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Alkyldihydroxyacetonephosphate Synthase Mechanism: ^{18}O Studies of Fatty Acid Release from Acyldihydroxyacetone Phosphate†

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ABSTRACT: Alkyldihydroxyacetonephosphate synthase (alkyl-DHAP synthase) catalyzes the exchange of the ester-linked fatty acid of 1-*O*-acyldihydroxyacetone phosphate (1-*O*-acyl-DHAP) for a fatty alcohol that is attached in an ether linkage to form 1-*O*-alkyldihydroxyacetone phosphate (1-*O*-alkyl-DHAP). In our continuing investigation of the mechanism of this enzyme, we have examined the fatty acid released during the reaction. In contrast to the reports of others using whole microsomes, we found that the cleavage of fatty acid by purified preparations of alkyl-DHAP synthase was dependent on the presence of the cosubstrate, fatty alcohol. Furthermore, the amount of fatty acid produced was equivalent to the alkyl-DHAP formed. Our previously proposed detailed mechanism for alkyl-DHAP synthase predicted that the fatty acid should retain both of the carboxyl ester oxygens upon cleavage. Reactions carried out with palmitoyl-[^{18}O]DHAP as substrate yielded [^{18}O]palmitic acid as the product in agreement with this scheme.

Alkyldihydroxyacetonephosphate synthase (alkyl-DHAP synthase)[1] catalyzes the formation of the ether bond found in alkyl and alk-1-enyl glycerolipids. In this reaction, the fatty acid ester of acyl-DHAP is cleaved and replaced with a fatty alcohol in an ether linkage (Hajra, 1970; Wykle et al., 1972). Concurrently, the *pro-R* hydrogen at the carbon of DHAP esterified to the fatty acid (DHAP C-1) is exchanged with the medium (Friedberg et al., 1971, 1972). Another feature of the reaction is the donation of the oxygen in the ether linkage by the fatty alcohol (Snyder et al., 1970); both oxygens in the fatty acyl ester of acyl-DHAP are lost during the formation

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[1] Abbreviations: DHAP, dihydroxyacetone phosphate; alkyl-DHAP synthase, alkyldihydroxyacetonephosphate synthase; alkyl-DHAP, alkyldihydroxyacetone phosphate; acyl-DHAP, acyldihydroxyacetone phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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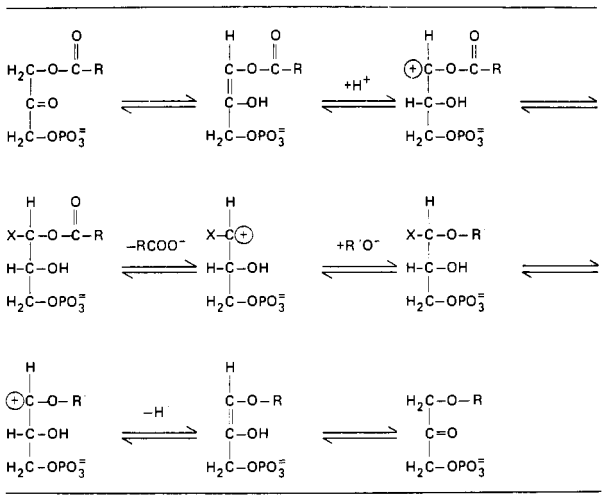

FIGURE 1: Previously proposed molecular reaction mechanism for the conversion of acyl-DHAP and fatty alcohols to alkyl-DHAP. R′ can only be an alkyl moiety and can never be replaced by an H; X designates the enzyme. See Brown & Snyder (1983) for additional details.

of alkyl-DHAP. Experimental documentation of the nature of this latter reaction is the subject of this report.

