

# Phosphorylation of rat muscle acetyl-CoA carboxylase by AMP-activated protein kinase and protein kinase A

W. W. WINDER,<sup>1</sup> H. A. WILSON,<sup>1</sup> D. G. HARDIE,<sup>2</sup> B. B. RASMUSSEN,<sup>1</sup> C. A. HUTBER,<sup>1</sup> G. B. CALL,<sup>1</sup> R. D. CLAYTON,<sup>1</sup> L. M. CONLEY,<sup>1</sup> S. YOON,<sup>1</sup> AND B. ZHOU<sup>1</sup>

<sup>1</sup>Department of Zoology, Brigham Young University, Provo, Utah 84602;

and <sup>2</sup>Department of Biochemistry, The University of Dundee, Dundee DD1 4HN, United Kingdom

**Winder, W. W., H. A. Wilson, D. G. Hardie, B. B. Rasmussen, C. A. Hutber, G. B. Call, R. D. Clayton, L. M. Conley, S. Yoon, and B. Zhou.** Phosphorylation of rat muscle acetyl-CoA carboxylase by AMP-activated protein kinase and protein kinase A. *J. Appl. Physiol.* 82(1): 219–225, 1997—This study was designed to compare functional effects of phosphorylation of muscle acetyl-CoA carboxylase (ACC) by adenosine 3',5'-cyclic monophosphate-dependent protein kinase (PKA) and by AMP-activated protein kinase (AMPK). Muscle ACC (272 kDa) was phosphorylated and then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography. Functional effects of phosphorylation were determined by measuring ACC activity at different concentrations of each of the substrates and of citrate, an activator of the enzyme. The maximal velocity ( $V_{max}$ ) and the Michaelis constants ( $K_m$ ) for ATP, acetyl-CoA, and bicarbonate were unaffected by phosphorylation by PKA. Phosphorylation by AMPK increased the  $K_m$  for ATP and acetyl-CoA. Sequential phosphorylation by PKA and AMPK, first without label and second with label, appeared to reduce the extent of label incorporation, regardless of the order. The activation constant ( $K_a$ ) for citrate activation was increased to the same extent by AMPK phosphorylation, regardless of previous or subsequent phosphorylation by PKA. Thus muscle ACC can be phosphorylated by PKA but with no apparent functional effects on the enzyme. AMPK appears to be the more important regulator of muscle ACC.

carnitine palmitoyl transferase; fatty acid oxidation by muscle; malonyl-CoA

IN LIVER, acetyl-CoA carboxylase (ACC) catalyzes the first committed step in lipogenesis (10). The product, malonyl-CoA, allosterically inhibits carnitine palmitoyl transferase 1 (CPT-1), thereby inhibiting fat oxidation when fatty acid synthesis is occurring at high rates (14, 16). The liver and adipose tissue isoforms of the enzyme can be phosphorylated by several different kinases, including adenosine 3',5'-cyclic monophosphate (cAMP)-dependent protein kinase (PKA), AMP-activated protein kinase (AMPK), and protein kinase C (PKC) (4, 10, 11). Phosphorylation of ACC by AMPK and PKA has been demonstrated to produce changes in functional characteristics of the enzyme, including decreases in maximal velocity ( $V_{max}$ ) and increases in the Michaelis constant ( $K_m$ ) (10, 11). At physiological concentrations of ATP, acetyl-CoA, bicarbonate, and citrate, the changes in these kinetic properties would be expected to produce marked decreases in ACC activity and the rate of malonyl-CoA synthesis.

Previous studies have demonstrated the presence of a 272- to 275-kDa isoform of ACC in rat and human skeletal muscle (2, 21, 22, 34), which is considered to be

a nonlipogenic tissue. The muscle ACC appears to be regulated differently than the principal liver isoforms, in that it does not increase from the condition of fasting to a refed state (with high-carbohydrate diet) (33). This system has been postulated to be present in muscle for the purpose of regulation of fatty acid oxidation. In support of this hypothesis, malonyl-CoA has been found to decrease in skeletal muscle during exercise and in response to fasting and electrical stimulation (8, 28). More recently, *in vitro* studies have demonstrated that avidin affinity column-purified skeletal muscle ACC can be phosphorylated by liver AMPK (31). This phosphorylation induces a decrease in ACC  $V_{max}$  and an increase in the citrate concentration required to produce half-maximal activation ( $K_{0.5}$ ) of the enzyme. Similar changes in kinetic constants of ACC occur in hindlimb muscle of the rat during a single bout of exercise. The increase in  $K_{0.5}$  for citrate and decrease in  $V_{max}$  of muscle ACC were accompanied by an increase in the activity of AMPK partially purified from muscle extracts of the exercised rats. These results imply that ACC is phosphorylated and inactivated in exercising skeletal muscle. The muscle and heart CPT-1 is even more sensitive to malonyl-CoA inhibition than is the liver CPT-1 (13–15, 25). It has been suggested (27, 28, 31) that the decrease in malonyl-CoA is one of the important signals for increased fatty acid oxidation (19) in muscle during exercise.

