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THE PURIFICATION AND PROPERTIES OF A PROTEIN KINASE AND THE PARTIAL PURIFICATION OF A PHOSPHOPROTEIN PHOSPHATASE THAT INACTIVATE AND ACTIVATE ACETYL-CoA CARBOXYLASE

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SUMMARY

The occurrence of a soluble fraction from rat liver that inactivates acetyl-CoA carboxylase was previously reported by this laboratory (1). The purification of this fraction is now reported, and we show that it behaves as a cAMP-independent kinase that inactivates acetyl-CoA carboxylase by phosphorylation. The kinase has a molecular weight of 160,000 and it requires ATP and Mg^{2+} for activity. A partial purification from rat liver cytosol of a Mg^{2+}-requiring phosphoprotein phosphatase of high molecular weight (greater than 200,000) which dephosphorylates phosphorylated acetyl-CoA carboxylase with the regeneration of enzyme activity is also reported. The kinase, phosphatase, and acetyl-CoA carboxylase are separable from each other by a combination of ammonium sulfate precipitation, DEAE-cellulose chromatography, and gel filtration.

Recently the phosphorylation-dephosphorylation of acetyl-CoA carboxylase (EC 6.4.1.2) and the ensuing effect on enzymatic activity have been studied (2-8). A number of the reported studies have been with tissues other than rat liver (4-7). Other studies by Carlson and Kim described a crude preparation from rat liver that phosphorylated and inactivated acetyl-CoA carboxylase but the putative kinase responsible for this action has not been further purified (2,3). Other soluble cAMP-independent kinases have been purified from rat liver, but these have not been tested on acetyl-CoA carboxylase (9,10). Likewise, dephosphorylation of acetyl-CoA carboxylase has been reported as occurring in crude liver extracts (2,3) or by preparations of phosphatase isolated from other tissues (6) or by a low molecular weight subunit of native enzyme (11).

The occurrence of a soluble fraction from rat liver that inactivates acetyl-CoA carboxylase was previously reported by this laboratory (1). The purification of this fraction is reported herein, and we show that it behaves as a cAMP-independent kinase that inactivates acetyl-CoA carboxylase by phosphorylation. Also reported is the partial purification from rat liver cytosol of a

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phosphoprotein phosphatase which dephosphorylates acetyl-CoA with the regeneration of enzyme activity.

MATERIALS AND METHODS

Acetyl-CoA carboxylase and the protein kinase and phosphatase that act on this enzyme were prepared from livers of male Holtzman rats (200 to 220 g) that were starved for 48 hr and then refed a fat-free diet (15750, United States Biochemical Corp.) for 48 hr. All operations in the isolation and purification of these enzymes were carried out at 4° except for the chromatographic separations. After homogenization in 50 mM Tris chloride, 20 mM potassium citrate, 5 mM β-mercaptoethanol, and 0.5 mM EDTA (buffer A) (pH 7.4), the homogenate was subjected to a low- (13,000 x *g*) and a high-speed (100,000 x *g*) centrifugation. The cytosolic supernatant solution was then used to prepare each of the three enzymes.

Acetyl-CoA carboxylase was purified by a modification of the procedure of Inoue and Lowenstein (12) in which ammonium sulfate precipitation (0 to 30% saturation, followed by a 0 to 25% saturation of the resuspended precipitate), DEAE-cellulose chromatography, and Sepharose 4B gel filtration were used. The final preparation had a specific activity of 10 units/mg protein (assayed at 30°). SDS gel electrophoresis of this purified enzyme showed a single band of protein of a molecular weight of 240,000. A complete report of this purification procedure will be published elsewhere.

The kinase was purified from the supernatant solution of the first ammonium sulfate precipitation (0 to 30% saturation) by DEAE-cellulose chromatography as reported previously (1). A second identical DEAE-cellulose chromatographic step was carried out, but in this step the buffer B (1) contained no citrate or glycerol (buffer B′). The peak tubes of kinase activity were again pooled, concentrated, and dialyzed in buffer B′. After dialysis the crude kinase was centrifuged and 4 ml aliquots (20 to 40 mg protein) were added to separate 2.3 x 60 cm Sephacryl S-300 superfine (Pharmacia) columns. Elution of protein from each column proceeded at the rate of 45 ml/hr and 4.0 ml fractions were collected. The peak tubes of kinase activity were pooled, dialyzed in 25 mM Tris chloride, 5 mM β-mercaptoethanol, and 0.5 mM EDTA (buffer D) (pH 7.4), and then added to a 0.7 x 5.5 cm ADP-hexylagarose (P-L Biochemicals) column that was washed and preequilibrated with the above buffer (but modified to contain 2 mM $MgCl_2$). After all of the kinase was bound and the contaminating protein was washed from the column, an eluting buffer, buffer D, modified with 2 mM $MgCl_2$ and 0.5 M NaCl was added. Fractions of 3 ml (45 ml/hr) were collected. The eluted kinase was concentrated and dialyzed in buffer A by vacuum dialysis in collodion bags (Schleicher and Schuell).

