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APPARENT PHOSPHORYLATION - DEPHOSPHORYLATION OF SOLUBLE PHOSPHATIDIC ACID PHOSPHATASE IN RAT LIVER.

Berglund, L.[1], Björkhem, I.[2x], and Einarsson, K.[3].

Department of, Surgery[1], Clinical Chemistry[2], and Medicine[3].

Karolinska Institutet, Huddinge Hospital, Huddinge, Sweden.

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Preincubation with alkaline phosphatase stimulated the activity of ammonium sulfate precipitated phosphatidic acid phosphatase from rat liver cytosol. Treatment with Mg-ATP inhibited the activity. The possibility is discussed that the soluble phosphatidic acid phosphatase, assumed to catalyze the rate-limiting step in the biosynthesis of triglycerides, is to some extent regulated by phosphorylation - dephosphorylation.

Phosphorylation - dephosphorylation reactions have been found to be of importance in the regulatory control of several rate-limiting enzymes. Among the more than 20 different enzymes presently known to be controlled by such a mechanism are several key enzymes such as glycogen phosphorylase, glycogen syntetase, hormone sensitive lipase, fructose 1,6-diphosphatase, phosphofructokinase, acetyl-CoA carboxylase and HMG CoA reductase (1).

In the present study, the possibility has been studied that the apparent rate-limiting enzyme in triglyceride biosynthesis, the soluble phosphatidic acid phosphatase (2) is regulated by phosphorylation - dephosphorylation.

Materials and Methods.

Preparation of phosphatidic acid phosphatase.

Male rats of the Sprague-Dawley strain were used. The rats were killed, the livers excised and homogenized in 4 volumes of

x) Correspondence should be addressed to this author.

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50 mM Tris-Cl buffer pH 7.4. A cytosolic fraction was prepared by centrifugation at 20,000 x g for 15 min followed by recentrifugation of the supernatant fraction at 100,000 x g for 1 h. This 100,000 x g supernatant fluid was directly used in some experiments. In most experiments, however, the 100,000 x g supernatant was precipitated by ammonium sulfate, essentially as described by Lamb and Fallon (3). The cytosol was treated with 0.23 g ammonium sulfate per ml solution for 10 min at 4°C. The mixture was then centrifuged at 10,000 x g for 10 min and the precipitate was washed gently with 50 mM Tris-Cl containing 0.23 M sucrose. It was then dissolved in 0.3 M sucrose and dialyzed over-night against 0.3 M sucrose containing 0.5 mM DTT. The dialyzed material was frozen and stored at -20°C prior to use. This storage did not affect the activity of the preparation during the time of the study.

Preparation of membrane bound and free ^{14}C-labeled phosphatidic acid.

Microsomal bound phosphatidic acid was prepared as described by Lamb and Fallon (3) by incubation of 1-^{14}C-labeled palmitate (obtained from Radiochemical Centre, Amersham, England) with rat liver microsomes in the presence of ATP, CoA, sn-glycerol-3-phosphate, albumin and NaF. The microsomal solution obtained contained about 2 mg of protein per ml. This preparation was frozen and stored at -20°C prior to use. Before incubation, the thawed suspension was heated at 85°C for 5 min in order to remove intrinsic microsomal activity.

More than 95% of the radioactivity in the final preparation was obtained in the phosphatidic acid fraction and less than 1% in the diglyceride fraction as judged by thin-layer chromatography of an extract of the microsomal suspension. Assuming no significant dilution with endogenous phosphatidic

acid, the specific radioactivity of the membrane bound phosphatidate was found to be 4.2×10^3 c.p.m. per nmol.

In some experiments, 1-^{14}C-palmitate of maximal specific radioactivity (Radiochemical Centre) was used for preparation of microsomal bound phosphatidic acid. The microsomal phosphatidic acid obtained was then extracted and isolated by preparative thin-layer chromatography. The material was finally diluted with dipalmitoyl phosphatidic acid (obtained from Sigma Chem. Co) to a specific radioactivity of 2×10^3 cpm/nmol.

Incubation procedures.

The standard incubation mixture contained phosphatidic acid phosphatase (60-100 ug protein), microsomal bound phosphatidate (39 nmol, 0.18×10^6 cpm) or free phosphatidic acid (500 nmol, 1×10^6 cpm), Tris-maleate, 60 umol, pH 6.9 and $MgCl_2$, 0.8 umol, in a total volume of 0.8 ml. In some experiments the incubation mixture was preincubated for 5 min (prior to addition of substrate) with 10 I.U. of alkaline phosphatase (isolated from E. Coli, Sigma Chem. Co.), dissolved in 240 ul of a solution containing 5 umol of imidazol buffer, pH 7.4 and 0.7 umol DDT. In the corresponding control experiments, the preincubation was performed only with the imidazol buffer and the DTT (Table 1). In some experiments ATP was added to a final concentration of 2 mmol/l. Under these conditions, the concentration of $MgCl_2$ was 4 mmol/l (Table 2). All incubations were performed at 37°C for 15 min and were terminated by additon of 4 ml of chloroform-methanol (2:1, v/v).

Analysis of incubation mixtures.

The chloroform phase obtained in the extraction of the incubation mixture with 4 ml of chloroform - methanol (2:1, v/v) was evaporated under nitrogen and the residue subjected to thin-

Table 1. Effect of addition of alkaline phosphatase on phosphatidic acid phosphatase activity.

For experimental details, see Materials and Methods.