Previous studies have also demonstrated that cAMP increases in skeletal muscle during prolonged exercise and that this increase is due to an increase in plasma epinephrine (27, 30). Hence, phosphorylation of ACC by PKA (as well as by AMPK) could also be responsible in part for the changes noted in ACC activity in extracts from exercising muscle. The purpose of the present study was to determine whether muscle ACC can be phosphorylated by PKA. We also studied the effects of PKA and AMPK phosphorylation on kinetic constants of ACC for each of the substrates and for the activator citrate. Possible interaction between effects of the two kinases was investigated by studying sequential phosphorylation by PKA and AMPK.

## MATERIALS AND METHODS

**Isolation of ACC.** ACC was isolated from quadriceps and gastrocnemius muscles of the rat hindlimb. Rats (body wt = 350–500 g) were anesthetized (pentobarbital sodium intraperitoneally) for at least 30 min before removing muscles. The muscles were cooled rapidly between aluminum blocks at near 0°C. Muscle groups were separated and fat and

connective tissue were removed. Muscle was minced with scissors and suspended in cold *buffer A* [(in mM) 225 mannitol, 75 sucrose, 10 tris(hydroxymethyl)aminomethane (Tris)·HCl, 0.05 EDTA, 5 potassium citrate, 2.5 MnCl<sub>2</sub>, pH 7.5, with 10 mg/l of leupeptin and antitrypsin and 10 ml/l of aprotinin (Sigma Chemical, St. Louis, MO)] in a ratio of 6–9 ml buffer per gram muscle. The muscle (from 10 to 12 rats) was then homogenized by using a Brinkmann PT-1000 tissue homogenizer using a PT-DA 3020/2 generator. After centrifugation at 17,000 *g* for 40 min, the supernatant was collected. The ACC was precipitated by adding 200 g ammonium sulfate/l and stirring at 4°C for 1 h. The precipitate was collected by centrifuging at 17,000 *g* for 30 min and then resuspended in minimal volume of buffer containing 100 mM Tris·HCl, 0.5 M NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, pH 7.5, to which was added 10 mg antitrypsin (Sigma Chemical), 10 mg leupeptin (Sigma Chemical), and 10.0 ml aprotinin (9 trypsin inhibitor units/ml) (Sigma Chemical) per liter. After centrifugation to remove insoluble material, the resuspended precipitate was dialyzed (by using Spectra/Por CE membrane; mol wt cut off: 15,000) for at least 5 h at 4°C against column buffer (100 mM Tris·HCl, 0.5 M NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol, pH 7.5) and then purified by avidin-Sepharose affinity chromatography, using Promega SoftLink Soft Release Avidin Resin (Fisher Scientific, Pittsburgh, PA). After addition of the resuspended precipitate to the column, the column was washed with 30 volumes of column buffer to remove nonbiotin-containing proteins. The ACC was then eluted with column buffer containing 5 mM biotin. Fractions containing ACC activity were pooled, mixed, and then stored frozen at –80°C in 100- $\mu$ l aliquots. Purity was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on each lot. The principal isoform was the 272-kDa species as previously reported (22).

**Phosphorylation of ACC.** For *in vitro* phosphorylation studies, globulin-free albumin (Sigma Chemical) was added (1  $\mu$ g/ $\mu$ l) to the purified enzyme (1–2  $\mu$ g protein in 0.1 ml), followed by an equal volume of cold, saturated ammonium sulfate. Poor yields were noted in the absence of albumin. The mixture was allowed to stand for 15 min on ice and was then centrifuged at 48,000 *g* for 15 min to collect the precipitate. After the supernatant was discarded, the precipitate was resuspended and utilized for phosphorylation studies. Final concentrations in the assay tubes were (in mM) 34 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 68 NaCl, 0.68 EDTA, 0.68 ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.68 dithiothreitol, 6.8% glycerol, 0.12 ATP, 3 MgCl<sub>2</sub>, pH 7.0. To this mixture was added 20  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP  $\pm$  6 units of the catalytic subunit of bovine heart PKA (Sigma Chemical). For the AMPK studies, the reaction mix was the same as for PKA with or without the addition of AMPK (5 U/ml) and 0.2 mM AMP. AMPK was isolated from rat liver as far as the gel filtration step (3). The final volume in the assay mix was 60  $\mu$ l. After incubating at 30°C for 1 h, the reaction was terminated by addition of 60  $\mu$ l saturated ammonium sulfate. The mixture was allowed to stand for 15 min and then was centrifuged at 48,000 *g* for 15 min. The supernatant was discarded, and the precipitate was washed with 1 ml 1:1 saturated ammonium sulfate, HEPES buffer. For electrophoresis, the final precipitate was resuspended in 25  $\mu$ l of HEPES buffer to which 50  $\mu$ l of electrophoresis sample buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 2.5% SDS, 5%  $\beta$ -mercaptoethanol, 0.025% bromphenol blue) were added. After being heated at 95°C for 4 min, the samples were subjected to PAGE with the Bio-Rad Mini-Protean II Dual Slab Vertical Electrophoresis System, using Mini-Protean II 4–15% gradient precast gels (Bio-Rad, Richmond, CA). Gels were run in the

presence of 0.1% SDS, 25 mM Tris, 192 mM glycine, pH 8.3 at 160 V for 75 min. Gels were stained by using a silver stain kit (Sigma Chemical) and were then dried for autoradiography (X-OMAT AR Scientific Imaging Film, Kodak).

