Chain Cleavage and Sulfoxidation of Thiastearoyl-ACP upon Reaction with Stearoyl-ACP Desaturase†

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ABSTRACT: The fatty acid analogues 9- and 10-thiastearate were converted to acyl-ACP derivatives by *in vitro* enzymatic synthesis and reacted with the reconstituted soluble stearoyl-ACP Δ9 desaturase complex. Electrospray ionization mass spectral analysis of the acyl chains purified from the reaction mixtures showed that 10-thiastearoyl-ACP was converted to the 10-sulfoxide as the sole product. In the presence of $^{18}O_2$, the sulfoxide oxygen was found to be derived exclusively from O_2. This result confirms the ability of the soluble stearoyl-ACP desaturase to catalyze O atom transfer in the presence of the appropriate substrate analogue. Inhibition studies showed that 10-thiastearoyl-ACP was a mixed-type inhibitor of 18:0-ACP, with an apparent K_I of ∼10 μM. Comparable reactions of the stearoyl-ACP desaturase complex with 9-thiastearoyl-ACP gave the 9-sulfoxide as ∼5% of the total products, with the O atom again exclusively derived from O_2. The remaining 95% of the total products arose from an acyl chain cleavage reaction between S-9 and C-10. Matrix-assisted laser desorption ionization time-of-flight mass spectral analysis showed that 9-thiastearoyl-ACP had a mass of 9443 amu while the acyl chain cleavage product had a mass of 9322 amu, corresponding to the calculated mass of 8-mercaptooctanoyl-ACP. Recovery of the acyl chain from the ACP product gave the disulfide of 8-mercaptooctanoate (mass of 349.1 amu), arising from the dimerization of 8-mercaptooctanoate during product workup. Gas chromatography−mass spectral analysis also showed the accumulation of nonanal in sealed reaction vials, accounting for the other product of the acyl chain cleavage reaction. The reactivity at both the 9 and 10 positions of the thia-substituted acyl-ACPs is consistent with the proximity of both positions to the diiron center oxidant in the enzyme−substrate complex. Moreover, the differential reactivity of the 9- and 10-thiastearoyl-ACPs also suggests position-dependent consequences of the reaction within the Δ9D active site. Mechanisms accounting for both sulfoxidation and acyl cleavage reactions by the stearoyl-ACP Δ9 desaturase are proposed.

Eukaryotes use either soluble or integral membrane desaturase complexes to catalyze the NADPH- and O_2-dependent insertion of double bonds into already-synthesized fatty acyl chains [Figure 1 (*1*)]. Although both desaturase homologues contain diiron centers (*2*), the duplicated EXXH ligand motif found in the soluble desaturases is distinct (*3*) from the eight-His ligand motif proposed for the membrane desaturases (*4*). Moreover, the soluble desaturases require acyl-ACP[1] as the catalytically relevant substrate [Figure 1 (*5*)], while the integral membrane desaturases utilize acyl chains attached to a variety of polar headgroups, including CoA, phospholipid, and diacylglycerols (*1*, *6*).

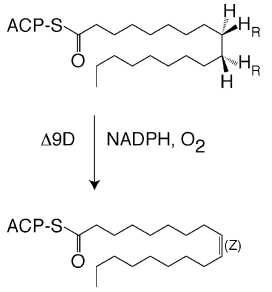

FIGURE 1: Schematic representation of the Δ9D reaction. The stereochemistry of H_R-atom removal from C-9 and C-10 was defined in ref *7*.

The structural distinctions between the soluble and membrane desaturases introduce questions regarding the degree to which often-assumed similarities in reactivity of the two homologues will be valid, and work to elucidate potential differences in reactivity has begun. The regio- and stereoselective desaturase reaction begins by H atom abstraction (*7*), and subsequent hydroxylation and dehydration is not a likely contributor to the reaction pathway. Kinetic isotope studies (*8*) with yeast stearoyl-CoA Δ9 desaturase using stereospecifically deuterated fatty acids showed that the initial, rate-limiting C−H abstraction occurs at the pro-*R*

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[1] Abbreviations: ACP, acyl carrier protein; 18:0-ACP, ACP with stearate covalently attached to ACP through a phosphopantetheine thioester bond; 18:1-ACP, ACP with oleate covalently attached to ACP through a phosphopantetheine thioester bond; Δ9D, 18:0-ACP Δ^9 desaturase; 9-thiastearate, 9-(octyl-1-sulfanyl)-nonanoate; 10-thiastearate, 8-(nonyl-1-sulfanyl)-octanoate; FdR, ferredoxin reductase; Fd, [2Fe-2S] ferredoxin; G6P, glucose 6-phosphate; G6PD, G6P dehydrogenase.

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position of C-9 followed by a kinetically silent reaction at the pro-*R* position of C-10, with a similar stereochemistry deduced for the soluble stearoyl-ACP Δ9 desaturase from castor plant (Δ9D) (*7*). Steady-state kinetic isotope effects were not observed with Δ9D (*9*), however, primarily because of a subsequently identified large forward commitment to catalysis and overall rate-limiting product release (*10*). This prevented an assessment of the position at which H atom abstraction might be initiated with Δ9D. In this regard, product distribution studies with Δ9D and acyloxy-ACPs with an O atom singly replacing the methylene groups at the 8−11 positions of a C-18 acyl chain (*11*) revealed a unique pattern of "register-shift" binding and quantitative acyl chain scission. One interpretation of these results is that the soluble desaturase reaction may begin at C-10, representing one mechanistic distinction between what have long been assumed to be functionally equivalent enzyme classes.