Two detailed molecular mechanisms have been proposed for the alkyl-DHAP synthase reaction. The first, that of Friedberg and co-workers, is a sequential mechanism in which both acyl-DHAP and fatty alcohol bind to the enzyme to form a ternary complex (Friedberg et al., 1980). The second, based on kinetic evidence and proposed by us (Brown & Snyder, 1982) and others (Davis & Hajra, 1977), is a ping-pong mechanism in which the enzyme first binds acyl-DHAP and subsequently cleaves the fatty acyl ester to produce an enzyme–substrate intermediate. This complex then reacts with the fatty alcohol to form alkyl-DHAP [see Figure 1 for the molecular mechanism of the alkyl-DHAP-catalyzed reaction, previously proposed by us (Brown & Snyder, 1983)]. Both the ping-pong and sequential mechanisms require that the two oxygens in the ester bond be retained by the fatty acid product, but no supportive evidence was available at the time the schemes were presented.

Addressing this question, Friedberg and co-workers synthesized [1-^{18}O]palmitoyl-DHAP to follow the fate of the carbinol oxygen of the ester linkage during alkyl-DHAP formation by Ehrlich ascites cell microsomes (Friedberg et al., 1983). They found that [^{18}O]palmitic acid was produced under conditions favoring alkyl-DHAP synthesis, whereas only [^{16}O]palmitic acid was recovered when Mg^{2+} or NADPH, each an inhibitor of alkyl-DHAP formation, was included in the reaction mixture. However, the production of [^{18}O]palmitic acid was not decreased by the absence of fatty alcohol. The latter observation was attributed to the previously proposed ability of alkyl-DHAP synthase to catalyze the hydrolysis of acyl-DHAP to DHAP, with water substituting for fatty alcohol as the cosubstrate (Friedberg & Gomillion, 1981). Evidence for this proposal was based on the exchange of the C-1 *pro-R* hydrogen of acyl-DHAP during this hydrolysis. We have subsequently demonstrated that this unusual hydrolase reaction is lost during solubilization and purification of alkyl-DHAP synthase (Brown & Snyder, 1983). It may be this microsomal hydrolase that is responsible for formation of [^{18}O]palmitic acid in the absence of fatty alcohol. Also, [^{18}O]palmitic acid may have been produced by the acyl exchange reaction catalyzed by alkyl-DHAP synthase in which the acyl group of acyl-DHAP is exchanged with free fatty acids in the reaction medium (Brown & Snyder, 1982; Davis & Hajra, 1977). Microsomes contain sufficient free fatty acids to allow significant acyl exchange.

In this paper, we have used a purified preparation of alkyl-DHAP synthase and short incubation times to compare the rate of fatty acid release from palmitoyl-DHAP ([9,10-^{3}H]palmitoyl or [1(*R*)-^{3}H]DHAP) to the C-1 *pro-R* hydrogen exchange and alkyl-DHAP formation. We have also synthesized palmitoyl-[^{18}O]DHAP to confirm our proposal that the fatty acid released by alkyl-DHAP synthase retains both oxygens of the acyl ester of the substrate.

Materials and Methods

Glycolic acid, chloroacetic acid, and alkaline phosphatase were purchased from Sigma Chemical Co.; [1-^{14}C]palmitic acid and [9,10-^{3}H]palmitic acid were from New England Nuclear; [^{18}O]water was from Merck; methoxylamine hydrochloride and *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane were from Pierce Chemicals; and oxalyl chloride was from Aldrich.

Enzyme. Alkyl-DHAP synthase from Ehrlich ascities cells was solubilized with Triton X-100, precipitated with acetone, and purified by chromatography on DEAE-cellulose, QAE-Sephadex, and Matrex Red as described previously (Brown & Snyder, 1982) followed by hydroxylapatite chromatography (Brown & Snyder, 1983) to yield three peaks of enzyme activity. The second peak of activity from the hydroxylapatite column with a specific activity of 65 nmol/(min·mg) (500-fold purification from microsomes) was used in these experiments. Alkyl-DHAP synthase was assayed during purification by the DEAE-paper disk method (Brown & Snyder, 1982), and products were confirmed as described before (Brown & Snyder, 1983).

Substrates. Unlabeled palmitoyl-DHAP and [9′,10′-^{3}H]-acyl-DHAP were synthesized by the method of Davis & Hajra (1979). Preparation of the latter substrate began with conversion of [9,10-^{3}H]palmitic acid to the acid chloride by refluxing with a 4-fold excess of oxalyl chloride in benzene for 1 h.

