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The low activity of acetyl-CoA carboxylase in basal and glucagon-stimulated hepatocytes is due to phosphorylation by the AMP-activated protein kinase and not cyclic AMP-dependent protein kinase

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Acetyl-CoA carboxylase purified from isolated hepatocytes is activated dramatically by protein phosphatase treatment, concomitant with a reduction of the phosphate content from 3.7 to 1.1 mol/subunit. Glucagon treatment of the cells produces a further inactivation of the enzyme that is totally reversed by phosphatase treatment, and is associated with an increase in phosphate content of 0.8 mol/subunit, distributed in two peptides which contain the sites phosphorylated in vitro by the cyclic AMP-dependent and AMP-activated protein kinases. Sequencing of these peptides shows that the low activity of acetyl-CoA carboxylase is due to phosphorylation by the AMP-activated protein kinase, and not cyclic AMP-dependent protein kinase, even after glucagon treatment.

Acetyl-CoA carboxylase; Hepatocyte; Glucagon; AMP-activated protein kinase; cyclic AMP-dependent protein kinase; (Rat)

1. INTRODUCTION

Acetyl-CoA carboxylase catalyses the first step committed to fatty acid biosynthesis, and is known to be regulated in vitro by allosteric effectors (e.g. activation by citrate) and by phosphorylation at multiple sites by a variety of protein kinases [1,2], including cyclic AMP-depdendent protein kinase [3,4] and a novel protein kinase from rat liver which also phosphorylates HMG-CoA reductase, and which we have termed the AMP-activated protein kinase [5,6]. Recently we have defined by amino acid sequencing the sites on acetyl-CoA carboxylase at which these two kinases inactivate the enzyme [7]. Phosphorylation by cyclic AMP-dependent protein kinase produces an increase in K_a for citrate and a modest depression of V_{max}, and is associated with the phosphorylation of sites 1 (RMSF) and 2 (SSMSGLHLVK). Phosphorylation by the AMP-activated protein kinase also increases the K_a for citrate but produces a much more dramatic decrease in V_{max}, and is associated with phosphorylation of sites 1 and 3, the latter (SSMSGLHLVK) being immediately adjacent to site 2 [7].

We have shown that hormones which increase cyclic AMP levels (glucagon in hepatocytes [8], glucagon and adrenaline in adipocytes [9,10]) lead to both inactivation and phosphorylation of acetyl-CoA carboxylase, although the enzyme is already in a highly phosphorylated form of low specific activity in unstimulated cells, particularly in hepatocytes. Phosphorylation in response to glucagon/adrenaline occurred within the same peptides which are phosphorylated by cyclic AMP-dependent protein kinase in vitro [8–10]. However, since the AMP-activated protein kinase phospho-

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Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HPLC, high-performance liquid chromatography

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rylates sites within these same peptides, in one case at a different serine residue, it was not clear which of the two kinases was responsible for the phosphorylation and low activity of the enzyme in basal or stimulated cells. We have therefore re-examined this question by sequencing of phosphopeptides derived from ^{32}P-labelled, isolated hepatocytes.

2. MATERIALS AND METHODS

Sources of materials have been described [7,8,11]. Hepatocytes were isolated from starved-refed rats in mid-dark phase, preincubated initially for 30 min, then for 60 min with or without [^{32}P]phosphate, and then treated with or without glucagon (10^{-7} M) for 15 min [8]. Acetyl-CoA carboxylase was purified by avidin-Sepharose chromatography as described [8] except that the buffers contained the protein phosphatase inhibitor Na pyrophosphate (5 mM) as well as NaF. For experiments in which the purified acetyl-CoA carboxylase was treated with protein phosphatase, the avidin-Sepharose column was washed with 5 vols buffer without NaF and pyrophosphate immediately before elution with the same buffer plus 2 mM biotin. The purified enzyme was treated with protein phosphatase-1 (400 U/ml [11]) in the biotin buffer in the presence of 1 mM $MnCl_2$ for 45 min at 37°C. Acetyl-CoA carboxylase was assayed using the ^{14}C-fixation assay [8]. For proteolytic digestion, the enzyme was precipitated with 300 μg bovine serum albumin using 55% saturated ammonium sulphate. The precipitate was collected in a microcentrifuge and resuspended in 50 mM Na Hepes (pH 7.0), 50 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM dithiothreitol, and digested for 16 h at 37°C with trypsin (1:50 by wt), chymotrypsin (1:50), or both proteinases together. The reaction was stopped by adding trichloroacetic acid to 10% (w/v). Undigested material was removed by centrifugation, trichloroacetic acid removed by ether extraction, and peptides were separated by reversed-phase HPLC in 0.1% (v/v) trifluoroacetic acid [7]. Automated Edman degradation was carried out on an Applied Biosystems 470A gas phase sequencer after derivatization of phosphoserine with ethanethiol [12]. Alternatively, samples were run without derivatization, and after various cycles of degradation, sections of the filter were removed [13], and the nature of the bound ^{32}P radioactivity analysed by thin-layer electrophoresis at pH 1.9 [7].

