Isolation and Characterization of a Novel Acetyl-CoA Carboxylase Kinase from Rat Liver*

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Acetyl-CoA carboxylase is regulated allosterically by citrate and covalently by a phosphorylation/dephosphorylation mechanism. We have isolated and purified from rat livers a novel kinase that phosphorylates and inactivates the carboxylase. This kinase is bound to the carboxylase and can be eluted in salt-rich solution. The native kinase exists as high molecular weight aggregates of a subunit that has a molecular weight of 40,000. The phosphorylation sites of the carboxylase were determined after tryptic and cyanogen bromide digestions of 32P-labeled carboxylase and separation of the peptides by various chromatographic procedures. Amino acid analyses of the phosphopeptides showed that the Ser77 and Ser1200 residues were the sites of phosphorylation. Treatment of the phosphorylated carboxylase with the Mn2+-dependent acetyl-CoA carboxylase phosphatase 2 removed the phosphate and reactivated the carboxylase. These results suggest that both this kinase and the acetyl-CoA carboxylase phosphatase 2 act at the same site(s) in the acetyl-CoA carboxylase molecule. Citrate dramatically inhibits the kinase-mediated phosphorylation of the carboxylase, suggesting that the allosteric modification and activation by citrate render the phosphorylation sites inaccessible to the kinase and therefore maintain high carboxylase activity. This observation indicates that there is a close interplay between the citrate effect on and phosphorylation of the carboxylase in regulating its activity.

Acetyl-CoA carboxylase (ACC), a cytoplasmic enzyme, catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, which is the rate-limiting step in the synthesis of long-chain fatty acids (1). Citrate, which is produced in the mitochondria and transported to the cytoplasm, is the precursor of acetyl-CoA and acts as a feed-forward allosteric activator of ACC (2). The mode of citrate activation is not clear; however, its action does lead to polymerization of the protein into aggregates with molecular weights greater than 105 (3). The carboxylase is also regulated by covalent modification of the protein by a phosphorylation/dephosphorylation mechanism. Several kinases have been found to phosphorylate the protein and reduce its activity (4). Kim and co-workers (5) isolated from rat liver a CAMP-independent kinase that phosphorylates and inactivates ACC. The phosphorylation mediated by this kinase is stimulated by CoA (5). Hardie and co-workers (6–10) also have employed many different protein kinases to phosphorylate ACC. The most notable of these are an AMP-activated protein kinase from rat livers and another protein kinase, acetyl-CoA carboxylase kinase 2, from rat mammary glands (10). The AMP-activated kinase is reported to have a molecular weight of 63,000 (8) and is not specific for ACC since it phosphorylates the β-hydroxy-β-methylglutaryl-CoA reductase, the hormone-sensitive lipase/cholesterol esterase, and possibly other enzymes (9, 11). Both kinases phosphorylate and inactivate ACC and are cAMP-independent enzymes (10). However, phosphorylation of the carboxylase by acetyl-CoA carboxylase kinase 2 changes the kinetic properties of the carboxylase in ways that are similar to those caused by phosphorylation mediated by the CAMP-dependent kinase (10). The AMP-activated protein kinase phosphorylates Ser77 (site 3), Ser1200 (site 1), and possibly Ser1215 (11), whereas cAMP-dependent protein kinase and CAMP-independent acetyl-CoA carboxylase kinase 2 both phosphorylate Ser77 (site 2) and Ser1200 (4, 10, 12). Protein kinase C can also phosphorylate the Ser77 of the ACC molecule (4, 10, 12). These three sites (Ser77, Ser79, and Ser1200) apparently are linked to the activity modulation of ACC (4, 9, 12).

In our studies of the regulation of ACC by phosphorylation, we noted that affinity-purified ACC undergoes "autophosphorylation" independent of added kinase. Further investigation revealed that this "autophosphorylation" varied from one preparation to another, suggesting the presence of an ACC-bound kinase. Herein, we report the isolation and characterization of a novel protein kinase from rat livers that phosphorylates and inactivates ACC. The phosphorylation of ACC mediated by this kinase is dramatically inhibited by citrate. This kinase does not require CAMP, AMP, or CoA. We propose that this kinase, bound to its substrate ACC, is involved in the activity modulation of ACC both in vivo and in vitro.

