Reductase, Phosphatase, and Kinase Activities in the Metabolism of Alkyldihydroxyacetone Phosphate and Alkyldihydroxyacetone*

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SUMMARY

Kinetic studies have been carried out with microsomal preparations from mouse preputial gland tumors that catalyze (a) the reduction of [1-14C]alkyldihydroxyacetone-P and [1-14C]alkyldihydroxyacetone; (b) the dephosphorylation of [1-32P]alkyldihydroxyacetone-P; and (c) the phosphorylation of [1-14C]alkyldihydroxyacetone. Our data demonstrate the existence of a pathway that permits the interconversion of metabolic intermediary ether-lipid products, i.e. alkyldihydroxyacetone-P, alkylglycerol-P, alkyldihydroxyacetone, and alkylglycerol. The labeled substrates used in these experiments, [1-14C]octadecyldihydroxyacetone-P and [1-14C]octadecyldihydroxyacetone, were chemically synthesized.

Alkyldihydroxyacetone-P is reduced to 1-alkyl-sn-glycerol-3-P by a microsomal reductase that is highly specific for NADPH and optimally active at pH 7.0. However, higher levels of NADH can substitute for the NADPH. The apparent $K_m$ for the alkyldihydroxyacetone-P reductase is 0.12 mM for NADPH and 3.2 mM for NADH. In contrast, only NADPH is utilized by the reductase that reduces alkyl-dihydroxyacetone to the alkylglycerol. With the alkylglycerol reductase, the apparent $K_m$ for NADPH is 0.38 mM.

The phosphatase present in the tumor microsomes is maximally inhibited at NaF concentrations greater than 40 mM; less than 4% of the alkyldihydroxyacetone-P was dephosphorylated during a 5-min incubation under these conditions. However, during longer incubation periods at low concentrations of NaF, significant quantities of alkyldihydroxyacetone and alkylglycerol are produced.

In the presence of ATP and Mg$^{2+}$, alkylhydroxyacetone is phosphorylated to alkylhydroxyacetone-P by a kinase in the tumor. The apparent $K_m$ for ATP was 3.6 mM. The highest specific activity for the alkylhydroxyacetone kinase was located in the microsomal fraction. These data demonstrate how alkylhydroxyacetone can re-enter the pathway responsible for the biosynthesis of complex ether lipids.

The enzymic synthesis of alkyl glycerolipids has been described for cell-free systems obtained from various sources (for review see Refs. 1–3). Alkyl-DHAP, the first detectable product, is formed by a reaction that involves the replacement of the acyl group of acyl-DHAP (4, 5) with a long chain fatty alcohol. Although the exact mechanism is not known, we have demonstrated that the oxygen (6) and the intact alkyl chain of the alcohol (7, 8) are incorporated into alkyl-DHAP, and Friedberg et al. (9) have shown that one of the hydrogens on the carbon of DHAP to which the acyl group is attached is lost during the formation of alkyl-DHAP. The alkyl-DHAP is then either reduced by microsomal enzymes in the presence of reduced pyridine nucleotides to yield alkylglycerol-P or dephosphorylated by microsomal phosphatases to yield alkylhydroxyacetone. In this paper, we describe kinetic data for the reductase(s) that reduces the ketone groups of alkyl-DHAP and alkylhydroxyacetone, the phosphatase(s) that hydrolyzes the phosphate groups of alkyl-DHAP and alkylglycerol-P, and the kinase that phosphorylates alkylhydroxyacetone. The availability of [1-14C]alkyl-DHAP and [1-14C]alkylhydroxyacetone synthesized in our organic laboratory (10) made this investigation possible.

EXPERIMENTAL PROCEDURE

Materials—The 1-[1-14C]octadecyldihydroxyacetone was prepared by adding a specific activity of 2.5 μCi per μmole) to the tumor, and the unlabeled alkyl-DHAP and alkylhydroxyacetone, used as thin layer chromatographic standards. Prepared according to the United States Atomic Energy Commission, Grant BC-70C from the American Cancer Society, Grant CA11949.03 from the National Cancer Institute, National Institutes of Health, and $K_2$ octadecyldihydroxyacetone was synthesized in our organic laboratory (10) made this investigation possible.