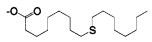
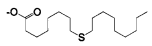
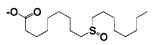
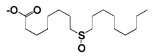
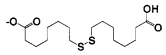
Stearate analogues with the 9 or 10 position of the acyl chain changed to sulfur have been previously used to probe the reactivity of the membrane desaturases (*12*). These compounds were found to be inhibitors of the desaturation of stearoyl-CoA in confluent 7800 C1 Morris hepatoma cells, cultured rat hepatocytes, and rat liver microsomes. After thiastearates had been fed to *Saccharomyces cerevisiae* cultures expressing stearoyl-CoA Δ9 desaturase, sulfoxides were recovered from the culture medium that had a high enantiomeric excess and a stereochemistry that matched the deduced pattern of H atom removal (*13*). This reactivity was designated "diverted" desaturation, and was the first demonstration of an alternative reaction for what is otherwise recognized as a high-fidelity and high-specificity catalytic site.

In this work, we report studies on the reaction of soluble acyl-ACP Δ9 desaturase with thiastearoyl-ACP analogues. One goal of this effort was to provide a comparative examination of the reactivity of the soluble desaturase with that previously reported for the integral membrane enzymes. The results presented here show a combination of sulfoxidation and position-specific acyl chain cleavage that further elaborates the differences in the reaction outcomes among these two desaturase superfamilies. The mechanistic implications of the differences in reactivity of the 9- and 10-thiastearoyl-ACP analogues are discussed.

MATERIALS AND METHODS

Enzymes. Δ9D was expressed, purified, and characterized as previously described (*14*). The concentration of Δ9D active sites was determined using an ϵ_{340} of 4200 M^{-1} cm^{-1} per diiron center, and cross correlated by determination of the protein concentration and the total amount of iron (*15*, *16*). For the preparations used in this work, these determinations indicate a >95% occupation of diiron active sites in the dimeric protein. The turnover number of the Δ9D preparations used in this work (0.5−0.7 s^{-1}) was comparable to that observed for the maximally active enzyme (*9*, *10*). Spinach apo-ACP isoform I was expressed in *Escherichia coli* BL21(DE3), converted to holo-ACP by *in vitro* reaction with holo-ACP synthase, and converted to acyl-ACPs by reaction with *E. coli* acyl-ACP synthase (*17*). Fatty acyl chains were reductively cleaved, extracted, derivatized, and analyzed by GC−MS as previously described (*5*). FdR (*18*) and Fd (*19*) were overexpressed in *E. coli*, purified by standard chromatographic methods, and quantified by optical spectroscopy (ϵ_{456} = 10 700 M^{-1} cm^{-1} for FdR; ϵ_{278} = 15 700 M^{-1} cm^{-1} for Fd).

Table 1: Names, Masses, and Structures of Compounds Considered in This Work

	mass (amu)[a]		Structure
1	301.2	10-thiastearate, [9-(octyl-1-sulfanyl)-nonanoic acid]	
2		9-thiastearate, [8-(nonyl-1-sulfanyl)-octanoic acid]	
3	317.2	10-sulfoxystearate, [8-(nonyl-1-sulfinyl)-octanoic acid]	
4		9-sulfoxystearate, [9-(octyl-1-sulfinyl)-nonanoic acid]	
5	175.1	8-mercaptooctanoic acid, [8-(nonyl-1-sulfanyl)-octanoic acid]	
6	349.1	7-carboxy-heptyl disulfide, 8-(7-carboxy-heptyldisulfanyl)-octanoic acid	
7	142.1	nonanal	

Chemicals. NADPH was from Sigma/Aldrich (St. Louis, MO) and was quantified by optical spectroscopy (ϵ_{340} = 6200 M^{-1} cm^{-1}). The fatty acids 18:0 and 18:1, superoxide dismutase, catalase, G6P, and G6PDH were from Sigma/Aldrich. Isotopically labeled $^{18}O_2$ (97% ^{18}O content) and $^{18}OH_2$ (97% ^{18}O content) were from ICON (Summit, NJ). Dichloromethane (Sigma/Aldrich, Milwaukee, WI) was distilled over molecular sieves immediately before use. MSTFA was purchased from Alltech (Deerfield, IL) and used immediately after its container had been opened. Ethyl acetate and methanol (Fisher Scientific, Fair Lawn, NJ) and all other chemicals were used without further purification.

Mass Spectral Instrumentation. The GC−EI-MS analyses were obtained on a Hewlett-Packard 6890 gas chromatograph equipped with a Hewlett-Packard 7683 auto injector and an HP-5MS column (30 m × 0.25 mm, film thickness of 0.25 μm) connected to a Hewlett-Packard 5973 electron ionization mass sensitive detector. The injector was maintained at 250 °C. During the analyses, the column temperature was maintained at 50 °C for 2 min, then increased at a rate of 35 °C/min to a temperature of 175 °C, held for 2 min at 175 °C, increased at a rate of 2 °C/min to 220 °C, increased at a rate of 45 °C/min to 250 °C, and then held at 250 °C for 7 min. The MALDI-TOF 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. The standard error for the MALDI-TOF MS studies of acyl-ACPs was ±18 amu (0.2%). The ESI-MS and ESI-MS/MS analyses were obtained with a Perkin-Elmer Sciex API 365 triple-quadrupole mass spectrometer. The standard error for the ESI-MS and ESI-MS/MS studies of fatty acid derivatives was better than ±0.1 amu.

