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PHOSPHORYLATION OF DIFFERENT SITES OF ACETYL COA CARBOXYLASE BY ATP-CITRATE LYASE KINASE AND CYCLIC AMP-DEPENDENT PROTEIN KINASE

Seethala RAMAKRISHNA and William B. BENJAMIN

Diabetes Research Laboratory,
Department of Physiology and Biophysics School of Medicine,
Health Sciences Center, State University of New York,
Stony Brook, New York 11794

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Native acetyl CoA carboxylase was phosphorylated by catalytic subunit of cyclic AMP-dependent protein kinase and ATP-citrate lyase kinase to 1 and 0.5 mol/subunit respectively. Both protein kinases added together increased acetyl CoA carboxylase phosphorylation additively. Partial proteolysis of ^{32}P-acetyl CoA carboxylase followed by electrophoretic analysis showed that the ^{32}P-phosphopeptides generated from acetyl CoA carboxylase phosphorylated with lyase kinase were different from the peptides obtained from the enzyme phosphorylated by cyclic AMP-dependent protein kinase. Mapping of tryptic ^{32}P-phosphopeptides by high performance liquid chromatography showed that the major phosphopeptides phosphorylated by ATP-citrate lyase kinase were different from the major phosphopeptides phosphorylated by cyclic AMP-dependent protein kinase. The results suggest that at least one different site on acetyl CoA carboxylase is preferentially phosphorylated by each protein kinase.

Acetyl CoA carboxylase (EC 6.4.1.2) is an important rate-limiting enzyme of fatty acid biosynthesis. One mechanism for the 'short term' control of enzyme activity is the covalent phosphorylation-dephosphorylation of specific sites on the enzyme (1,2). Recently it has been found in mammalian tissues that hormones which raise the cyclic AMP level increase the net phosphorylation of acetyl CoA carboxylase and decrease the enzyme activity (3-5). However, acetyl CoA carboxylase is phosphorylated at multiple sites by both cyclic AMP-dependent protein kinase and cyclic AMP-independent protein kinases (6-11). Analysis of the tryptic peptides derived from *in vitro* and *in vivo* phosphorylated acetyl CoA carboxylase suggested that the enzyme is phosphorylated at the same sites by cyclic AMP- dependent protein kinase *in vitro* and by treatment of fat cells with epinephrine (3). It has been reported that insulin action increases enzyme activity

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(5). This increase in enzyme activity was presumed to be due to the dephosphorylation of the enzyme. However, insulin was found to stimulate the phosphorylation of acetyl CoA carboxylase in both hepatocytes and adipose tissue (12-14). Insulin stimulated phosphorylation occurs on a specific site that is different from the epinephrine stimulated phosphorylation sites. Based on the suggestion of Benjamin and Singer (15) and these results these results Denton et al (16) proposed that enzyme activation of acetyl CoA carboxylase by insulin action could involve an insulin directed cyclic AMP-independent protein kinase. Because ATP-citrate lyase kinase could be a physiologically significant adipose tissue protein kinase as the sites phosphorylated on ATP-citrate lyase by ATP-citrate lyase kinase in vitro are identical to the sites phosphorylated on ATP-citrate lyase when adipose tissue is incubated with $^{32}P_i$ (17). To determine if ATP-citrate lyase kinase plays a more general role in the phosphorylation of key enzymes of intermediary metabolism it is important to study the effects of this protein kinase on acetyl CoA carboxylase phosphorylation and on enzyme activity. To begin this study we determined if ATP-citrate lyase kinase phosphorylates acetyl CoA carboxylase independently of cyclic AMP-dependent protein kinase and whether the site(s) phosphorylated is(are) separate from that phosphorylated by cyclic AMP-dependent protein kinase.

