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Dephosphorylation and Activation of Acetyl-CoA Carboxylase by Phosphorylase Phosphatase

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Summary

Acetyl-CoA carboxylase from rat epididymal fat tissue is activated by phosphorylase phosphatase, a reaction which is inhibited by phosphatase inhibitor-1. This activation is accompanied by a corresponding loss of ^{32}P from the labelled enzyme. These results establish that dephosphorylation of the enzyme causes its activation.

INTRODUCTION

Recently there has been increasing evidence for the regulation of acetyl-CoA carboxylase by covalent modification; phosphorylation inactivates the enzyme and dephosphorylation activates it (1-3). Experiments to establish such a regulatory mechanism have been carried out with partially purified enzyme preparations containing the endogenous kinase and phosphatase. As a result, an unequivocal demonstration of such a mechanism has been difficult. For example, it has been known for some time that limited proteolysis of liver acetyl-CoA carboxylase increases the activity of the carboxylase (4, 5), and it has been difficult to establish that the apparent dephosphorylation which correlates with the increase in the activity is not due to the partial proteolysis of the phosphorylated segment of the carboxylase, as is the case with yeast glycogen synthase (6). In addition, it has been reported that the degree of phosphorylation of the chick liver carboxylase does not correlate with the enzyme activity (7).

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In this communication, we present evidence that dephosphorylation of the carboxylase from epididymal fat tissue by phosphoprotein phosphatase activates the carboxylase, and that this dephosphorylation is inhibited by a specific inhibitor of phosphoprotein phosphatase. This indicates that acetyl-CoA carboxylase from epididymal fat tissue is regulated not only by the phosphorylation and dephosphorylation mechanism, as reported for the liver enzyme, but the present studies provide compelling evidence that the covalent modification mechanism of phosphorylation-dephosphorylation is causatively related to the activity of the carboxylase.

MATERIALS AND METHODS

Chemicals - Acetyl-CoA, phenylmethylsulfonyl fluoride, crystalline bovine serum albumin, fraction V bovine serum albumin, and ATP were obtained from Sigma. Carrier-free [^{32}P]P_i and NaH[^{14}C]O_3 (59.3 mCi/mmol) were purchased from Amersham/Searle; enzyme grade ammonium sulfate from Schwarz/Mann; acrylamide and sodium dodecyl sulfate from Bio-Rad; glass fibre membranes from Reeve Angel; complete Freund's adjuvant from Difco.

Animals - Male Wistar rats, weighing 210-255 g, were raised in the departmental rat colony and fed ad libitum with a commercial animal diet.

Preparation of [^{32}P]-Acetyl-CoA Carboxylase - Two grams of adipose tissue (from 10 animals) were preincubated in 20 ml of Krebs-Ringer bicarbonate medium containing ^{32}P-phosphate (200 µCi/g tissue), 3% BSA, and 1 mg/ml of glucose for 3h. The gas phase was 95% O_2 and 5% CO_2. The preincubated tissues were then homogenized in two volumes of buffer containing 0.25M sucrose, 50 mM Tris, 1 mg/ml BSA, 10 mM ß-mercaptoethanol, and 0.2 mM PMSF, pH 7.2 (buffer A), and acetyl-CoA carboxylase was partially purified through the 45% ammonium sulfate step, as described previously (8).

Assay of Acetyl-CoA Carboxylase and Phosphoprotein Phosphatase - Acetyl-CoA carboxylase was assayed by the method previously described (8), but citrate was omitted. For the assay of phosphoprotein phosphatase, the partially purified carboxylase was preincubated for 15 min at 30°, and the phosphatase reaction was initiated with the addition of an appropriate amount of the enzyme. One unit of phosphatase is defined as the amount of enzyme which converts 1 nmole of phosphorylase a dimer per minute (9).

Enzymes - Rat liver phosphorylase phosphatase-C, purified through the second DEAE - Sephadex A-50 step according to Brandt et al (9), was the gift of D. M. Gibson, Indiana University School of Medicine (10). Purified phosphatase inhibitor-1 (in its active, phosphorylated mode) was the gift of P. Cohen, University of Dundee (11).