The phosphatase can be isolated from the cytosolic fraction from which acetyl-CoA carboxylase and kinase have been removed. However, livers from animals that were fasted for one day and then refed a fat-free diet for one day were generally used for this purpose. Livers were blended (4 x 5 sec, full speed) in a Waring blender and then homogenized (3 strokes in a Potter-Elvehjem glass homogenizer) in buffer A containing no citrate (buffer A′) (2:1 v/w). After low- (13,000 x *g*) and high-speed (100,000 x *g*) centrifugations, the filtered supernatant solution was brought to 40% saturation with ammonium sulfate. After removal of the precipitate by centrifugation, the resulting supernatant solution was brought to 65% saturation with ammonium sulfate. The precipitate was collected by centrifugation and resuspended in buffer A′. The protein concentration of this solution was adjusted to 20 to 30 mg of protein/ml and the pH of the solution was lowered to 5.8 by slowly adding 1 N acetic acid. After cen-

trifugation the clear supernatant solution was raised to pH 7.4 with 0.1 M Tris base and the soluble protein was dialyzed against several changes of buffer A′. The dialyzed solution was added to a DEAE-cellulose column (DE 23 from Whatman and acid-base washed according to their specifications) of 2.4 x 25 cm that had been washed and equilibrated with buffer A′. Nonbinding protein was washed from this column until the absorbance was less than 0.01. Phosphatase activity was then eluted from the column by application of a linear gradient (each 3 x the column void volume) of buffer A′ and buffer A′ containing 0.5 M NaCl. Two closely overlapping peaks of activity for dephosphorylation of acetyl-CoA carboxylase were eluted from the column and then separately pooled and dialyzed in buffer A′ to remove NaCl.

Acetyl-CoA carboxylase activity is determined by a modification of the coupled fatty acid synthetase-acetyl-CoA carboxylase spectrophotometric system of Numa (13) that we have described in detail elsewhere (14). The production of malonyl-CoA by acetyl-CoA carboxylase is coupled with the oxidation of NADPH by the fatty acid synthetase complex. Kinase or phosphatase modulation of the activity of acetyl-CoA carboxylase activity can be followed and quantitated by adding either of these enzymes before or after the substrates of the coupled assay system have been added. Pigeon liver fatty acid synthetase was used in the coupled spectrophotometric assay and it was purified according to the procedure of Muesing and Porter (15).

Immunoglobulin to acetyl-CoA carboxylase was raised and purified according to the methods of Livingston (16). The immunoprecipitation procedures of Lornitzo *et al.* (14) were followed to remove acetyl-CoA carboxylase from an incubation mixture. In these assays the immunoglobulin acetyl-CoA carboxylase mixture was incubated at 37° for 1 hr, followed by 16 hr at 4°, centrifuged, and the precipitate was analyzed for radioactivity.

The radioactive labeling experiments utilized [γ^{32}P]ATP, 5 to 15 μCi/μmole (Amersham), and they were carried out in buffer A that was modified to 5 mM potassium citrate, 5 mM Mg^{2+}, and 2 mM ATP. Further details of some of the individual experiments utilizing [^{32}P]ATP are reported in Shiao *et al.* (17). Protein determinations were made according to the methods of Gornall (18), Lowry (19), and Bradford (20). The gel electrophoresis procedures that were followed were those of Laemmli (21).

The specific activities of the enzymes are expressed in units/mg. One unit of acetyl-CoA carboxylase activity corresponds to 1 μmole of malonyl-CoA produced per min. One unit of kinase or phosphatase activity is equal to one unit of acetyl-CoA carboxylase inactivated or reactivated, respectively.

