Regulation of purified rat liver acetyl CoA carboxylase by phosphorylation

John B. Allred, Gloria J. Harris, and Jonathan Goodson
Department of Food Science and Nutrition, The Ohio State University, Columbus, OH 43210

Abstract Acetyl CoA carboxylase was purified from liver of fasted-refed rats to near homogeneity, based on electrophoretic analysis and biotin content. These preparations contained an endogenous protein kinase that catalyzed the transfer of radioactive phosphate from \([\gamma-^{32}P]ATP\) to acetyl CoA carboxylase, accompanied by a decrease in acetyl CoA carboxylase activity. Phosphate incorporated into acetyl CoA carboxylase was removed when the preparation was incubated with partially purified phosphorylase phosphatase catalytic subunit with regain of enzymatic activity. This endogenous protein kinase was shown not to be affected by either cyclic-AMP-dependent protein kinase inhibitor, EGTA, or trifluoperazine. The addition of either cyclic-AMP or purified cyclic-AMP-dependent protein kinase catalytic subunit to the purified acetyl CoA carboxylase preparation increased protein phosphorylation but had no further effect on acetyl CoA carboxylase activity. Purified acetyl CoA carboxylase was shown to act as an ATPase during the phosphorylation reaction.


Supplementary key words cyclic-AMP-independent protein kinase • cyclic-AMP-dependent protein kinase • calcium-independent protein kinase • ATPase activity

Fatty acid biosynthesis and the activity of acetyl CoA carboxylase have been shown to be rapidly decreased by glucagon or cyclic-AMP or its derivatives in a variety of rat liver preparations (1–9). Catecholamines or their analogs, working through the \(\alpha\)-adrenergic receptors in a calcium-dependent process, have also been shown to reduce the rate of lipogenesis and the activity of acetyl CoA carboxylase in isolated rat hepatocytes (10). The simplest interpretation of these observations is that acetyl CoA carboxylase can be phosphorylated and inactivated by either cyclic-AMP-dependent or a calcium-dependent protein kinase. The possibility that a cyclic-AMP-dependent protein kinase can play a regulatory role was strengthened by the observation that treatment of isolated hepatocytes with glucagon in the presence of radioactive inorganic phosphate resulted in increased phosphorylation of acetyl CoA carboxylase, accompanied by a decrease in its catalytic activity (11).

Although a number of reports (see 12, 13 for review) following the original observation of Carlson and Kim (14) have shown that the activity of acetyl CoA carboxylase in broken rat liver cell preparations can be regulated by phosphorylation-dephosphorylation, the phosphorylation is reported to be catalyzed by a cyclic-AMP-independent protein kinase (14, 15). The addition of cyclic-AMP to relatively crude preparations of rat liver decreased the activity (4, 15, 16) and increased phosphorylation of acetyl CoA carboxylase (15, 16) but this effect can be mimicked by 5'-AMP and has been attributed to an alteration of energy charge (16). In contrast, acetyl CoA carboxylase isolated from rat mammary gland has been shown (17) to be phosphorylated and inactivated by purified cyclic-AMP-dependent protein kinase catalytic subunit. The work reported here was initiated to study regulation by phosphorylation of a similarly prepared enzyme from rat liver.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 150–250 g were fasted for 2 days and refed a high carbohydrate diet for 2 days. They were maintained in a controlled environment with a 7 AM to 7 PM dark cycle. At approximately 9 AM, rats were stunned by a blow on the head and decapitated, and livers were quickly removed and cooled on ice.

Enzyme purification

Liver was homogenized with a motor-driven Potter-Elvehjem homogenizer in 1.5 volumes of cold 0.3 M mannitol that contained 15 mM \(\beta\)-mercaptoethanol and the following proteolytic enzyme inhibitors at the indicated concentrations: leupeptin, 0.4 \(\mu\)g/ml; pepstatin, 0.4 \(\mu\)g/ml; trypsin inhibitor, 0.1 \(\mu\)g/ml; phenylmethane sulfonil fluoride, 0.1 mM; tosyl-lysine chloromethyl ketone.