EXPERIMENTAL PROCEDURES

Materials: Acetyl CoA carboxylase was purified from rat liver and adipose tissue (18,19), the catalytic subunit of cyclic AMP-dependent protein kinase was purified from rabbit muscle (20) and ATP-citrate lyase kinase from rat liver (21,22). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham. TPCK-trypsin[1] was purchased from Worthington. Staphylococcus aureus protease was obtained from Miles Laboratories. All other reagents were the best grade obtainable and were purchased from standard suppliers.

Phosphorylation of Acetyl CoA Carboxylase: Acetyl CoA carboxylase phosphorylation by ATP-citrate lyase kinase or catalytic subunit of

[1] The abbreviations used are: TPCK-trypsin, L-1-tosylamido-2-phenylethyl methyl ketone-treated trypsin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

cyclic AMP-dependent protein kinase was measured by incubating each protein knase separately or together at 30°C in a 50 µl reaction mixture containing 50 mM MES buffer (pH 6.7), 0.3 mM EGTA, 0.5 mM EDTA, 8 mM magnesium acetate, 0.05 mM [γ-^{32}P]ATP (150-250 cpm/pmol), and 1 mg/ml of bovine serum albumin. The reaction was terminated by adding 17 µl of sample buffer and boiling for 3 min. Boiled samples (45 µl) were analyzed on 6% polyacrylamide gels in the presence of 0.1% SDS. The gels were stained with 0.1% Coomassie blue G in 50% methanol, 7% acetic acid destained with 10% methanol, 7% acetic acid and dried. The stained band of acetyl CoA carboxylase was cut from the dried gel and the radioactivity associated with the protein was determined.

Limited Proteolysis and Peptide Mapping: Acetyl CoA carboxylase was phosphorylated as described above and the reaction was terminated by the addition of sample buffer to give a final concentrations of 50 mM Tris-HCl, pH 6.8, 0.5% SDS, 10% glycerol and 0.001% bromophenol blue and heated at 100°C for 5 min. Proteolysis of the phosphorylated acetyl CoA carboxylase and peptide mapping on SDS polyacrylamide gels was by a modification of the method of Cleveland *et al* (23) as described previously (24).

HPLC of Acetyl CoA carboxylase Phosphopeptides: Acetyl CoA carboxylase was phosphorylated using [γ-^{32}P]ATP and either protein kinase for 3 h. The reaction mixture was passed through a Sephadex G-150 superfine column (0.8 x 10 cm). Phosphorylated acetyl CoA carboxylase free of protein kinase and ATP was recovered in the void volume. Phosphorylated acetyl CoA carboxylase was reduced, carboxymethylated and digested with TPCK-trypsin for 18 h at 30°C by the addition of 0.25 mg/ml of TPCK-trypsin followed by an additional 0.25 mg/ml 12 h later. The tryptic digest was lyophylized, dissolved in 55 µl of 0.2 M phosphate buffer, pH 3 and centrifuged. Sample (50 µl) was injected into a reversed phase C_{18} column (Beckman Ultrasphere ODS C_{18} column, 0.46 x 15 cm) equilibrated with 5% acetonitrile containing 0.2 M phosphate buffer. The column was washed for 5 min with 5% acetonitrile, and peptides were eluted with linear gradients of 5-10% acetonitrile in 0.2 M phosphate buffer for 10 min followed by 10-40% acetonitrile in 0.2 M phosphate buffer for 80 min and then by 40% acetonitrile buffer for 5 min. The flow rate was 1 ml/min and peptides were monitered by absorbance at 210 nm. Radiolabeled phosphopeptides were located by collecting 1-min fractions and measuring their Cerenkov radiation.

