

Insulin stimulates the dephosphorylation and activation of acetyl-CoA carboxylase

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ABSTRACT The mechanism underlying the ability of insulin to acutely activate acetyl-CoA carboxylase [acetyl-CoA: carbon-dioxide ligase (ADP-forming), EC 6.4.1.2; AcCoA-Case] has been examined in Fao Reuber hepatoma cells. Insulin promotes the rapid activation of AcCoA-Case, as measured in cell lysates, and this stimulation persists to the same degree after isolation of AcCoA-Case by avidin-Sepharose chromatography. The insulin-stimulated enzyme, as compared with control enzyme, exhibits an increase in both citrate-independent and -dependent activity and a decrease in the K_m for citrate. Direct examination of the phosphorylation state of isolated ^{32}P -labeled AcCoA-Case after insulin exposure reveals a marked decrease in total enzyme phosphorylation coincident with activation. The dephosphorylation due to insulin appears to be restricted to the phosphorylation sites previously shown to regulate AcCoA-Case activity. All of these effects of insulin are mimicked by a low molecular weight autocrine factor, tentatively identified as an oligosaccharide, present in conditioned medium of hepatoma cells. These data suggest that insulin may activate AcCoA-Case by inhibiting the activity of protein kinase(s) or stimulating the activity of protein phosphatase(s) that control the phosphorylation state of the enzyme.

The activity of acetyl-CoA-carboxylase [acetyl-CoA:carbon-dioxide ligase (ADP-forming), EC 6.4.1.2; AcCoA-Case], a rate-limiting enzyme for fatty acid biosynthesis, may be rapidly modulated by several hormones. Epinephrine in rat adipose tissue and glucagon in rat liver and adipose tissue promote the inactivation of AcCoA-Case (1-3). This inactivation persists after purification of the enzyme to homogeneity and can be accounted for by agonist-stimulated increase in AcCoA-Case phosphorylation (1, 3). This hormone-mediated inactivation can be entirely mimicked by phosphorylation of the enzyme *in vitro* by the cAMP-dependent protein kinase and some other kinases active on AcCoA-Case (1, 3, 4).

The mechanism(s) by which insulin rapidly activates AcCoA-Case has proven to be elusive. Insulin in rat adipose tissue and adipocytes stimulates the activity of AcCoA-Case as measured in crude cellular extracts (1, 5-7). However, this stimulation has not persisted when the enzyme was isolated by avidin-Sepharose chromatography (1, 7, 8). Contrary to the expected dephosphorylation of AcCoA-Case in response to insulin, the only observed effect of insulin on the phosphorylation state of AcCoA-Case has been an increase in phosphorylation at a site distinct from that phosphorylated in response to epinephrine or glucagon (1, 5, 7, 8). It has been alternatively suggested that the insulin stimulation of AcCoA-Case is not due to a change in the phosphorylation state but rather to an unidentified allosteric regulator (7). In addition, insulin appears to promote the polymerization of the enzyme in rat adipose tissue (9, 10).

The relationship between insulin activation of AcCoA-Case and changes in its phosphorylation state has been clarified in the present study. Our results indicate that both insulin and an insulinomimetic oligosaccharide promote the dephosphorylation of AcCoA-Case coincident with its activation in Fao Reuber ("Fao") hepatoma cells.

MATERIALS AND METHODS

$^{32}\text{P}_i$ (carrier free) and $\text{NaH}^{14}\text{CO}_3$ were purchased from ICN. ^{125}I -labeled goat anti-mouse Ig was purchased from New England Nuclear. Cell culture media were obtained from GIBCO, and calf serum was from HyClone (Logan, UT). Insulin was a gift of Ronald Chance (Eli Lilly). All other chemicals were obtained from Sigma. Nitrocellulose was obtained from Schleicher & Schuell.

Cell Culture. Fao hepatoma cells were a kind gift of C. Ronald Kahn (Harvard Medical School). Fao cells were grown in RPMI 1640 medium supplemented with 2.5% fetal calf serum, 2.5% calf serum, and penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2 /95% air. All experiments were performed under serum-free conditions, as detailed below. H-35 hepatoma cells (a gift of Michael Czech), used for the production of the autocrine factor, were grown in Dulbecco's minimal essential medium supplemented with identical medium additions.

