ACYL DIHYDROXYACETONE PHOSPHATE: PRECURSOR OF ALKYL ETHERS

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Summary: 1-0-Alkyl dihydroxyacetone phosphate is biosynthesized in guinea pig liver mitochondria and in rat brain microsomes by direct reaction of a long chain alcohol and acyl dihydroxyacetone phosphate. ATP and Mg^{++} are stimulatory but coenzyme A is not necessary for the reaction.

1-0-Alkyl dihydroxyacetone phosphate (alkyl DHAP)[1] was shown to be biosynthesized from long chain alcohol and DHAP with ATP, CoA, Mg^{++} and NaF as necessary cofactors (1,2). The requirement of CoA was surprising because the formation of a thioester intermediate could not easily be envisioned. Various preincubation and kinetic studies indicated that an intermediate containing DHAP was a precursor of alkyl DHAP (3). In this report evidence is presented that acyl DHAP, a lipid known to be formed in the same preparations that can form alkyl ethers, reacts with the long chain alcohol to form alkyl DHAP. CoA is needed only for the formation of acyl DHAP from endogenous fatty acid (4). Formation of acyl DHAP in the alkyl DHAP synthesizing enzyme systems has already been reported (1).

MATERIALS AND METHODS

1-C^{14}-Hexadecanol was prepared by reducing the methyl ester of 1-C^{14}-palmitate by RED-Al [sodium bis (2-methoxy ethoxy)-aluminum hydride, Aldrich, Milwaukee, Wisc.]. ^{32}P-Labeled palmitoyl DHAP was prepared biosynthetically by incubating palmitate and ^{32}P-DHAP with guinea pig liver mitochondria and necessary cofactors (4). The labeled lipid was purified by chromatography on silicic acid. ^{32}P-Acyl GP was prepared by reducing ^{32}P-acyl DHAP with $NaBH_4$

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[1]Abbreviations used: DHAP - dihydroxyacetone phosphate; GP - glycerol-3-phosphate.

in ethanol. Unlabeled palmitoyl DHAP was chemically synthesized (5). 1-O-Hexadecoxy DHAP was chemically synthesized from chimyl alcohol as outlined below. Chimyl alcohol is oxidized by a mixture of $NaIO_4$ and $KMnO_4$ (6) to 2-hexadecoxy glycolic acid which is converted to 1-hexadecoxy diazoacetone by condensing the acid chloride with diazomethane (5). Hexadecoxy DHAP was obtained by decomposing the diazo compound with H_3PO_4 (5).

Guinea pig liver mitochondria were frozen and thawed and the particulate residue was washed with 0.25 M sucrose. Young rat (18 days old) brain microsomal fraction was prepared by differential centrifugation (18,000xg - 100,000xg, 60 min) and was washed once with 0.25 M sucrose. The lipid emulsions (long chain alcohol, acyl DHAP, etc.) were made by drying down the lipids and emulsifier (Triton-X-100) together and then dispersing the dried residue in dilute (0.05 M) Tris buffer, and incubating as described in Table 1. The incubation was stopped by adding 4.5 ml of $CHCl_3$-methanol (1:2) then 1.5 ml of 2 M KCl containing H_3PO_4 (0.2 M) and 1.5 ml of additional $CHCl_3$ (7,8) were added. After mixing and centrifuging, an aliquot of the lower layer was dried and the radioactivity in the ^{32}P-labeled alkali-stable lipid was determined as described previously (1). When ^{14}C-labeled hexadecanol was used as the precursor, the ^{14}C-labeled alkyl DHAP was separated from the radioactive precursors and other products by column and thin layer chromatography. After alkaline methanolysis (1), the lipid was put on a 6 cm x 0.5 cm silicic acid column (Unisil, Clarkson Chemical Co., Pa.) with 0.1 - 0.2 mg of carrier non-radioactive synthetic alkyl DHAP. The neutral lipids were eluted with 10 ml of $CHCl_3$ and the alkyl DHAP was eluted out with 10 ml of $CHCl_3$-methanol (4:1). The second fraction was dried and an aliquot was applied to a commercial, precoated, thin layer plate (E. Merck, Darmstadt, obtained through Brinkmann, N.Y.) and the radioactivity in the alkyl DHAP spot was determined after development with acetic acid containing solvents with or without bisulfite (9,10). Other methods and materials were as described before (1,4,5,10).

Table 1. Requirements for the formation of alkali-stable radioactive lipid from ^{32}P-DHAP or ^{32}P-palmitoyl DHAP in liver mitochondria or brain microsomes.

The whole system contained Tris-HCl (pH 7.4, 50 mM), ATP (8.6 mM), NaF (8.6 mM), $MgCl_2$ (4.3 mM), CoASH (87 μM), glutathione (4.3 mM), hexadecanol (50 μg emulsified by 50 μg Triton-X-100) and enzyme containing 1 mg of protein in a total volume of 1.2 ml. The radioactive substrates added are: palmitoyl ^{32}P-DHAP or ^{32}P-DHAP or ^{32}P-acyl GP (each 3 nmoles, 105,000 cpm). The tubes were incubated at 37° for 30 min and the radioactivity in the alkali-stable lipid was determined.

