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The short-term regulation of hepatic acetyl-CoA carboxylase during starvation and re-feeding in the rat

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Rapid inhibition of acetyl-CoA carboxylase (ACC) activity in rat liver in response to 6 h starvation and rapid re-activation in response to 2–6 h of re-feeding chow were shown to be due to changes in the expressed activity of existing enzyme. Decreases and increases in ACC concentration occurred at later stages of the transitions, i.e. 6–48 h starvation and 8–24 h re-feeding respectively. The decrease in expressed activity of ACC was due primarily to changes in its phosphorylation state, demonstrated by a significantly decreased $V_{max.}$ and significantly increased K_a for citrate of enzyme purified by avidin–Sepharose chromatography from 6 h- or 48 h-starved rats. These effects were totally reversed within 2–4 h of chow re-feeding. Changes in the activity of purified ACC closely correlated with reciprocal changes in the activity of AMP-activated protein kinase (AMP-PK) over the fed to starved to re-fed transition. Increases in the activity ratio of cyclic-AMP-dependent protein kinase in response to starvation lagged behind the increase in AMP-PK and the decrease in ACC activity. Changes in AMP-PK and ACC activities of rat liver closely correlated with changes in plasma insulin concentration in response to time courses of starvation and re-feeding.

INTRODUCTION

Fatty acid synthesis in the major lipogenic tissues of the rat is stringently regulated according to nutritional status. Although long-term changes in lipid synthesis can be effected through changes in enzyme concentration [1], short-term regulation is achieved via changes in the expressed activity of one or more regulatory enzymes in the pathway. There are a number of potential regulatory steps in the pathway of fatty acid synthesis from carbohydrate precursors, one of which is the reaction catalysed by acetyl-CoA carboxylase (ACC). Control of this enzyme may be particularly important in liver, where the supply of precursors for fatty acid synthesis (which include lactate [2] and amino acids) is not necessarily subject to the regulatory influences of hepatic glycolysis.

ACC is allosterically activated by citrate and inhibited by long-chain fatty acyl-CoA [3]. In addition, it can be inactivated *in vitro* via phosphorylation by the catalytic subunit of cyclic-AMP-dependent protein kinase (cAMP-PK) purified from bovine heart or an AMP-activated protein kinase (AMP-PK) isolated from rat liver [4]. In each case $V_{max.}$ is decreased and the concentration of citrate required to achieve half-maximal enzyme activation (K_a for citrate) is increased [4]. Amino acid sequencing of phosphopeptides reveals that, although both kinases phosphorylate serine-1200 of ACC, cAMP-PK phosphorylates serine-77 and AMP-PK phosphorylates serine-79 [4]. Experiments show that only serine-1200 and serine-79 are phosphorylated in isolated hepatocytes [5], implying that AMP-PK is the physiologically important kinase. AMP-PK is itself phosphorylated and activated by a separate kinase kinase activity [6] and, although this has proved not to be cAMP-PK, it has been suggested that cAMP-PK may be involved somewhere in the cascade [3,7].

Reversible phosphorylation and inactivation of ACC in rat liver in response to prolonged periods of starvation and re-feeding have been reported [8]. In the present study we have measured temporal changes in the activity of hepatic ACC in extracts of, and purified from, rat liver, in response to starvation and chow re-feeding. The changes in purified ACC activity are compared with changes in the activities of cAMP-PK and AMP-PK.

MATERIALS AND METHODS

Animals

Female albino Wistar rats (180–220 g) subjected to a 12 h-light/12 h-dark cycle (light from 08:30 h) were fed *ad libitum* on standard rodent diet (52% digestible carbohydrate, 16% protein, 2% lipid and 30% non-digestible residue, by wt.). Experiments were started at 08:30 h, at which time food was removed for either 6 h or 48 h. Starved rats (48 h) were re-fed *ad libitum* with chow and were sampled at intervals thereafter. Liver was freeze-clamped *in situ* while the rat was under sodium pentobarbital anaesthesia such that motor activity had ceased (5 min; 6 mg/100 g body wt.), and stored in liquid N_2.

