published elsewhere (*33*). Thus, Δ9D-catalyzed reaction with O-9 ACP yields 8-hydroxyoctanoyl-ACP and 1-nonanal as the exclusive products via an acyl chain scission reaction.

Reaction of O-10 ACP. Figure 4 shows the structures of products obtained from reaction of O-10 ACP. As in reaction with O-9 ACP, GC/EI-MS characterization revealed no evidence for a long-chain acyloxy product, and HPLC separation followed by MALDI-MS analysis showed only 9-hydroxynonanoyl-ACP (Figure 4B, where O-10 ACP has an expected *m*/*z* = 9116 and 9-hydroxynonanoyl-ACP has an expected *m*/*z* = 9003). The assignment of the ACP-bound fragment as 9-hydroxynonanoate was further corroborated by ESI-MS analysis of the hydrolyzed acyl group, which gave the predicted *m*/*z* = 173 (not shown). Figure 4C shows the GC/EI-MS analysis of the volatile products of the O-10 ACP reaction. This mass spectral pattern is indistinguishable from that obtained from an authentic sample of 1-octanal (*33*). Thus, as observed for O-9 ACP, the Δ9D-catalyzed reaction with O-10 ACP yielded an acyl chain scission reaction as the exclusive catalytic result. *In this case, however, the masses of the products demanded that the acyl chain was broken exclusively between the O-10 and C-11 positions.* This result is contrary to the expected reactivity between C-9 and O-10 if the positional specificity observed for either 18:0- or O-9 ACPs was matched with O-10 ACP. Thus, the result with O-10 ACP consitutes a "register shift" in binding, where the C-9, O-10, and C-11 atoms of O-10 ACP most likely shift to positions within the active site equivalent to those occupied by the C-8 through C-10 atoms of either 18:0-ACP or O-9 ACP in order to permit catalysis and yield the observed products.

DISCUSSION

Mechanism of Acyl Chain Scission. Acyl chain scission is consistent with an oxidative attack on the acyl chain at a carbon position adjacent to the ether functional group. Rearrangements of hemiacetals created by oxidative catalysis have been proposed in O-dealkylation reactions catalyzed by P450 (*34*), diiron (*35*−*37*), and other iron-containing oxygenases (*38*), providing substantial precedent for the proposed rearrangement leading to acyl chain scission. The absence of other products observed from reaction of O-9 ACP indicates that only a single binding configuration, which situates the O-9 and C-10 atoms of the acyloxy chain in vicinity to the catalytic center, can lead to catalysis. Figure 5A indicates potential reaction pathways beginning with a discrete C−H bond breakage (*39, 40*), while Figure 5B indicates a potential reaction pathway of direct OH^+ insertion