RESULTS AND DISCUSSION

Previous investigations of the inactivation of acetyl-CoA carboxylase utilized an inactivator that was subjected to a single DEAE-cellulose chromatographic step (1). A similar second step which removes additional contaminating protein and results in less interference in the coupled spectrophotometric assay is used in the present procedure. Sephacryl S-300 gel filtration further purifies the kinase to an activity of 0.5 unit/mg of protein (Fig. 1). The ADP-affinity column binds the kinase at low ionic strength (Fig. 2), and the traces of phosphatase activity that were present with the kinase added to this column

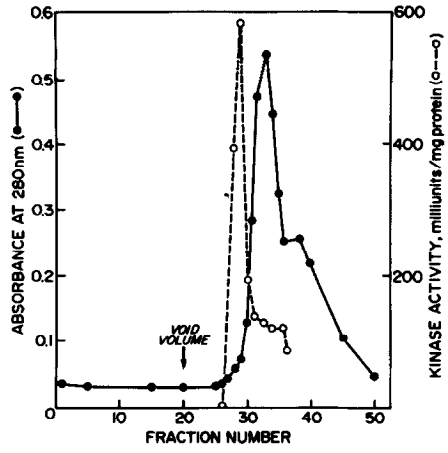

Fig. 1. Sephacryl S-300 gel filtration of the kinase. Two ml (7.5 mg of protein/ml) of kinase activity that were concentrated from the second DEAE-cellulose chromatographic step were added to a Sephacryl S-300 column (2.3 x 60 cm). Protein was eluted from the column with buffer B′ and fractions of 4 ml were collected at a rate of 0.75 ml/min. Fractions were assayed for absorbance at 280 nm and kinase activity as described in the section on Materials and Methods.

are found in the unbound fractions. No kinase activity is found in these fractions. The pattern of elution of kinase activity from Sephacryl S-300 or Sepharose 4B gel filtration shows an apparent molecular weight of 160,000 to 200,000. A heavy band corresponding to 90,000 molecular weight along with two lighter bands of 40,000 and 60,000 daltons are seen on SDS gel electrophoresis (Fig. 3). The latter two bands vary in intensity in different preparations.

Incorporation of phosphate into acetyl-CoA carboxylase by the kinase is demonstrated with [γ^{32}P]ATP (17). This incorporation of radioactivity into the enzyme approaches a maximum between 2 and 5 min after kinase addition. The inactivation of acetyl-CoA carboxylase observed in the spectrophotometric assay is also extremely fast, and a rapid decrease in absorbance is observed if kinase is added to an acetyl-CoA carboxylase reaction already underway. Adding more acetyl-CoA carboxylase after the plateau of phosphorylation has been reached results in a new surge of phosphorylation of protein. Immunoprecipitation of [^{32}P]acetyl-CoA carboxylase indicates that 4 to 5 moles of phosphate are incorporated per mole of enzyme protomer of molecular weight of 480,000.

Some phosphorylation of kinase occurs as there is TCA-precipitable radioactivity present in the supernatant after the immunoprecipitated protein is removed (17). Based on a 160,000 molecular weight for the kinase, 0.1 to 0.2 moles of phosphate are bound per mole of kinase. The phosphorylation of immuno-

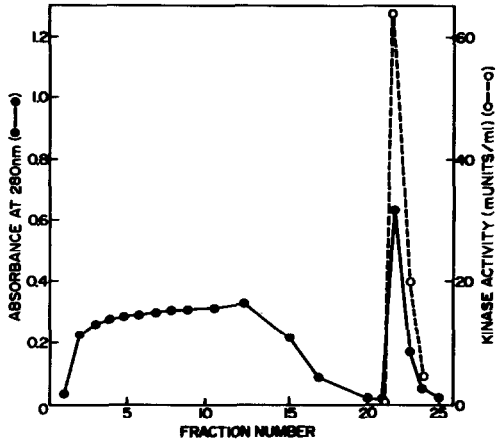

Fig. 2. ADP-hexylagarose affinity chromatography of the kinase. Purified kinase, 20 mg of protein, from the Sephacryl S-300 column was added to an agarose-hexyl-adenosine 5′-diphosphate affinity column (0.7 x 5.5 cm). The column was washed, eluted, and fractions were assayed for absorbance at 280 nm and kinase activity as described in the section on Materials and Methods.

globulin and bovine serum albumin controls ranged from 0.02 to 0.07 moles of phosphate per mole of protein.

Acetyl-CoA carboxylase and kinase activities are separated from each other by Sepharose 4B gel filtration, Fig. 4. The molecular weight of the phosphorylated enzyme is not that of the polymeric form, however, but that of the protomeric enzyme having a molecular weight of 480,000. This result is in agreement with Lent *et al.* (22) who previously reported the depolymerization of phosphorylated acetyl-CoA carboxylase. SDS gel electrophoresis of this antibody-precipitated phosphorylated acetyl-CoA carboxylase demonstrates that the radioactivity comigrates with authentic enzyme (17). The elution of phosphorylated kinase on gel filtration coincides with that of kinase activity of the unphosphorylated enzyme. Only one band of radioactivity, which corresponds to that of the kinase, appears upon neutral gel electrophoresis of the phosphorylated kinase fraction concentrated after elution from Sepharose 4B.