Biochemicals

Sources of materials for enzyme assays and purification were as described in [9]. $NaH^{14}CO_3$ and [γ-^{32}P]ATP were obtained from ICN Flow, High Wycombe, Bucks., U.K. Kemptide and the specific peptide inhibitor of cAMP-PK were obtained from Sigma Chemical Co., Poole, Dorset, U.K. The specific peptide substrate HMRSAMSGLHLVK for AMP-PK assay was synthesized by Dr. Gabor Toth and Dr. Paolo Mascagni, using an ABI peptide synthesizer in the School of Pharmacy. Poly(ethylene glycol) 6000 was from BDH Chemicals, Poole, Dorset, U.K. The catalytic subunit of protein phosphatase 2A was purified as far as the polylysine–Sepharose step as described in [10].

Abbreviations used: ACC, acetyl-CoA carboxylase; cAMP-PK, cyclic AMP-dependent protein kinase; AMP-PK, AMP-activated protein kinase.

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Insulin assays

Plasma insulin levels were measured as described in [11].

ACC assay in poly(ethylene glycol)-precipitated protein pellets

Powdered frozen liver (0.1 g) was homogenized (30 strokes of Dounce homogenizer) in 10 vol. of 0.3 M-mannitol/100 mM-Tris/HCl buffer (pH 7.4 at 4 °C)/2 mM-EDTA/50 mM-NaF/2 mM-$Na_4P_2O_7$ containing 1 mM-benzamidine, 2 μg of soya-bean trypsin inhibitor/ml and 1 mM-phenylmethanesulphonyl fluoride. The homogenate was centrifuged (12000 *g*, 1 min) and poly(ethylene glycol) was added to the supernatant to a final concentration of 6% (w/v). The mixture was vortex-mixed, left on ice for 2 min and then centrifuged (12000 *g*, 3 min). The supernatant was discarded and the poly(ethylene glycol) pellets were frozen in liquid N_2.

For initial-activity measurements, the poly(ethylene glycol) pellet was resuspended in 150 μl of 0.15 M-mannitol/100 mM-Tris/HCl (pH 7.4 at 37 °C)/1 mM-EDTA/1 mM-dithiothreitol/100 mM-NaF/2 mM-$Na_4P_2O_7$ containing 1 mM-benzamidine and 2 μg of soya-bean trypsin inhibitor/ml. A 10 μl sample was assayed for ACC activity as described in [9]. For measurement of total activity the pellet was resuspended in 200 μl of the same buffer but without 100 mM-NaF or 2 mM-$Na_4P_2O_7$, but containing 20 mM-sodium citrate, 20 mM-$MgCl_2$ and 20 units of the catalytic subunit of protein phosphatase-2A/ml, and incubated for 30 min at 37 °C. ACC activity was then assayed as described in [9]. This poly(ethylene glycol) fractionation recovered > 90% of the ACC activity in the supernatant.

Purification and assay of ACC

ACC was purified by avidin–Sepharose affinity chromatography as described in [9], except that 2 mM-$Na_4P_2O_7$ was included in all buffers and ACC was eluted from the column in buffer containing no glycerol and assayed immediately. The kinetic parameters of pure ACC were determined as described previously [9].

Assay of cAMP-PK and AMP-PK

Assay of cAMP-PK was based on a modification of that described in [12]. Frozen liver powder was homogenized in 10 vol. of 10 mM-phosphate buffer, pH 6.8, containing 1 mM-EDTA, 1 mM-isobutylmethylxanthine and 1 mM-dithiothreitol by using 30 rapid strokes of a Teflon/Pyrex hand-held homogenizer. The homogenate was diluted 1:10 with 10 mg of BSA/ml and a 5 μl sample was immediately transferred to a 25 μl incubation mixture containing 10 mM-Hepes, pH 7.0, 150 μM-Kemptide, 0.2 mM-[γ-^{32}P]ATP [sp. radioactivity (2–4) × 10^6 c.p.m./nmol], 4 mM-$MgCl_2$ and 0.2 mM-EDTA. Initial cAMP-PK activity was measured as the linear incorporation of ^{32}P into Kemptide over a 4 min incubation at 37 °C from which incorporation in the presence of the specific peptide inhibitor of cAMP-PK (100 ng/ml) had been subtracted. The total cAMP-PK activity was measured in a similar manner but in the presence of 10 μM-cyclic AMP. At the end of each incubation, reactions were stopped with excess EDTA (5 mM) to chelate Mg^{2+}, and a sample of the reaction mixture was spotted on to a phosphocellulose P81 paper square.