EXPERIMENTAL PROCEDURES

Materials—Sprague female retired breeder rats were purchased from Harlan Co. Polyethylene glycol (PEG) 8000, phenylmethanesulfon-yl fluoride, biotin, phosphorylase b, histone, benzamidine, leupeptin, aprotonin, trypsin inhibitor, CAMP protein kinase (from porcine heart), cAMP protein kinase peptide inhibitor, CAMP, and all other chemicals and biochemicals were purchased from Sigma. [14C]Carbonate and ammonium sulfate were purchased from ICN Biochemicals, and [32P]ATP from DuPont NEN or Amersham Corp.

Preparation of Acetyl-CoA Carboxylase—ACC was prepared from the livers of fasted and refed rats as described previously (13) with the following modifications: ACC was precipitated at 5.5% PEG instead of 5%, and the protein was precipitated at 40% saturation of ammonium sulfate. The unbound (avidin flow-through) material was collected before the column was washed and saved as the source of carboxylase kinase (see below).

Acetyl-CoA Carboxylase Assay—ACC was assayed by using a modification of the [14C]Carbonate fixation assay as described by Thampany and Wakil (14). One unit of activity is defined as 1 nmol of malonyl-CoA formed/min at 37°C. Specific activity is defined as 1 unit/mg protein.

Carboxylase Inactivation Assay—The inactivation assay mixture (1 ml) contained 50 mm HEPES (pH 7.5), 10 mm MgCl2, 1 mm dithio-
threitol, 1 mg of bovine serum albumin, 0.1 mm ATP, and 20 μg of affinity-purified ACC. The reaction was started by adding 0.1-5 μg of kinase to the mixture. After incubating the mixture for 30-60 min at 37 °C, an aliquot containing 0.2 μg of ACC was removed and assayed for carboxylase activity by using the procedure described above.

Carboxylase Phosphorylation Assay—Phosphorylation of ACC in the presence of γ-[32P]ATP and kinase was performed as described for the inactivation assay, except that bovine serum albumin was omitted and 250 μg of affinity-purified ACC, 5 μg of kinase, and 0.1 μm of γ-[32P]ATP (1 μCi/μmol) were used. After incubation, the reaction was stopped by adding Laemmli solubilization buffer (15). The ACC was separated in 5% SDS-PAGE (13) and visualized by autoradiography. For quantitation, the carboxylase band was excised from the dried gel, cut into pieces, soaked in a solution containing 0.5 ml of water and 4.5 ml of scintillation fluid, and the radioactivity was determined. Alternatively, after the ACC was incubated with the kinase, aliquots were withdrawn and layered onto Whatman 3-mm paper squares (2 cm x 2 cm) that had been presoaked in 500 μl cold ATP and dried. The paper discs were dried at 40 °C and washed three times in cold 10% trichloroacetic acid (5-10 ml/disc/wash). The paper discs were then immersed in ethanol, rinsed with ether, and air-dried, and the radioactivity was counted in a liquid scintillation spectrometer (16).

Other Methods—The amount of protein was estimated by using the dye-binding method (17), and silver staining of the gels was done as previously described (18).