1 Alkyl and acyl terms used in this paper are all O-substituted.
2 The abbreviation used is: DHAP, dihydroxyacetone phosphate.
to the procedure of Piantadosi et al. (11). We purchased the NADP⁺, NAD⁺, NADPH, NADH, glucose-6-P, glucose-6-P dehydrogenase, ATP, and CoA from Nutritional Biochemicals, Cleveland, Ohio.

Unlabeled alkylglycerol P, used as a standard, was prepared from alkyl-DHAP by a modification of a procedure described by Hajra and Agranoff (12). Five milligrams of 1-octadecyl-DHAP were dissolved in 5 ml of ethanol in a flask, and 1 ml of 0.1 M NaBH₄ in ethanol was added. After 30 min of mixing in a wrist action shaker at room temperature, an additional 0.5 ml of the NaBH₄ solution was added; the flask was then placed back on the shaker for 30 min more. Then 0.5 ml of glacial acetic acid was added, followed by an addition of 7.5 ml of chloroform and 6 ml of water. The mixture was shaken, centrifuged, and the lower layer removed. The solvent was evaporated to dryness and the residual white solid was dissolved in chloroform and stored at −27°C until used. The other lipid standards used were hexadecylglycerol and hexadecylcarnitine (Analabs, North Haven, Conn.) and hexadecanol (Applied Science Labs, State College, Pa.).

Source of Enzymes—Microsomes used in these experiments were obtained from preputial gland tumors (ESR-586) grown subcutaneously for a period of 4 weeks in C57BL/6 mice. The preparation of enzyme fractions and the tumor transplantation procedure have been described previously (7, 13). All microsomal preparations were washed three times with 0.25 M sucrose by centrifugation (100,000 × g for 1 hour) in a Beckman ultracentrifuge (model L3-50), and the pellet was suspended in 0.25 M sucrose by mixing in a tube with a Potter-Elvehjem homogenizer. The microsomes were stored at −27°C until used. Total protein content of each sample was determined by the procedure of Lowry et al. (14).

Reductase and Phosphatase Assays—The complete incubation mixtures contained the following components unless otherwise stated: [1-¹⁴C]alkyl-DHAP (8.3 µM, 0.062 µCi) or [1-¹⁴C]alkyl-dihydroxyacetone (8.3 µM, 0.062 µCi), NaF (40 mM), NADH (9 mM) or an NADPH generating system consisting of glucose-6-P (6.7 mM), glucose-6-P dehydrogenase (2 units), NADP⁺ (2 mM), and microsomes from preputial gland tumors (0.07 mg of protein) in a total volume of 3.0 ml of 0.1 M Tris-maleate buffer (pH 7.0). The incubations were carried out for 5 min. The alkyl-DHAP (Peak 3, A) and alkylglycerol-P (Peak 1, A) were separated in Solvent System I, which consisted of chloroform-methanol-glacial acetic acid (90:10:10, v/v/v). Thin layers (250 µm) of Silica Gel G were used for the separation of neutral lipids in Solvent System II, which consisted of hexane-diethyl ether-methanol-glacial acetic acid (70:30:5:1, v/v/v). The separated components on the chromatoplates were sprayed with 2',7'-dichlorofluorescein (0.2% solution in ethanol) and visualized under ultraviolet light. Radioactivity distributions were determined by zonal profile scanning (2-mm) and area scraping procedures (17, 18). Identification of all products was based on methods previously described (8, 19).

RESULTS AND DISCUSSION

Reductase Studies—Fig. 1 shows a typical zonal profile scan of the phospholipids (A) and phosphorus-free lipids (B) in the total lipid extract that was obtained after incubating [1-¹⁴C]alkyl-DHAP, the NADPH generating system, NaF, and the washed microsomes from preputial gland tumors for a period of 5 mm. The alkyl-DHAP (Peak 2, A) and alkylglycerol-P (Peak 1, A) are extremely difficult to resolve but they can be detected as two distinct peaks after chromatography on Silica Gel HR layers in Solvent System I. Although radioactivity did not occur in any other phospholipids, several lipids containing no phosphorus were also formed during the incubations. In addition to the expected alkylidihydroxyacetone (Peak 3, B) and alkylglycerol (Peak 2, B), it can be seen from the zonal profile scan in Fig. 1B that fatty alcohols (Peak 4, B) and wax esters (Peak 5, B) are also present. The alcohol is a degradative product of alkyl-DHAP (19) and the wax esters originate from the esterification of the labeled alcohols with endogenous fatty acids.