For the assay of AMP-PK, frozen liver powder was homogenized in 10 vol. of 0.25 M-mannitol/100 mM-Tris/HCl (pH 7.2 at 4 °C)/50 mM-NaF/1 mM-EDTA containing 1 mM-benzamidine, 1 μg of soya-bean trypsin inhibitor/ml and 1 mM-phenylmethanesulphonyl fluoride by using 30 rapid strokes of a Teflon/Pyrex hand-held homogenizer. The homogenate was diluted 10-fold with 10 mg of BSA/ml and a 5 μl sample was transferred to 25 μl of incubation mixture containing 10 mM-Hepes, pH 7.0, 100 μM synthetic peptide HMRSAMSGLHLVK, 0.2 mM-[γ-^{32}P]ATP [sp. radioactivity (2–4) × 10^6 c.p.m./nmol), 4 mM-$MgCl_2$, 50 mM-NaF and 0.2 mM-EDTA. Incubations proceeded for 4 min at 37 °C, after which time they were stopped with excess EDTA (5 mM) and a sample of each reaction mixture was spotted on to a phosphocellulose P81 paper square.

The P81 papers from cAMP-PK or AMP-PK assays were washed three times in 75 mM-H_3PO_4, and the bound ^{32}P-labelled peptide was counted for radioactivity by immersion in 4 ml of toluene-based scintillant in an LKB-Rackbeta liquid-scintillation counter. The dilutions of supernatants into the assay were the same for all samples assayed. One unit of protein kinase activity represents incorporation of 1 nmol of ^{32}P into peptide/min. The activity of cAMP-PK was expressed as the ratio of initial activity (in the absence of cyclic AMP):total activity (in the presence of 10 μM-cyclic AMP).

RESULTS AND DISCUSSION

ACC activity in poly(ethylene glycol) pellets

The initial activity of ACC measured at 0.5 mM- or 10 mM-citrate (Fig. 1*a*) decreased by 50–70% in response to 48 h starvation, with the major proportion of this decrease occurring within the first 6 h. The initial activity of ACC in the tissue rapidly increased again within 2 h of chow re-feeding and increased steadily, such that ACC activity was 100% greater in livers of 24 h-re-fed rats compared with fed controls. There was no significant change in total ACC activity in the first 6 h of starvation, but by 48 h starvation it had declined by 60% (Fig. 1*c*). Total activity did not increase over the first 6 h of re-feeding, but by 24 h was higher than that of fed controls. These changes in ACC concentration agree with most previous observations ([13,14], but see also [15]), and are relatively slow, as might be expected for an enzyme with a half-life in excess of 24 h [16]. Such changes are probably due to changes in rates of ACC synthesis or degradation, since Zammit and co-workers [15] were unable to find evidence for the storage or recruitment of mitochondrial ACC forms in response to starvation or re-feeding as previously reported [14].

When initial activity is expressed as a percentage of total ACC activity (Fig. 1), it becomes clear that there is marked inactivation of the existing ACC protein within the first 6 h of starvation, but little further change over the subsequent 42 h. This suggests that the decreases in initial activities observed between 6 h and 48 h starvation (Fig. 1*a*) are due largely to decreases in enzyme concentration. Inactivation of hepatic ACC has previously been observed after 24 h starvation [15], but the present data suggest that inactivation occurs more rapidly than this.

The re-activation of ACC in response to re-feeding was rapid and complete within 6–8 h of re-feeding. Some 60% of this re-activation occurred during the first 2 h (Fig. 1*b*). This re-activation was clearly observed in a part of the time course during which ACC concentration had not changed (Fig. 1*c*). This rapid response returned the initial ACC activity to within 70–80% of the fed value (Fig. 1*a*) within 2 h re-feeding and preceded the re-activation of pyruvate dehydrogenase, which is refractory over this period [17]. Hence the increase in hepatic lipogenesis in the first 2 h of re-feeding in the absence of increased pyruvate dehydrogenase activity [18] may be the result of this ACC re-activation.

This study clearly shows that the rapid changes in ACC activity from 0–6 h starvation, and from 0–8 h re-feeding after 48 h starvation, are the result of changes in the intrinsic activity of existing ACC enzyme.