Palmitoyl-[1(*R*)-^{3}H]DHAP was synthesized as previously reported (Brown & Snyder, 1983). Palmitoyl-[^{18}O]DHAP was prepared by the method of Davis & Hajra (1979) starting with [2-^{18}O]glycolic acid obtained by heating 1 mmol of chloroacetic acid and 2 mmol of NaOH in 1 mL of $H_2{}^{18}O$ for 2 h at 100 °C. In addition, [9,10-^{3}H]palmitic acid at 12.5 Ci/mol was used as the acyl chain of palmitoyl-[^{18}O]DHAP. The final product was purified by thin-layer chromatography and analyzed as outlined by Friedberg et al. (1983). For product analysis, the palmitoyl-[^{18}O]DHAP (0.5 mg) was sonicated into 1 mL of 100 mM Tris-HCl, pH 8.5, and incubated for 2 h at 37 °C following addition of 50 units of alkaline phosphatase. The resulting palmitoyldihydroxyacetone (palmitoyl-DHA) was purified by thin-layer chromatography with diethyl ether as the developing solvent and treated with methoxylamine hydrochloride and then with *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane to form the trimethylsilyl (Me_3Si) *O*-methyloxime derivative (Friedberg et al., 1983).

The amount of ^{18}O in the palmitoyl-DHAP was determined by mass spectrometry. The Me_3Si *O*-methyloxime derivative was introduced into a Kratos MS25 mass spectrometer on the direct insertion probe. Figure 2 shows the mass spectra of the natural DHAP (top) and the ^{18}O-enriched sample (bottom). The relative intensities of the m/z 191 and 193 ions in the enriched sample were used to calculate the percent enrichment in ^{18}O. The amount of palmitoyl-DHAP enriched in ^{18}O was

Table I: Comparison of Experimental and Theoretical Mass Values for Ions Used To Determine Site of ^{18}O Incorporation

formula	exptl	theoretical
$C_{23}H_{47}NO_3{}^{18}OSi$	431.3314	431.3316
$C_{23}H_{47}NO_4Si$	429.3282	429.3274
$C_{16}H_{31}O$	239.2375	239.2375
$C_7H_{15}NO_2Si$	173.0874	173.0872
$C_4H_{11}OSi$	103.0579	103.0579
$C_4H_7NO^{18}O$	103.0514	103.0519
$C_4H_7NO_2$	101.0468	101.0477

determined to be 80.5%. This number was confirmed with high-resolution mass measurements on an AEI MS50 mass spectrometer.

High-resolution measurements were taken to confirm the site of the ^{18}O in the enriched sample. Four fragment ions can be used to deduce the position of the ^{18}O atom. Table I lists the experimental and theoretical mass measurements for these ions and the molecular ions. The ion at m/z 239 is the acylium ion resulting from cleavage between the DHAP C-1 oxygen and carbonyl carbon. Since it is not shifted up in mass in the lower spectrum in Figure 2, the carbonyl oxygen can be ruled out as being the ^{18}O. The ion at m/z 173 results from a hydrogen transfer and cleavage between DHAP C-1 and its oxygen. Again this ion does not shift in the lower spectrum, indicating that the ^{18}O is neither the DHAP C-3 oxygen nor the *O*-methyloxime oxygen. Further evidence that the DHAP C-3 oxygen is not ^{18}O comes from the m/z 103 ion, which is the Me_3Si group plus the DHAP C-3 carbon and oxygen. This ion does not shift to m/z 105 in the lower spectrum. The ion at m/z 101 in the top spectrum does shift up 2 mass units to m/z 103 in the bottom spectrum. The m/z 101 ion results from loss of the carbonyl group from the DHAP C-1 end of the molecule and loss of *O*-Me_3Si from the DHAP C-3 end. Thus it contains the DHAP C-1 oxygen and the *O*-methyloxime oxygen. High-resolution measurements show that the lower spectrum in Figure 2 contains two ionic species at m/z 103, the ^{18}O analogue of the m/z 101 ion and the m/z 103 ion discussed above. Since it has already been concluded that the *O*-methyloxime oxygen was not the ^{18}O, the DHAP C-1 oxygen must be where the ^{18}O is located in the m/z 103 ion that shifted from m/z 101, thus confirming the position of the ^{18}O.