The optimum pH for the reduction of alkyl-DHAP was approximately 7.0. At pH 7.0, the alkyl-DHAP reductase activities were approximately 40% higher in 0.1 M Tris-maleate buffer than in the 0.1 M potassium phosphate buffer; the relative activities of the reductase were maintained for the entire pH range tested (1.2 to 9.0) for both buffers. Formation of alkylglycerol-P with both buffer solutions decreased rapidly at basic pH, whereas the formation of the dephosphorylated neutral lipids (alkylglycerol and alkylidihydroxyacetone) increased; this is due to the presence of phosphatase in the preparations (see next section on phosphatase).

Fig. 2 shows the time course of the enzymic reduction of alkyl-DHAP, using NADPH or NADH. Formation of the product, alkylglycerol-P, was not increased by either NADPH or NADH after 20 min of incubation. However, after longer incubation periods, considerable dephosphorylation of the alkyl-DHAP and alkylglycerol-P occurred. The reaction rate for the reduction of alkyl-DHAP in the presence of NADPH or NADH was constant with microsomal protein concentrations above 0.2 mg in incubations carried out for 5 min (Fig. 3). At the lower protein concentrations, the NADPH was more efficient than NADH (at equimolar levels) as the hydrogen source for the reaction. The apparent Kₘ values were 0.12 mm for NADPH and 3.2 mg for NADH (Fig. 4).

Alkylidihydroxyacetone was also enzymically reduced by NADPH but essentially no reduction was observed in the presence of NADH. Fig. 5 shows the NADPH concentration curve for the reduction of alkylidihydroxyacetone; the apparent Kₘ for NADPH was 0.38 mm. The maximum rate of reduction of alkylidihydroxyacetone by NADPH was slower than that of the NADPH-linked reduction of alkyl-DHAP by the same enzyme preparation.
FIG. 1. 14C Zonal profile scans (2-mm) of total lipids extracted from an incubation mixture of [1-14C]alkyl-DHAP, an NADPH generating system, NaF, and microsomes from preputial gland tumors as described under "Reductase and Phosphatase Assays" under "Experimental Procedure." The phospholipid classes (A) were separated on Silica Gel G layers in Solvent System II, and the products were identified as alkylglycerol-P (Peak 1, A), alkyl-DHAP (Peak 2, A), and neutral lipids (Peak 3, A). The neutral lipid classes (B) were separated on Silica Gel HR layers in Solvent System I, and the products were identified as phospholipids (Peak 1, B), alkylglycerol (Peak 2, B), alkylidihydroxyacetones (Peak 3, B), fatty alcohol (Peak 4, B), and waxes (Peak 5, B). The unlabeled standards used for identification migrated at the positions labeled for each peak.

FIG. 2. Time course for the reduction of alkyl-DHAP by NADPH and NADH. Conditions are described under "Reductase and Phosphatase Assays" under "Experimental Procedure."

FIG. 3. Effect of enzyme protein concentration on the reduction of alkyl-DHAP by NADPH- and NADH-linked reductase(s). Conditions are described under "Reductase and Phosphatase Assays" under "Experimental Procedure."

FIG. 4. Effect of NADPH and NADH concentration on the reduction of alkyl-DHAP. Conditions are described under "Reductase and Phosphatase Assays" under "Experimental Procedure."

FIG. 5. Effect of NADPH concentration on the reduction of alkylidihydroxyacetone. Conditions are described under "Reductase and Phosphatase Assays" under "Experimental Procedure."
Glycerol-P (Table I).

Ehrlich ascites cells. The reduction product formed with either reduced pyridine nucleotide as the cofactor is alkylglycerol-P, in the reduction of alkyl-DHAP by microsomal enzymes from preputial gland tumors. Systems I and II. Values are given for duplicate incubations. Values are increased, the extent of dephosphorylation also increases in the presence of NaF (40 mM).

Earlier reports (4, 8, 19) showed that both alkyl-DHAP and alkylidihydroxyacetone are reduced by an NADPH-linked reductase, and more recently Wykle et al. (5) reported that under specified conditions NADH could also substitute for NADPH in the reduction of alkyl DHAP by microsomal enzymes from Ehrlich ascites cells. The reduction product formed with either reduced pyridine nucleotide as the cofactor is alkylglycerol-P, which can be acylated by acyltransferases to yield alkylacylglycerol-P (Table I).