3. RESULTS

3.1. *Effect of protein phosphatase treatment on acetyl-CoA carboxylase purified from control or glucagon-treated hepatocytes*

Fig.1 shows that, as reported previously [8], glucagon treatment of isolated hepatocytes produced an inactivation of acetyl-CoA carboxylase which was stable during purification to homogeneity in the presence of protein phosphatase inhibitors. In three experiments glucon decreased the V_{max} of the enzymes from 0.97 ± 0.24 to 0.45 ± 0.09 μmol/min per mg (mean ± SE), while it increased the K_a for the allosteric activator, citrate, from 5.65 ± 0.86 to 8.07 ± 3.3 mM. Fig.1 shows that protein phosphatase treatment dramatically activated enzymes from both control and glucagon-treated cells, so that their activities became indistinguishable. At a physiological citrate concentration (0.6 mM), the control enzyme was activated 13-fold and the 'glucagon' enzyme 26-fold (mean of 3 experiments). For control and glucagon samples respectively, the V_{max} values increased to 3.19 ± 0.19 and 3.35 ± 0.06 μmol/min per mg, and the K_a values decreased to 1.1 ± 0.4 and 1.4 ± 0.1 mM.

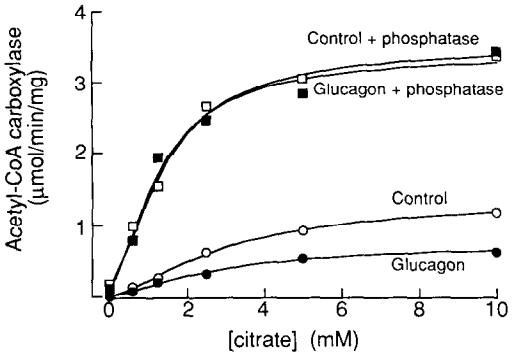

Fig.1. Activity, expressed as a function of citrate concentration, of acetyl-CoA carboxylase purified from control (open symbols) or glucagon-treated (filled symbols) hepatocytes. Square symbols show the activity after protein phosphatase treatment of the purified enzymes.

When cells were ^{32}P-labelled for 60 min prior to hormone treatment, the inactivation produced by glucagon was found to be associated with a 16 ± 2% (8) increase in the specific activity of purified acetyl-CoA carboxylase. In 2 experiments we estimated the actual phosphate content of the enzyme by determining the specific activity of the intracellular nucleotides, and obtained values of 3.92 and 3.56 mol/subunit (control) and 4.71 and 4.27 mol/subunit (glucagon), which are slightly lower than values obtained in a previous, more extended series of measurements [8]. Protein phosphatase treatment reduced the phosphate content to 1.1–1.3 mol/subunit in every case.

3.2. *Phosphopeptide analysis of acetyl-CoA carboxylase from control and glucagon-treated hepatocytes*

Acetyl-CoA carboxylase purified from ^{32}P-labelled, control or glucagon-treated hepatocytes was digested with proteinases and the radioactive phosphopeptides analysed by reversed-phase HPLC. Fig.2 shows that glucagon produced small increases in phosphorylation within a single tryptic peptide (T1), or within two peptides generated by simultaneous digestion with trypsin plus chymotrypsin (TC1, TC2). Each of these peptides comigrated (fig.2) with previously sequenced peptides [7] containing sites phosphorylated by the cyclic AMP-dependent and AMP-activated protein kinases: T1 corresponds to the peptide SSMSGLHLVK, containing sites 2 and 3; TC1 corresponds to the peptide SSMSGL, also containing sites 2 and 3; and TC2 corresponds to the peptide RMSF, containing site 1. We have shown previously that glucagon increased ^{32}P labelling of a single chymotryptic peptide, comigrating with the single major phosphopeptide (C1) produced by chymotryptic digestion of acetyl-CoA carboxylase phosphorylated by cyclic AMP-dependent protein kinase [8]. Peptide C1 has now been shown by sequencing to be identical with TC2 [7].

