

Negative interactions between phosphorylation of acetyl-CoA carboxylase by the cyclic AMP-dependent and AMP-activated protein kinases

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We have reported previously that cyclic AMP-dependent protein kinase phosphorylates two sites on acetyl-CoA carboxylase (site 1: Arg-Met-Ser(P)-Phe, and site 2: Ser-Ser(P)-Met-Ser-Gly-Leu), while the AMP-activated protein kinase also phosphorylates site 1, plus site 3 (Ser-Ser-Met-Ser(P)-Gly-Leu), the latter being two residues C-terminal to site 2. We now report that prior phosphorylation of site 2 by cyclic AMP-dependent protein kinase prevents the subsequent phosphorylation of site 3 and the consequent large decrease in V_{\max} produced by the AMP-activated protein kinase. Similarly, prior phosphorylation of site 3 by the AMP-activated protein kinase prevents subsequent phosphorylation of site 2 by cyclic AMP-dependent protein kinase.

Acetyl-CoA carboxylase; cyclic AMP-dependent protein kinase; AMP-activated protein kinase; Phosphorylation site; Interaction; (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-dependent protein kinase [3,4] and a 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 (Arg-Met-Ser(P)-Phe) and 2 (Ser-Ser(P)-Met-Ser-Gly-Leu). 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 this is associated with phosphorylation of site 1 and site 3, the latter (Ser-Ser-Met-Ser(P)-Gly-Leu) being 2 residues C-terminal to site 2 [7]. Comparison with the complete sequence of chicken acetyl-CoA carboxylase predicted from the recently described cDNA sequence [8] shows that site 1 is in the centre of the polypeptide (residue 1193), while sites 2 and 3 are close to the N-terminus (residues 78 and 80, respectively).

The close proximity of sites 2 and 3 suggested the possibility that there may be interactions between phosphorylation events at these sites. In this paper we report that phosphorylation of acetyl-CoA carboxylase by the cyclic AMP-dependent and AMP-activated protein kinases are not additive, and that phosphorylation at sites 2 and 3 appear to be mutually exclusive.

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2. MATERIALS AND METHODS

2.1. Materials

Acetyl-CoA carboxylase was purified from rat mammary gland and dephosphorylated with protein phosphatase-2A prior to use [7]. The catalytic subunit of cyclic AMP-dependent protein kinase was purified from bovine heart [9]. The AMP-activated protein kinase (formerly called acetyl-CoA carboxylase kinase-3) was purified 1000-fold from rat liver in the presence of 50 mM NaF and 5 mM Na pyrophosphate to a final specific activity of 50 units/mg as described in [6]. Sources of other radioisotopes and biochemicals were as described [7].

2.2. Methods

Acetyl-CoA carboxylase (0.72 mg/ml) was phosphorylated at 30°C in incubations containing AMP (100 μ M), Na HEPES, pH 7.0 (50 mM), glycerol (10%, v/v), NaCl (50 mM), NaF (50 mM), EDTA (1 mM), dithiothreitol (1 mM), $MgCl_2$ (4 mM) and $[\gamma\text{-}^{32}P]ATP$ (0.2 mM, $1\text{--}2 \times 10^5$ cpm/nmol) and protein kinases as specified in the figure legends. Incorporation of phosphate into protein was measured by trichloroacetic acid precipitation [10]. Acetyl-CoA carboxylase from these incubations was also precipitated using ammonium sulphate to remove $[\gamma\text{-}^{32}P]ATP$, resuspended, and digested with proteinases as in [7]. Labelled peptides were separated by reverse-phase high-performance liquid chromatography (HPLC) in 0.1% (v/v) trifluoroacetic acid [7], dried in a centrifugal vacuum concentrator, and analysed by thin layer isoelectric focussing [11].

Parallel experiments were carried out using unlabelled ATP at the same concentration. Aliquots (10 μ l) were removed, diluted 50-fold in 0.1 M Tris-HCl, pH 7.4, and acetyl-CoA carboxylase was assayed at 10 mM citrate as in [12].