Dephosphorylation of the Carboxylase - The determination of ^{32}P released from the labelled enzyme following phosphoprotein phosphatase treatment was carried out as follows. Following the addition of the phosphatase, 1.0 ml samples were taken at the indicated times. These samples were precipitated with 45% ammonium sulfate and resuspended in a

buffer containing 50 mM Tris, 0.15 M NaCl, 1 mM EDTA, and 5 mM β-mercaptoethanol. To these samples immunoprecipitation studies were carried out with antiserum to the fat pad carboxylase, using 2.5 times the equivalence point of the antibody. The mixture was incubated 20 min at 30°, then 16 to 20 h at 4°. The antibody-antigen complex obtained by centrifugation at 3000 g for 15 min was then washed two times with cold 0.15 M NaCl and 0.3 mM PMSF. The immunoprecipitates were suspended in a buffer containing 50 mM Tris, 7% sucrose, 2% SDS, 5% β-mercaptoethanol, 0.5 mM EDTA, and 0.2 mM PMSF at pH 7.2, and heated in a boiling water bath for 15 min. These samples were subjected to 5% acrylamide SDS gel electrophoresis (12); the gels were sliced into 2 mm segments, and the carboxylase band was counted in an aqueous scintillation fluid.

Autoradiographical Studies - Alternatively, the incorporation of ^{32}P into the carboxylase was measured as follows. Following the addition of the phosphatase, 100 µl samples were taken at the indicated times and were added to 20 µl 10% SDS with 50% β-mercaptoethanol. These samples were boiled 15 min, and 100 µl samples were subjected to electrophoresis on slab gels prepared by a modification of the method of Galagher and Ryrie (13): 5% acrylamide resolving gels (750 mM Tris, pH 8.9) with stacking gels of 4% acrylamide (68 mM Tris, pH 6.7). The running buffer was 25 mM Tris, 0.2 M glycine, 0.1% SDS, pH 8.6. After staining and destaining, the gels were dried and exposed to Kodak Safety Film IRO with Cronex intensifying screens (DuPont) for 72 to 96 h. The autoradiograms were analyzed by densitometric scanning. The peak corresponding to carboxylase was removed and weighed for the relative amount of radioactivity in the carboxylase.

RESULTS AND DISCUSSION

As shown with the rat liver enzyme preparation, the partially purified acetyl-CoA carboxylase of rat epididymal fat tissue is slowly activated at 30°. This temperature-dependent activation reaches a maximum within 10 min, and no further activation is observed in subsequent incubation (Fig. 1). The cause of this temperature-dependent enzyme activation is not known; however, this activation is independent of citrate and has been observed also with liver enzyme preparations (14). When phosphoprotein phosphatase is added to the enzyme preparation which had been preincubated for 10 min, the carboxylase is activated further as shown in Fig. 1.

To demonstrate that this phosphoprotein phosphatase-dependent activation of acetyl-CoA carboxylase is due to dephosphorylation of the enzyme, we have prepared partially purified [^{32}P]-acetyl-CoA carboxylase from fat tissue which had been incubated with ^{32}P for 3 hours as described in the Methods section. The partially purified enzyme is preincubated for 15 min, and then the purified phosphoprotein

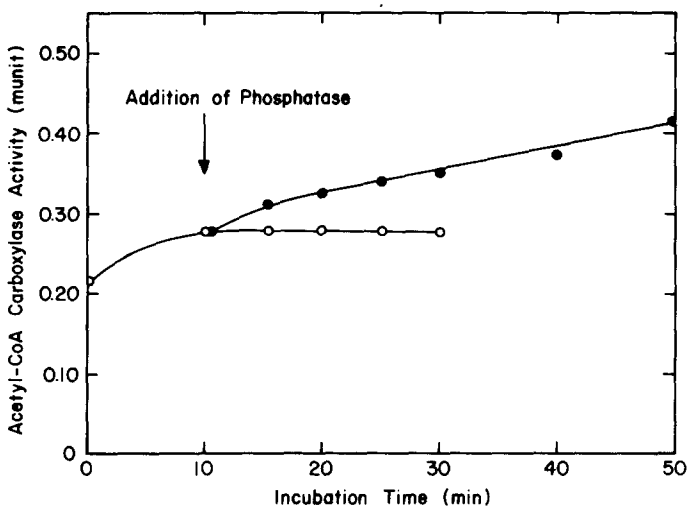

Figure 1 - Effect of Phosphorylase Phosphatase on Acetyl-CoA Carboxylase. The partially purified acetyl-CoA carboxylase preparation was preincubated 10 min at 30° to allow for endogenous activation. At this point exogenous phosphorylase phosphatase (3 munits) was added and the mixture incubated further at 30°. At various times 20 µl aliquots were taken, and the carboxylase was assayed for 2 min at 30°. •-•, represents exogenous activation; o-o, represents heat activation.

phosphatase is added. Four samples are taken at different times as indicated; one sample is used for ^{32}P determination and another sample is used to assay the carboxylase activity. In the case of the carboxylase assay, the ^{14}C-malonyl-CoA formed is determined following complete decay of ^{32}P, as well as under the proper settings of the discriminators of the scintillation counter. A third aliquot is precipitated with the use of antibody to the carboxylase. The enzyme-antibody complex is subjected to SDS gel electrophoresis, and the ^{32}P content in the carboxylase is then determined. A fourth sample is subjected to SDS slab gel electrophoresis, dried, and used for autoradiography as described in Methods.