Syntheses of Substrates and Products. The substrates and products considered in this work are shown in Table 1. 10-Thiastearic acid (**1**) was synthesized by oxidation of 9-bromononanol to 9-bromononanoic acid followed by condensation with octanethiol (*20*). The ^1H NMR and GC−EI-MS parameters of the synthesized 10-thiastearic acid matched those reported in the literature. The sulfoxystearate isomers (**3** and **4**) were synthesized from the corresponding thiastear-

ates by oxidation with 1 equiv of H_2O_2 in methanol (*20*). The synthesized sulfoxystearate isomers had ^{1}H NMR parameters matching previously published values. The synthesized **3** had ESI-MS/MS peaks at 204 amu [$^-$OOC$(CH_2)_8$S(O)], 187 amu [$^-$OOC$(CH_2)_7$CHS], and 155 amu [$^-$OOC$(CH_2)_7$CH], while the synthesized **4** had ESI-MS/MS peaks at 190 amu [$^-$OOC$(CH_2)_7$S(O)], 173 amu [$^-$OOC$(CH_2)_6$CHS], and 141 amu [$^-$OOC$(CH_2)_6$CH]. 8-Mercaptooctanoic acid (**5**) was synthesized by sulfination of 8-bromooctanoic acid (*20*), and 7-carboxyheptyl disulfide (**6**) was prepared from **5** by duplicating the conditions of the sample preparation used for ESI-MS/MS analysis (evaporation from CH_2Cl_2 at 40 °C). The disulfide exhibited the following ^{1}H NMR resonances (200 MHz): δ 1.25−1.43 (20 H, m, methylene envelope), 2.37 (4 H, br s, C*H*$_2$-S-S-C*H*$_2$), and 2.67 (4 H, t, HOOC-C*H*$_2$); ESI-MS/MS peaks with masses of 349.2, 331.4, 175.2, 173.2, and 141.2 amu.

Multiple-Turnover Catalysis. Reactions were performed in 250 μL of 50 mM Hepes buffer (pH 7.8) containing 35 mM NaCl. The reaction mixtures contained 8 μM FdR, 37 μM Fd, 40 units/mL superoxide dismutase, 800 units/mL catalase, 40 units/mL G6PD, 14 mM G6P, 200 μM thiastearoyl-ACP, and 6.7 μM Δ9D. The reactions were initiated by the addition of Δ9D and the mixtures incubated in a rotary shaker at 150 rpm and 25 °C for the appropriate time period up to 1 h.

For reaction mixtures subjected to MALDI-TOF MS analysis, aliquots of the reaction mixture (250 μL) were frozen until they were needed. For other analyses, reactions were rapidly quenched by injection of the mixtures into 100 μL of tetrahydrofuran. The products derived from thiastearoyl-ACPs were isolated by hydrolysis upon the addition of 15 μL of 10 M NaOH and incubation at 40 °C for 10 min. The hydrolyzed sample was placed in a screw-cap vial along with 200 μL of 1 M HCl saturated with NaCl and 250 μL of a saturated NaCl solution and extracted three times with 750 μL of freshly distilled CH_2Cl_2. The combined extracts were dried under N_2 at 40 °C and dissolved in 50 μL of CH_3OH for ESI-MS analysis. Nonanal was recovered by performing the enzyme reaction in a septum-sealed 5 mL glass vial, followed by extraction of the unquenched reaction in the sealed vial with 100 μL of hexane. The hexane extract was analyzed by GC−EI-MS (*11*). The oleoyl-ACP product was recovered by reduction of the acyl thioester using $NaBH_4$ (*5*). The fatty alcohols 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, and the derivatized fatty alcohols were analyzed by ESI-MS.

The disappearance of 10-thiastearoyl-ACP was assessed by normalization of the peak obtained from MSTFA-derivatized 10-thiastearol with the area of a similarly derivatized peak of heptadecan-1-ol added as an internal standard to hexane. The ability of 8-mercaptooctanoyl-ACP to inhibit the Δ9D reaction was assessed from assays containing both 18:0-ACP and 8-mercaptooctanoyl-ACP at 100 μM and other reaction constituents as described above. Reaction aliquots were taken starting immediately after the addition of NADPH at 30 s intervals. The enzymatic conversion of 18:0-ACP to 18:1-ACP was determined as previously described (*5*). An additional control experiment was performed by substituting apo-ACP for 8-mercaptooctanoyl-ACP.

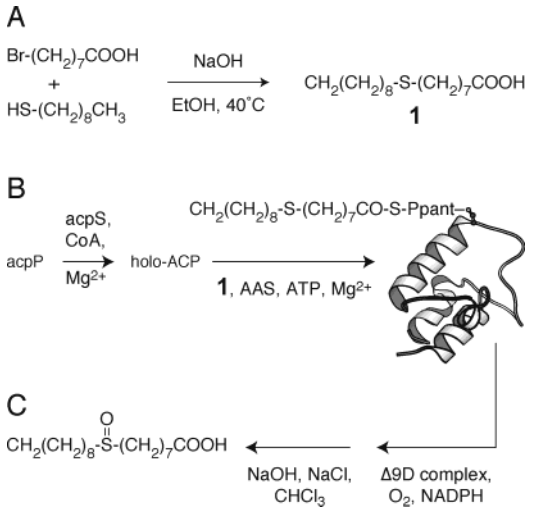


FIGURE 2: A summary of the chemical synthesis of 10-thiastearoyl-ACP and the conditions used for reaction with Δ9D. A, condensation of 9-bromononanoate with octanethiol. B, enzymatic phosphopantetheinylation of apo-ACP to form holo-ACP and enzymatic acylation of holo-ACP with 10-thiastearate by acyl-ACP synthetase (*17*). C, Δ9D reaction and acyl chain recovery (*5*).