RESULTS AND DISCUSSION

Acetyl CoA carboxylase was purified from rat liver and adipose tissue by avidin-Sepharose affinity chromatography (18,19) and when analyzed by SDS-PAGE showed a single Coomassie blue staining band with a molecular weight of 250,000 (18). Purified acetyl CoA carboxylase was phosphorylated to 1.0 mol/subunit when the enzyme was phosphorylated with catalytic subunit of cyclic AMP-dependent protein kinase and to 0.4-0.5 mol/subunit when phosphorylated with ATP-citrate lyase kinase. Acetyl CoA carboxylase in contrast to ATP-citrate lyase (25) was phosphorylated additively to 1.5 mol/subunit when the enzyme was

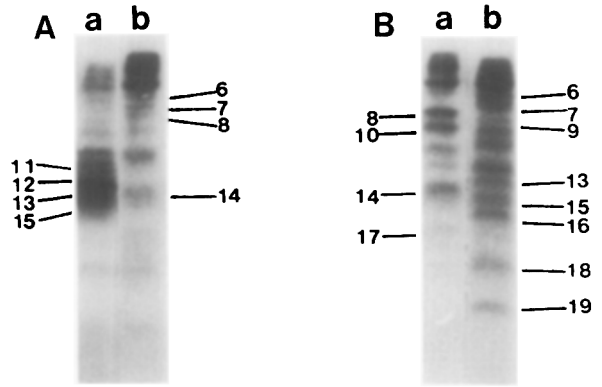

Fig. 1 Autoradiogram of phosphorylated acetyl CoA carboxylase after limited proteolysis. Proteolysis was for 30 min with S. aureus protease (A) or TPCK-trypsin (b). Lanes a : acetyl CoA Carboxylase phosphorylated by ATP- citrate lyase kinase and lanes b : acetyl CoA carboxylase phosphorylated by catalytic subunit of cyclic AMP-dependent protein kinase. The concentration of S. aureus protease was 10 µg/ml and TPCK-trypsin was 50 µg/ml.

phosphorylated by both protein kinases added together to the reaction mixture.

To demonstrate that the sites phosphorylated by each protein kinase were different, purified acetyl CoA carboxylase phosphorylated with either protein kinase or both was digested at 37°C for 30 min with TPCK-trypsin or S. aureus protease. Fig. 1 is an autoradiogram of the proteolytic products resolved by SDS-PAGE and Table I is an analysis of these results. Some radioactive bands were present or absent in the sample phosphorylated by ATP-citrate lyase kinase that were absent or present in the sample phosphorylated by cyclic AMP-dependent

TABLE I: Distribution of ^{32}P-proteolytic products of acetyl CoA carboxylase phosphorylated by different protein kinases

Treatment	ATP-citrate lyase kinase	cyclic AMP-dependent protein kinase	Both protein kinases
1. S. aureus protease :			
bands 11,12,13,15	+	-	+
bands 6,7,8,14	-	+	+
2. TPCK-trypsin :			
bands 8,10,14,17	+	-	+
bands 6,7,9,13,15,16,18,19	-	+	+

Bands present in one sample and absent in the other are represented by + and - respectively.

protein kinase. All radioactive bands were present when acetyl CoA carboxylase was phosphorylated with both protein kinases added together.

It is concluded that many of the radioactive fragments generated by trypsin or protease digestion of acetyl CoA carboxylase phosphorylated with ATP-citrate lyase kinase were different from those generated from the enzyme phosphorylated with catalytic subunit of cyclic AMP-dependent protein kinase. The observed differences in the proteolytic fragments in lanes a and b (Fig. 1) were not due to protease contamination of any of the added protein kinases as samples treated similarly but without trypsin and protease did not show any proteolytic peptides other than those present at zero time. Again, the presence of the complete complement of peptides in the sample treated with both protein kinases suggest that the observed differences in the radioactive fragments was not due to conformational changes related to the level of phosphorylation but was due to phosphorylation of different sites by lyase kinase and cyclic AMP-dependent protein kinase.