An additional experiment was done to make certain the PKA was responsible for the phosphorylation of ACC. PKA inhibitor (rabbit sequence, Sigma Chemical) (2  $\mu$ g) was added to the phosphorylation medium before addition of PKA. After a 60-min incubation, the mixture was precipitated with ammonium sulfate, resuspended, and subjected to SDS-PAGE followed by autoradiography.

Two other kinases, phosphorylase kinase and PKC (Sigma Chemical), were also tested to determine whether ACC is a substrate. Conditions were the same as for PKA except for addition of calcium (1.5 mM for phosphorylase kinase and 2.0 mM for PKC). PKC tubes also had 50  $\mu$ g/ml phosphatidyl serine and 10  $\mu$ M diacylglycerol.

**ACC activity measurements.** To determine functional effects of phosphorylation, ACC was incubated with and without PKA or with and without AMPK and AMP in the same reaction mix in the absence of radiolabeled ATP. ACC activity was determined at the end of the phosphorylation, with no ammonium sulfate precipitation. ACC activity was determined by measuring rate of incorporation of [<sup>14</sup>C]bicarbonate into acid-stable compounds (malonyl-CoA) at 37°C for 2 min. Final concentrations of reagents were (in mM) 50 HEPES buffer, pH 7.5, 1.5 MgSO<sub>4</sub>, 2 dithiothreitol, 0.25 acetyl-CoA, 4 ATP, 12.5 KHCO<sub>3</sub>, 2  $\mu$ Ci [<sup>14</sup>C]bicarbonate, and 0.75 mg/ml fatty acid free bovine serum albumin. Citrate and magnesium acetate were added in equimolar concentrations ranging from 0 to 20 mM. Substrate concentrations were varied singly (with other substrates at saturating concentrations) for determination of kinetic constants. ATP/MgSO<sub>4</sub> concentrations were varied between 0 and 4 mM. In the case of ATP, an additional precipitation of the enzyme was required after the phosphorylation reaction for removal of ATP from the medium before ACC activity measurement. Alternatively, the ATP added to the incubation tube along with the ACC was accounted for in the final calculation of concentration. Bicarbonate was varied between 0 and 12.5 mM. Acetyl-CoA was varied between 0 and 500  $\mu$ M. The reaction was started by addition of ACC. The final reaction volume was 200  $\mu$ l. The reaction was stopped by addition of 50  $\mu$ l of 5 N HCl. After centrifugation, 200  $\mu$ l were transferred to a scintillation vial and evaporated to dryness at 80°C. The residue was dissolved in 0.4 ml water and then mixed with 5.5 ml Scintiverse (Fisher Scientific) or Ecolite (ICN) for determination of radioactivity. Preliminary experiments indicated linearity with time and enzyme concentration in this range. The citrate data were fitted to the Hill equation using the Grafit program (Sigma Chemical). This program allows determination of the activation constant ( $K_a$ ) for citrate, the maximal activity as a function of citrate concentration ( $V_{max}$ ), and the citrate concentration required for  $K_{0.5}$  of ACC. The enzyme activity data for substrates were fitted to the Michaelis-Menton equation;  $V_{max}$  and  $K_m$  were determined using the Grafit software. Kinetic constants for both nonphosphorylated and phosphorylated ACC (from the same lot of ACC) were determined in parallel on any 1 day. Because the calculated  $V_{max}$  varied, depending on the protein content and activity of each lot of enzyme, means of kinetic constants for the two treatment groups were compared, by using Student's *t*-test for paired observations.

**Sequential phosphorylation by PKA and AMPK.** When it was found that PKA phosphorylation had no effect on activity of ACC, a series of sequential phosphorylations was done to determine whether prior PKA phosphorylation would influence effects of AMPK phosphorylation on the enzyme. In this