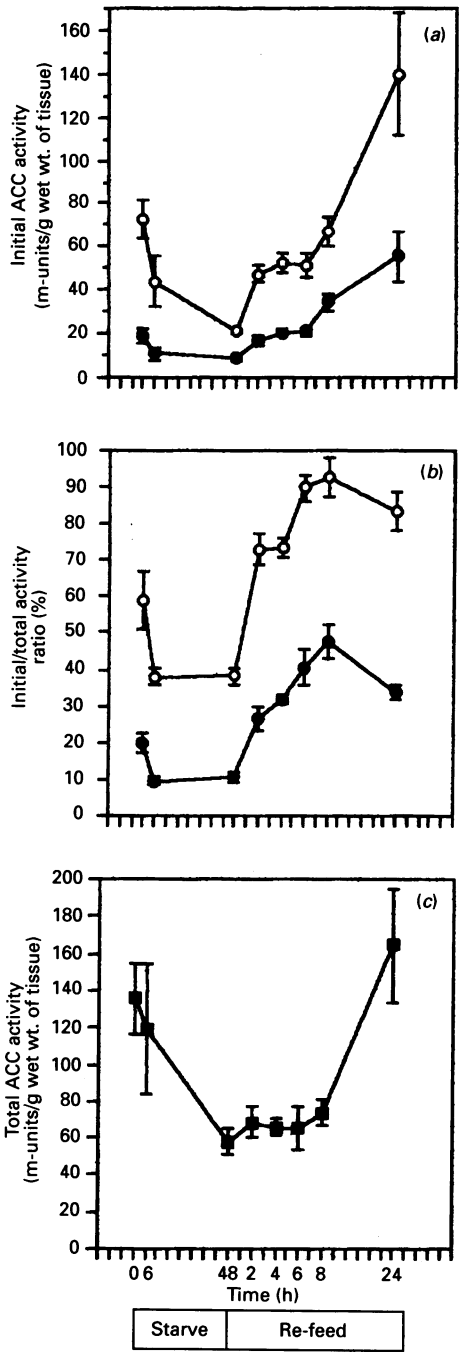

Fig. 1. ACC activity in poly(ethylene glycol) pellets from rat liver

Initial and total ACC activities were measured in resuspended poly(ethylene glycol) pellets as described in the Materials and methods section. (*a*) Initial activity measured at (●) 0.5 mM- and (○) 10 mM-citrate. (*b*) Initial activity at (●) 0.5 mM- and (○) 10 mM-citrate expressed as a percentage of total activity. (*c*) Total ACC activity. Each point is the mean of at least six observations, with S.E.M. represented by vertical bars.

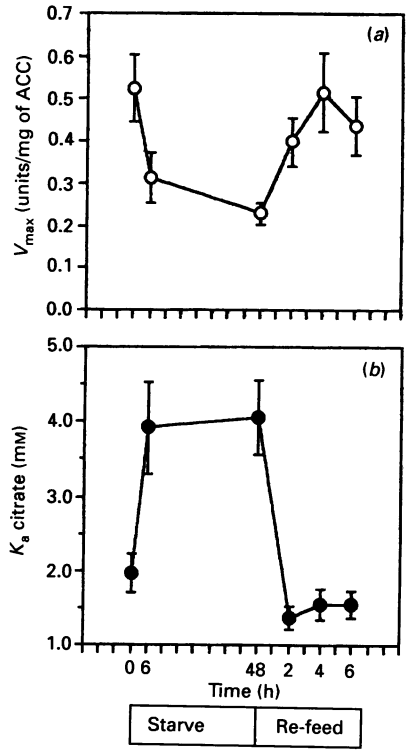

Fig. 2. Kinetic parameters of ACC purified from rat liver

ACC was purified by avidin–Sepharose affinity chromatography at the times of starvation and re-feeding indicated. At each time point, $V_{max.}$ and the concentration of citrate required for half-maximal activation, K_a citrate, were measured as described in the Materials and methods section. Each value represents the mean of at least four separate preparations at each time point, with S.E.M. represented by vertical bars.

Mechanism of ACC inactivation

Differences in enzyme activity after the purification by avidin–Sepharose chromatography are solely the result of differences in phosphorylation state, since this procedure requires the monomerization of the enzyme, and removes endogenous citrate or fatty acyl-CoA. The absolute values of K_a for citrate (2.0 mM) and $V_{max.}$ (0.6 unit/mg) for avidin–Sepharose-purified ACC from the livers of fed rats (Fig. 2) compare favourably with reported values for rat hepatocytes [5], although the $V_{max.}$ value is slightly lower.