[1-^{14}C]Hexadecanol was prepared by reduction of [1-^{14}C]palmitic acid (Snyder et al., 1971).

Incubations. Stoichiometry experments were carried out under conditions of linear time and protein concentration and of saturating substrate concentrations. The incubations consisted of 50 μL of purified enzyme in 200 mM potassium phosphate, pH 7.4, 20% ethylene glycol, 0.2% Triton X-100, and 1 mM dithiothreitol and 50 μL of 250 mM sucrose, 100 mM Tris-HCl, pH 7.4, 100 mM NaF, and 100 mM KCl containing 12 nmol of palmitoyl-DHAP (a mixture of [9′,10′-^{3}H]palmitoyl-DHAP and palmitoyl-[1(*R*)-^{3}H]DHAP at final specific activities of 70 and 35 Ci/mol, respectively) and 10 nmol of [1-^{14}C]hexadecanol (52 Ci/mol). Reactions were initiated by adding the enzyme to the sonicated substrate mixture and stopped after 30 min at 37 °C by adding 0.9 mL of 0.001 N HCl; 0.5 mL of this was applied to a 1-mL column of Dowex 50×8, which was then washed with 1.5 mL of water. The lipid substrates and products adsorbed to the resin while the ^3H_2O passed through (Brown & Snyder, 1983). The remainder of the reaction was extracted by the method of Bligh & Dyer (1959) with 1 N HCl in the aqueous phase. Half of the extract was chromatographed on silica gel G thin-layer plates developed in diethyl ether/hexane/acetic acid (50:50:1 v/v), and the fatty acid band, visualized by iodine staining of the oleic acid internal standard, was scraped and counted. The remaining extract was chromatographed on silica gel HR plates developed in chloroform/methanol/acetic acid (90:10:10 v/v), and the [^{14}C]hexadecyl-DHAP was measured by scraping the acyl-DHAP/alkyl-DHAP band and counting under conditions that eliminated ^{3}H spillover into the ^{14}C channel (56% efficiency).