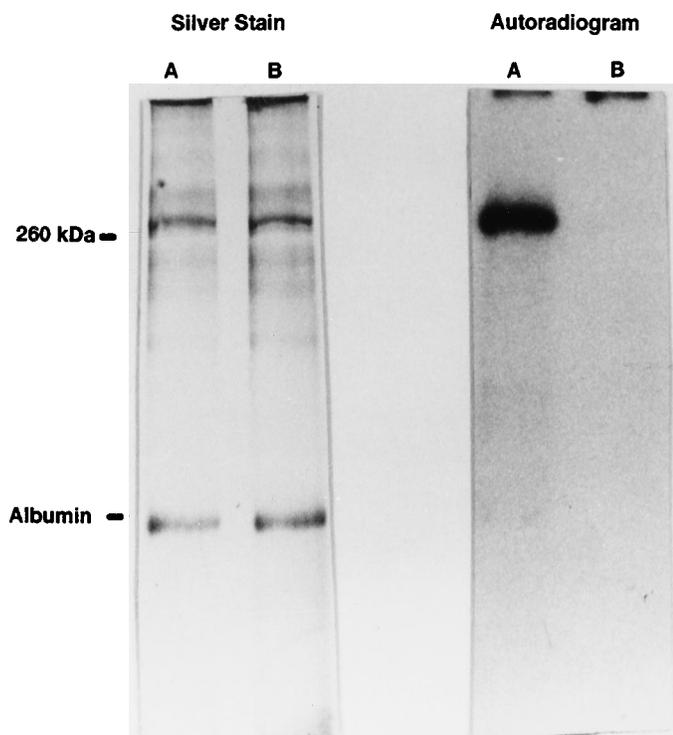


Fig. 1. Silver-stained gel and autoradiograph of the dried gel from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of acetyl-CoA carboxylase (ACC) isolated from hindlimb skeletal muscle by avidin-Sepharose affinity chromatography and subjected to phosphorylation for 60 min, as described in MATERIALS AND METHODS. *Lane A*: ACC was exposed to all reagents in the phosphorylation mixture, including protein kinase A (PKA). *Lane B*: ACC was exposed to all reagents except PKA. Similar results were observed on 4 different preparations of ACC.

series, the same incubation conditions were used as described above except that the labeled ATP was not included for the first 30 min of incubation with or without PKA or AMPK. At the end of 30 min, the other kinase was added along with labeled ATP. This design allows determination whether prior phosphorylation by one kinase alters extent of phosphorylation by the other kinase. If the two kinases share one or more phosphorylation sites, or if phosphorylation by one kinase sterically interferes with phosphorylation by the other kinase, we would expect to see a diminished label incorporation by the second kinase. This can be detected after SDS-PAGE followed by autoradiography. The same protocol was followed for a study on citrate dependence to determine whether sequential phosphorylation has functional effects on the enzyme.

## RESULTS

*In vitro phosphorylation studies.* Figure 1 shows the silver-stained SDS-PAGE gels (*A* and *B*, left) and autoradiographs (*A* and *B*, right) of corresponding lanes after the phosphorylation reaction. Little phosphorylation was noted in the absence of PKA (*B*). Addition of PKA to the medium resulted in significant incorporation of the labeled phosphate into the 272-kDa-band region, indicated on the autoradiograph (Fig. 1, *A*, right). No significant accumulation of labeled phosphate was seen at any other position on the gel, with the exception of some that remained at the origin in both PKA and control lanes. PKA inhibitor (rabbit sequence) completely inhibited phosphorylation of ACC by PKA (autoradiographs not shown).

Figure 2 shows the autoradiograph of six lanes of a dried SDS-PAGE gel. *Lanes A*, *C*, and *E* represent ACC incubated with the phosphorylation mix without kinase. *Lane B* shows phosphorylation of ACC with PKA. *Lane D* shows phosphorylation of ACC with PKC.