Experimental Design and Cell Labeling with $^{32}\text{P}_i$. Fao cells were seeded into P-100 plates (Corning) at 1×10^6 cells per plate and grown to 60-70% confluence over 3-4 days. The cells were then washed three times with 4 ml of ice-cold phosphate-buffered saline and then incubated for 1 hr in serum-free medium. This cycle of washing and serum-free incubation was then repeated two more times. The medium was again changed, and hormone additions were made after an additional 15 min. After 30 min of hormone exposure, the cells were washed twice with ice-cold phosphate-buffered saline and then lysed with a digitonin-containing buffer. Cell lysates were prepared by the addition of 400 μl of a buffer (4°C) containing 50 mM Tris chloride (pH 7.5), 1 mM EDTA, 0.1 M NaF, 10 mM 2-mercaptoethanol, 0.25 M sucrose, 0.4 mg of digitonin per ml, and seven protease inhibitors, as in ref. 11. The plates were rocked manually for 2 min; the lysate was removed and added directly to 0.1 vol of the same buffer without digitonin and containing 60 mg of fatty acid-free bovine serum albumin per ml on ice. This method of lysis was shown to remove quantitatively all of the AcCoA-Case, as judged by immunoblotting of lysates and residual cell pellets (data not shown). In each of the experiments reported here, lysates from six P-100 plates for each experimental condition were pooled in the same tube. An aliquot of this mixture was then removed for determination of AcCoA-Case activity. The remainder was then dialysed overnight against the avidin-Sepharose chromatography buffer (see below) used for isolation of AcCoA-Case.

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Abbreviation: AcCoA-Case, acetyl-CoA-carboxylase.
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^{32}P -labeling was done under identical conditions except that phosphate-free serum-free medium was used in the preliminary washing cycles, and the intermittent washings were done with Krebs-Ringer buffer containing 10 mM sodium Hepes (pH 7.4). Prior to hormone addition, the cells were additionally equilibrated with carrier-free $^{32}\text{P}_i$ (0.2 mCi/ml; 1 Ci = 37 GBq) for 2 hr. After hormone stimulation, lysates were prepared as with the unlabeled cells. Preliminary experiments established that these changes in the cell incubation protocol did not affect the hormonal stimulations previously observed in unlabeled cells.

AcCoACase Isolation. After dialysis, the extracts were applied to 1.0-ml monomeric avidin-Sepharose columns (11) equilibrated in 50 mM Tris chloride, pH 7.5/0.5 M NaCl/0.1 M NaF/2 mM EDTA/10 mM 2-mercaptoethanol/0.02% NaN_3 /10% (vol/vol) glycerol. The columns were then washed with >50 column vol of buffer; AcCoACase was then eluted in 2.5 ml of the same buffer containing 2 mM biotin directly into a plastic tube containing 0.25 ml of the same buffer containing 10 mg of bovine serum albumin per ml. The latter was added to diminish adsorption of the small amounts of eluted protein to the tube. These eluates and aliquots of the original lysates were then dialyzed against the original lysis buffer (without digitonin) overnight to diminish the high salt concentration, which interferes with the assay of enzyme activity. After dialysis, the fractions were assayed for AcCoACase activity, and samples of the eluates were prepared either for immunoblotting (unlabeled extracts) or for NaDodSO₄ gel electrophoresis and radioautography (labeled extracts).

AcCoACase Activity and Immunoblotting. The activity of AcCoACase was determined by $\text{H}^{14}\text{CO}_3^-$ fixation in the presence of various concentrations of citrate (0–10 mM) (12) modified by the substitution of acetate salts of Tris and Mg^{2+} for the chloride ones. AcCoACase mass in the avidin-Sepharose eluates of unlabeled extracts was determined by quantitative immunoblotting with an AcCoACase monoclonal antibody after NaDodSO₄ gel electrophoresis and transfer to nitrocellulose as in ref. 13. As shown in the autoradiogram in Fig. 1 *Upper*, a single band of M_r 240,000 in purified rat liver AcCoACase is recognized by this antibody.