To demonstrate conclusively that different sites are phosphorylated in response to different protein kinases, acetyl CoA carboxylase phosphorylated with either protein kinase was separated from the protein kinases by chromatography on Sephadex G-150. The phosphorylated acetyl CoA carboxylase was treated with trypsin 0.5 mg/ml for 24 h and the resulting tryptic fragments were processed for analysis by HPLC. As illustrated in Fig. 2, quantitative differences in the distribution of ^{32}P-peptides of acetyl CoA carboxylase were observed between the samples phosphorylated with cyclic AMP-dependent protein kinase and ATP-citrate lyase kinase. In addition, possible qualitative differences were noted between the radioactive peptide samples. A peak of ^{32}P-phosphopeptide (peak C) generated from acetyl CoA carboxylase phosphorylated by ATP-citrate lyase kinase was eluted at

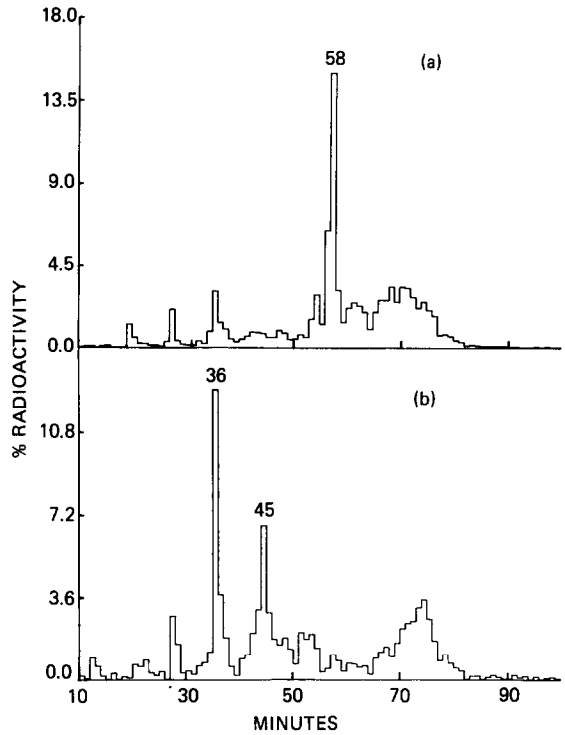

Fig. 2 HPLC maps of ^{32}P-acetyl CoA carboxylase peptides. Purified acetyl CoA carboxylase (30 µg) phosphorylated in vitro with [γ-^{32}P]ATP and either ATP-citrate lyase kinase (a) or catalytic subunit of cyclic AMP-dependent protein kinase (b) was treated with TPCK-trypsin (0.5 mg/ml). The tryptic digest was chromatographed on a reversed phase C_{18} column. The column was washed with 5% acetonitrile in 0.2 M phosphate buffer for 5 min and peptides were eluted with linear gradients of 5-10% acetonitrile in 0.2 M phosphate buffer for 10 min followed by 10-40% acetonitrile in 0.2 M phosphate buffer for 80 min. Radioactivity injected on to the column was 32,200 cpm in (a), and 4900 cpm in (b). The overall recovery of radioactivity injected into the C_{18} column was over 90%.

58 min (Fig. 2a) whereas two major ^{32}P-peaks generated from the enzyme phosphorylated by cyclic AMP-dependent protein kinase were eluted at 36 min (peak A) and 45 min (peak B) (Fig. 2b). In addition, three broad ^{32}P-peaks eluting between 61-65, 66-69 and 70-77 min (peaks D, E and F respectively) were present in both samples. The per cent radioactivity distributed in the peaks A-F in the samples phosphorylated by ATP-citrate lyase kinase or by cyclic AMP-dependent protein kinase was peak A, 5% and 20%; peak B, 3% and 17%; peak C, 25% and 2%; peak D, 12% and 3%; peak E, 11% and 4%; peak F, 20% and 19% respectively.