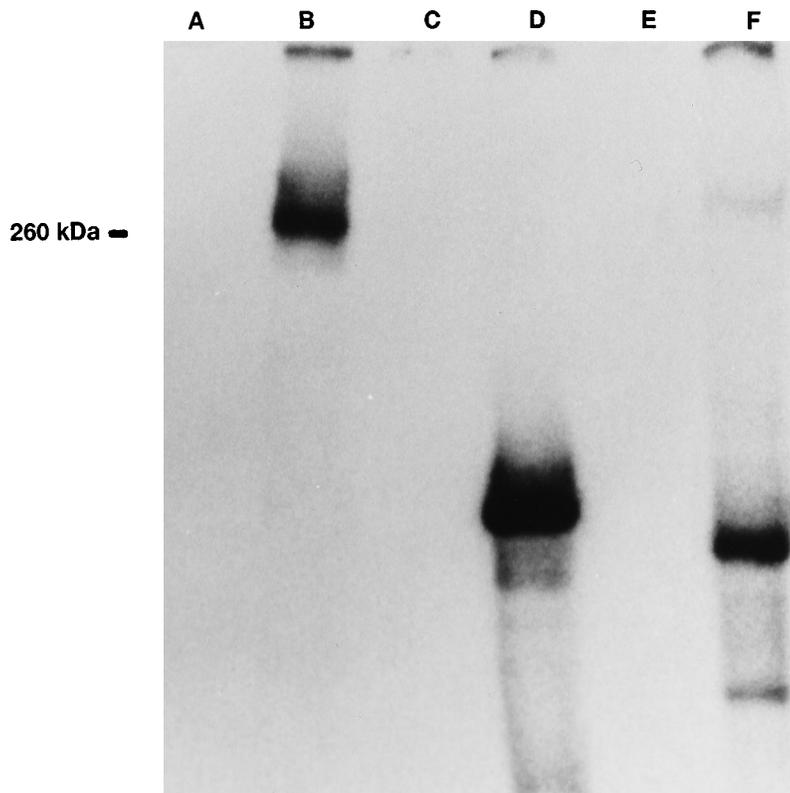
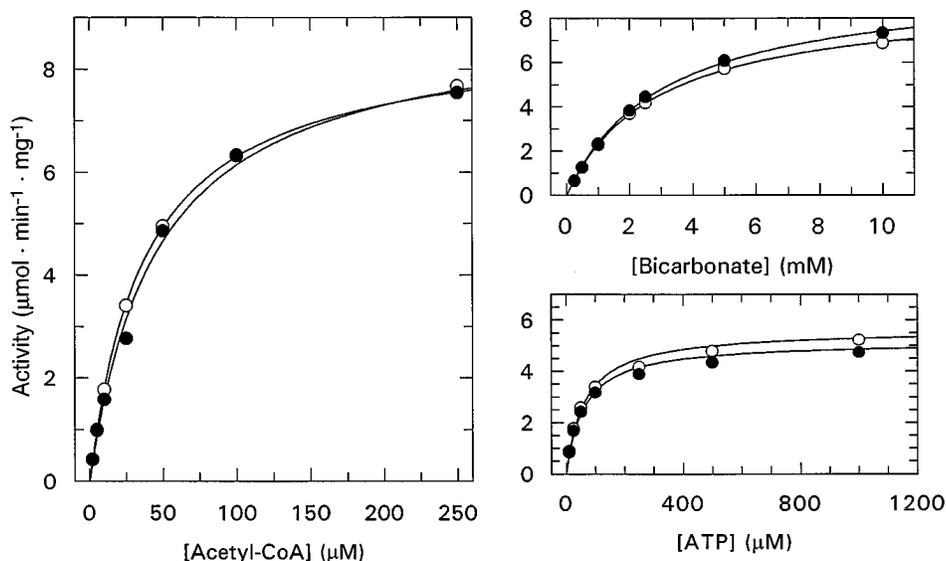


Fig. 2. Autoradiograph of dried gel from SDS-PAGE of ACC isolated from hindlimb muscle by avidin-Sepharose affinity chromatography and subjected to phosphorylation for 60 min, as described in MATERIALS AND METHODS. *Lanes A*, *C*, and *E*: ACC was treated with phosphorylation mixture in absence of kinase. *Lane B*: ACC was treated with PKA. *Lane D*: ACC was treated with phosphorylase kinase. *Lane F*: ACC was treated with PKC.

Fig. 3. Effect of phosphorylation by PKA (●) on muscle ACC activity in presence of variable concentrations of substrate. ○, No PKA treatment. Each point represents 5 determinations. The fitted curves are generated from the Michaelis-Menton equation, using Graftit.



Lane D indicates that ACC is not a substrate for phosphorylase kinase under these conditions. Lane F shows a slight degree of phosphorylation of skeletal muscle ACC by PKC. The dark bands indicating labeled phosphate incorporation into lower molecular weight proteins show that both phosphorylase kinase and PKC were active under these conditions.

Figure 3 shows the effect of phosphorylation by PKA on ACC activity in the presence of variable concentrations of each substrate. The curves represent the theoretical relationship between substrate concentration and activity, based on the Michaelis-Menton equation. Each point on each curve represents five determinations. For clarity, SE are not shown on these curves, but means and SE for kinetic constants are shown in Table 1. It seems clear that no significant changes occurred in any of the kinetic constants for any of the substrates in response to phosphorylation by PKA.

Figure 4 shows the effect of phosphorylation by AMPK on ACC activity in the presence of variable concentrations of the substrates. In contrast to the effects of PKA, marked changes in the  $K_m$  for ATP and acetyl-CoA occurred in response to phosphorylation by AMPK (Table 2).

Figure 5 shows the effect of sequential phosphorylation by PKA and AMPK. Prior treatment of muscle ACC

with PKA in the absence of labeled ATP (nonlabeled ATP in reaction mix) resulted in diminished incorporation of label by AMPK (compare lanes A and B). Radioactivity in the 272-kDa band was reduced by 31%. Similarly, prior treatment of ACC with AMPK in the absence of labeled ATP caused decreased incorporation of label (–36%) into ACC catalyzed by PKA (compare lanes C and D). Incorporation of label into the 272-kDa band by PKA was 57% of that induced by AMPK.

Figure 6 shows functional effects of sequential phosphorylation on citrate dependence of muscle ACC. Note that AMPK phosphorylation produces a marked change in the  $K_a$  for citrate activation and that PKA does not. AMPK phosphorylation produces nearly identical changes in  $K_a$  for citrate activation, regardless of the order of kinase treatment.