Abbreviations: PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; SEM, standard error of the mean.
Precipitated enzyme, collected by centrifugation, was resolubilized in dialysis buffer and reprecipitated with solid ammonium sulfate with stirring to 35% saturation. The precipitated enzyme was further purified by the method of Hardie and Cohen (19, 20) except that dialysis (against 0.1 M potassium phosphate, 25 mM citrate, 15 mM β-mercaptoethanol buffer, pH 7.0, containing the proteolytic enzyme inhibitors used in the homogenization media at the same concentrations) was for 4 instead of 6 hr. PEG-6000 was used at a final concentration of 5% to precipitate the enzyme. The precipitated enzyme, collected by centrifugation, was resolubilized in dialysis buffer and reprecipitated with 5% PEG (final concentration). After a third PEG precipitation, the enzyme was resolubilized in dialysis buffer and, after insoluble protein was removed by centrifugation, the supernatant was chromatographed at 0–4°C on a Sepharose-6B column equilibrated with 0.12 M Tris-acetate, 0.2 M potassium acetate, 2 mM potassium citrate, and 15 mM β-mercaptoethanol, pH 7.5, and eluted with the same buffer. Fractions were collected and assayed for acetyl CoA carboxylase activity using a coupled spectrophotometric assay (21). Fractions with a specific activity greater than 4 U/mg protein (coupled assay, 25°C) were combined and dialyzed overnight at 0–4°C against column buffer to which glyceraldehyde phosphate (final concentration, 10%) had been added. The dialyzed enzyme was stable when stored frozen for several months. The specific activity of seven different rat liver preparations, assayed by radioactive bicarbonate into acid-stable form in 1 min (mean ± SEM) U/mg of protein. Biotin analysis gave a value of 4.25 ± 0.21 (n = 4) nmol of biotin/mg of protein. This compares well with the value of 4.23 nmol/mg protein reported by Inoue and Lowenstein (22) for purified rat liver enzyme of specific activity 14 units/mg protein. Other analyses

Acetyl CoA carboxylase assay

Acetyl CoA carboxylase activity was measured in the phosphorylation experiments by the conversion of radioactive bicarbonate into acid-stable form in 1 min (18). A unit of enzyme activity is defined as the conversion of 1 μmol/min of radioactive bicarbonate to an acid-stable form.

Enzyme phosphorylation

A concentrated solution of ATP-magnesium acetate, neutralized with Tris, was added to nine volumes of purified enzyme in the final dialysis buffer to give a final concentration of either 0.2 mM or 1.0 mM ATP (as indicated in the results) and 2 mM magnesium acetate. A sample was taken immediately for assay of acetyl CoA carboxylase activity (0 time) before incubation at 37°C. Samples were taken at intervals indicated in the figures for acetyl CoA carboxylase activity determination. Control tubes had an equivalent amount of water added in place of the ATP-magnesium acetate solution.

Identical phosphorylation reaction mixtures were used for measurements of phosphate incorporation except that [γ-32P]ATP was added to give a specific activity of 200 to 300 cpm per pmol ATP. Control tubes had radioactive phosphate but no enzyme. Duplicate aliquots (20 μl) were removed at indicated times after the addition of radioactive ATP and placed on a 1-cm square of Whatman ET-31 filter paper that was immersed in 10% TCA as suggested by Hathaway et al. (25). The filter papers were washed with four changes of TCA, then with petroleum ether, and finally acetone. Acetone was evaporated in an oven at 80°C and the filter papers were placed in 10 ml of scintillation solution (18) and assayed for radioactivity.

Other analyses

Protein was determined by dye binding (26) using bovine serum albumin as a standard. Biotin was determined by a modification of the radioactive biotin-avidin method (27). ATPase activity was determined by measuring radioactive inorganic phosphate extracted as the phosphomolybdic acid complex (28) from an aliquot of the supernatant of the phosphorylation reaction mixture after TCA precipitation of protein. Activities of commercial preparations of cyclic-AMP-dependent protein kinase catalytic subunit (29) and the inhibitor of this subunit (30) were assayed as described above for acetyl CoA carboxylase phosphorylation except that histone (Type Iib, 2 mg/ml) was used as the substrate.

Reagents

Biochemicals were purchased from Sigma Chemical Co., St. Louis, except that radioactive ATP was obtained from New England Nuclear, Boston, and radioactive biotin was from Amersham Corporation, Arlington Heights, IL. Trifluoperazine was generously supplied by Smith, Kline, and French Laboratories, Philadelphia. Purified rabbit mammary gland fatty acid

Electrophoresis

SDS and β-mercaptoethanol were added to the enzyme to give a final concentration of 2.0% and 75 mM, respectively. The samples were placed in a boiling water bath for 4 min and aliquots were then subjected to SDS-5% polyacrylamide disc (23) or slab (24) gel electrophoresis.