Inhibition Analysis. Reactions were performed in the reaction mixtures described above. The following concentrations of substrate and inhibitors were used: 0, 12.5, 25, 50, and 100 μM 18:0-ACP, 0, 12.5, 25, and 50 μM 9-thiastearoyl-ACP, and 0, 12.5, 25, and 50 μM 10-thiastearoyl-ACP. The reactions were initiated by the addition of Δ9D, and the mixtures were incubated in a rotary shaker at 150 rpm and 25 °C. At the appropriate time points, aliquots of the reaction mixture (43 μL) were withdrawn and the reactions quenched by addition of the mixtures to 100 μL of tetrahydrofuran. Initial rates for the formation of 18:1-ACP were determined as previously reported (*21*). Linear least-squares fitting was used to evaluate double-reciprocal plots at different, fixed concentrations of thiastearoyl-ACPs and varied concentrations of 18:0-ACP.

Single-Turnover Catalysis. Reduced Fd was made by placing a solution of oxidized Fd in a septum-sealed cuvette modified to contain a two-way stopcock. This solution was made anaerobic by repeated cycling between vacuum and re-fill with O_2-free Ar. The oxidized Fd was reduced by titration with a solution of ~0.1 M $Na_2S_2O_4$ prepared in anaerobic 1 M sodium phosphate buffer (pH 7.0) until the A_{398}/A_{422} ratio was ≥1.2. Four equivalents of reduced Fd was mixed with an aerobic solution containing Δ9D (2 equiv of diferric active sites) and 2 equiv of 9-thiastearoyl-ACP. After 0.5 h, the acyl chains were cleaved, extracted, and analyzed by ESI-MS.

RESULTS

Substrate Syntheses. Figure 2 summarizes the combination of chemical and enzymatic syntheses required to produce 10-thiastearoyl-ACP. An analogous procedure was used to produce 9-thiastearoyl-ACP after substitution of commercially available 9-thiastearate for **1** in step 2B. The overall protocol allowed production of ~50 mg batches of the thiastearoyl-ACPs. The 8-mercaptooctanoyl-ACPs could also be produced from this method by starting with the corresponding acyl compound.

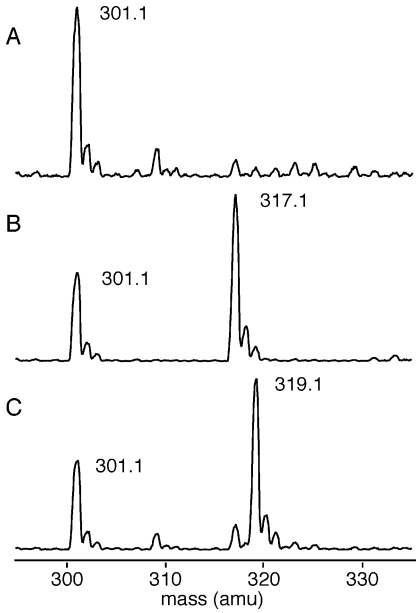


FIGURE 3: Results of an ESI-MS analysis of the sulfoxide produced by reaction of 10-thiastearoyl-ACP with Δ9D. A, control reaction containing FdR, Fd, NADPH, G6PD, G6P, superoxide dismutase, and catalase but lacking Δ9D. B, reaction containing Δ9D and other components described in 3A was performed in the presence of $^{16}O_2$. 10-sulfoxystearate has an expected mass of 317.2 amu; the observed mass was 317.1 amu. C, reaction performed in the presence of $^{18}O_2$.

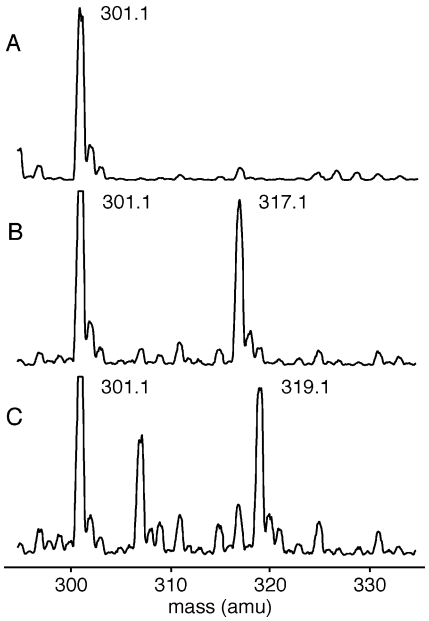


FIGURE 4: Results of an ESI-MS analysis of the sulfoxide produced from reaction of 9-thiastearoyl-ACP with Δ9D. A, control reaction containing FdR, Fd, NADPH, G6PD, G6P, superoxide dismutase, and catalase but lacking Δ9D. B, reaction containing Δ9D and the other components described in 4A performed in the presence of $^{16}O_2$. 9-sulfoxystearate has an expected mass of 317.2 amu; the observed mass was 317.1 amu. C, reaction performed in the presence of $^{18}O_2$.

Control Experiments. In the absence of the NADPH regeneration system, the thiastearoyl-ACPs were stable over a 0.5 h time period and **1** and **2** could be hydrolyzed from the corresponding thiastearoyl-ACP and recovered from the reaction mixtures in essentially quantitative yield. In the presence of the reconstituted electron transfer chain, ESI-MS analysis showed that a small, variable amount of sulfoxystearate (**3** and **4**) was produced in the absence of Δ9D. This adventitious sulfoxidation likely arose from uncoupled electron transfer reactions, which would be consistent with the chemical synthesis of either **3** or **4** in the presence of H_2O_2 (*20*). In control reaction mixtures containing superoxide dismutase and catalase and lacking Δ9D, the adventitious sulfoxidation was completely suppressed, and intact **1** (Figure 3A, 301.1 amu) and **2** (Figure 4A, 301.1 amu) were recovered in high yield. The addition of Δ9D to the reaction mixtures containing NADPH, superoxide dismutase, and catalase yielded sulfoxide products.