Miscellaneous Methods. AcCoACase was isolated from the livers of Sprague-Dawley rats who had been fasted for 48 hr and then refed for 48 hr with a low-fat high-carbohydrate diet as described (11). Casein kinase II was purified from rat liver as in ref. 14. HPLC mapping of ^{32}P -labeled AcCoACase tryptic peptides was performed as described (1). The insulinomimetic oligosaccharide was partially purified from H-35 conditioned medium through the P2 gel filtration step as described (15). NaDodSO₄ polyacrylamide gels were performed by the method of Laemmli (16).

RESULTS

AcCoACase Activation in Fao Cells. As measured in cell lysates, both insulin and the insulinomimetic oligosaccharide factor increased the activity of AcCoACase; the largest effect was evident in the citrate-independent activity, although there was also an increase in citrate-dependent activity (Table 1). The degree of stimulation of AcCoACase by insulin is typical of that observed in extracts of other cells (1, 2, 5–7, 12). After purification of AcCoACase by the highly specific monomeric avidin-Sepharose method, the insulin- and factor-stimulated increases persisted to the same extent. Because of the low amounts of AcCoACase isolated in these experiments (1–2.5 μg from each pool of six P-100 plates) and their dilution in an albumin-containing buffer, we cannot claim that this is a homogeneous preparation based on protein assay or staining of NaDodSO₄ gels, although homogenous preparations are obtained from rat liver by the same isolation technique

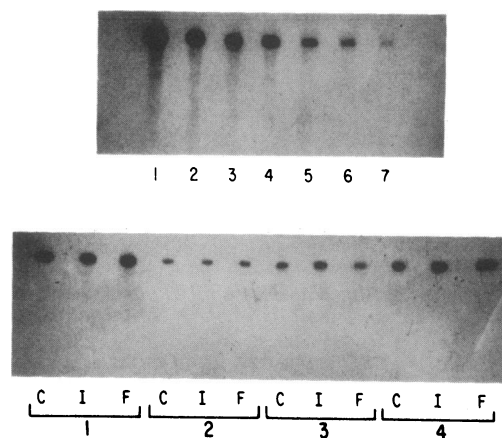


FIG. 1. Immunoblot analysis of purified AcCoACase derived from rat liver and Fao hepatoma cells. AcCoACase content in avidin-Sepharose isolates from Fao cells was determined by immunoblotting with AcCoACase monoclonal antibody as detailed. (*Upper*) Representative immunoblot derived from NaDodSO₄ gel electrophoresis (7.2% gels) and electrotransfer to nitrocellulose of various amounts of purified rat liver AcCoACase. The following amounts of AcCoACase were loaded in respective lanes: 1, 200 ng; 2, 160 ng; 3, 100 ng; 4, 80 ng; 5, 40 ng; 6, 20 ng; and 7, 10 ng. These bands were then excised, and radioactivity was determined to construct a standard curve. (*Lower*) Immunoblot derived from the analysis of avidin-Sepharose isolates from control (lanes C), insulin-stimulated (lanes I), and factor-stimulated (lanes F) Fao cells in four experiments (1–4); 16 μl of each eluate fraction was loaded on each lane of the NaDodSO₄ gel. The content of AcCoACase in each was then determined by excision of the labeled band, assay of ^{125}I , and comparison to the standard curve.

(11). However, the immunoblot technique does allow a precise estimate of AcCoACase content in the isolates. As shown in Fig. 1 *Lower*, a single band of M_r 240,000 that comigrates with rat liver AcCoACase was detected in the avidin-Sepharose eluates, and there were no significant differences in the recovery of this protein between control and agonist-stimulated isolations in four experiments (see also Table 1). This immunoblot technique allowed us to express the data as units of enzyme activity per mg of AcCoACase protein (Table 1); these estimates of specific activity were in good agreement with those of AcCoACase isolated from whole rat liver under a variety of nutritional conditions (11, 17). In addition, there was no significant difference in the percentage of enzyme activity recovered upon purification from control and stimulated lysates.

Comparison of the kinetic properties of AcCoACase isolated from control and stimulated cells indicates three changes that occurred with activation (Table 1 and Fig. 2). The largest effect of insulin and the factor was on citrate-independent activity (2- to 3-fold). There was also a 50% decrease in the K_a for citrate and a 50% increase in maximal activity measured at saturating citrate concentrations.