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synthetase used as a molecular weight marker was given to us by A. D. McCarthy and D. G. Hardie. The catalytic subunit of phosphorylase phosphatase was purified from rat liver through the second ammonium sulfate precipitation step of the method of Brandt, Capulong, and Lee (31).

RESULTS AND DISCUSSION

When purified acetyl CoA carboxylase was incubated with \([\gamma^{32}P]ATP\), acid-precipitated protein contained increasing amounts of radioactive phosphate over time up to 15 min (Fig. 1), which indicates that this preparation contained a protein kinase. There was an increasing loss of catalytic activity over the same time period. Control enzyme, incubated in the absence of ATP, lost a very small amount of activity. This active, endogenous protein kinase was shown not to be previously activated cyclic-AMP-dependent protein kinase catalytic subunit since the addition of purified inhibitor of this catalytic subunit (30) did not affect either phosphorylation or activity changes under our assay conditions.

In addition to a cyclic-AMP-independent protein kinase, this enzyme preparation apparently also contained cyclic-AMP-dependent protein kinase since the addition of cyclic-AMP increased the incorporation of radioactive phosphate into acid-precipitated protein. In contrast to the effect of the cyclic-AMP-independent protein kinase, protein phosphorylation catalyzed by the cyclic-AMP-dependent enzyme was not accompanied by a further decrease in acetyl CoA carboxylase catalytic activity. Similarly prepared purified acetyl CoA carboxylase from rabbit mammary gland has been shown to contain both endogenous cyclic-AMP-dependent and -independent protein kinases but their effects on activity were not reported (20).

Since the phosphorylation data in Fig. 1 were based on the incorporation of radioactive phosphate into acid-precipitated protein, it was important to establish that it was indeed acetyl CoA carboxylase undergoing phosphorylation rather than a contaminant of the preparation. The data in Fig. 2 show that the major radioactive peak coincided with acetyl CoA carboxylase stained with Coomassie Brilliant Blue. The recovery of radioactive phosphate on the gels was within 5% of that expected from the radioactivity in acid-precipitated protein. Protein staining showed that there were two protein bands but since these were separated by less than 2 mm (Fig.

Fig. 1. Phosphorylation and activity changes of purified acetyl CoA carboxylase incubated with ATP-Mg++. Purified rat liver acetyl CoA carboxylase (0.6 mg/ml, final concentration) was incubated with water (Δ) or an equivalent volume of ATP-magnesium acetate to produce final concentrations of 0.2 mM and 2.0 mM, respectively. Catalytic activity (solid lines) was measured at the indicated times. Phosphate incorporation into acid-precipitated protein (dashed lines) was measured at the same time points in an identical reaction mixture except that it included [\(\gamma^{32}P\)]ATP. Additions were: none (○); 10 U/ml (final concentration) cyclic-AMP-dependent protein kinase catalytic subunit inhibitor (X); and 0.05 mM (final concentration) cyclic AMP (○).
Dye Front

Fig. 3. Radioautography of phosphorylated acetyl CoA carboxylase. Acetyl CoA carboxylase was phosphorylated as in Fig. 2 and subjected to slab SDS-PAGE which, after drying, was exposed to Kodak XRP-1 X-ray film.

2), slicing the gels into 2-mm segments did not give sufficient resolution to determine whether both hands contained radioactive phosphate. Subsequent experiments in which the phosphorylated proteins were subjected to electrophoresis on slab gels followed by radioautography (Fig. 3) showed that both bands were labeled and confirmed that, except for a very light band corresponding to fraction 15, Fig. 2, the acetyl CoA carboxylase bands were the only ones labeled. The reason for the double band observed with purified acetyl CoA carboxylase has yet to be established. It is consistently observed by us on disc or slab gels and has been reported by others (20). When purified rabbit mammary gland fatty acid synthetase was used as a marker [subunit molecular weight 250 kdalton (19)], its migration was between that of the two acetyl CoA carboxylase bands.