Reaction of 10-Thiastearoyl-ACP. Figure 3B shows the ESI-MS analysis of the acyl chain isolated from a reaction mixture containing the reconstituted Δ9D complex, superoxide dismutase, catalase, and 50 nmol of 10-thiastearoyl-ACP. In the presence of $^{16}O_2$, the 10-thiastearoyl chain was converted to a single product with a mass of 317.1 amu. ESI-MS/MS of the 317.1 amu feature revealed a fragmentation pattern matching that of chemically synthesized 10-sulfoxystearate (**3**, fragments with masses of 204, 187, and 155 amu, not shown), indicating that a single ^{16}O atom was incorporated as a sulfoxide group. Figure 3C shows an ESI-MS analysis of the acyl chains isolated from the reaction performed in the presence of $^{18}O_2$. In this case, the mass of sulfoxystearate was 319.1 amu, corresponding to the incorporation of one ^{18}O atom from $^{18}O_2$.

Reaction with 9-Thiastearoyl-ACP. The multiple-turnover reaction of Δ9D with 9-thiastearoyl-ACP yielded 9-sulfoxystearate (mass of 317.1 amu, Figure 4B), and this product also contained ^{18}O when the reaction was performed in the presence of $^{18}O_2$ (mass of 319.1 amu, Figure 4C; Figure 5A). However, Figure 5A shows that 9-sulfoxystearate accounted for only ∼5−10% of the total products, while an additional product with a mass of 349.1 amu was recovered as the major product of the enzyme reaction (>90%, the peaks with masses of 337.2 and 355.2 amu are the HCl adducts of 9-thiastearate and 9-sulfoxystearate, respectively). The 349.1 amu product was not observed in reaction mixtures lacking Δ9D, and no comparable alternative product was observed when 10-thiastearoyl-ACP was used as the substrate. The use of $^{16}O_2$ did not change the mass of this product. However, Figure 5C shows that when the reaction was performed in a buffer having ∼75% enrichment of $^{18}OH_2$, multiple solvent-derived ^{18}O atoms were incorporated into the majority product.

Figure 6 shows a MALDI-TOF MS analysis of the acyl-ACPs present in the reaction mixture obtained without hydrolysis of the acyl chain from the protein. The substrate 9-thiastearoyl-ACP gave an observed mass of 9443 amu (Figure 6A). Upon reaction with Δ9D, the mass of the acyl-ACP product was observed to decrease by ∼125 amu to 9322 amu (Figure 6B). This change was consistent with loss of the terminal C-9 portion of 9-thiastearoyl-ACP, leaving 8-mercaptooctanoyl-ACP as the major protein-bound product. During the acyl chain hydrolysis and workup required for ESI-MS analysis, the hydrolyzed 8-mercaptooctanoate (**5**) underwent a dimerization reaction to give 7-carboxylheptyl disulfide (**6**, 349.1 amu). The ESI-MS/MS spectra of synthetic **6** and the product isolated from the enzyme reaction were identical and assigned as follows: 349.2 amu for $^{-}OOC(CH_2)_7$-S-S-$(CH_2)_7$-COOH, 331.4 amu for OC═CH-$(CH_2)_6$-S-S-$(CH_2)_7COO^{-}$, 175.2 amu for $^{-}OOC(CH_2)_7$-SH,

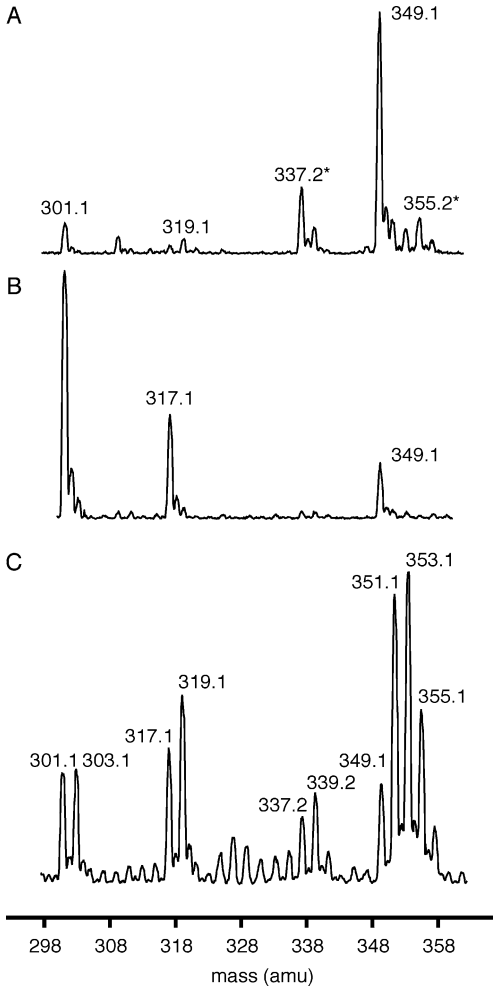


FIGURE 5: Results of an ESI-MS analysis of the majority acyl product isolated from two different reactions of 9-thiastearoyl-ACP. A, multiple turnover reaction in the presence of $^{18}O_2$. The peak with mass of 319.1 amu is 9-sulfoxystearate containing ^{18}O, while the majority product has mass of 349.1 amu. B, single turnover reaction of 9-thiastearoyl-ACP in the presence of Fd-reduced Δ9D and $^{16}O_2$. The peak with mass of 317.1 amu is 9-sulfoxystearate containing ^{16}O. C, majority product of the reaction of 9-thiastearoyl-ACP after recovery by hydrolysis in a buffer containing ~75% enrichment of $^{18}OH_2$.