In the same 8 experiments in which the total specific activity of acetyl-CoA carboxylase increased by $16 \pm 2\%$, the increases in labelling of peptides TC1 and TC2 were 28 ± 6 and $40 \pm 5\%$, respectively. All of these effects of glucagon were significant ($p < 0.05$) by the paired t-test.

3.3. *Analysis of the serine residue phosphorylated within peptide T1*

Since peptides C1 and TC2 are identical and contain a single phosphorylatable serine residue, it is clear from this and our previous studies [8] that glucagon treatment of hepatocytes (and adrenaline or glucagon treatment of adipocytes [10]) increases phosphorylation of site 1. However, peptides T1 and TC1 contain three serine residues, at least two of which (sites 2,3) can be phosphorylated by distinct protein kinases in vitro. Peptide T1 was purified from control hepatocytes and subjected to automated Edman degradation in the gas-phase sequencer after derivatization with ethanethiol to convert phosphoseine to ethylcysteine [12]. The results (not shown) confirmed that the peptide was

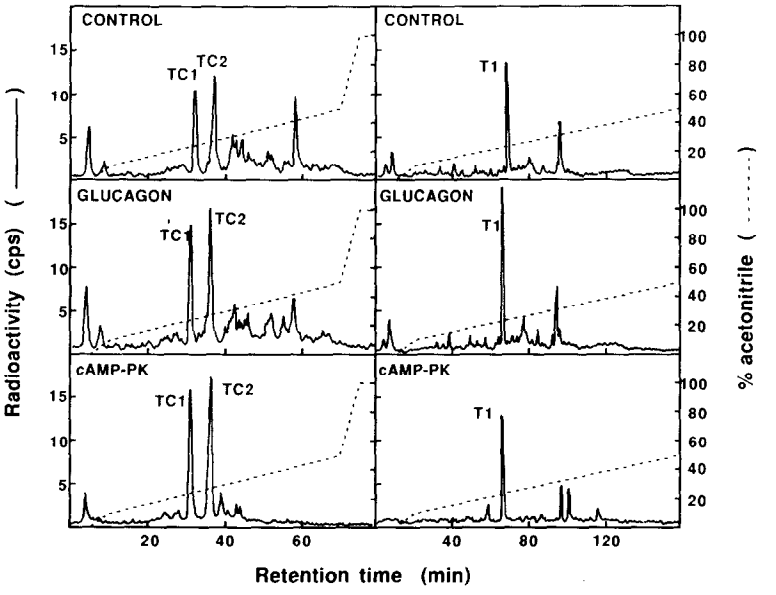

Fig.2. Reversed-phase HPLC analysis of ^{32}P-labelled peptides derived from digestion of acetyl-CoA carboxylase by digestion with trypsin + chymotrypsin (left) or trypsin alone (right). Acetyl-CoA carboxylase was either purified from ^{32}P-labelled, control or glucagon-treated hepatocytes, or was purified from lactating mammary gland and phosphorylated in vitro using [γ-^{32}P]ATP and the purified catalytic subunit of cyclic AMP-dependent protein kinase.

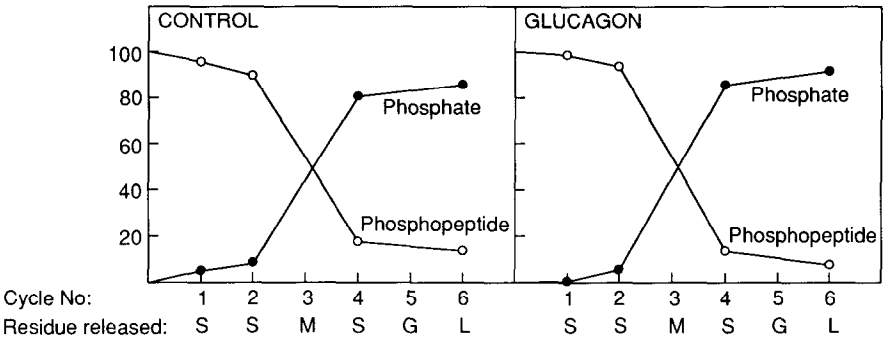

Fig.3. Recovery of radioactivity eluted from the glass fibre filter after various cycles of Edman degradation of peptide T1 in the gas-phase sequencer. Peptide T1 was isolated from ^{32}P-labelled, control or glucagon-treated hepatocytes. Samples were analysed by thin-layer electrophoresis at pH 1.9, and results are expressed as the % of the total radioactivity recovered migrating as free phosphate or as phosphopeptide. The total radioactivity recovered was 500–600 cpm (Cerenkov) in each case.