Changes in AcCoACase Phosphorylation. The persistence of AcCoACase activation after dialysis against high-salt buffers and, most importantly, after isolation by avidin-Sepharose chromatography suggests strongly that the insulin- and factor-stimulated change in AcCoACase activity is due to a covalent modification. While proteolysis can lead to AcCoACase activation (18), no evidence of altered molecular size was evident on the immunoblot (Fig. 1 *Lower*). Therefore, we next examined the effects of these stimulators on the phosphorylation state of AcCoACase as isolated from ^{32}P -labeled cells. A single [^{32}P]phosphoprotein of M_r 240,000, which comigrated with AcCoACase isolated from rat liver, was detected in the avidin-Sepharose eluates (Fig. 3 *Left*). The apparent ^{32}P content of AcCoACase was markedly diminished in isolates from insulin- and factor-stimulated

Table 1. AcCoACase activity in Fao cell lysates and avidin-Sepharose isolates

	Control	Insulin-stimulated	Factor-stimulated
Cell lysates V_{10} , mU/mg of protein	7.76 ± 0.56	10.39 ± 1.13	11.47 ± 1.33
V_0 , mU/mg of protein	0.98 ± 0.11	2.01 ± 0.21	2.23 ± 0.21
AcCoACase isolates V_{10} , U/mg of ACC	1.88 ± 0.18	2.67 ± 0.06	2.95 ± 0.28
V_0 , U/mg of ACC	0.24 ± 0.04	0.63 ± 0.15	0.88 ± 0.17
K_a citrate, mM	1.13 ± 0.28	0.43 ± 0.06	0.51 ± 0.07
AcCoACase protein, % control	100	109 ± 28	119 ± 32
AcCoACase activity recovery	25.4 ± 7.7	25.6 ± 5.0	25.5 ± 10.4

AcCoACase activity was determined in lysates of Fao cells after stimulation with insulin (100 nM) or the autocrine factor for 30 min in six experiments with separate platings of cells as detailed. In these experiments, the lysate was not added to the excess concentration of bovine serum albumin to permit precise estimation of its protein content. These lysate data are expressed as the means (±SD) of AcCoACase activity (milliunits per mg of lysate protein) measured in the absence (V_0) or presence (V_{10}) of a saturating (10 mM) citrate concentration. In a separate series of four experiments, AcCoACase activity was measured after isolation of the enzyme from lysates by avidin-Sepharose chromatography. AcCoACase content in these isolates was determined by quantitative immunoblotting by excision and counting of the ¹²⁵I-labeled band on blots with comparison to a standard curve constructed with isolated AcCoACase. The results are expressed as units of AcCoACase activity per mg of AcCoACase protein (means ± SD). The K_a for citrate (±SEM) was determined in these same isolates by enzyme assay at variable citrate concentrations; these data were analyzed by linear double reciprocal plots of $1/V - V_0$ versus $1/[citrate]$, and the K_a was determined by linear regression analysis. The recovery of AcCoACase protein through isolation is depicted as percent control recovery (±SD), as judged by immunoblotting. The total recovery varied between experiments with separate cell platings between 1 and 2.5 μg of AcCoACase protein per total volume of the eluate but did not differ significantly between control, insulin-, or factor-stimulated isolates within each experiment (see also Fig. 1 Lower). AcCoACase activity recovery was calculated based on assays of the lysates and the final eluates, as measured at a maximally stimulating citrate concentration; these data are expressed as % total lysate activity (±SD) recovered in the avidin-Sepharose eluates. The differences in AcCoACase activity parameters between control and agonist-stimulated are all significant at $P < 0.005$, as determined by Student's *t* test.

cells. In this same experiment, AcCoACase activity was enhanced in the insulin and factor-stimulated isolates coincident with this decrease in total ³²P content (Fig. 3 Right).