	Liver mitochondria		Brain microsomes	
	From ^{32}P-acyl DHAP	From ^{32}P-DHAP	From ^{32}P-acyl DHAP	From ^{32}P-DHAP
	Radioactivity in alkali-stable lipid			
	cpm	cpm	cpm	cpm
Whole system	15,000	780	8,200	410
-ATP	3,800	32	4,200	24
-CoA	23,000	120	10,500	28
-ATP, -CoA	5,600	21	6,500	23
-NaF	400	31	200	-
$-MgCl_2$	13,500	-	6,900	-
-ROH	1,000	38	800	-
*Control	300	-	405	-
^{32}P-α-acyl GP†	380	-	410	-

*The mitochondria or microsomes were heated at 100° for 10 min before incubation.
†^{32}P-acyl DHAP addition was omitted, instead DL-^{32}P-α-palmitoyl GP was added.

RESULTS

a) Formation of alkali-stable ^{32}P-lipid from ^{32}P-acyl DHAP.

In Table 1 the conditions for the formation of alkali-stable lipid from acyl DHAP and DHAP are given. It is seen that the conversion of acyl DHAP to alkali-stable lipid is more efficient than the corresponding conversion of DHAP. Also, CoA is not required when acyl DHAP is used and in many instances CoA is actually found to be inhibitory for the reaction. This may be due to the formation of acyl CoA, since the addition of palmitoyl CoA was also found to inhibit the reaction. Long chain alcohol is needed for the reaction and acyl DHAP cannot be replaced by α-acyl GP (Table 1). Addition of Mg^{++} is

Table 2. Incorporation of 1-C^{14}-hexadecanol into alkyl DHAP.

The whole incubation mixture has the same composition as described under Table 1 except that 1-C^{14}-hexadecanol (50 μg, 8.95x10^5 cpm) was added and no CoA, ^{32}P-acyl DHAP or ^{32}P-DHAP were present. The alkali-stable lipid was fractionated on a silicic acid column and alkyl-DHAP in fraction 2 was further purified by thin layer chromatography (see text).

	Liver mitochondria	Brain microsomes
	Labeled alkyl DHAP formed	
	cpm	cpm
Whole system	1,800	800
+Palmitoyl DHAP (30 μg)	52,000	13,200
+Hexadecoxy DHAP	4,500	1,200
+1-palmitoyl GP (DL)	2,100	750

only slightly stimulatory, however addition of EDTA completely inhibits the reaction and can be reversed by the addition of Mg^{++} or Mn^{++}.

b) Conversion of 1-C^{14}-hexadecanol to alkyl DHAP.

In Table 2 the data for the formation of alkyl DHAP from 1-C^{14}-hexadecanol and palmitoyl DHAP are given. Acyl GP could not replace acyl DHAP but a small stimulation of alkyl DHAP formation by hexadecoxy DHAP was observed.

Both the ^{32}P- and ^{14}C-labeled alkali-stable products were identified as alkyl DHAP by various methods as described previously (1). On chromatography, the lipid migrated with carrier alkyl DHAP in various solvents with or without bisulfite (10). The labeled product was stable to alkaline hydrolysis and was also stable to acid hydrolysis (1 N HCl, 100°, 10 min) after reduction by $NaBH_4$. After $NaBH_4$ reduction the properties of the lipid became similar to monoalkyl GP (1). The C^{14}-labeled lipid after treatment with $LiAlH_4$ (11) was converted to C^{14}-labeled chimyl alcohol which was verified by cochromatography with the authentic compound and also by oxidation with $NaIO_4$ and by conversion to the isopropylidine derivative (12).

c) Inhibition studies with DHAP and acyl DHAP.

In Fig. 1A the effect of acyl DHAP on the incorporation of DHAP to alkali-stable lipid is shown. At a low concentration acyl DHAP was found to be somewhat stimulatory. However, at higher concentrations, acyl DHAP, as expected, was inhibitory of the conversion of DHAP to alkyl ethers. DHAP, in the absence of CoA, did not inhibit the formation of alkali-stable lipid from acyl DHAP (Fig. 1B). But, in the presence of CoA, DHAP was inhibitory. This is to be expected since acyl DHAP formed from DHAP would lower the specific activity of the added radioactive precursor.