3. RESULTS

3.1. Sequential phosphorylation and inactivation by the two protein kinases

Fig.1 shows that in the absence of added kinase there was no significant phosphorylation (A) or inactivation (B) of acetyl-CoA carboxylase during incubation for 60 min with MgATP. However in the presence of cyclic AMP-dependent protein kinase, there was incorporation of 1.4 mol phosphate per subunit (C), accompanied by a modest inactivation ($\sim 15\%$) of acetyl-CoA carboxylase (D). The small effect on acetyl-CoA carboxylase activity is due to the fact that the assays were performed using a near saturating citrate concentration (10 mM), when only the small effect of cyclic AMP-dependent protein kinase on V_{max} is observed [7]. Addition of the AMP-activated protein kinase to the controls (A,B) after 60 min of incubation produced a large phosphorylation (1.5 mol/subunit) which correlated with a large inactivation ($\sim 70\%$), consistent with the known ef-

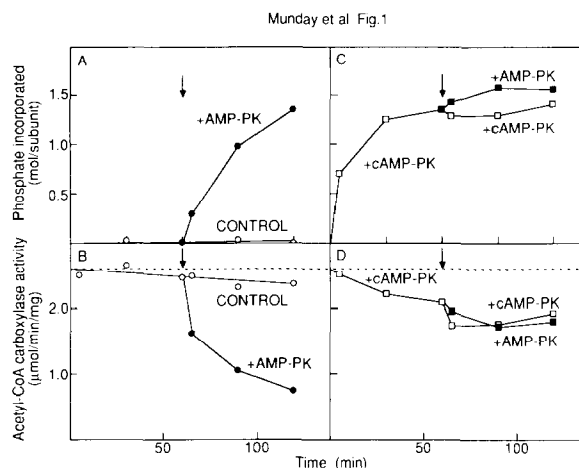


Fig.1. Sequential phosphorylation of acetyl-CoA carboxylase by cyclic AMP-dependent protein kinase and the AMP-activated protein kinase. Acetyl-CoA carboxylase was incubated with $[\gamma\text{-}^{32}P]ATP$ either with (open squares) or without (open circles) the catalytic subunit of cyclic AMP-dependent protein kinase (10 U/ml). After 60 min (arrow), the following additions were made: either AMP-activated protein kinase (filled symbols, 1 U/ml), a further identical aliquot of cyclic AMP-dependent protein kinase (open squares), or buffer only (open circles). At various times, aliquots were removed for determination of incorporation of phosphate into protein (A,C) or, from parallel incubations containing unlabelled ATP, for determination of acetyl-CoA carboxylase activity at 10 mM citrate (B,D).

fect of this kinase on the V_{max} of acetyl-CoA carboxylase [7]. By contrast, addition of the AMP-activated protein kinase after prior phosphorylation by cyclic AMP-dependent protein kinase (C,D) produced only a slight additional phosphorylation (<0.2 mol/subunit) and no additional inactivation.

Fig.2 shows data for the converse experiment. It is clear that prior phosphorylation by the AMP-activated protein kinase completely prevents additional phosphorylation by cyclic AMP-dependent protein kinase. Phosphorylation by the AMP-activated protein kinase was associated with a large decrease in acetyl-CoA carboxylase activity similar to that shown in fig.1B: subsequent addition of cyclic AMP-dependent protein kinase produced no further inactivation (not shown).

3.2. Analysis of phosphorylation sites

Acetyl-CoA carboxylase that had been incubated with $[\gamma\text{-}^{32}P]ATP$ and one protein kinase

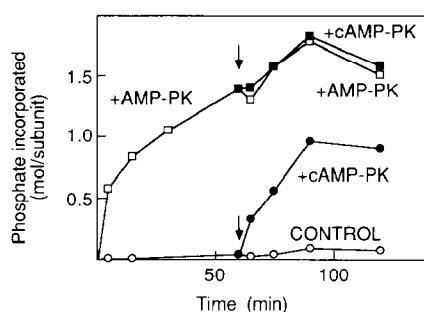


Fig. 2. Sequential phosphorylation of acetyl-CoA carboxylase by the AMP-activated protein kinase and cyclic AMP-dependent protein kinase. The experiment was identical with that in fig. 1A,C except that the order of addition of the kinases was reversed.