The ³²P-labeling protocol did not alter the stimulations of AcCoACase activity observed in unlabeled cells (compare Table 1 and Fig. 3). We were unable to measure the mass of AcCoACase in the ³²P-labeling experiments because of interference of ³²P with the ¹²⁵I-labeled immunoblot. The results presented in Table 1 derived from unlabeled cells argue strongly that the difference in ³²P radioactivity seen in the cell-labeling experiments was not due to a difference in AcCoACase mass in the isolates. Because of our inability to quantitate AcCoACase mass in the ³²P-labeled isolates, we also have presented the results in these experiments both as ³²P content per total eluate and as ³²P content per microunit

of AcCoACase activity in the absence and presence of citrate, the latter ratio being independent of enzyme mass (Table 2). Both total ³²P content in the eluate and this ratio were decreased in insulin- and factor-stimulated isolates, the

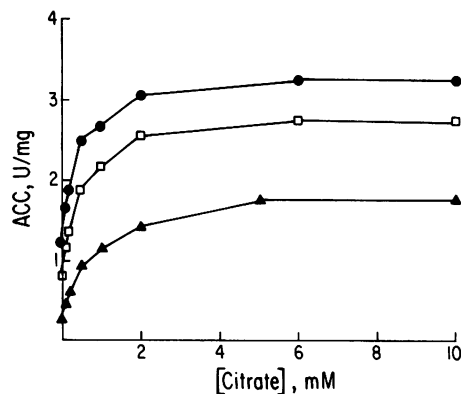


FIG. 2. AcCoACase activity as a function of citrate concentration in control, insulin-stimulated, and factor-stimulated AcCoACase isolates. Shown is a representative experiment in which AcCoACase activity was determined at variable concentrations of citrate in the assay in avidin-Sepharose isolates from control (▲), insulin-stimulated (□), and factor-stimulated (●) cells. The AcCoACase activity is expressed as units per mg of AcCoACase protein; the latter was determined by quantitative immunoblotting, as in Fig. 1.

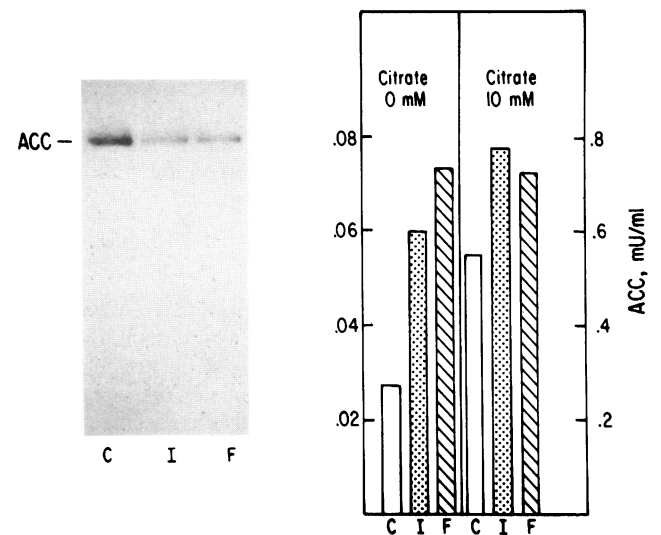


FIG. 3. Analysis of ³²P-labeled AcCoACase by radioautography and enzyme assay. (Left) Representative autoradiogram of ³²P-labeled avidin-Sepharose isolates separated on a NaDodSO₄/7.2% gel. Isolates (32 μl) from control (lane C), insulin-stimulated (lane I), and factor-stimulated (lane F) preparations were loaded on the gel in this experiment. The ³²P-labeled band comigrates with the Coomassie blue-stained M_r 240,000 subunit of rat liver AcCoACase (indicated to left). (Right) AcCoACase activity measured in the presence and absence of a saturating citrate concentration (10 mM) of these three isolates (□, control; ▨, insulin-stimulated preparation; and ▩, factor-stimulated preparation) in the same experiment. These results are expressed as milliunit per ml of isolate, since AcCoACase content could not be determined in these experiments by immunoblotting (see text).