## DISCUSSION

Previous studies have demonstrated that PKA phosphorylates liver ACC at serines –77 and –1200 (7, 11). Phosphorylation at these sites was accompanied by an increase in  $K_{0.5}$  for citrate activation and a decrease in  $V_{max}$  for ACC as a function of citrate concentration. The critical site for PKA-induced changes in activity of the 260-kDa isoform of ACC appears from site-directed mutagenesis studies to be serine 1200 (9). A more recent report giving the partial amino acid sequence of human 272-kDa ACC shows serine 1200 to be missing in this isoform (26). In intact liver cells in culture, the inactivation of ACC by glucagon was found to be due to phosphorylation by the AMPK (which phosphorylates at serines 79, 1200, and 1215) instead of by the PKA (17). Hence, the role of the PKA in regulation of liver ACC is also not well defined.

Heart ACC (280 kDa) is also inactivated by phosphorylation, evidenced by an increase in activity after treatment with a phosphatase (20). AMPK increases in isolated perfused working rat hearts concurrently with a decrease in ACC activity (12). In isolated perfused rat hearts, the malonyl-CoA content correlates negatively

Table 1. Effect of phosphorylation of skeletal muscle acetyl-CoA carboxylase by PKA on kinetic constants for substrates

	Nonphosphorylated	Phosphorylated with PKA
$V_{max}$	$7.17 \pm 0.81 \mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$	$7.27 \pm 0.76 \mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$
$K_m$ , acetyl-CoA	$37.1 \pm 2.5 \mu\text{M}$	$37.7 \pm 2.9 \mu\text{M}$
$K_m$ , ATP	$61.7 \pm 2.4 \mu\text{M}$	$59.4 \pm 2.8 \mu\text{M}$
$K_m$ , bicarbonate	$2.87 \pm 0.23 \text{ mM}$	$2.95 \pm 0.24 \text{ mM}$

Values are means  $\pm$  SE for 5–6 determinations except for maximum velocity ( $V_{max}$ ) of enzyme reactions, where  $n = 22$ . PKA, protein kinase A;  $K_m$ , Michaelis constant. No significant differences were noted using Student's *t*-test for paired observations.

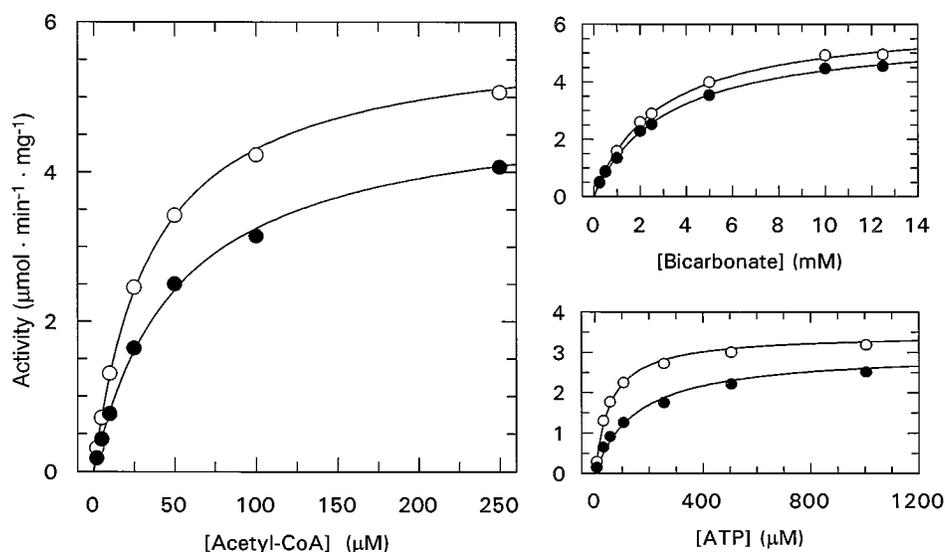


Fig. 4. Effect of phosphorylation by AMP-activated protein kinase (AMPK; ●) on muscle ACC activity in presence of variable concentrations of each substrate. ○, No AMPK treatment. Each point represents 5–6 determinations. Fitted curves are generated from the Michaelis-Menten equation, using Grafit.

with the rate of fatty acid oxidation (15). Fatty acid oxidation in isolated myocytes increases in response to inclusion of epinephrine in the medium (containing insulin and glucose), implying a role for PKA in regulation of rat heart ACC (1). The possibility of epinephrine regulation at other sites, such as malonyl-CoA decarboxylase modulation, is also recognized.

It is clear from the present study that the muscle ACC (272 kDa) is a substrate for PKA under in vitro conditions. It was anticipated that with phosphorylation by PKA, conformational changes would occur in the ACC, resulting in alteration of accessibility of substrates to the active site. This would be expected to alter the kinetic properties of the enzyme with respect to the three substrates and to the activator citrate. The fact that the  $V_{max}$ ,  $K_m$  for substrates, and the  $K_{0.5}$  for citrate activation were not significantly altered implies that neither the active site nor the citrate-binding domain of ACC was affected by PKA phosphorylation.