Addition	Activity with membrane bound substrate	Activity with free substrate
	%	
None	100	100
Alkaline phosphatase, 10 I.U.	172 ± 25*	136 ± 9*

* Mean ± S.E.M., n = 6

layer chromatography using diisopropylene ether - acetic acid (24:1, v/v) as solvent. The solvent was allowed to move only 10 cm. The chromatoplate was then dried in air for 30 min and immediately subjected to a second chromatogrpahy, using hexane - diethyl ether - acetic acid (80:20:1, v/v/v) as solvent. The converison of labeled phosphatidic acid into diglyceride was measured by scanning of the chromatoplates with a radioscanner (Berthold, Wildbad, West Germany).

The conversion in each experiment was compared to the conversion in the corresponding control experiment, which was set to 100%. Protein was determined according to the Lowry procedure (4).

Results.

In accordance with our previous results (5), the conversion of phosphatidic acid into diglyceride was linear with time as well as with enzyme concentration under the standard conditions used for assay. When using microsomal bound phosphatidate, the enzyme could not be saturated with substrate. In contrast, with 500 nmol of added free phosphatidic acid, there was a substrate saturation.

Addition of alkaline phosphatase was found to stimulate the activity of ammonium sulfate precipitated phosphatidic acid

Table 2. Effect of addition of ATP on phosphatidic acid phosphatase activity.

For experimental details, see Materials and Methods

Addition	Activity with membrane bound substrate	Activity with free substrate
	%	
Mg^{++}, 4 mmol/l	100	100
Mg^{++}, 4 mmol/l + ATP, 2 mmol/l	53 ± 12*	28 ± 9*

* Mean ± S.E.M., n = 6

phosphatase when using membrane bound phosphatidate as substrate (Table 1). In six different experiments, using different preparations of phosphatidic acid phosphatase of varying activities, the degree of stimulation varied from about 40% up to about 180% (mean 72%). In general, it was not possible to demonstrate such a stimulatory effect of alkaline phosphatase when using a crude cytosolic fraction as source of phosphatidic acid phosphatase.

When using free phosphatidic acid as substrate, the degree of stimulation was lower, and varied between 10 and 60% (mean, 36%) (Table 1).

Increasing the amount of alkaline phosphatase above 10 I.U. did not increase the degree of stimulation.

Following addition of ATP to a preparation of ammonium sulfate precipitated phosphatidic acid phosphatase, there was a significant inhibition of the enzyme activity (Table 2). In six different experiments, using different preparations of phosphatase, the degree of inhibition with 2 mmol/l of ATP varied between 20 and 100% (mean, 47%). Similar results were obtained also when using a crude cytosolic fraction as source of phosphatidic acid phosphatase.

When using free phosphatidic acid as substrate, the degree of inhibition by ATP varied between 30 and 90% (mean 72%).

Addition of cyclic AMP, in final concentrations of 10^{-3}–10^{-5} moles/l did not increase the degree of inhibition obtained with ATP, and cyclic AMP in itself had no significant effect on the activity.

Discussion.

Phosphatidic acid phosphatase seems to catalyze the rate-limiting step in the biosynthesis of triglycerides in the liver (2,6,7). In vitro studies have shown that the rate of conversion of phosphatidic acid into diglyceride is lower than the rate of any other step in the sequence. Furthermore, this enzyme activity varies in parallel with changes in the triacylglycerol biosynthesis under different dietary and hormonal conditions (2). Phosphatidic acid phosphatase is present both in the soluble and the microsomal fraction of a liver homogenate. The enzyme present in the microsomal fraction is less active than the corresponding activity in the cytosol. However, in most cases the microsomal enzyme responds in parallel to the soluble enzyme under similar conditions (6). In our laboratory attempts to study the microsomal activity under enzymological conditions have failed as yet, and in the present work only the soluble activity was investigated.

In accordance with the results obtained by others, more reproducible results were obtained after precipitation of the cytosolic phosphatidic acid phosphatase with ammonium sulfate than when using the crude cytosol (3). It has been suggested that membrane bound phosphatidic acid is a more physiological substrate for the soluble enzyme than is free phosphatidic acid (7). On the other hand, it is not possible to obtain substrate saturation with microsomal bound phosphatidic acid making it more

difficult to evaluate kinetical experiments. In the present work both types of substrate were used, with essentially similar results.

As described above, alkaline phosphatase was found to stimulate phosphatidic acid phosphatase activity, whereas Mg-ATP inhibited the enzyme activity. The degree of stimulation and inhibition was of the same magnitude as that obtained with crude microsomal HMG-CoA reductase, when tested under similar conditions (8). A further support for involvement of a phosphoprotein kinase in an ATP-mediated inactivation of a specific enzyme is a demonstration that the activity can be restored by removal of Mg^{++} by addition of excess EDTA to the incubation mixture (1). Since the phosphatidic acid phosphatase activity in itself is dependent upon Mg^{++}, however, this approach was not possible to use here. A further evidence for involvement of a phosphoprotein phosphatase in the activation of a specific enzyme is a demonstration of an inhibition with sodium fluoride (1). Sodium fluoride inhibits most phosphatases, however, including phosphatidic acid phosphatase (9).

From the results obtained, we suggest that phosphatidic acid phosphorylase to some extent is reversibly modulated by phosphorylation - dephosphorylation. Definite evidence for such a mechanism can be obtained first after extensive purification of the enzyme. As yet, the soluble phosphatidic acid phosphorylase has only been purified about 15 to 20-fold (9). Attempts to further purify the enzyme are now in progress.

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