Phosphorlase Analysis and Phosphopeptide Sequencing—The [32P]-labeled ACC generated by the aforementioned procedure was separated from the reaction mixture by precipitating it with ammonium sulfate. The [32P]ACC was digested with trypsin at an enzyme to substrate ratio of 1:200 for 12 h. The dialyzed solution was centrifuged to remove denatured proteins and loaded onto a DEAE-Bio-Gel column (1) (2.5 x 25 cm) that was equilibrated with kinase buffer. After extensive washing with kinase buffer, most of the protein was eluted in 0.5 M NaCl in the same buffer. Ammonium sulfate was added to the eluate to 50% saturation, and the precipitated protein was removed by centrifugation, redissolved in kinase buffer, and dialyzed against the same buffer for 12 h. The dialyzed solution was clarified by centrifugation, assayed for kinase activity, and loaded onto a second DEAE-Bio-Gel column (II) (2.5 x 25 cm). The proteins were eluted by a linear gradient consisting of 200 ml each of kinase buffer and of 0.5 M NaCl in the same buffer, and fractions of 8.3 ml each were collected and assayed for kinase activity by using the [32P]ATP assay procedure (see "Experimental Procedures"). As shown in Fig. 1 (A and B), the kinase eluted in fractions 5-30. However, we found that the early fractions (5-11) contained nearly pure kinase, whereas later fractions were contaminated with many proteins as shown by Coomassie Blue staining of gels (data not shown). Further purification of the kinase was counterproductive, since the purified enzyme was unstable and each step introduced resulted in rapid loss of kinase activity. Hence, we opted to use fractions 5-11 (Fig. 1A) in one-step purification by using a DEAE-Affi-Gel Blue column. These fractions were pooled, and the protein was precipitated with ammonium sulfate at 50% saturation. The precipitated protein was isolated by centrifugation, redissolved in kinase buffer, and dialyzed against the same buffer for 4 h. The dialyzed mixture was loaded onto a small DEAE-Affi-Gel Blue column (5 ml). The column was washed with 30 ml of kinase buffer, and the proteins were eluted in successive washings with 5 ml each of kinase buffer containing 50 mm NaCl, 10 mm ADP, and 2 m NaCl. Fractions were collected separately and tested for kinase activity (Fig. 2, A and B). Analysis of the 50 mm NaCl eluate of the DEAE-Affi-Gel Blue-purified kinase on SDS-PAGE showed a single protein band with an estimated molecular weight of 40,000 (Fig. 3). However, when the active kinase fractions were concentrated and analyzed by chromatography on a Superox 6-FPLC in the presence of 0.1 m Tris-HCl (pH 7.5) and 0.1 m NaCl, most of the kinase eluted in the void volume, suggesting that the kinase exists as high molecular weight aggregates under these conditions (data not shown).

Generally, the highly purified kinase was labile and lost activity within 5 days when stored at -70 °C. However, the partially purified fractions that eluted at about 150 m NaCl in the DEAE Bio-Gel column (II) (fractions 12-25) were stable for several months when stored at -70 °C. As summarized in Table 1, this procedure overall purified the kinase by more than 500-fold.

**RESULTS**

**Purification of Acetyl-CoA Carboxylase Kinase**—The carboxylase kinase was first detected as an ACC-associated protein after the ACC had been precipitated with 5.5% PEG and fractionated with 40% ammonium sulfate (see "Experimental Procedures"). This association persisted even after ACC bound to the avidin-Sepharose column. However, when the bound ACC-avidin column was washed with avidin column buffer containing 0.5 m NaCl, most of the kinase was released in the eluate. Eluates from several preparations (equivalent to 60 rat livers) were pooled, and enough ammonium sulfate was added to achieve 50% saturation. The precipitated protein was separated by centrifugation, redissolved in kinase buffer (50 mm Tris-HCl (pH 7.5), 1 mm EDTA, 1 mm dithiothreitol, 0.1 mm phenylmethylsulfonyl fluoride, and 5% glycerol), and dialyzed against the same buffer for 12 h. The dialyzed solution was centrifuged to remove denatured proteins and loaded onto a DEAE-Bio-Gel column (1) (2.5 x 25 cm) that was equilibrated with kinase buffer. After extensive washing with kinase buffer, most of the kinase was eluted in 0.5 m NaCl in the same buffer. Ammonium sulfate was added to the eluate to 50% saturation, and the precipitated protein was removed by centrifugation, redissolved in kinase buffer, and dialyzed against the same buffer for 12 h. The dialyzed solution was clarified by centrifugation, assayed for kinase activity, and loaded onto a second DEAE-Bio-Gel column (II) (2.5 x 25 cm). The proteins were eluted by a linear gradient consisting of 200 ml each of kinase buffer and of 0.5 m NaCl in the same buffer, and fractions of 8.3 ml each were collected and assayed for kinase activity by using the [32P]ATP assay procedure (see "Experimental Procedures"). As shown in Fig. 1 (A and B), the kinase eluted in fractions 5-30. However, we found that the early fractions (5-11) contained nearly pure kinase, whereas later fractions were contaminated with many proteins as shown by Coomassie Blue staining of gels (data not shown). Further purification of the kinase was counterproductive, since the purified enzyme was unstable and each step introduced resulted in rapid loss of kinase activity. Hence, we opted to use fractions 5-11 (Fig. 1A) in one-step purification by using a DEAE-Affi-Gel Blue column. These fractions were pooled, and the protein was precipitated with ammonium sulfate at 50% saturation. The precipitated protein was isolated by centrifugation, redissolved in kinase buffer, and dialyzed against the same buffer for 4 h. The dialyzed mixture was loaded onto a small DEAE-Affi-Gel Blue column (5 ml). The column was washed with 30 ml of kinase buffer, and the proteins were eluted in successive washings with 5 ml each of kinase buffer containing 50 mm NaCl, 10 mm ADP, and 2 m NaCl. Fractions were collected separately and tested for kinase activity (Fig. 2, A and B). Analysis of the 50 mm NaCl eluate of the DEAE-Affi-Gel Blue-purified kinase on SDS-PAGE showed a single protein band with an estimated molecular weight of 40,000 (Fig. 3). However, when the active kinase fractions were concentrated and analyzed by chromatography on a Superox 6-FPLC in the presence of 0.1 m Tris-HCl (pH 7.5) and 0.1 m NaCl, most of the kinase eluted in the void volume, suggesting that the kinase exists as high molecular weight aggregates under these conditions (data not shown).