The experimental results relating the dephosphorylation and the carboxylase activation is shown in Fig. 2. It is clear that activation of the carboxylase by the phosphoprotein phosphatase is related to dephosphorylation of the carboxylase. Further evidence that dephosphorylation is

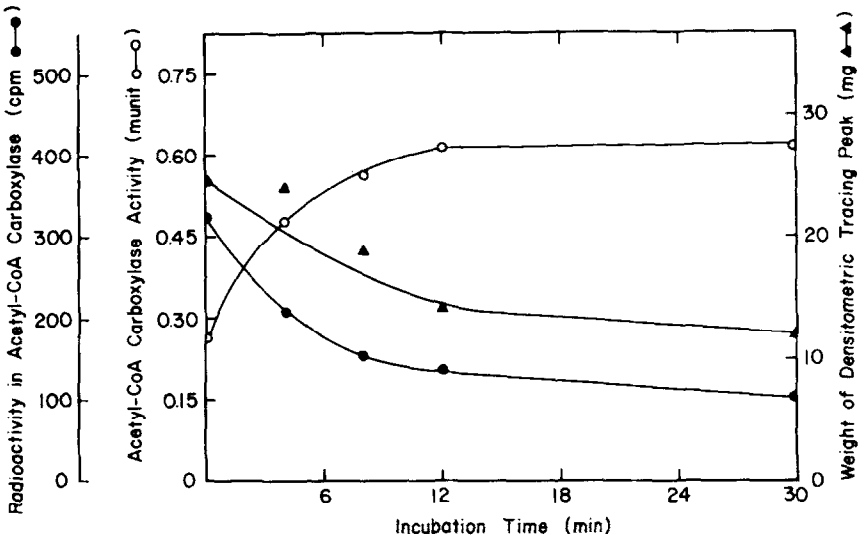

Figure 2 - Dephosphorylation and Activation of Acetyl-CoA Carboxylase by Phosphorylase Phosphatase. The labelled enzyme was preincubated for 10 min at 30° for endogenous activation. Phosphorylase phosphatase (3 munits) was added to the preincubated enzyme preparation; at the indicated times aliquots were withdrawn to assay the carboxylase activity (20 µl), to determine ^{32}P content in the carboxylase (20 µl), and to prepare for autoradiography (100 µl) and antibody precipitation (1.0 ml). o represents the assay of the carboxylase, ● represents the cpm localized in the carboxylase band of the SDS gel after precipitating with antibody; ▲ represents the relative radioactivity measured by the weight of the peak off the densitometric tracing of the autoradiogram.

the cause of the carboxylase activation comes from the use of the specific inhibitor protein of the phosphoprotein phosphatase (11). As shown in Table I, activation of the carboxylase by the phosphoprotein phosphatase is completely inhibited by four units of inhibitor protein-1. Along with inhibition of the phosphatase, there is no release of ^{32}P from the labelled enzyme (data not shown). A similar effect of phosphoprotein phosphatase on the enzyme from the rabbit mammary gland has recently been reported (15).

The carboxylase activation during the preincubation period is also accompanied by dephosphorylation; however, protein inhibitors of the phosphatase had no effect on this activation or dephosphorylation (data not shown).

TABLE I

Effect of the Inhibitor Protein-1 on the Activation of Acetyl-CoA Carboxylase by Phosphoprotein Phosphatase

Addition	Acetyl-CoA Carboxylase (munits)	% Inhibition
None[a]	.36	--
Phosphoprotein Phosphatase (3 munits)	.53	--
Phosphoprotein Phosphatase (3 munits) plus inhibitor protein		
0.4 unit	.49	22
1.0 unit	.44	52
2.0 unit	.42	63
4.0 unit	.36	98

[a]The partially purified enzyme preparation was preincubated for 15 min at 30°. This incubation activated the carboxylase from .29 milliunit to .36 milliunit.

Table I - The partially purified acetyl-CoA carboxylase preparation was preincubated 15 min at 30° to allow for endogenous activation. At this point phosphorylase phosphatase was added, and the mixture was preincubated a further 30 min at 30° with varying amounts of phosphatase inhibitor-1. After the preincubation samples were assayed for 2 min at 30°.

These data, therefore provide compelling evidence that dephosphorylation of the carboxylase causes its activation.

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