The current study is consistent with results obtained from adrenomedullated exercising rats (29). To determine the role of epinephrine in causing the decrease in malonyl-CoA in muscle during exercise, rats were adrenomedullated or sham operated and then subjected to treadmill running. The adrenomedullated rats, which showed no increase in plasma epinephrine during exercise, had the same decrease in malonyl-CoA as sham-operated rats, which showed an increase in epinephrine during exercise. Previous studies (30, 32) have demonstrated that the rise in muscle cAMP seen during exercise or during insulin-induced hypoglycemia is completely prevented in adrenomedullated rats. Thus the increase in epinephrine is not only unessential for decreasing malonyl-CoA, but the present study indicates that PKA has no detectable effect on catalytic properties of ACC. It thus appears that the AMPK is more likely responsible for the regulation of ACC activity in muscle.

The AMPK gene is highly expressed in skeletal muscle (18, 23), although the enzyme activity in skeletal muscle extracts is quite low (5). During exercise,

the AMPK is activated in skeletal muscle (31). This activation of AMPK is accompanied by a decreased ACC activity and a decrease in malonyl-CoA. We postulated that in response to a contraction-induced increase in free calcium or free 5'-AMP in the muscle, an AMPK kinase is activated which then phosphorylates and activates the AMPK (31). A similar mechanism has already been described for activation of liver AMPK (24). It has also been determined recently that 5'-AMP decreases the rate of inactivation of AMPK by protein phosphatase-2C and by protein phosphatase-2A (6). AMPK activation by whatever mechanism would ultimately result in the decrease in malonyl-CoA and an increase in fatty acid oxidation.

Two calcium-activated kinases were tested with respect to capacity for phosphorylating muscle ACC. The results indicate that if phosphorylase kinase is involved in control of ACC, it is not likely to be via direct phosphorylation of the enzyme. Possible phosphorylation of the AMPK has not been ruled out by these experiments. The small degree of phosphorylation by the PKC is unlikely to have regulatory effects (see Ref. 7), although this has not been examined carefully.

A previous report from our laboratories showed marked effects of phosphorylation by AMPK on citrate

Table 2. Effect of phosphorylation of skeletal muscle acetyl-CoA carboxylase by AMPK on kinetic constants for substrates

	Nonphosphorylated	Phosphorylated with AMPK
$V_{max}$	$5.84 \pm 0.83 \mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$	$4.92 \pm 0.61 \mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$
$K_m$ , acetyl-CoA	$35.5 \pm 1.3 \mu\text{M}$	$51.5 \pm 2.5 \mu\text{M}$
$K_m$ , ATP	$54.4 \pm 2.3 \mu\text{M}$	$147.1 \pm 11.0 \mu\text{M}$
$K_m$ , bicarbonate	$2.84 \pm 0.07 \text{mM}$	$3.15 \pm 0.15 \text{mM}$

Values are means  $\pm$  SE for 5–6 determinations. AMPK, AMP-activated protein kinase. All values for phosphorylated acetyl-CoA carboxylase (ACC) are significantly different from those of the nonphosphorylated ACC ( $P < 0.05$ ) using *t*-test for paired observations.

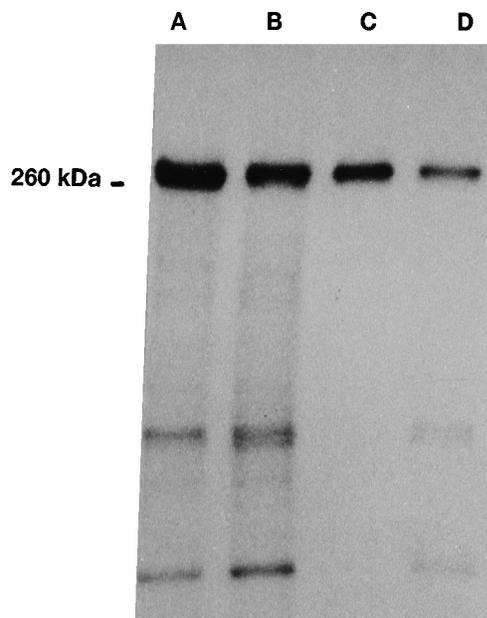
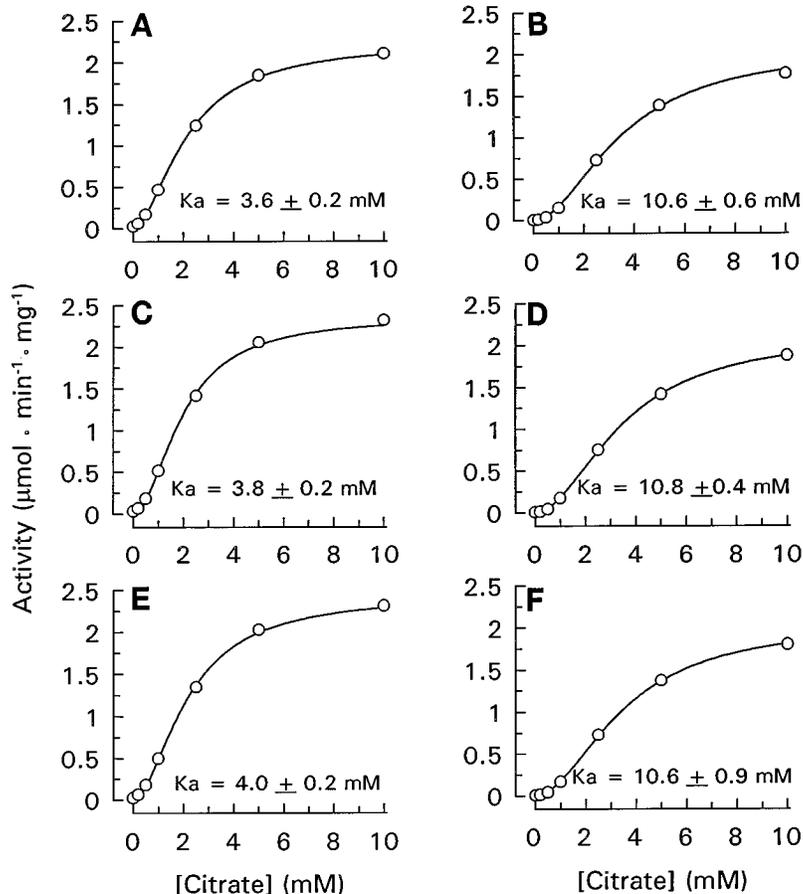