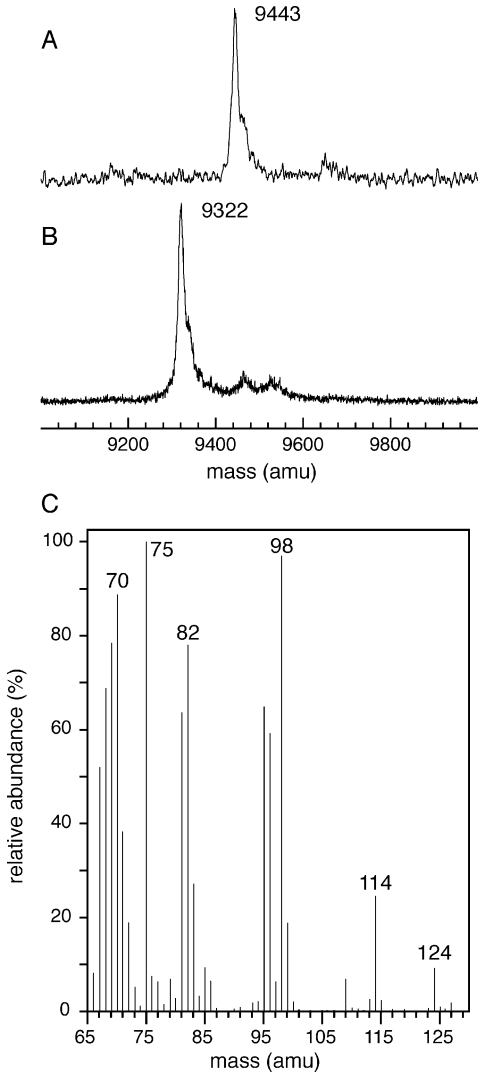


FIGURE 6: Analysis of products from the reaction of 9-thiastearoyl-ACP with Δ9D. A, MALDI-MS analysis of 9-thiastearoyl-ACP, which has an expected mass of 9459 amu and gave an observed mass of 9443 amu. B, the protein-bound product 8-mercaptooctanoyl-ACP has an expected mass of 9332 amu; while the observed mass was 9322 amu. C, GC/EI-MS spectrum of 1-nonanal produced from the proposed acyl chain cleavage of 9-thiastearoyl-ACP between S-9 and C-10.

173.2 amu for $S{=}CH(CH_2)_6COO^-$, and 141.2 amu for $CH_2{=}CH(CH_2)_5COO^-$.

The acyl chain cleavage reaction suggested by panels A and B of Figure 6 must also correspond with the formation of a C-9 fragment. This product was recovered by extraction of the contents of a sealed reaction vial with 100 μL of hexane and analysis of the organic fraction using GC−MS (*11*). Figure 6C shows the ESI-MS/MS spectrum obtained from this product. This fragmentation pattern was indistinguishable from that obtained from an authentic sample of nonanal and published elsewhere (*22*), including our previous studies of the acyl chain cleavage of *O*-9-acyloxy-ACP upon reaction with Δ9D (*11*).[2]

Single-Turnover Reaction of 9-Thiastearoyl-ACP. Recent studies have shown that preincubation of the substrate complex of Δ9D with reduced Fd prior to mixing with aerobic buffer yielded catalytically competent single-turnover production of 18:1-ACP (*10*). Furthermore, by using defined amounts of reduced Fd, the stoichiometry of reaction could be effectively limited to less than one catalytic turnover per Δ9D active site. Figure 5B shows the results of a single-turnover reaction of with 9-thiastearoyl-ACP in the presence of $^{16}O_2$. Under these conditions, the unreacted substrate (301.1 amu) and products from both sulfoxidation (317.1 amu) and acyl chain cleavage and subsequent dimerization (349.1 amu) were recovered from the single-turnover reaction.

Catalytic and Inhibitory Properties of Thiastearoyl-ACPs. A steady-state turnover number of 0.15 ± 0.5 s^{-1} was determined by monitoring the disappearance of 10-thiastearoyl-ACP (100 μM). This rate was ~4-fold slower than that measured for conversion of 18:0-ACP to 18:1-ACP [0.6 s^{-1} (*5*, *10*)]. Preliminary investigations of the inhibition of 18:0-ACP desaturation by 10-thiastearoyl-ACP indicated a

[2] Control reactions showed that nonanal was converted into 1-nonanol by independent action of FdR, Fd, and NADPH during the time period (0.5 to 1 h) required to accumulate sufficient products from the slow reaction of 9-thiastearoyl-ACP. No comparable reduction was observed with acyloxy-ACPs, which reacted on a shorter time scale (*10*).

mixed-type inhibition, with an apparent K_I of ~10 μM. Because of the complexity of this kinetic system, further evaluations were not undertaken. Comparable evaluations indicated that the steady-state turnover rate for 9-thiastearoyl-ACP must be 40−100 fold slower that the conversion of 18:0-ACP to 18:1-ACP, which also precluded a more detailed kinetic analysis. When 18:0-ACP desaturation assays were performed in buffer containing either 8-mercaptooctanoyl-ACP (product of the acyl chain cleavage reaction) or apo-ACP (nonfunctional form of ACP lacking phosphopantetheine), no change in the rate of formation of 18:1-ACP was observed relative to the rates from control experiments lacking these latter two protein derivatives.