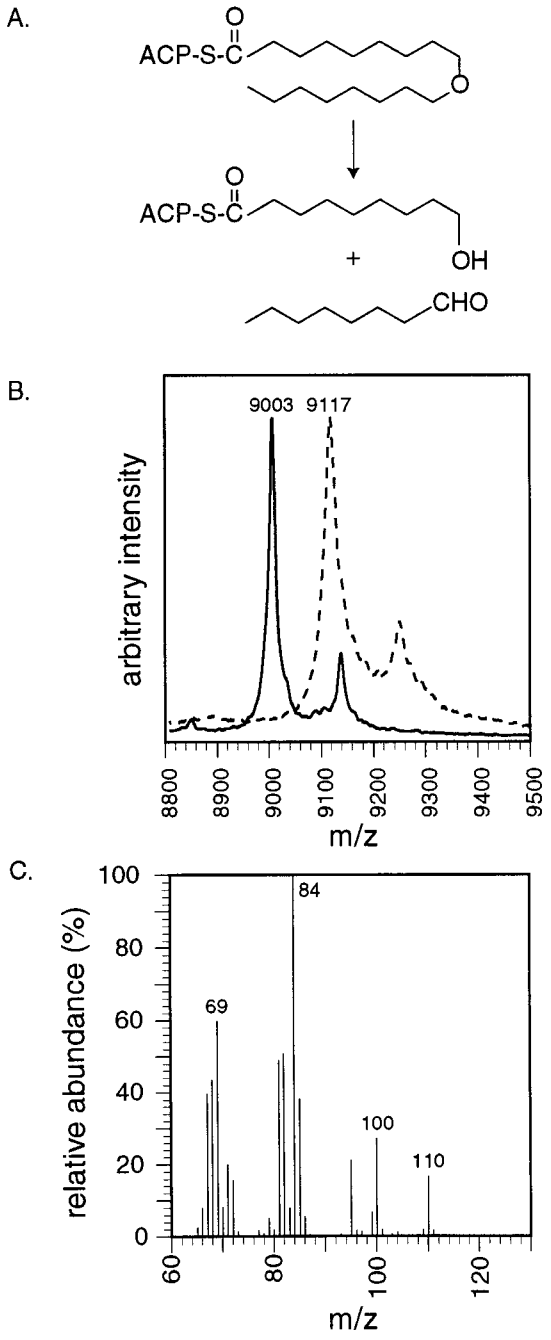

FIGURE 4: Mass spectral data obtained on the products of the reaction of O-10 ACP with Δ9D. (A) Reaction scheme showing structures of the substrate and products. (B) MALDI-MS analysis of the HPLC-purified acyl-ACP product. Dashed line, O-10 ACP, observed *m*/*z* is 9117, expected *m*/*z* is 9116. Solid line, 9-hydroxynonanoyl-ACP, observed *m*/*z* is 9006, expected *m*/*z* is 9003. (C) GC-EIMS spectrum of 1-octanal produced from chain cleavage of O-10 ACP. See Figure 3 for other details.

into a C−H bond (*41*, *42*). Either of these pathways is postulated to yield an unstable long-chain hemiacetal that ultimately rearranges to the observed hydroxyalkanoyl-ACP and long-chain aldehyde (Figure 4C).

An intramolecular isotope effect observed for hydrogen abstraction at the C-10 position by 9D (*43*) supports the C−H abstraction pathway of Figure 5A (*44*). For reaction of the acyloxy-ACPs, the putative radical intermediate of Figure 5A would be stabilized by electron donation from the adjacent lone pair orbitals of the adjacent oxygen. Figure

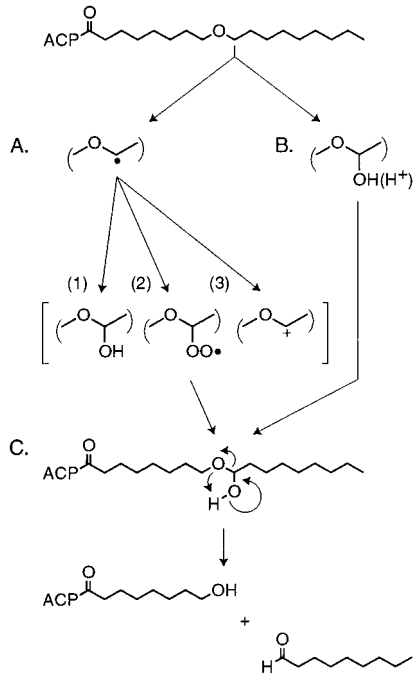

FIGURE 5: Potential mechanisms for Δ9D-catalyzed scission of the acyloxy ether of O-9 ACP between the O-9 and C-10 positions. (A) C−H bond abstraction leading to the formation of a transient substrate radical intermediate. Subsequent reactions for the substrate radical intermediate, including (1) O-atom rebound, (2) quench by O_2 to generate an unstable α-hydroperoxyl ether radical, or (3) $1e^-$ oxidation to generate a transient substrate carbocation intermediate. (B) Direct insertion of OH^+. (C) Decomposition of a common hemiacetal intermediate to yield 8-hydroxyoctanoyl-ACP and 1-nonanal.