During the progress of our investigation, LaBelle and Hajra (20) demonstrated that the microsomal enzyme from Ehrlich ascites cells that reduces alkyl-DHAP is specific for NADPH. Our data show that at higher concentrations of NADH (2 mM), it can donate hydrogen as efficiently as NADPH for the reduction of alkyl-DHAP (see Table I, Figs. 2 and 3). The primary differences in the experimental conditions between our work and that of LaBelle and Hajra (20) were that their concentrations of alkyl-DHAP (100 µM) were 12 times higher than ours and they used much lower quantities of pyridine nucleotides (their lowest concentration (40 mM)). Therefore, we carried out the experiment reported in Table II. These data, which were obtained with higher levels of alkyl-DHAP (three times that used in our other incubations), and lower levels of NADPH and NADH (40 µM) with the same concentration used by LaBelle and Hajra (20)) demonstrate that alkyldihydroxyacetone was so different, it is conceivable that two different enzymes might be involved. Unlike LaBelle and Hajra (20), our results show that significant quantities of alkyldihydroxyacetone can be reduced to form alkylglycerols by the preputial gland tumor microsomes. Furthermore, it is important to note when alkyl-DHAP is incubated for longer than 5 min that the high phosphatase activity in most microsomal preparations, even in the presence of fluoride ions (see “Phosphatase Studies”), produces significant quantities of alkyldihydroxyacetone which can then be reduced to form alkylglycerols by the NADPH-linked reductase. It is obvious that this phosphatase activity cannot be ignored when assaying the enzymic reduction of alkyl-DHAP.

The present kinetic studies on the reduction of alkyl-DHAP by the microsomal enzyme from preputial gland tumors support the earlier results (4, 8, 19) and clear up some of the questions concerning the reported utilization of NADH as a substrate for NADPH in these reactions. In addition, we have been able to demonstrate that alkyldihydroxyacetone is also a substrate for an NADPH-dependent reductase. Since the enzyme specificity of NADPH and NADH in the reduction of alkyl-DHAP and alkylidihydroxyacetone was so different, it is conceivable that two different enzymes might be involved. Unlike LaBelle and Hajra (20), our results show that significant quantities of alkyldihydroxyacetone can be reduced to form alkylglycerols by the preputial gland tumor microsomes. Furthermore, it is important to note when alkyl-DHAP is incubated for longer than 5 min that the high phosphatase activity in most microsomal preparations, even in the presence of fluoride ions (see “Phosphatase Studies”), produces significant quantities of alkyldihydroxyacetone which can then be reduced to alkylglycerols by the NADPH-linked reductase. It is obvious that this phosphatase activity cannot be ignored when assaying the enzymic reduction of alkyl-DHAP.

Phosphatase Studies—When alkyl-DHAP is formed or incubated in the presence of tumor microsomes, significant quantities of alkyldihydroxyacetone are also produced (see Refs. 2 and 3). This has also been confirmed in the present study with the labeled alkyl-DHAP as substrate (Fig. 1 and Table I).

Our initial studies (21) showed that NaF can suppress the alkyl-DHAP phosphatase activities in similar tumor preparations. However, our present results reveal that as the incubation time is increased, the extent of dephosphorylation also increases in the presence of 40 mM NaF (Fig. 6). The decrease in labeled
Fig. 7. Effect of NaF concentration on the phosphatase activity. The dotted line designates alkyl-DHAP (Part A) and alkylglycerol-acetone (Part B). The solid line designates alkylglycerol-P (Part A) and alkylglycerol (Part B). Conditions are described under "Reductase and Phosphatase Assays" under "Experimental Procedure." Solvent System I was used to obtain data for the curves in Part A and Solvent System II was used to obtain the data for the curves in Part B.