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**General Properties of the Acetyl-CoA Carboxylase Kinase**

Incubating ACC with the purified kinase in the presence of [γ-32P]ATP resulted in time-dependent incorporation of radioactivity into the carboxylase (data not shown). Under these conditions, maximum incorporation of 32P into the ACC protein ($M_r = 260,000$) was achieved after 60 min of incubation. The maximum incorporation of 32P was quite variable, being in the range of 0.1–0.5 mol of P_i/mol of carboxylase (data not shown). The $K_m$ values of the carboxylase kinase for ATP and for ACC were 30 μM and 300 nm, respectively. Another protein band ($M_r$,
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Fig. 3. SDS-PAGE analysis of DEAE-Affi-Gel Blue-purified acetyl-CoA carboxylase kinase. Kinase samples (100 μl) were obtained as described in Fig. 2, analyzed in SDS-PAGE (10%), and stained with silver nitrate (18). Lane 1, DEAE-Affi-Gel Blue column load; lanes 2 and 3, 50 mM NaCl eluate; lanes 4 and 5, 10 mM ADP eluate (see legend of Fig. 2).

= 125,000), which was also labeled with 32P by the kinase, was identified as a proteolyzed fragment of ACC based on the positive reaction of the bands with anti-ACC antibodies in a Western blot assay (data not shown).

Kinase-mediated phosphorylation of ACC resulted in loss of carboxylase activity, which also was time-dependent (Fig. 4). The inactivation of ACC was ATP-dependent; after incubating 60 min with the kinase and ATP, ACC lost more than 75% of its activity (Fig. 4). Incubating the ACC with the kinase in the absence of ATP resulted in some loss of activity (<20%), which may have been due to nonspecific denaturation.

Effects of Citrate on the Phosphorylation of Acetyl-CoA Carboxylase by the Kinase—The rat liver ACC that was purified by avidin-Sepharose affinity chromatography was activated instantaneously when citrate was added to the assay mixture (no preincubation was required; Ref. 24). As reported earlier (13), the presence of 5 mM citrate in the reaction mixture resulted in 4-fold activation of ACC. Pretreating ACC with kinase resulted in pronounced inactivation, even when the carboxylase was assayed in 5 mM citrate (Fig. 5A). The kinase-phosphorylated ACC can be reactivated by treating it with partially purified Mn2+-dependent acetyl-CoA carboxylase phosphate 2 (14). Likewise, when the phosphatase-treated and activated ACC was phosphorylated with the kinase, the carboxylase activity was markedly reduced (Fig. 5B). These observations are consistent with the concept that ACC is regulated by a phosphorylation/dephosphorylation mechanism and that the kinase and the Mn2+-dependent carboxylase phosphatase operate in the reverse direction in regulating ACC activity. We presume that the two enzymes act at the same phosphorylation site of ACC. However, further information is needed to substantiate this presumption.