5A(1) shows hydroxylation of a transient substrate radical through a "rebound" mechanism similar to that proposed for the P450 (*45*) and diiron hydroxylases (*39*, *40*), yielding the putative hemiacetal intermediate (Figure 5C). For the natural acyl-ACP substrates, a transient hydroxylation followed by dehydration has overall been considered unlikely (*46*, *47*), although products derived from $^{18}O_2$-derived ^{18}O-atom transfer have been reported for reactions of nonnatural thiastearoyl-ACPs (*48*, *49*) and 9(*R*)-fluorostearoyl-ACP (*43*).

Figure 5A(2) postulates that a substrate radical generated by C−H bond abstraction could also react with diffusible O_2, possibly within the active site, to generate a transient *gem*-hydroperoxyl radical ether similar to that observed in the lipoxygenase reaction (*50*, *51*). Rearrangement of a *gem*-hydroperoxyl radical ether produced from oxidation of an acyloxy-ACP would then yield a hemiacetal (Figure 5C) and water, perhaps facilitated by the excess reducing equivalents present in the multiple turnover reaction conditions.

Figure 5A(3) shows a second $1e^-$ oxidation yielding a substrate carbocation. Hydride transfer from an acyloxy-ACP to the metallooxidant could also directly yield a substrate carbocation (pathway not shown). Carbocations have been proposed as intermediates in reactions of the prostaglandin prostacyclin, and in thromboxane synthases (*52*, *53*), the methane monooxygenase with "radical-clock" and other substrates (*41*, *54*−*56*), P450 enzymes (*57*), and ammonia-oxidizing bacteria (*58*). In the reaction of an acyloxy fatty acid, formation of a carbocation would be stabilized by the adjacent lone pair orbitals of the ether group. Quenching of a carbocation, either by water or by an iron-bound hydroxy or aqua group, would then yield a hemiacetal (Figure 5C).

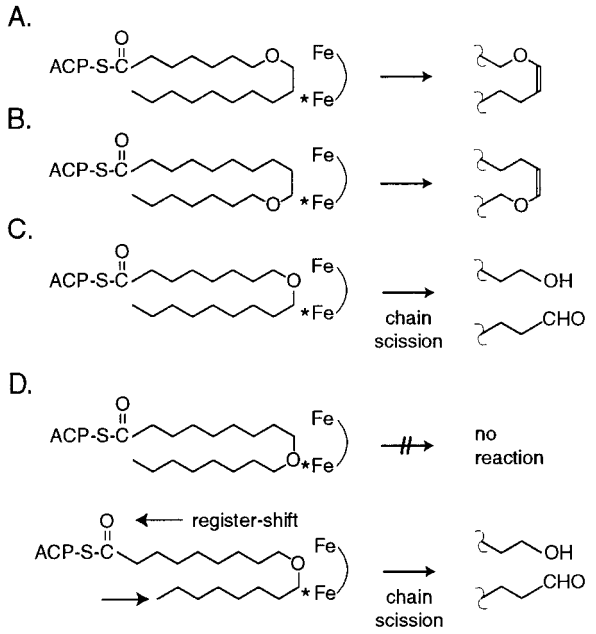

FIGURE 6: Summary of experimental results leading to the assignment of an obligate requirement for "register-shift" binding of O-10 ACP and exclusive acyl chain cleavage between the O-10 and C-11 positions. The combined reactivity patterns for all analogues indicate that the initial steps of reaction occur at the 10-position, as indicated by the asterisk.