In previous studies we found that ATP-citrate lyase was phosphorylated by ATP-citrate lyase kinase at a serine and threonine residue in peptide b whereas the enzyme was phosphorylated by cyclic AMP-dependent protein kinase at a serine residue in peptide a (24, 25). No overlap between the phosphorylation of sites of ATP-citrate lyase phosphorylated by ATP-citrate lyase kinase and cyclic AMP-dependent protein kinase was noted because incomplete proteolysis of ATP-citrate lyase phosphorylated with cyclic AMP-dependent protein kinase gave a larger ^{32}P-peptide a' which on further proteolysis was converted to peptide a whereas the enzyme phosphorylated by ATP-citrate lyase kinase gave ^{32}P-peptide b which was converted after extensive proteolysis to smaller peptides still containing both radiolabeled phosphoserine and phosphothreonone (25). To determine whether peak C generated from acetyl CoA carboxylase phosphorylated by ATP-citrate lyase kinase could be an incompletely digested tryptic peptide and whether further treatment with trypsin would give rise to peaks A, and B or other small peptides, peak C was pooled desalted, concentrated and rehydrolyzed with 0.5 mg/ml of trypsin for 24 h and chromatographed. As depicted in Fig. 3 about 65% of the radioactivity was recovered in peak C and only approximately 10% of the total counts were found in a broad peak eluting between 44-50 min. This result suggests that peak C generated from the sample phosphorylated by ATP-citrate lyase kinase was a final tryptic peptide. All the major peaks generated by trypsin treatment of acetyl CoA carboxylase phosphorylated with cyclic AMP - dependent protein kinase contained phosphoserine. Peak C generated from acetyl CoA carboxylase phosphorylated by ATP-citrate lyase kinase contained in addition to phosphoserine trace amounts of phosphothreonine (<5%).

Brownsey and Denton (14) using two dimensional peptide mapping found that insulin and epinephrine both act to increase the phosphorylation of acetyl CoA carboxylase in rat adipose tissue. No unique

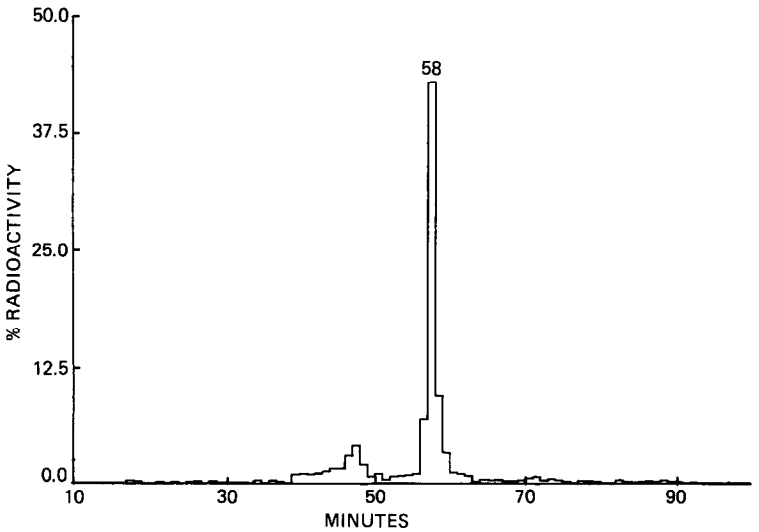

Fig. 3 HPLC map of peak C peptide of acetyl CoA carbpxylase. Fractions 57-59 of peak C from Fig. 2 were pooled, desalted, lyophylized and dissolved in 0.25 ml 50 mM ammonium bicarbonate, pH 8.0. Trypsin was added to a final concentration of 0.5 mg/ml and incubated at 37°C for 24 h. Sample was lyophylized, dissolved in 55 µl of phosphate buffer. 50 µl sample (4100 cpm) was chromatographed on C_{18} column as described in Fig. 2.

phosphorylation site could be ascribed to the action of these hormones. Insulin treatment did increase the phosphorylation of only one specific site without effect on the phosphorylation of other sites whereas epinephrine treatment increased the phosphorylation of a few peptides without increasing the phosphorylation of the site affected by insulin action (14). We are currently investigating the effect of insulin and β-adrenergic agonists on the sites phosphorylated on acetyl CoA carboxylase *in vivo* by HPLC analysis to compare these with the sites phosphorylated *in vitro* by various protein kinases including ATP-citrate lyase kinase in an effort to identify the putative insulin sensitive protein kinase.

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