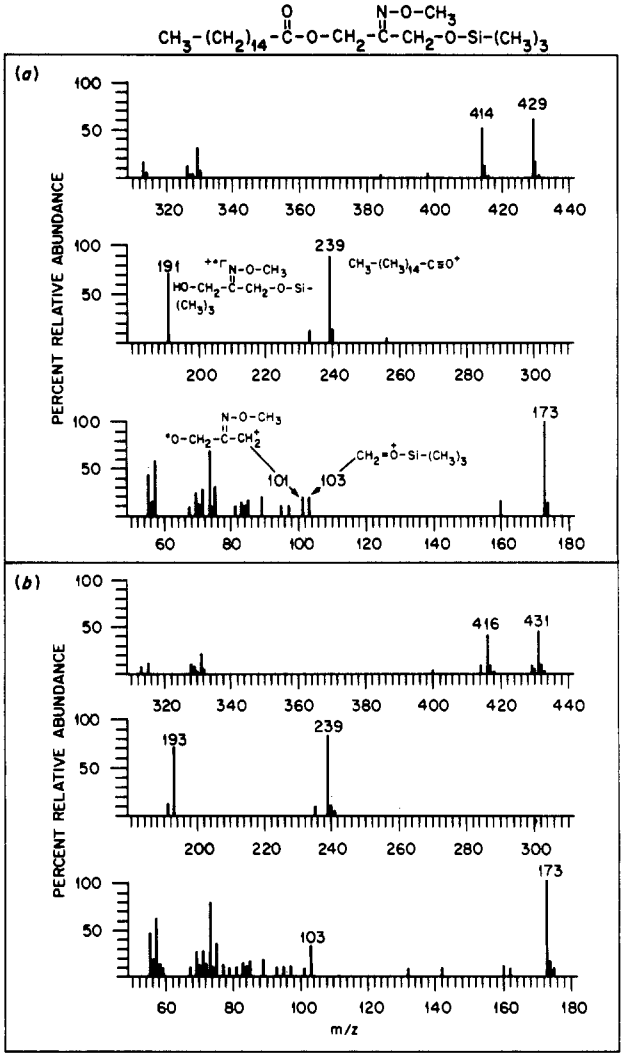

FIGURE 2: Mass spectra of Me_3Si *O*-methyloxime derivatives of natural (top) and ^{18}O-enriched (bottom) palmitoyl-DHAP. Natural and ^{18}O-enriched palmitoyl-DHAP were synthesized, derivatized, and analyzed by GC/MS as outlined under Materials and Methods. ^{18}O was found to be exclusively in the DHAP C-1 of the palmitoyl-DHAP at an abundance of 80.5%.

Experiments to determine the site of cleavage of palmitoyl-DHAP by using the ^{18}O-labeled substrate were done as follows. The incubations contained 250 μL of purified enzyme in 200 mM potassium phosphate, pH 7.4, 20% ethylene glycol, 0.2% Triton X-100, and 1 mM dithiothreitol and 250 μL of substrate mixture consisting of 60 nmol of [9′,10′-^{3}H]palmitoyl-[^{18}O]DHAP and 75 nmol of [1-^{14}C]hexadecanol sonicated into 250 mM sucrose, 100 mM KCl, 100 mM NaF, and 100 mM Tris-HCl, pH 7.4. Reactions were initiated by addition of enzyme, incubated at 37 °C for 30 min, and stopped by addition of 0.5 mL of 0.1 N HCl. The acidified reactions were then extracted by the method of Bligh & Dyer (1959), the chloroform extracts were dried, and aliquots were chromatographed on thin-layer plates as described above for quantitation of [^{3}H]palmitic acid and [^{14}C]hexadecyl-DHAP.

Table II: Stoichiometry of Products of Alkyl-DHAP Synthase Reaction

enzyme	[^{14}C]hexadecanol	^3H_2O (nmol)		[^{3}H]palmitic acid (nmol)		[^{14}C]hexadecyl-DHAP (nmol)	
		total[a]	net[b]	total[a]	net[b]	total[a]	net[b]
−	−	0.44 ± 0.01		0.10 ± 0.01			
+	−	1.04 ± 0.04	0.60	0.36 ± 0.01	0.26		
−	+	0.42 ± 0.02		0.10 ± 0.01		0.01	
+	+	2.60 ± 0.02	2.18	2.06 ± 0.05	1.96	2.03 ± 0.15	2.02

[a] Total reaction products. [b] Net enzymatically produced products.

The remaining extract was dissolved in 3 mL of hexane, 3 mL of methanol/0.1 N HCl (1:1) was added, and the fatty acid fraction was extracted into the hexane phase. The aqueous methanol phase was reextracted twice with hexane, and the extracts were pooled, dried, and taken up in 3 mL of hexane. Three milliliters of methanol/0.001 N NaOH was added, and the extraction with hexane was repeated to remove the hexadecanol. Finally, the aqueous methanol phase was acidified with 1 N HCl and extracted 3 times with hexane to recover the fatty acid. After methylation with diazomethane, recoveries wre calculated by measuring methyl [^{3}H]palmitate, and the samples were analyzed by gas chromatography (GC) and mass spectrometry (MS) to determine the quantity of [^{18}O]palmitic acid produced in the incubations.

Quantitation was done with ethyl palmitate as an internal standard. A ratio of the peak heights of the ions resulting from the McLafferty rearrangement, m/z 74 and 88, was used (peak areas gave only minimal improvement in precision). The samples were spiked with an amount of ethyl palmitate close to the expected yield of methyl palmitate. Correction for differences in response of the methyl and ethyl palmitates was made by using a correction factor determined by running samples of prepared ratios of the two palmitates.