In the phosphorylation experiment shown in Fig. 1 as well as in several other experiments using different enzyme preparations, phosphorylation and loss of catalytic activity reached a plateau after about 20 min even though considerable acetyl CoA carboxylase activity remained. In these experiments, the concentration of ATP used in the phosphorylation reaction was 0.2 mM since much higher concentrations of ATP are reported to inhibit phosphorylation of acetyl CoA carboxylase (15). On the other hand, acetyl CoA carboxylase can act as an ATPase in the presence of small amounts of bicarbonate present in buffers, presumably because of the ATP-dependent half-reaction of carbon dioxide fixation to the enzyme followed by dissociation of the biotin-bound carbon dioxide in the absence of acetyl CoA (28). Thus, it was considered possible that depletion of ATP by ATPase activity could have limited protein phosphorylation and loss of acetyl CoA carboxylase activity. Consequently a similar experiment was conducted with an ATP concentration of 1.0 mM (Fig. 4). As in the experiment in Fig. 1, there was an increase in protein phosphorylation catalyzed by the endogenous protein kinase and a decrease in catalytic activity of acetyl CoA carboxylase over time, both of which leveled off after 20 min. It should be noted that in this experiment the effect of cyclic-AMP-dependent protein kinase on phosphorylation and loss of acetyl CoA carboxylase activity was determined by adding purified cyclic-AMP-dependent protein kinase catalytic subunit rather than activating the endogenous enzyme by the addition of cyclic-AMP. As shown in Fig. 4, the purified catalytic subunit catalyzed additional phosphate incorporation but did not further affect acetyl CoA carboxylase activity, in agreement with the results of the experiment shown in Fig. 1.

Inorganic phosphate released, measured during the phosphorylation reaction, showed that there was substantial ATPase activity (Fig. 4) which resulted in the hydrolysis of about 50% of the initial ATP added by the end of 40 min. It was apparent from the data in Fig. 4, however, that the loss of ATP through hydrolysis did not account for the plateaus observed in protein phosphorylation or loss of acetyl CoA carboxylase catalytic activity since the quantity of ATP not accounted for by hydrolysis was higher even after 40 min than the initial concentration of ATP in the experiment shown in Fig. 1. The plateau in phosphorylation is not likely due to the production of ADP by the ATPase since ADP is
reported to increase phosphorylation of acetyl CoA carboxylase, at least with partially purified enzyme (15). If our purified enzyme preparation contained endogenous protein phosphatase as well as protein kinase, the two enzymes together could act as an ATPase and, if protein phosphorylation-dephosphorylation reached a steady state after 20 min, this could explain the plateaus observed. However, the purified enzyme preparation did not contain measurable amounts of protein phosphatase activity as determined with \(^{32}\)P-labeled phosphorylase "a" as a substrate (data not shown). It is possible that if a protein phosphatase were present, phosphorylase "a" may not be a usable substrate or the protein phosphatase may be so tightly bound that it would not dephosphorylate any added substrate.

Since ADP is formed by ATPase during the phosphorylation reaction, the possibility must be considered that the loss of catalytic activity could be due to ADP inhibition of acetyl CoA carboxylase rather than protein phosphorylation. However, the addition of either ATP, ADP, or combinations of the two to purified acetyl CoA carboxylase with 2 mM magnesium acetate immediately prior to enzyme activity assay, in proportions that would occur during the phosphorylation reaction, had no effect on acetyl CoA carboxylase activity (data not shown). More direct evidence that the loss of catalytic activity was related to protein phosphorylation was obtained by the experiment shown in Fig. 5 in which the reversibility of the phosphorylation reaction was demonstrated. Purified acetyl CoA carboxylase was incubated with either radioactive ATP or ATP as in Fig. 1 and protein phosphorylation and loss of acetyl CoA carboxylase activity was measured over time. After 20 min, the reaction mixture was treated with Sephadex G-25 to remove unreacted ATP. Phosphorylase phosphatase catalytic subunit (15 U/ml, final concentration) was added and changes in catalytic activity and protein phosphorylation were again measured (panel B). The data in panel B were corrected for the 10% loss of protein during the Sephadex G-25 treatment and for dilution by the phosphatase preparation. The inset in each panel shows the section of a radioautograph corresponding to acetyl CoA carboxylase when a sample of the reaction mixture taken at each assay time-point during phosphorylation (panel A) and dephosphorylation (panel B) was subjected to SDS-PAGE.