The partial purification of the phosphatase that we report avoids the use of an ethanol treatment which dissociates high molecular weight phosphatases to a catalytic subunit of 35,000 (23). The molecular weight range of phosphatase activity of our most active fraction of DEAE-cellulose purified enzyme is between 200,000 and 250,000, as judged from Sepharose 4B gel filtration.

In the routine spectrophotometric assay of phosphatase activity, acetyl-CoA carboxylase is first phosphorylated and inactivated by kinase. The dephosphorylation is then followed by the rate of change in light absorption at 340 nm. There is no effect of phosphatase on either pure fatty acid synthe-

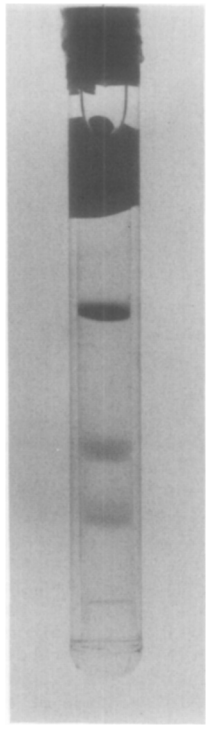

Fig. 3. SDS gel electrophoresis of the kinase. Twenty μg of protein of the peak fraction of kinase activity obtained from an ADP-affinity chromatography column were subjected to electrophoresis in a 7.5% polyacrylamide gel according to the procedure of Laemmli (21). Protein bands were stained with Coomassie blue.

tase or pure acetyl-CoA carboxylase. Dephosphorylation of phosphorylated acetyl-CoA carboxylase occurs very rapidly (17). Only 0.1 mole of [^{32}P]Pi per mole of enzyme protomer, as measured by immunoprecipitation, remains after incubation of the phosphorylated enzyme with phosphatase for 3 min.

The possibility of an acetyl-CoA carboxylase-ATP-Mg^{2+} complex formation is eliminated by using high dilution technique and G-25 gel filtration after incubation of the enzyme with ATP-Mg^{2+}. Inactivation of acetyl-CoA carboxylase occurs only in the presence of the kinase. Similar experiments have shown that Mg^{2+} is necessary for enzyme reactivation in the presence of added phosphatase. Fluoride treatment of phosphorylated acetyl-CoA carboxylase also prevents phosphatase reactivation of acetyl-CoA carboxylase.

Thus the results presented here report the first purification of a rat cytosolic kinase that inactivates acetyl-CoA carboxylase by phosphorylation and the partial purification of a high molecular weight phosphatase which reactivates this enzyme by dephosphorylation. Further investigations on the

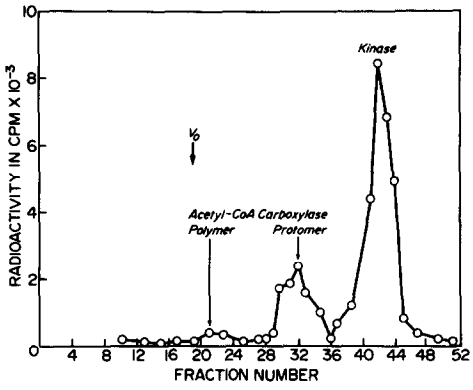

Fig. 4. Separation of phosphorylated acetyl-CoA carboxylase and kinase by gel filtration. Purified acetyl-CoA carboxylase (20.5 pmoles) and Sephacryl S-300 purified kinase (80 milliunits) were incubated with Mg^{2+} and [γ^{32}P]-ATP (11 μCi/μmole). After one and three-fourths min the reaction was stopped with EDTA (final concentration, 10 mM). Approximately one-third of the incubation mixture was added to a 0.9 x 26 cm Sephadex G-25 column to remove unreacted [^{32}P]ATP and Mg^{2+}. Three ml of eluate-containing protein were pooled, and purified acetyl-CoA carboxylase was added, and this mixture was then added to a Sepharose 4B column (1.5 x 70 cm). The column was eluted and acetyl-CoA carboxylase activity and radioactivity were determined on eluate fractions as described in the section on Materials and Methods. Previously, purified acetyl-CoA carboxylase and purified kinase were charged separately and together to the same column and the positions of elution of these activities were found by assay.

mechanisms of kinase and phosphatase actions and on their activities in animals in different nutritional and hormonal states as a means of short-term control of acetyl-CoA carboxylase *in vivo* are in progress.

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