Citrate not only activates ACC, it also causes polymerization of the enzyme into large aggregates (2, 4, 25). When kinase-mediated phosphorylation of ACC was carried out in the presence of citrate, phosphorylation of the ACC protein was totally abolished (Fig. 6, lane 5). This inhibition of ACC phosphorylation by the kinase apparently is dependent on the citrate concentration (Fig. 7). Other anions, such as NaCl, did not profoundly affect ACC phosphorylation (Fig. 7). We could not attribute this citrate-mediated inhibition of ACC phosphorylation to the complexing of citrate with Mg2+, which would reduce the effectiveness of the Mg2+-ATP complex in the reaction. For the citrate-mediated inhibition occurred even when the Mg2+ concentration was raised to 35 mM, which exceeds the citrate concentration. Moreover, citrate, at the concentration used in the ACC phosphorylation assay, had no effect on the phosphorylation of phosphorylase b kinase by cAMP-dependent protein kinase when it was used as a control.

The data presented in Fig. 6 also show that the presence of cAMP (0.2 mM), AMP (0.5 mM), or CoA (0.4 mM) in the ACC-phosphorylation reaction mixture did not affect the phosphorylation of ACC by this kinase. Previously, these factors were considered necessary for the activity of their respective kinases in phosphorylating ACC (5–8). Moreover, cAMP-dependent protein-kinase peptide inhibitor (16) did not inhibit nor did the presence of palmitoyl-CoA enhance ACC phosphorylation (Fig. 6). Hardie and co-workers (26) reported that palmitoyl-CoA enhanced the phosphorylating activity of purified AMP-activated kinase; however, they attributed this increase to activation of a contaminating kinase-kinase activity in their preparation. No such activation was noted in our kinase preparation. In addition, the kinase described herein did not phosphorylate phosphorylase b or histone (data not shown). Altogether, these observations and the size of the kinase subunit strongly suggest that the kinase we isolated is not the one isolated by Lent and Kim (5) nor is it the AMP-activated kinase reported by Hardie and co-workers (6, 7).

Incubating the carboxylase with [32P]ATP in the absence of the carboxylase kinase resulted in the incorporation of a small but relatively significant amount of radioactivity into the protein (Fig. 6, lane 2), suggesting that this "autophosphorylation" is due to the bound kinase. This incorporation varied from preparation to preparation and depended upon the extent to which the carboxylase-bound avidin-Sepharose column was washed with the salt-rich buffer during the preparation of ACC (see "Experimental Procedures"). Extensive washings in the salt-rich buffer disrupted the binding of the kinase to ACC, thus stripping the kinase from the carboxylase.

Phosphorylation Sites on Acetyl-CoA Carboxylase—The sites of phosphorylation of ACC by the kinase were determined by labeling the carboxylase with [32P]ATP and then isolating and analyzing for phosphoamino acids and labeled peptides. As described under "Experimental Procedures," the radiolabeled carboxylase was hydrolyzed with HCl, the 32P-labeled amino acids were separated by two-dimensional high voltage electrophoresis, and the labeled amino acids were identified by their mobility with respect to standard derivatives (i.e. phosphoserine, phosphothreonine, and phosphotyrosine). The results showed that the labeling was associated with phosphoserine (data not shown).

The 32P-labeled ACC was digested with either trypsin or chymotrypsin and cleaved by cyanogen bromide; the resulting phosphopeptides were purified as described under "Experimental Procedures." Tryptic digestion yielded a pure 32P-labeled peptide whose amino acid sequence was determined as SS7MSGHHLVK (Fig. 8). All the radioactivity in this peptide was associated with Ser77. Chymotryptic and cyanogen bromide cleavage yielded two labeled peptides (C-CN1 and C-CN2; Fig. 9). Both peptides were rechromatographed on the same column, and their amino acid sequences were determined as S1200FASNLNHY and S1200FASNLNHYGM, respectively. The sequences of the two peptides overlap and represent carboxylase residues 1200–1210 (27). All the radioactivity in the two labeled peptides was associated with Ser1200.
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Table I

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<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
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<td>20.50</td>
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</tbody>
</table>

* Fractions 5–11 from the DEAE-Bio-Gel (II) column (see Fig. 1B) were used in this purification step.