Table 2. ^{32}P content and AcCoACase activity of isolates from ^{32}P -labeled cells

	Exp.	Control	Insulin-stimulated	Factor-stimulated
^{32}P , cpm per eluate	1	6150 (100)	3860 (63)	3440 (56)
	2	3864 (100)	2026 (52)	1936 (50)
^{32}P , cpm per microunit of AcCoACase Citrate (0 mM)	1	228 (100)	81 (36)	46 (20)
	2	77 (100)	34 (44)	30 (39)
(10 mM)	1	9.2 (100)	5.6 (61)	4.2 (46)
	2	6.6 (100)	3.8 (57)	2.1 (32)

Shown are two representative experiments derived from isolation of AcCoACase from ^{32}P -labeled cells after exposure to the control vehicle, insulin (100 nM), and factor for 30 min as detailed. In a third experiment, the same changes in ^{32}P content were observed, but activity was not determined. ^{32}P content is expressed as cpm per total volume of the dialyzed eluate; all radioactivity was precipitable with 25% trichloroacetic acid, and a single radiolabeled band was detected on NaDodSO₄ gel electrophoresis and radioautography (see Fig. 3). Because AcCoACase content could not be determined in these experiments (see text), the data have been normalized to the measurement of AcCoACase activity at 0 and 10 mM citrate in these same isolates and are presented as cpm of ^{32}P per microunit of AcCoACase activity. In parentheses is shown the percentage of each parameter relative to the control (= 100).

latter at each concentration of citrate. These data indicate that, per unit of enzyme activity, the AcCoACase isolated from insulin- and factor-stimulated cells is relatively dephosphorylated.

Preliminary mapping of tryptic phosphopeptides by reverse-phase HPLC from ^{32}P -labeled cells revealed a pattern of site-directed phosphorylation similar to that previously observed in both rat adipocytes and hepatocytes and after *in vitro* phosphorylation of AcCoACase with various protein kinases (not shown) (1, 3, 4). The agonist-stimulated dephosphorylation appeared to occur on a peptide that could be labeled *in vitro* on incubation of isolated rat liver AcCoACase with the cAMP-dependent protein kinase and an endogenous 5'-AMP-stimulated kinase present in highly purified preparations of AcCoACase.† No change in the phosphorylation of a second peptide that can be labeled *in vitro* with casein kinase II was seen either in response to insulin or the oligosaccharide. Thus, the agonist-mediated dephosphorylation of AcCoACase appeared to occur in the phosphorylation site(s) that is phosphorylated in the intact cell in response to agonists, such as glucagon and epinephrine, which inactivate the enzyme in intact cells and *in vitro* upon incubation with AcCoACase-inactivating protein kinases.

DISCUSSION

The results of the present study indicate that enzyme dephosphorylation is the mechanism by which insulin and an insulinomimetic autocrine factor produced by Reuber hepatoma cells acutely increase the activity of AcCoACase in Fao cells. Persistence of the agonist-induced activity change with purification of the enzyme coupled with changes in ^{32}P content argue persuasively for this underlying covalent change being responsible for the alteration in catalytic activity.

Several laboratories, including our own, have previously described an increase in AcCoACase phosphorylation in response to insulin in rat adipocytes and hepatocytes (1, 5, 7, 8). However, the insulin-stimulated change in AcCoACase activity detectable in crude cellular extracts has not persisted heretofore to the same extent on purification of the enzyme,

making linkage between this phosphorylation change and changes in catalytic activity uncertain. Our current experiments indicate that the dephosphorylation of AcCoACase is largely confined to one or more phosphorylation sites that are phosphorylated *in vitro* by two protein kinases (the cAMP-dependent protein kinase and an endogenous 5'-AMP-stimulated protein kinase) accompanied by enzyme inactivation. Therefore, dephosphorylation of these sites would be expected to increase AcCoACase activity. This same phosphorylation site(s) also appears to be phosphorylated in response to glucagon and epinephrine in rat cells coincident with enzyme inactivation (1, 3)

Dephosphorylation of AcCoACase *in vitro* by various protein phosphatases has long been recognized to lead to enzyme activation (4, 7, 11, 17, 20–23). This mechanism also has been demonstrated directly to occur *in vivo* in rat mammary tissue with alterations in nutrition (23) and has been postulated to occur *in vivo* in response to an acute glucose challenge (17, 24). The alterations in citrate-independent activity, maximal enzyme velocity (at maximal stimulating citrate concentrations), and K_a for citrate observed in the present study in response to insulin and the factor are entirely consistent with the demonstration of enzyme dephosphorylation, based on analogy to the protein phosphatase experiments. Each of these kinetic parameters has been shown to be influenced in the same way by *in vitro* dephosphorylation of AcCoACase (7, 17, 20–24).