### TABLE III

<table>
<thead>
<tr>
<th>System</th>
<th>% Alkyl-DHAP</th>
<th>% Alkylglycerol-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Completeb</td>
<td>5.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Complete minus ATP</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>Complete minus Mg2+</td>
<td>1.9</td>
<td>0</td>
</tr>
<tr>
<td>Complete minus NaF</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>Complete minus ATP, Mg2+</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>Completec</td>
<td>1.7</td>
<td>13.0</td>
</tr>
</tbody>
</table>

- Based on radioassay of products after separation in Solvent System I.
- The complete system using the postmitochondrial fraction (0.5 mg of protein) was the same as described under "Kinase Assays" under "Experimental Procedure."
- After the initial 30 min of incubation, NADPH (2 mM) and CoA (0.1 mM) were added and incubated for another 30 min.

Phosphorylated products after 20 to 60 min of incubation is due to this phosphatase activity (Fig. 2).

Fig. 7 shows the effect of NaF concentration on the inhibition of phosphatase activity in the microsomes when alkyl-DHAP was incubated in the presence of NADPH for a period of 5 min. Under these conditions, maximum inhibition of phosphatase activity occurred with 40 mM or higher levels of NaF, i.e. less than 4% of the alkyl-DHAP was dephosphorylated.

The specificity of alkaline and acid phosphatases for hydrolyzing phosphate groups from lipid substrates has previously been demonstrated by our laboratory (22). Alkaline phosphatase was only active when position 2 of the glycerolipids was unoccupied by an acyl moiety, whereas the acid phosphatase was active with all glycerolipids possessing a free phosphate group.

### TABLE IV

<table>
<thead>
<tr>
<th>System</th>
<th>[3C]Alkyl-DHAP per mg protein %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>0.31</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.45</td>
</tr>
<tr>
<td>Microsomes</td>
<td>1.30</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.12</td>
</tr>
</tbody>
</table>

- Conditions are described under "Kinase Assays" under "Experimental Procedure."
- The samples were incubated for 30 min.
- Based on radioassay of product after separation in Solvent System I.

Fig. 8. Effect of ATP concentration on the formation of alkyl-DHAP from alkylglycerol-acetone. Conditions are described under "Kinase Assays" under "Experimental Procedure."

Kinase Studies—The preparations of the preputial gland tumors also contain an alkylglycerol-acetone kinase (Tables III and IV; Fig. 8). These data demonstrate that ATP and Mg2+ are required for the phosphorylation step. The small quantity of alkylacylglycerol-P (2%) produced under these conditions presumably occurs after reduction of the ketone group by the small endogenous quantity of NADPH present. The formation of alkylacylglycerol-P was greatly increased when NADPH and CoA were added to the system (Table III).
These data indicate that the alkyl-DHAP formed was reduced by the NADPH-linked enzyme to form alkylglycerol-P which was then acylated; it is also possible for the alkyldihydroxyacetone to be reduced by the NADPH-linked enzymes to form alkylglycerol, which could then participate as a substrate (16) for the kinase.

Fig. 8 shows the effect of ATP concentration on the formation of alkyl-DHAP from alkyldihydroxyacetone; the apparent $K_m$ was 3.6 mM. The activity of the alkyldihydroxyacetone kinase was lower than the values that we previously observed for the alkylglycerol (16) with similar microsomal preparations. The highest specific activity for the alkyldihydroxyacetone kinase (Table IV) was in the microsomes.

CONCLUSIONS

The following reactions, catalyzed by microsomal enzymes from preputial gland tumors, have been elucidated in this investigation:

\[
\begin{align*}
\text{Alkyl-DHAP phosphatase} & \quad \text{Alkyldihydroxyacetone} \\
\text{NADPH} \quad \text{(high levels of NAD)} & \quad \text{NADPH} \\
\text{Alkylglycerol-P phosphatase} & \quad \text{Alkylglycerol} \\
\text{ATP, Mg}^{2+} \quad \text{(Ref. 10)} & \quad \text{(Ref. 10)}
\end{align*}
\]

These reactions appear to be important in the regulation of the biosynthesis and degradation of ether lipids. Alkylglycerol-P can be acylated by acyltransferases (8) and then proceed to form complex phospholipids (23). On the other hand, the alkylglycerol can be degraded in tissues containing a tetrahydropteridine-requiring alkyl cleavage enzyme (24-26). In addition, both the alkylglycerol and alkyldihydroxyacetone can re-enter the biosynthetic pathways via the kinase reactions.

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Reductase, Phosphatase, and Kinase Activities in the Metabolism of Alkyldihydroxyacetone Phosphate and Alkyldihydroxyacetone
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