DISCUSSION

The reactions of soluble stearoyl-ACP Δ9 desaturase (Δ9D) with 9- and 10-thiastearoyl-ACPs provide a revealing counterpoint to the previously studied reactivity of the integral membrane stearoyl-CoA desaturase with thistearoyl-CoAs. Here we have shown that the major reaction pathway for Δ9D with 9-thiastearoyl-ACP is not sulfoxidation but is instead an acyl chain cleavage between S-9 and C-10. This is the second example of acyl chain cleavage catalyzed by Δ9D when confronted with a heteroatom-substituted acyl chain. The simplest interpretation of these results and the previous studies of reactions with acyloxy-ACPs (*11*) is that the desaturation reaction catalyzed by Δ9D is initiated at the C-10 position. In contrast, no comparable acyl chain cleavage reactions have been reported for the integral membrane stearoyl-CoA Δ9 desaturase, which ostensibly catalyzes the same double bond insertion in a reaction initiated at the C-9 position as evidenced by kinetic isotope effect studies (*8*, *23*).

O Atom Transfer. In previous studies of the reaction of thiastearates with integral membrane desaturases, stereospecific sulfoxides were recovered in high enantiomeric excess (*13*). This result strongly argues for a role of the enzyme active site in the reaction. However, since the origin of the incorporated O atom was not determined, an important aspect of the reaction chemistry was left unresolved. For example, O atom transfer from the diiron oxidant or attack of a water molecule on a cationic intermediate within a stereospecific confinement of the enzyme active site could lead to the observed result. In this context, the first "diverted" monooxygenase reaction reported for the diiron enzyme ribonucleotide reductase (*24*) was later shown to be derived from solvent quenching of a putative Tyr208 cation radical (*25*).

Experiments presented in this work (Table 1) establish that sulfoxidation of both 9- and 10-thiastearoyl-ACP can be catalyzed by Δ9D. Furthermore, isotopic labeling studies show that the O atom in the sulfoxide functional group arises exclusively from O_2, implicating the role of an activated complex derived from O_2 and the diiron center in catalysis. O atom transfer has been suggested in reactions of chiral fluorinated stearates with Δ9D (*7*), and the results presented here confirm the ability of Δ9D to perform O atom transfer chemistry, *in the presence of the appropriate substrate.* Moreover, the observed O atom transfer supports mechanistic linkages between the different catalytic classes of diiron enzymes: acyl-ACP desaturases; bacterial hydroxylases, including methane monooxygenase and toluene 4-monooxygenase; and ribonucleotide reductase (*2*, *24*−*29*). Figure 7 shows a schematic representation of diiron intermediates that may be involved in the reactions of these enzymes. Although it is not possible to ascertain the nature of the diiron intermediate(s) used by Δ9D from the experiments presented here, either a peroxo or diferryl intermediate is reasonable (*30*−*32*).

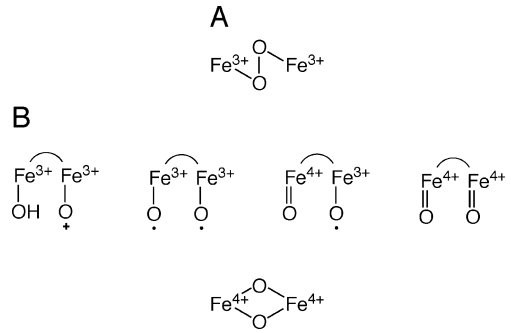


FIGURE 7: Plausible diiron intermediates for reactions observed from Δ9D with thiastearoyl-ACPs. A, μ-1,2 peroxodiiron(III). B, alternative representations of a high-valent intermediate obtained by breakage of the O−O bond in A.

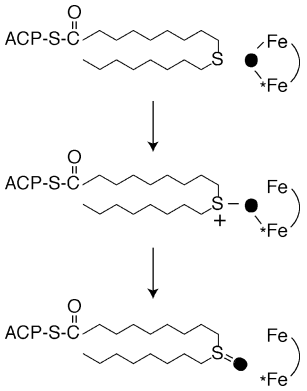


FIGURE 8: Minimal mechanism for sulfoxidation of 10-thiastearoyl-ACP by Δ9D. The minor product, 9-sulfoxystearoyl-ACP, may arise from a similar reaction involving the 9-position.

The single-turnover results of Figure 5C indicate that both sulfoxidation and acyl chain cleavage can occur as the result of a single O_2 activation event. Thus, it is likely that these two reactions arise as the result of a partition between alternative catalytic outcomes of the thiastearoyl group bound within the enzyme active site.

Positional Specificity of the Thiastearoyl-ACP Reaction. Reactions of Δ9D with *O*-10-acyloxy-ACP yielded 9-hydroxynonanoyl-ACP and octanal, corresponding to an alternative binding of the acyloxy chain prior to an acyl chain cleavage reaction between the O-10 and C-11 positions (*11*). Upon the basis of these experiments, register shift was defined as a mechanistically relevant misalignment of acyl chain binding that resulted in Δ9D reactions at positions other than the 9 and 10 positions of the acyl chain. For the *O*-10-acyloxy analogue, the shift was required because an O-10 substitution would not be susceptible to a proposed oxidative attack initiated at the 10 position.