The inability to maintain the insulin-stimulated increase in AcCoACase activity through enzyme purification has led others to suggest that insulin regulation occurs through an allosteric mechanism because of some unidentified allosteric regulator (7). This observation was based on the loss of insulin-stimulated AcCoACase activity after desalting of crude cell extracts in the presence of high salt concentrations. In the present study, we have been able to maintain the insulin-stimulated increase in activity after dialysis against a high-salt buffer, extensive high-salt washing after enzyme immobilization of avidin-Sepharose, and subsequent dialysis of biotin eluates of these columns. These observations argue strongly against such an allosteric mechanism.

Our previous inability and that of others (1, 5, 7, 8) to demonstrate insulin-stimulated dephosphorylation of AcCoACase coincident with enzyme activation is not explainable based on the present results. Some studies have been carried out in collagenase-isolated rat cells rather than in cultured cells. It is possible that collagenase isolation influences the experimental results. Alternatively, the hepatoma cells in culture might display unique regulatory properties. The technique of digitonin lysis used in this study allows

†Highly purified rat liver AcCoACase preparations contain an inactivating protein kinase (11). Recent experiments have determined that the activity of this kinase towards AcCoACase is markedly stimulated (6- to 10-fold) by 5'-AMP; half-maximal stimulation occurs at 20 μM 5'-AMP, with maximal stimulation at 200 μM (A. Bianchi and L. A. W., unpublished observations). This kinase is likely identical to that described by others (19).

rapid release of the cytosolic contents into a buffer containing both phosphatase and protease inhibitors; this rapid "quenching" may be necessary.

The present results suggest that attention should be drawn to insulin's ability to inhibit a cellular protein kinase that leads to AcCoACase inactivation or to activate a protein phosphatase that leads to site-specific dephosphorylation. Such kinases include the cAMP-dependent protein kinase and other cAMP-independent kinases (1, 4, 10, 19, 24, 25). While the kinases have some site specificity, the protein phosphatases active on AcCoACase in rat liver apparently do not discriminate between phosphorylation sites labeled by the cAMP-dependent protein kinase, casein kinase I, and casein kinase II (26). This nonselectivity makes it difficult to explain a discrete site-specific dephosphorylation in response to insulin as being due to activation of one of these phosphatases. These observations make it reasonable to speculate that the effect of insulin and the autocrine factor could be due to inhibition of one or more of the protein kinases active on AcCoACase.

We also have not observed an increase in phosphorylation in response to insulin in the AcCoACase peptide observed in previous experiments. This phosphorylation may be mediated by casein kinase II (1, 8). Phosphorylation of AcCoACase *in vitro* by this kinase has no effect on its kinetic properties (4, 14). It is possible that this phosphorylation occurs in adipocytes and in hepatocytes from fasted/refed rats because of the relative abundance of AcCoACase in these cells, as opposed to the cultured hepatoma cells. Alternatively, it is possible that the ability of insulin to dephosphorylate and activate AcCoACase is the result of a concerted phosphorylation/dephosphorylation occurring at separate phosphorylation sites. Sommercorn and co-workers have shown that insulin can cause a rapid activation of casein kinase II in 3T3-L1 adipocytes and Reuber hepatoma cells (27). In addition, this laboratory has reported that when AcCoACase is phosphorylated *in vitro* by this kinase, the rate of dephosphorylation of a separate site phosphorylated by the cAMP-dependent protein kinase is accelerated (28). We cannot exclude that such a concerted mechanism has not occurred in our cell lines, since the phosphorylation measurements were only done at a single time point (30 min).

The effects of insulin in these experiments were entirely mimicked by the autocrine factor, which has been partially purified from the conditioned medium of Reuber hepatoma cells (15). This factor may be identical to a polar oligosaccharide isolated after digestion of a glycopospholipid with a phosphatidylinositol-specific phospholipase C and after exposure of BC3H1 myocytes to insulin; these oligosaccharides have several other insulinomimetic actions in both whole cells and *in vitro* (29–33). While we have no evidence at present that this factor is generated in hepatoma cells in response to insulin, its ability to mimic the action of insulin to stimulate and dephosphorylate AcCoACase has provided a valuable probe that could be useful in identifying the locus of insulin regulation.