Within the first 6 h of starvation, the $V_{max.}$ of ACC decreased and the K_a for citrate increased dramatically (Fig. 2). There was little change in either parameter in response to a further 42 h of starvation (Fig. 2). This suggests that ACC is maximally inactivated within the first 6 h of starvation, via phosphorylation. After 48 h starvation, the $V_{max.}$ of hepatic ACC had decreased by 53% and the K_a for citrate increased by 100%. Re-activation of ACC in liver in response to chow re-feeding occurred rapidly. Increases in $V_{max.}$ (Fig. 2*a*) and decreases in the K_a for citrate (Fig. 2*b*) of purified hepatic ACC had occurred within 2 h re-feeding. However, these were not entirely coincident, since the full restoration of $V_{max.}$ to fed control levels required 4 h re-feeding, whereas K_a for citrate was fully restored within 2 h (Fig. 2). This might suggest that $V_{max.}$ and K_a for citrate are controlled by different phosphorylation sites on ACC that are dephosphorylated at different rates. The data of Hardie and co-workers appear to rule out the possibility that one of these is serine-1200 [19], but there are at least six other phosphorylation sites in the *N*-terminus of the molecule [3]. Phosphorylation/inactivation and dephosphorylation/activation of ACC purified by other methods has been reported in livers of 2-day-fasted/2-day-re-fed rats in response to subsequent prolonged periods of starvation and re-feeding, respectively [8]. Witters and co-workers have reported a more rapid (and apparently cAMP-PK or AMP-PK site-specific) dephosphorylation and activation of ACC in Fao Reuber hepatoma cells in response to insulin [20].

Comparison of Figs. 1(*b*), 2(*a*) and 2(*b*) suggests that, although

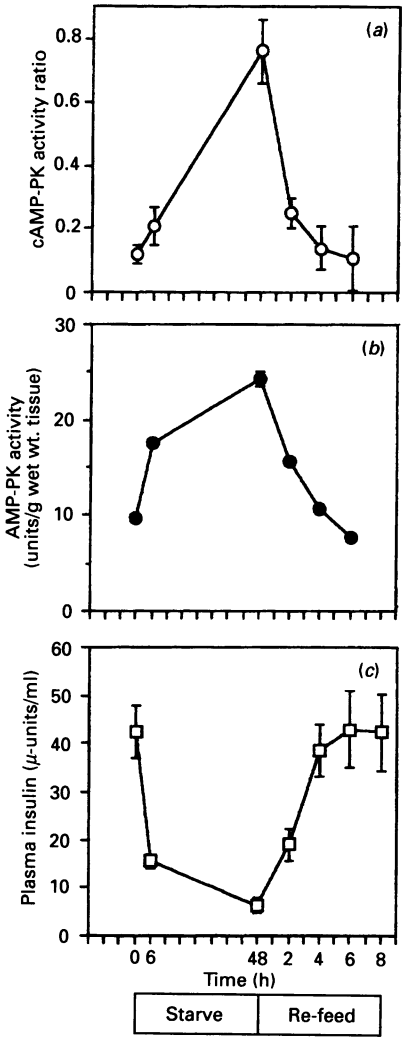

Fig. 3. Hepatic cAMP-PK and AMP-PK activity and plasma insulin concentrations

(*a*) cAMP-PK activity was measured in the absence and presence of 10 μM-cyclic AMP at intervals during starvation and re-feeding as described in the Materials and methods section, and the ratio of activity in the absence to that in the presence of cyclic AMP is shown. (*b*) AMP-PK activity was measured with HMRSAM-SGLHLVK as substrate as described in the Materials and methods section. (*c*) Arterial plasma insulin concentration was measured as described in the Materials and methods section. Each point represents the mean of at least four values $\pm$ S.E.M.

dephosphorylation of the inhibitory phosphorylation sites and activation of ACC may occur within the first 2–4 h of re-feeding (Fig. 2), there is further activation of the enzyme between this period and the increase in enzyme concentration at 8 h re-feeding (Fig. 1*b*). Denton and co-workers have shown that insulin treatment of adipocytes promotes the polymerization and activation of ACC [21]. It has been reported that hepatic ACC undergoes a protomer-to-polymer transition when 48 h-fasted rats are re-fed on chow for 24 h [8], and re-feeding was also observed to produce a gradual increase in the citrate-independent activity of ACC in starved rat liver [16]. Citrate-independent ACC activity was not measured in the poly(ethylene glycol) pellets of the present study, but in avidin–Sepharose-purified ACC it was negligible and did not vary independently of the kinetic parameters. It has been suggested that avidin–Sepharose purification may select against certain pools of ACC, particularly the citrate-independent polymeric form [21]. These data suggest that the rapid re-activation of hepatic ACC in the early stages of chow re-feeding may comprise a number of components, including dephosphorylation and activation, accompanied or preceded by polymerization.