Unlike the acyloxy analogues, the thiastearoyl analogues are susceptible to oxidative attack without register shift, and thus provide an alternative way of probing position-specific reaction outcomes. Figure 8 shows a minimal mechanism for the reaction of Δ9D with 10-thiastearoyl-ACP leading

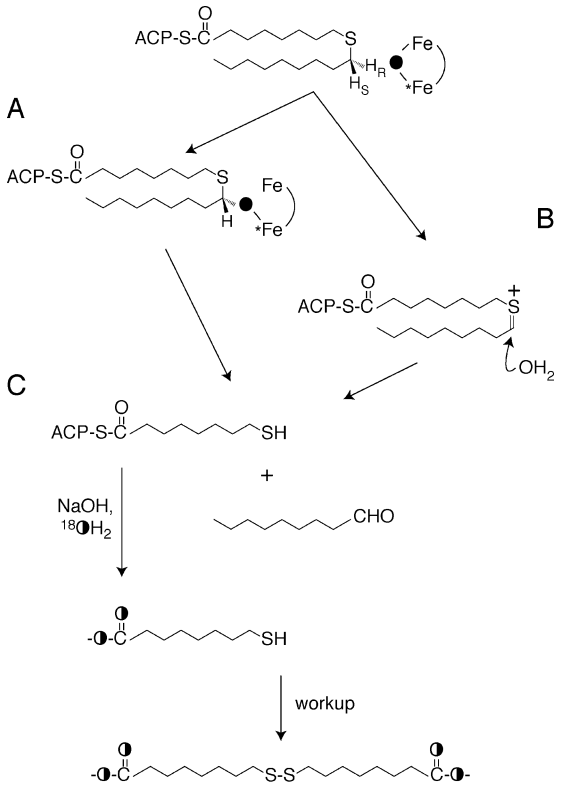


FIGURE 9: Alternative mechanisms leading to the Δ9D-catalyzed cleavage of 9-thiastearoyl-ACP. A, hydroxylation and rearrangement of a hemiacetal. B, $2e^-$ oxidation to a sulfenium cation intermediate followed by addition of water and rearrangement. C, hydrolysis of 8-mercaptooctanoyl-ACP in $^{18}OH_2$-enriched buffer resulting in partial labeling of the carboxyl group, which corresponds to the origin of the heterogeneous isotopic incorporation observed in **6** (Figure 5C).

to S-10 sulfoxidation. Since $2e^-$ oxidation required from the reaction stoichiometry can be obtained from S-10 without the involvement of any other acyl chain position, register shift was not necessary and diverted oxidations at either C-9 or C-11 did not occur. Oxidation at the S-9 position by a mechanism analogous to that shown in Figure 8 would yield the minority sulfoxide product observed from the reaction with 9-thiastearoyl-ACP, while a reaction involving the C-10 position will be required to achieve the observed acyl chain cleavage.

Figure 9 shows two alternative mechanisms leading to the acyl chain cleavage observed with 9-thiastearoyl-ACP. Figure 9A suggests that the reaction may begin at the C-10 position, as we have proposed for the acyloxy-ACPs and, by analogy, for the saturated fatty acids. Hydroxylation at C-10 would generate an unstable adduct capable of rearranging to the observed products 8-mercaptooctanoyl-ACP and nonanal. Although O atom transfer reactions are not observed from Δ9D with natural, saturated fatty acids, alterations in either the reactivity or stability of substrate intermediates may arise from sulfur or other heteroatom substitutions. This may possibly facilitate an O atom transfer. The O atom transfer suggested in Figure 9A could reasonably arise from the activation of O_2 by a diiron center, but it is noted that this suggestion cannot be ascertained due to exchange of the aldehyde in solution.

Figure 9B shows an alternative reaction sequence at C-10 that could also give rise to an acyl chain cleavage. In this reaction, the removal of $2e^-$ required by the desaturase reaction stoichiometry would be satisfied by a reaction initiated and completed at C-10. The presence of the adjacent S-9 atom would facilitate the oxidation, and may give rise to a relatively stabilized sulfenium cation intermediate bound within the active site. In this case, an active site water molecule or an iron-bound water or hydroxide may be able to react with this unusual double bond. This type of addition reaction has been proposed for the reaction of a sulfenium cation with acetate anion during the Pummerer rearrangement (*33*). Ultimately, the addition reaction proposed in Figure 9B would also result in rearrangement and cleavage of the 9-thiastearoyl-ACP substrate and formation of the observed products. As indicated in Figure 9C, partial isotopic incorporation into the 8-mercaptooctanoate product would be expected upon hydrolysis in buffers enriched with $^{18}OH_2$, giving rise to the heterogeneous distribution of isotopic label observed in the dimerized product (Figure 5C). Conversely, isotopic incorporation from $^{18}O_2$ would not be expected for products derived from this reaction sequence, as was also observed.

Inhibition of Δ9D by Thiastearoyl-ACPs. The relatively favorable rate of reaction with 10-thiastearoyl-ACP ($k_{cat} \approx 0.15\ s^{-1}$) and the appearance of a single reaction product suggest that this substrate may overall reproduce the energetic inputs required for Δ9D catalysis, including a slow k_{off} for the enzyme−substrate complex (*34*), sufficient hydrophobic binding energy to promote catalysis (*5*), and a sufficiently high rate of product release to allow the continued function of the catalytic cycle (*10*). In contrast, one or more of these inputs must be altered during the reaction with 9-thiastearoyl-ACP, which leads to a considerably slower reaction and two distinct product formation pathways. The predominant acyl chain cleavage reaction described here most likely occurs within the active site. Consequently, inhibition may arise from coordination of the nascent thiol group to an iron atom of the diiron center or from deposition of nonanal in the interior reaches of the substrate channel. Without a linkage to the outside solvent provided by the ACP and phosphopantetheine moieties of the natural substrate, this internal occupation of the active site could give rise to an unusually stable product-bound state. In any case, the position-specific introduction of a heteroatom into the active site has given further insight into the distinct reactivities of the soluble and integral membrane classes of desaturases.

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