Fig. 4. Inactivation of acetyl-CoA carboxylase by the kinase as a function of time. ACC (25 μg) was phosphorylated with 0.1 mM ATP at 37 °C by partially purified kinase (0.5 μg; eluted at about 50 mM NaCl) (fraction 7 in Fig. 1A) as described under "Experimental Procedures." Aliquot containing 0.2 μg of carboxylase were withdrawn at the indicated times and assayed for ACC activity in the presence of 1 mM potassium citrate. The plots represent ACC plus ATP without kinase (△), ACC plus kinase without ATP (○), and ACC plus kinase plus ATP (●).

DISCUSSION

Rat liver ACC is a phosphoprotein that contains about 4–6 mol of phosphate (4, 14). Liver transfusion experiments showed that at least 1 mol of phosphate undergoes rapid turnover under physiological conditions (14). To understand the process and metabolic significance of phosphorylation and dephosphorylation of ACC, we isolated ACC, protein phosphatase, and kinase from the same animal species and the same tissue. Although other investigators (6–9) for convenience have mixed and matched different tissues from different animal species (e.g., rat liver kinase acting upon mammary gland carboxylase from rats and other animals), we used the same tissue from the same animal species to avoid extrapolative inferences. With this approach, we isolated and characterized an Mn²⁺-dependent protein phosphatase, acetyl-CoA carboxylase phosphatase 2 (14), that acts upon rat liver ACC with relatively high affinity (Kₘ ≈ 0.2 μM) and yields a partially dephosphorylated enzyme with an 8–10-fold increase in carboxylase activity. Moreover, dephosphorylation by this phosphatase renders ACC citrate-independent, as compared to the phosphorylated form, which is citrate-dependent.

A comparable highly active and citrate-independent carboxylase (specific activity of 1–3 μmol of malonyl-CoA formed · min⁻¹ · mg⁻¹ of protein) was isolated from rat livers by using the freeze-clamp procedure of Thampy and Wakil (13). Adding citrate to this ACC enhanced its activity by an additional 3–6-fold. By using this form of ACC as a substrate, we isolated a novel carboxylase kinase that phosphorylates the protein. "Auto-phosphorylation" of affinity-purified rat liver ACC, which had not been sufficiently washed in salt-rich (0.5 mM NaCl) buffer, gave us the initial clue that a kinase co-purified with the carboxylase. This observation was similar to that of Kim and co-
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FIG. 6. Autoradiogram showing the effects of citrate, cAMP, AMP, palmitoyl-CoA, and cAMP-dependent protein kinase inhibitor on the phosphorylation of acetyl-CoA carboxylase. Reaction mixtures contained ACC (25 μg), kinase (0.5 μg; fraction 7 in Fig. 1A), and 0.1 μM [32P]ATP (specific activity of 1 μCi/nmol) and were incubated at 37 °C for 60 min. Analyses were carried out as described in Fig. 1B, lanes 1 and 2, controls without ACC or kinase, respectively; and lanes 3 and 4, reaction mixtures incubated at 0 and 60 min, respectively. The reaction mixtures contained the following: lane 5, 20 mM potassium citrate; lane 6, 0.2 mM cAMP; lane 7, cAMP-dependent protein-kinase inhibitor peptide (10 μM); lane 8, 0.5 mM AMP; lane 9, 0.4 mM CoA; lane 10, 20 μM palmitoyl-CoA. Similar results were obtained when 10 μM of the native CAMP-dependent protein-kinase inhibitor (26 kDa) was used in place of the peptide inhibitor (lane 7).

workers (5); however, the kinase they purified had an estimated molecular weight of 170,000 and showed negligible activity in the absence of CoA. When they added CoA (100 μM) to their assay mixture, maximum phosphorylation and inactivation of the carboxylase resulted. Our enzyme apparently does not require CoA since it was not activated by CoA, even at a concentration of 400 μM (Fig. 6, lane 9). Kim and co-workers (5) showed that their CoA-activated kinase phosphorylated 1 mol of P/mol of carboxylase. In contrast, Jamil and Madsen (28) prepared the same CoA-activated kinase from rat liver and showed that it incorporated 0.45 mol of P/mol of carboxylase after a prolonged incubation of 240 min, with a concomitant loss of about 80% of carboxylase activity. The carboxylase kinase, under our assay conditions, also incorporated 0.5 mol of P/mol of carboxylase within 60 min of incubation at 37 °C and lost about 80% of its activity; further incubation did not improve the incorporation of P. The precise mechanism by which only a fraction of the ACC molecules are phosphorylated while losing most of their activity is intriguing. We speculate that the ACC polymers depolymerize upon phosphorylation, undergo massive structural changes, and mask the phosphorylation sites of other molecules; alternatively, this process may be caused by steric hindrance between the polymeric substrate and...
carboxylase and the high aggregation of the kinase molecules under the conditions of the low ionic strength assay.