Register-Shift in Acyl Chain Binding and Reactivity. The specificity for binding of long-chain biopolymers was originally elaborated by study of sequence-specific proteases (*59*) and hydrolytic enzymes such as lysozyme (*60, 61*). By the established convention, a complete binding site can be subdivided into a series of localized subsites situated both before and after the active site, where chemistry occurs. In principle, a similar spatial definition for substrate binding can be applied to the fatty acid desaturases, but with an understanding that the repeated methylene groups of a saturated acyl chain provide only minimal structural variation which might be used to indicate the proper register of binding. Consequently, total binding energy provides a major input to specificity and catalysis (*14*).

Products consistent with a register-shift have been observed with other acyl-ACP substrates. For example, during Δ9D reaction with the substrate analogue trans-Δ^7-18:1-ACP (*15*), 20% of the total product was trans-Δ^7,cis-Δ^{10}-18:2, a register-shift product, in addition to the expected trans-Δ^7,-cis-Δ^9-18:2 product that arose from a correct register of binding. The most likely origin of the Δ^{10}-18:2 product was a restriction of complete entry of the substrate into the active site, which thus produced the register-shift. Likewise, a mutated Δ9D isoform with an occluded binding site gave ~5% of a Δ^{10}-18:1-ACP product (*62*). The reaction of 9(*R*)-fluorostearoyl-ACP also gave several products that apparently arise from a misalignment of substrate in the active site, followed by a "register-shift" reaction between C-10 and C-11 (*43*). In combination with the present results, these other examples validate the importance of considering register-shift in the interpretation of desaturase product distributions.

Implications for Mechanisms of Desaturation. Figure 6 summarizes results obtained from the study of acyloxy-ACPs. This work provides insight into the position of initial C−H bond abstraction catalyzed by Δ9D. The presence of the ACP moiety likely prevents the acyl chain from being drawn into the substrate channel sufficiently to permit reaction between C-8 and C-9. This is consistent with the absence of desaturation at C-8 or closer to the carboxyl terminal. Furthermore, the exclusive and overall efficient reactivity of both O-8 and O-11 ACPs between C-9 and C-10 (see Figure 2 and compare k_{cat} values in Table 1) indicates that the ether functional group is able to attain the correct binding register and geometry of CH_2 groups required for double bond insertion (Figure 6A,B). These results with O-8 and O-11 ACP imply that both O-9 and O-10 ACP should minimally be able to attain the correct binding register. Indeed, for reaction with O-9 ACP, an acyl chain scission reaction corresponding to singular reactivity between O-9 and C-10 was the only observed outcome (Figure 6C).

For the reaction of O-10 ACP (Figure 6D), binding of C-9 and O-10 in the active site without a register shift would require that catalysis must initiate at the C-9 position, since the presence of O-10 would likely block oxidative attack. However, in this case, an initial reaction at the C-9 position would then yield 8-hydroxyoctanoyl-ACP and 1-nonanal, *products that were clearly not obtained.* Therefore, we propose that the true products, 9-hydroxynonanoyl-ACP and 1-octanal, arise from a register-shift of substrate binding followed by initial attack at C-11, since the O-10 position would still not be susceptible to oxidative attack, *even if it were shifted to an active site position expected to be occupied by C-9*. The absence of acyl chain scission products from reaction with O-11 ACP further indicates that once the initial substrate intermediate is formed, subsequent oxidation steps leading to desaturation must be orders of magnitude faster than a putative hydroxylation at C-10, which would lead to chain cleavage.

The possibility that Δ9D-catalyzed desaturation begins by initial attack at the C-10 position contrasts with results obtained from the integral membrane desaturases (*22*). For example, studies on the yeast stearoyl-CoA Δ^9 desaturase suggest that the initial, rate-determining C−H abstraction occurs from the carbon atom of the nascent double bond closest to the carboxyl end (*20*). Therefore, the unique and quantitative reaction products observed from O-9 and O-10 ACPs provide important experimental evidence that Δ9D may initiate catalysis at the C-10 position. This represents a new mechanistic distinction between what have long been assumed to be functionally equivalent enzyme classes.

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BI020306D