Fig. 5. Sequential phosphorylation of muscle ACC. Muscle ACC was treated with no kinase (*lane A*) or with PKA (*lane B*) for 30 min in absence of  $^{32}\text{P}$ -labeled ATP and then for an additional 30 min with AMPK in presence of  $^{32}\text{P}$ -labeled ATP. ACC was treated with no kinase (*lane C*) or with AMPK (*lane D*) for 30 min in absence of  $^{32}\text{P}$ -labeled ATP and then with PKA for an additional 30 min in presence of  $^{32}\text{P}$ -labeled ATP. Similar results were obtained with 6 additional determinations on 3 different enzyme preparations.

dependence of ACC (31). Particularly marked decreases in ACC activity occurred at physiological concentrations of citrate. The current studies demonstrate marked effects of phosphorylation of ACC by AMPK on  $K_m$  for acetyl-CoA and ATP. The changes in kinetic properties of ACC for these substrates may also contribute to inactivation of ACC and decrease in malonyl-CoA production in skeletal muscle during exercise.

Sequential phosphorylation of liver ACC by PKA followed by AMPK results in decreased phosphorylation at the serine-79 site, one that is unique for the AMPK (7). Presumably, phosphorylation of serine 77 by the PKA interferes sterically with phosphorylation of serine 79 by AMPK. The decrease in ACC activity seen with AMPK phosphorylation is partially attenuated when ACC is first treated with PKA. Although specific phosphorylation sites have not been identified for the muscle isoform of ACC, the possibility remained that AMPK effects may be modulated by PKA. In searching for a physiological role for muscle ACC phosphorylation by PKA, we considered the possibility of modulation of AMPK effects. The mutual interference of each kinase on phosphorylation by the other implies either the existence of at least one phosphorylation site common to both PKA and AMPK or possibly closely adjacent sites which sterically interfere. The data showing activity changes only with AMPK, regardless of the phosphorylation order, clearly indicate the existence of a unique site for AMPK that is uninflu-

Fig. 6. Effect on citrate dependence curves and activation constants ( $K_a$ ) for citrate (mM) of phosphorylation of muscle ACC by PKA alone (second 30 min; *E*), AMPK alone (second 30 min; *B*), and in sequence with AMPK first (30 min; *D*) and then with PKA first (30 min) followed by incubation with the other kinase an additional 30 min (*F*). *A*: purified enzyme not incubated with phosphorylation medium. *B*: enzyme incubated with AMPK. *C*: enzyme incubated for 60 min without kinase. Curves and  $K_a$  were generated from activity data, using a modified form of Hill equation. Each point on each curve represents the mean of 4 separate determinations on 2 different enzyme preparations. All  $K_a$  for AMPK-treated ACC are significantly different ( $P < 0.001$ ) from those not treated with AMPK. ACC treated with PKA as well as with AMPK (*D* and *F*) showed no significant differences from ACC treated with AMPK alone (*B*).



enced by phosphorylation by PKA at a common site or at a site unique to PKA. The muscle ACC appears to differ in this respect from the liver ACC.

In summary, PKA phosphorylates ACC isolated from rat skeletal muscle without any detectable change in catalytic function. AMPK phosphorylation of muscle ACC is accompanied by increases in  $K_m$  for acetyl-CoA, ATP, and bicarbonate, and a more than twofold increase in the  $K_a$  for citrate activation. Phosphorylation by PKA has no detectable modulatory effects on AMPK regulation of muscle ACC. We conclude that phosphorylation of ACC by AMPK is more important for regulation of malonyl-CoA and hence fatty acid oxidation in skeletal muscle than is phosphorylation by PKA.

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Address for reprint requests: W. W. Winder, Dept. of Zoology, 545 WIDB, Brigham Young Univ., Provo, UT 84602 (E-mail: winderw@acd1.byu.edu).

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