Hardie and co-workers (6–8, 12) have tested many kinases, most notably, the cAMP-independent protein from rat livers. This kinase has a subunit molecular weight of 63,000, and its activity is enhanced 3–5-fold in the presence of 200 μM AMP. The carboxylase kinase we isolated has a subunit molecular weight of 40,000, and the presence of AMP does not affect its activity (Fig. 3; Fig. 6, lane 8).

Hardie and co-workers (2, 12) also have studied extensively the sites of phosphorylation of the ACC molecule by different kinases. They found that there are 8 serine residues in rat liver ACC that can be phosphorylated by different kinases in vitro or in vivo; 6 of these residues reside in the first 100 amino acid residues at the NH₂-terminal end. Ser₂⁵⁳ apparently is phosphorylated nonspecifically by any kinase, whereas Ser₂⁵⁵ is phosphorylated by calmodulin-dependent protein kinase, Ser₂⁹ by casein kinase-2, Ser₇⁷ by cAMP-independent protein kinase, Ser₉⁷ by AMP-activated protein kinase, and Ser₁⁰⁶ by protein kinase C. Of the remaining two sites, Ser₁²⁰⁰ is phosphorylated by both cAMP-dependent protein kinase and AMP-activated protein kinase; the latter also phosphorylates Ser₁²¹⁸. Although residue-specific phosphorylation at the various sites by different kinases is possible, cross-phosphorylation cannot be ruled out. Depending on the interplay of the protein kinases and phosphatases and the metabolic state of the cell, the serine residues that are available for phosphorylation may be quite variable within a given time. The kinase we isolated phosphorylates Ser₇⁷ and Ser₁²⁰⁰ (Figs. 8 and 9). In this aspect, our enzyme resembles the cAMP-independent acetyl-CoA carboxylase kinase 2 isolated from rat and rabbit mammary glands by Hardie and co-workers (10).

The most interesting feature of the carboxylase kinase we isolated is the dramatic inhibition of ACC phosphorylation by millimolar concentrations of citrate that occurred when the kinase was present in the reaction mixture (Fig. 6, lane 5; Fig. 7). The function of citrate as a feed-forward activator of ACC is well documented; citrate activates ACC with subsequent aggregation of the protein molecules, whereas phosphorylation of ACC results in its inactivation and deggregation (1, 2). The results presented in Fig. 6 demonstrate that citrate can alleviate all, or at least most, of the ACC inhibition caused by phosphorylation, suggesting that when citrate is present the kinase is either totally inhibited or cannot access the phosphorylation sites in the ACC molecule. This situation does not result from depletion by citrate of the Mg²⁺ required for the carboxylation reaction, since adding more Mg²⁺ to the phosphorylation assay did not prevent the profound inhibition of carboxylase phosphorylation we observed. It is possible that the action of citrate on ACC may be due to its interaction with the enzyme at two different domains through different mechanisms.

These results point to a unique role for citrate in regulating ACC activity; citrate induces conformational changes in the protein that result in enhancement of ACC activity and subsequent polymerization of the protein (24). This type of reversible citrate-induced activation and polymerization of ACC occurred in intact chicken hepatocytes in monolayer culture and closely corresponded to the rate of fatty acid synthesis (29). These citrate-induced conformational changes may also be important in modulating the phosphorylation of ACC and the enzyme's activity. The interplay between citrate and the carboxylase kinase may therefore determine the activity of ACC and consequently the rate of de novo fatty acid synthesis. These observations, together with the fact that the kinase is associated with ACC, tempt us to postulate that this kinase is physiologically important in modulating the acetyl-CoA carboxylase activity.

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REFERENCES