The spiked samples were run on a MS25/DS55 GC/MS system. A 25-m Hewlett-Packard OV-101 fused-silica capillary column with a flow rate of 2 mL/min of helium was used for the GC separation. The temperature program for the column was as follows: 30 °C for 1200 s (to allow the solvent to pass), 140 °C for 600 s (to pass impurities, etc.), and then a ramp of 6 °C/min to a final temperature of 280 °C, during which the palmitates eluted. The injector, transfer lines, and mass spectrometer interface were all held at 220 °C. The mass spectra were obtained under electron ionization conditions at a scan rate of 1 s/decade. The spectrometer operating conditions were as follows: source temperature, 180 °C; electron energy, 70 eV; trap current, 100 μA; accelerating voltage, 2000 V; source pressure, 2×10^{-5} torr.

Results and Discussion

Stoichiometry of Fatty Acid Cleavage. In response to reports by Friedberg and co-workers that alkyl-DHAP synthase can catalyze the cleavage of fatty acid from acyl-DHAP in the absence of fatty alcohol, we compared the production of fatty acid to the exchange of the DHAP C-1 *pro-R* hydrogen and the formation of alkyl-DHAP. For these experiments, we used purified (500-fold) alkyl-DHAP synthase, [9′,10′-^{3}H]palmitoyl-DHAP, palmitoyl-[1(R)-^{3}H]DHAP, and [1-^{14}C]hexadecanol. Incubations were limited to 30 min at 37 °C, conditions under which product formation was linear with time and exchange of the acyl ester of the palmitoyl-DHAP with the free fatty acid that was produced in the reaction was minimal. Table II shows the amounts of ^3H_2O [from exchange of the 1(R)-^{3}H], [^{3}H]palmitic acid, and [^{14}C]hexadecyl-DHAP produced under these conditions. In the presence of hexadecanol, these products were made in equivalent amounts. When hexadecanol was omitted, [^{3}H]palmitic acid and ^3H_2O formation decreased 87% and 72%, respectively. Inhibition of alkyl-DHAP synthase with 1 mM dinitrofluorobenzene or 1 mM 5,5′-dithiobis(2-nitrobenzoate) inhibited the formation of all three products completely (data not shown). Thus, with purified alkyl-DHAP synthase, it is clear that cleavage of fatty acid from acyl-DHAP requires fatty alcohol.

Table III: Fatty Acid Products of Alkyl-DHAP Synthase Reaction

product	−hexadecanol	+hexadecanol
[^{14}C]hexadecyl-DHAP[a] (nmol)		10.55 ± 0.11
[^{3}H]palmitic acid[a] (nmol)	0.87 ± 0.02	12.00 ± 0.12
methyl [^{18}O]palmitate[b] (nmol)	0.62 ± 0.26	7.49 ± 1.35

[a] Net enzymatically formed product quantitated by thin-layer chromatography as described under Materials and Methods [b] Net enzymatically produced [^{18}O]palmitate after correction for recovery during workup and for ^{18}O abundance in substrate.

Mode of Fatty Acid Cleavage. The proposed molecular mechanisms for alkyl-DHAP synthase predict that the fatty acid ester of acyl-DHAP is cleaved between the carbinol oxygen and DHAP C-1. This unusual cleavage can be demonstrated by substituting with ^{18}O in the carbinol oxygen and analyzing the fatty acid produced in the reaction by mass spectrometry. We have synthesized palmitoyl-[^{18}O]DHAP, and analysis of the trimethylsilyl *O*-methyloxime derivative of the palmitoyl-DHA produced by alkaline phosphatase confirmed the identity of the product and indicated an ^{18}O content of 80.5% (Figure 2). Cleavage of this substrate between the carbinol oxygen and the carbonyl carbon of the acyl group would yield palmitic acid containing only ^{16}O, whereas cleavage between DHAP C-1 and the carbinol oxygen would yield palmitic acid with one ^{18}O and one ^{16}O. Incubations with [9′,10′-^{3}H]palmitoyl-[^{18}O]DHAP (specific activity of ^{3}H = 12.5 Ci/mol) were carried out as for the stoichiometry experiments but scaled up 5-fold to obtain sufficient material for analysis. Aliquots were taken after the initial extractions to quantitate [^{3}H]palmitic acid and [^{14}C]hexadecyl-DHAP as before. As seen in Table III, the formation of these two products was again equivalent when hexadecanol was present, and the palmitic acid cleavage was relatively small in the absence of hexadecanol. The palmitic acid was purified as described under Materials and Methods and methylated for analysis by gas chromatography/mass spectrometry. The label in the methyl palmitate was used to calculate recoveries during the workup procedure.