identical with peptide T1 phosphorylated by cyclic AMP-dependent protein kinase, and the recovery of ethylcysteine in the 4th cycle showed that site 3 was at least partially phosphorylated in the intact cells. To study this more quantitatively, we analysed the underivatized peptides from control and glucagon-treated cells by the method of Wang et al. [13], in which sections of the filter containing aliquots of the sample are removed after various cycles of degradation, eluted, and the radioactive products analysed for the presence of phosphopeptide or free phosphate. The results (fig.3) showed that only ~5% of the radioactivity was recovered as free phosphate in cycles 1 and 2, as against 80–90% after cycle 4. These results show that only site 3 (Ser-Ser-Met-Ser(P)-Gly-Leu) was significantly phosphorylated in T1 in both control and glucagon-treated hepatocytes.

4. DISCUSSION

Our results strongly suggest that the low V_{max} of acetyl-CoA carboxylase isolated from hepatocytes is due to phosphorylation at site 3 by the AMP-activated protein kinase. Of seven purified protein kinases with which we have demonstrated phosphorylation of acetyl-CoA carboxylase in vitro, the AMP-activated protein kinase is the only one which phosphorylates site 3 [7], and the only one which produced a large (>75%) depression of V_{max} [5,6] similar to that observed in intact hepatocytes. Apart from a partial phosphorylation of site 1, the only other major phosphopeptide observed under basal conditions was peptide T4. Phosphorylation of this peptide increases in response to insulin and EGF [14], and we have shown by amino acid sequencing that, at least in isolated adipocytes, the sites phosphorylated in T4 correspond to those phosphorylated in vitro by casein kinase-2 and the calmodulin-dependent multiprotein kinase [15]. Neither of these protein kinases produces a significant change in the kinetic properties of acetyl-CoA carboxylase in vitro [16,17], and it therefore seems unlikely that phosphorylation within this peptide would explain the low basal activity of the enzyme.

The finding that the increase in phosphorylation of acetyl-CoA carboxylase after glucagon treatment was confined to sites 1 and 3 was unexpected, and shows that the glucagon-mediated inactivation of acetyl-CoA carboxylase is due to changes at the sites phosphorylated by the AMP-activated protein kinase and not due to direct phosphorylation by the cyclic AMP-dependent protein kinase as we previously suggested [8]. Site 2 is not significantly phosphorylated in the isolated hepatocytes even after glucagon treatment, showing that acetyl-CoA carboxylase is not a substrate for cyclic AMP-dependent protein kinase in intact cells. Although acetyl-CoA carboxylase is a substrate for this kinase in vitro, at a protein substrate concentration of 2 μM it is phosphorylated at only ~10% of the rate of phosphorylase kinase [3], and sites 1 and 2 contain only one N-terminal arginine residue rather than the two adjacent basic residues normal-

ly considered to be specificity determinants for this kinase [18].

How phosphorylation at sites 1 and 3 is stimulated by glucagon is not clear. Lent and Kim [19] reported that a hepatic acetyl-CoA carboxylase kinase they had purified was activated by phosphorylation by cyclic AMP-dependent protein kinase. Although the kinase of Lent and Kim appears similar to the AMP-activated protein kinase in many respects, we have been unable to show activation of the AMP-activated protein kinase using purified cyclic AMP-dependent protein kinase. An alternative possibility is that glucagon could cause inhibition of protein phosphatases acting on either acetyl-CoA carboxylase or the AMP-activated protein kinase or both. Possible mechanisms include inhibition of protein phosphatase-1 by inhibitor-1 [18] or by phosphorylase [20]. Both of these are potent inhibitors of the phosphatase in their phosphorylated forms, into which they would be converted after glucagon treatment. It is however not certain that phosphatase-1, which appears in the cell to be largely bound to glycogen particles and/or membranes [18], is responsible for dephosphorylation of these substrates in vivo.

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