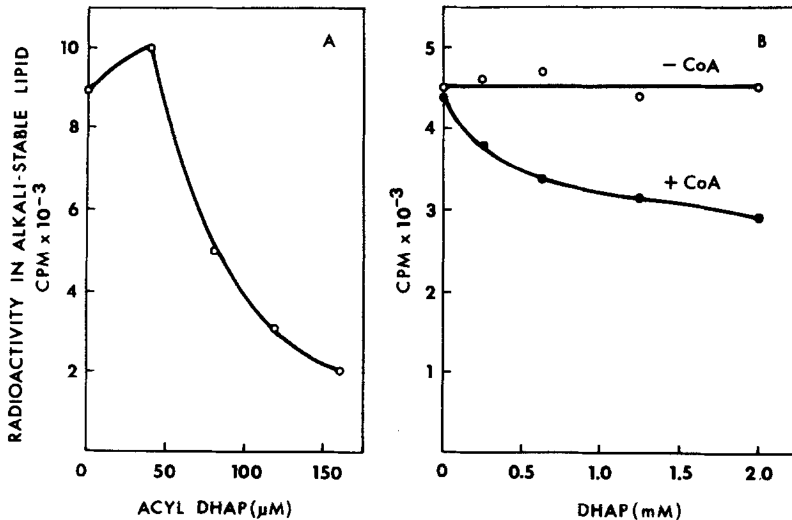


Fig. 1. A. Inhibition of radioactive alkali-stable lipid formed from ^{32}P-DHAP in brain microsomes by increasing concentrations of palmitoyl DHAP. The incubation condition is the same as described under Table 1 except that 120 nmoles (3.2×10^6 cpm) of ^{32}P-DHAP was used. B. Inhibition of radioactive alkali-stable lipid formed from ^{32}P-acyl DHAP (34 nmoles, 80,000 cpm) in brain microsomes by increasing concentrations of DHAP. The incubation condition is similar to that described in Table 1. Upper curve without CoA and lower curve with CoA.

d) Other properties.

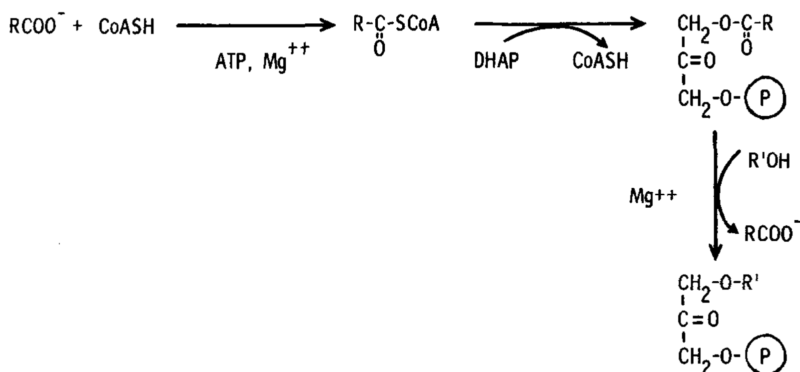
Palmitic acid at low concentration (5 μg/1.2 ml) was found to stimulate the incorporation of DHAP to alkyl ethers. However, at higher concentrations

(> 20 μg/1.2 ml) palmitic acid was inhibitory. In contrast, palmitic acid at all concentrations was inhibitory of the conversion of acyl DHAP to alkyl DHAP.

Free fatty acid was identified as a product of the reaction between acyl DHAP and long chain alcohol. Using ^{14}C-palmitoyl ^{32}P-DHAP, the molar ratio of the long chain alcohol-dependent release of fatty acid to the amount of alkyl ether formed was found to be 1.1. The slight stimulation of alkyl ether formation by the addition of alkyl DHAP (Table 2) indicated that the reaction may be reversible. However, in a preliminary experiment, no alkali-unstable lipid was found after incubating ^{32}P-alkyl DHAP and palmitic acid.

DISCUSSION

These data suggest the pathway for the formation of alkyl DHAP shown in Scheme 1. Endogenous fatty acid forms acyl DHAP and is again released after the reaction of acyl DHAP with long chain alcohol. CoA and ATP are needed for the acylation of DHAP. F^- possibly protects the phosphomonoester bond of both acyl and alkyl DHAP by inhibiting lipid phosphomonoesterase (13). Mg^{++} (or other metal ions) may be required for the ether bond formation. The stimulatory effect of ATP on the ether bond formation from acyl DHAP is still not



Scheme 1. Proposed pathway of biosynthesis of 1-O-alkyl dihydroxyacetone phosphate.

explained. However, the effect of ATP may not be very specific. β,γ-Methylene adenosine triphosphate (Miles Lab., Ind.) was also found to be somewhat stimulatory and only little stimulation by ATP was observed when phosphate buffer was used instead of Tris. The inhibition of alkyl ether biosynthesis by NADPH when added from the beginning of the reaction (1,14) can also be explained by the above scheme. Addition of NADPH would reduce the acyl DHAP to 1-acyl GP (15), which would no longer serve as a precursor to alkyl ethers.

The reaction is somewhat unusual in the sense that an ether bond is formed by replacing an ester bond. The closest analogous biochemical reaction might be the formation of various glycosidic linkages from phosphate esters. The presence of a carbonyl and an ester group adjacent to carbon-1 possibly facilitate the reaction with alcohol with the release of fatty acid.

Acyl DHAP may be enzymatically reduced and acylated to form phosphatidate (10) or as shown here, form ether lipids which may also be reduced and acylated (1). Alkenyl ethers may also arise from alkyl ether lipids (16). Hence, the acylation of DHAP may play a key role in various aspects of cellular lipid metabolism.

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