The ^{18}O content of the methyl palmitate recovered from the incubations was based on the relative intensities of the ions at m/z 74 and 76 resulting from the McLafferty rearrangements of methyl palmitate containing only ^{16}O and methyl palmitate with one ^{16}O and one ^{18}O, respectively. The amounts of methyl [^{18}O]palmitate produced in the presence and absence of hexadecanol are shown in Table III. The 7.49 nmol of methyl [^{18}O]palmitate found in samples from incubations containing hexadecanol is 71% of that expected on the basis of the quantity of hexadecyl-DHAP produced; this somewhat less than stoichiometric amount may be due to exchange of the carboxyl oxygens during the workup. The data indicate,

though, that cleavage of the fatty acid ester by alkyl-DHAP synthase is between DHAP C-1 and the carbinol oxygen. The small amount of enzymatically produced palmitic acid, mostly ^{18}O, produced in the absence of fatty alcohol was probably due to the acyl exchange reaction that is catalyzed by alkyl-DHAP synthase in the absence of fatty alcohol and presence of fatty acid (Brown & Snyder, 1982; Davis & Hajra, 1977). Our purified preparation probably contained trace amounts of free fatty acid, and some was formed chemically from palmitoyl-DHAP during the incubation (see Table II), but at the short reaction time, acyl exchange was minimal relative to the alkyl-DHAP formation. On the other hand, the whole microsomes used by Friedberg et al. (1983) contained considerable free fatty acid (80 nmol of palmitic acid alone per 5 mL of reaction mixture containing 1.5 mg of microsomal protein), and during the 2-h incubation a large amount of acyl exchange would have been expected and could have accounted for the [^{18}O]palmitic acid produced in the absence of hexadecanol. Another possibility for the formation of [^{18}O]palmitic acid observed by Friedberg et al. (1983) is the hydrolase we have observed in whole microsomes that cleaves palmitoyl-[1-(*R*)-^{3}H]DHAP to DHAP that has lost the 1(*R*)-^{3}H. This hydrolase may also cleave palmitoyl-DHAP between the carbinol oxygen and DHAP C-1; however, this hydrolase activity is removed when alkyl-DHAP synthase is purified (Brown & Snyder, 1983).

The data in this paper further support our previous studies of the reaction mechanism of alkyl-DHAP synthase. The reaction starts with binding of acyl-DHAP to the enzyme followed by the release of the fatty acid, which retains both ester oxygens, and exchange of the *pro-R* hydrogen of C-1 of the DHAP portion of the molecule. The resulting complex reacts with fatty alcohol to produce alkyl-DHAP or with fatty acid to regenerate acyl-DHAP (acyl exchange reaction). In addition, it seems clear from our data presented here and previously that the complex reacts much more slowly with water to form DHAP. However, the existence of a similar enzyme in Ehrlich ascites cell microsomes that produce DHAP by this mechanism cannot be ruled out. Such an enzyme would be of great interest with respect to the evolutionary origin of alkyl-DHAP synthase and ether lipids.

Registry No. Palmitoyl-DHAP, 17378-38-0; hexadecyl-DHAP, 32113-53-4; alkyl-DHAP synthase, 64060-42-0; $CH_3(CH_2)_5OH$, 36653-82-4; H_2, 1333-74-0; palmitic acid, 57-10-3.

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