Hepatic AMP-PK activity

AMP-PK is thought to be the kinase responsible for phosphorylation and inactivation of ACC in response to glucagon in isolated hepatocytes [5]. The activity of AMP-PK increased by 75% in response to 6 h starvation and by 140% after 48 h starvation (Fig. 3*b*), and rapidly decreased upon re-feeding. This decrease was evident within 2 h, and activity returned to fed levels after 4 h (Fig. 3*b*). These rapid changes in AMP-PK activity occurred in parallel with the rapid changes in activity of purified ACC, demonstrating a remarkable inverse correlation with the pattern of changes in ACC $V_{max.}$ (Fig. 2*a*) and providing yet further support for a major physiological role of AMP-PK in regulating ACC activity *in vivo*.

The observed changes in AMP-PK activity are unlikely to be due directly to changes in cellular AMP concentration, since they survive a 500-fold dilution into the assay. Changes are more likely to be due to changes in the phosphorylation and activation state of the AMP-PK, the concentration of AMP-PK or the concentration or activity of one or more inhibitors of AMP-PK within the cell. Each possibility requires further investigation.

Hepatic cAMP-PK activity

It has been suggested that cAMP-PK participates in the phosphorylation cascade mediating the activation of AMP-PK by glucagon [3]. In the present study, as the activities of hepatic AMP-PK and cAMP-PK did not increase in parallel after 6 h starvation (Figs. 3*a* and 3*b*), we were unable to obtain clear confirmation of this putative role. However, such participation is not necessarily excluded, since apparently insignificant rises in cyclic AMP concentration in hepatocytes can give rise to approx. 90% of the maximal increase in phosphorylase *a* activity [22]. The relatively minor response of cAMP-PK to 6 h starvation (as opposed to 48 h starvation, where the activity ratio had increased 7-fold; Fig. 3*a*) is in keeping with the reported gradual increase in hepatic cyclic AMP concentrations (14% after 6 h starvation, 90% after 48 h starvation) [23]. The increase in activity ratio of cAMP-PK was rapidly reversed by re-feeding, reaching fed control levels within 4 h (Fig. 3*a*), in parallel with AMP-PK activity.

Changes in AMP-PK activity in response to starvation and re-feeding closely and inversely correlated with changes in the plasma insulin concentration (Fig. 3*c*). This suggests that insulin may regulate AMP-PK activity in rat liver *in vivo*. An insulin-stimulated inactivation of AMP-PK would facilitate rapid dephosphorylation of inhibitory phosphorylation sites.

Conclusions

The results of this study clearly demonstrate that marked ACC inactivation occurs within the first 6 h of starvation. This is likely to be the result of increased phosphorylation in response to a parallel increase in AMP-PK activity. The magnitude of the decrease in $V_{max.}$ for pure ACC observed after 48 h starvation in this study (53%) strongly suggests phosphorylation by AMP-PK (which produces a $V_{max.}$ decrease of 81% *in vitro*) rather than by cAMP-PK (which decreases $V_{max.}$ by 13% *in vitro*) [4]. The greater decrease in $V_{max.}$ by AMP-PK is achieved by phosphorylation of serine-79 on the ACC polypeptide chain compared with serine-77 phosphorylated by cAMP-PK [4]. Phosphorylation of these two sites is mutually exclusive [24], and only serine-79 is phosphorylated when ACC is inactivated by glucagon treatment of hepatocytes [5,19].

The re-activation of ACC in response to re-feeding is accompanied by decreased AMP-PK and cAMP-PK activity and increased plasma insulin. The ACC response occurs in at least three phases; the initial rapid phase is apparent as an increased $V_{max.}$ and decreased K_a for citrate of pure ACC, suggestive of enzyme dephosphorylation. The second phase may involve insulin-stimulated polymerization of the enzyme, and the third is a longer-term increase in the concentration of ACC protein. The observation that ACC re-activation precedes that of pyruvate dehydrogenase when starved rats are re-fed may represent a shift in the control point in the pathway in anticipation of an increased supply of carbohydrate precursors. Assuming that insulin is the signal for activation of both enzymes, it will be interesting to discover how the individual mechanisms of regulation achieve differential responses.

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