Fig. 5. Reversible inactivation of acetyl CoA carboxylase by phosphorylation. Purified acetyl CoA carboxylase (1 mg/ml, final concentration) was incubated with ATP or \([\gamma^{32}\text{P}]\)ATP-Mg\(^{++}\) as in Fig. 1 and the changes in acetyl CoA carboxylase activity (solid line) and \(^{32}\)P incorporated into acid-precipitated protein (dashed line) were measured over a 20-min period (panel A). The phosphorylation reaction mixture was then treated with Sephadex G-25 to remove unreacted ATP. Phosphorylase phosphatase catalytic subunit (15 U/ml, final concentration) was added and changes in catalytic activity and protein phosphorylation were again measured (panel B). The data in panel B were corrected for the 10% loss of protein during the Sephadex G-25 treatment and for dilution by the phosphatase preparation. The inset in each panel shows the section of a radioautograph corresponding to acetyl CoA carboxylase when a sample of the reaction mixture taken at each assay time-point during phosphorylation (panel A) and dephosphorylation (panel B) was subjected to SDS-PAGE.
Purified acetyl CoA carboxylase was incubated with \([\gamma-^{32}P]ATP-Mg^{2+}\) as described in Fig. 1 with additions at final concentrations indicated. Radioactive phosphate incorporated into acid-precipitated protein was measured at 0, 3, and 6 min, and phosphorylation rates were calculated.

cyclic-AMP-independent protein kinase that can phosphorylate and inactivate acetyl CoA carboxylase. It was recently reported that a calcium-dependent protein kinase, activated through the \(\alpha\)-adrenergic receptor system of rat hepatocytes can phosphorylate and inactivate acetyl CoA carboxylase (10). However, initial rates of phosphorylation of acetyl CoA carboxylase by the endogenous protein kinase in our preparation were found to be unaffected by a calcium chelator, EGTA, or a calmodulin antagonist, trifluoperazine (Table 1). As in previous experiments, the addition of an inhibitor of the cyclic-AMP-dependent protein kinase catalytic subunit did not affect initial phosphorylation rates while the purified catalytic subunit increased the rate of phosphorylation. Further, EGTA was found not to affect the rate or extent of phosphorylation of acetyl CoA carboxylase over a 30-min incubation period and had no effect on the loss of acetyl CoA carboxylase activity (data not shown). These data indicate that the endogenous protein kinase is neither calcium- nor cyclic-AMP-dependent.

Two previous reports have appeared concerning a liver protein kinase that will phosphorylate and inactivate acetyl CoA carboxylase (32, 33); its properties appear to be similar to the endogenous cyclic-AMP-independent enzyme present in our purified acetyl CoA carboxylase. Our data indicate that this endogenous protein kinase catalyzes the phosphorylation of a regulatory site of acetyl CoA carboxylase. If it is assumed that the phosphorylated enzyme is inactive (34), the stoichiometry of the phosphorylation can be calculated on the basis of the change in specific activity per nmol of phosphate incorporated. The results of such calculations from four different experiments gave a value of 1.95 \pm 0.2 mol (mean \pm SEM, \(n = 14\)) of phosphate per 480 kdalton protomer. Cyclic-AMP-dependent protein kinase catalytic subunit almost doubled phosphate incorporation into protein (Figs. 1 and 3) and therefore likely catalyzes the phosphorylation of a second site that is nonregulatory under the conditions of our assay. It is not known whether cyclic-AMP-dependent protein kinase could catalyze the phosphorylation of the regulatory site in the absence of the endogenous protein kinase as it does in rat mammary gland preparations (17) because we have yet to obtain an active preparation of acetyl CoA carboxylase devoid of this endogenous enzyme. In our hands, even acetyl CoA carboxylase purified using an avidin affinity column (34) contained measurable acetyl CoA carboxylase kinase activity (data not shown). If this endogenous protein kinase were active in vivo, it would be expected to severely limit lipogenesis. If however, it is artifically released and/or activated during tissue homogenization, its activity may mask the effect of cyclic-AMP-dependent protein kinase and account for the discrepancy between the effect of cyclic-AMP in intact cells compared to broken-cell preparations. The origin and regulation of this endogenous protein kinase is clearly of interest but has yet to be established.

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