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1. Witters, L. A., Tipper, J. P. & Bacon, G. W. (1983) *J. Biol. Chem.* **258**, 5643–5648.
2. Brownsey, R. W., Hughes, W. A. & Denton, R. M. (1979) *Biochem. J.* **184**, 23–32.
3. Holland, R., Witters, L. A. & Hardie, D. G. (1984) *Eur. J. Biochem.* **140**, 325–333.
4. Munday, M. R. & Hardie, D. G. (1984) *Eur. J. Biochem.* **141**, 617–627.
5. Brownsey, R. W. & Denton, R. M. (1982) *Biochem. J.* **202**, 77–86.
6. Haystead, T. A. J. & Hardie, D. G. (1986) *Biochem. J.* **234**, 279–284.
7. Haystead, T. A. J. & Hardie, D. G. (1987) *Biochem. J.* **240**, 99–106.
8. Holland, R. & Hardie, D. G. (1985) *FEBS Lett.* **181**, 308–312.
9. Borthwick, A. C., Edgell, N. J. & Denton, R. M. (1987) *Biochem. J.* **241**, 773–782.
10. Borthwick, A. C., Edgell, N. J. & Denton, R. M. (1986) *Biochem. Soc. Trans.* **14**, 563–565.
11. Tipper, J. P. & Witters, L. A. (1982) *Biochim. Biophys. Acta* **715**, 162–169.
12. Witters, L. A., Moriarty, D. & Martin, D. B. (1979) *J. Biol. Chem.* **254**, 6644–6649.
13. Evans, J. A. & Witters, L. A. (1988) *Arch. Biochem. Biophys.*, in press.
14. Tipper, J. P. & Witters, L. A. (1983) *Arch. Biochem. Biophys.* **227**, 386–396.
15. Witters, L. A. & Watts, T. D. (1988) *J. Biol. Chem.* **263**, 8027–8036.
16. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
17. Jamil, H. & Madsen, N. B. (1987) *J. Biol. Chem.* **262**, 638–642.
18. Guy, P. S. & Hardie, D. G. (1981) *FEBS Lett.* **132**, 67–70.
19. Carling, D., Zammit, V. A. & Hardie, D. G. (1987) *FEBS Lett.* **223**, 217–222.
20. Krakower, G. R. & Kim, K.-H. (1981) *J. Biol. Chem.* **256**, 2408–2413.
21. Ingebritsen, T. S., Blair, J., Guy, P., Witters, L. A. & Hardie, D. G. (1983) *Eur. J. Biochem.* **132**, 275–281.
22. Thampy, K. G. & Wakil, S. J. (1985) *J. Biol. Chem.* **260**, 6318–6323.
23. Munday, M. R. & Hardie, D. G. (1986) *Biochem. J.* **237**, 85–91.
24. Jamil, H. & Madsen, N. B. (1987) *J. Biol. Chem.* **262**, 630–637.
25. Lent, B. & Kim, K.-H. (1982) *J. Biol. Chem.* **257**, 1897–1901.
26. Witters, L. A. & Bacon, G. W. (1985) *Biochem. Biophys. Res. Commun.* **130**, 1132–1138.
27. Sommercorn, J., Mulligan, J. A., Lozeman, F. J. & Krebs, E. G. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8834–8838.
28. Sommercorn, J., McNall, S. J., Fischer, E. H. & Krebs, E. G. (1987) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **46**, 2003 (abstr.).
29. Saltiel, A. R., Fox, J. A., Sherline, P. & Cuatrecasas, P. (1986) *Science* **233**, 967–971.
30. Saltiel, A. R. & Cuatrecasas, P. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5793–5797.
31. Mato, J. M., Kelly, K. L., Ablner, A. & Jarett, L. (1987) *J. Biol. Chem.* **262**, 2131–2137.
32. Kelly, K. L., Mato, J. M., Merida, I. & Jarett, L. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6404–6407.
33. Low, M. G. & Saltiel, A. R. (1988) *Science* **239**, 268–275.