alone for 60 min, and enzyme that had been incubated with one kinase for 60 min and then the other kinase added for a further 60 min (as in figs 1 and 2), was digested with trypsin alone, or with trypsin plus chymotrypsin, and the labelled peptides analysed by reverse-phase HPLC. The phosphopeptide profiles were very similar to those observed previously after treatment with individual kinases [7]. After phosphorylation for 60 min with cyclic AMP-dependent protein kinase or the AMP-activated protein kinase alone, the ratios of radioactivity obtained in tryptic/chymotryptic peptides TC1 and TC2 were 0.96 and 1.12, respectively. This is consistent with the fact that there is one site for each kinase on these peptides (TC1 is Ser-Ser-Met-Ser-Gly-Leu, containing sites 2 and 3; TC2 is Arg-Met-Ser-Phe, containing site 1). If there was no interaction between sites 2 and 3, one would expect the ratio of radioactivity (TC1:TC2) to increase from 1:1 to 2:1 as the additional site was filled by the second protein kinase. However, these ratios did not change significantly (1.00 and 0.82, respectively). Both kinases yielded one major tryptic peptide (T1, corresponding to the peptide Ser-Ser-Met-Ser-Gly-Leu-His-Leu-Val-Lys [7]: site 1 is not recovered by HPLC after trypsin digestion) and labelling of T1 did not increase on incubation for a further 60 min in the presence of the second kinase (not shown).

The failure of addition of a second kinase to increase labelling of TC1 or T1 suggested that it was possible to label these peptides in site 2 or site 3, but not both. However, small amounts of

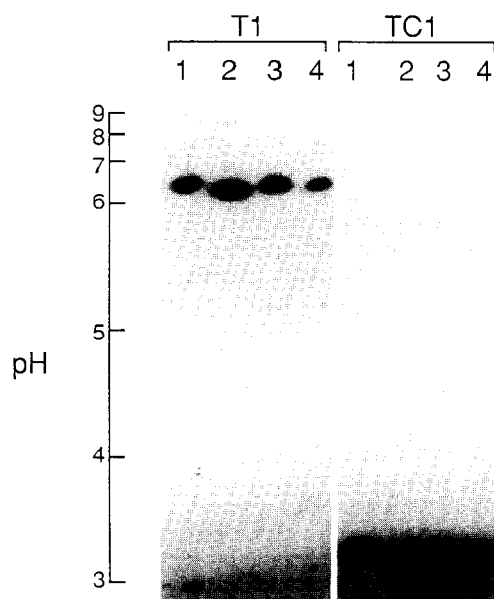


Fig. 3. Thin layer isoelectric focussing of peptides T1 and TC1. Peptides were derived by digestion with trypsin (T1) or trypsin + chymotrypsin (TC1), and partially purified by HPLC, from acetyl-CoA carboxylase that had been incubated with (lane 1) AMP-activated protein kinase for 60 min; (2) cyclic AMP-dependent protein kinase for 60 min; (3) AMP-activated protein kinase for 60 min, then cyclic AMP-dependent protein kinase for 60 min; (4) cyclic AMP-dependent protein kinase for 60 min, then AMP-activated protein kinase for 60 min (as described for figs 1 and 2). The photograph shows an autoradiogram of the dried gel. The approximate pI values were estimated using coloured protein isoelectric point markers (BDH Ltd., Poole, England).

diphosphopeptides would not be readily detected in the presence of monophosphopeptides in our HPLC system, so we reanalysed the various forms of TC1 and T1 by thin layer isoelectric focussing, in which different phosphorylated forms are dramatically separated [13]. Fig. 3 shows that in every case TC1 and T1 exhibited a single isoelectric point, ruling out the existence of even trace amounts of diphosphopeptides.

4. DISCUSSION

These data show unequivocally that site 2, phosphorylated by cyclic AMP-dependent protein kinase, and site 3, phosphorylated by the AMP-activated protein kinase, are mutually exclusive and cannot both be phosphorylated in the same

molecule of acetyl-CoA carboxylase, at least using these two protein kinases. The evidence may be summarised as follows: (i) prior phosphorylation by cyclic AMP-dependent protein kinase, which produces ~15% inactivation of acetyl-CoA carboxylase measured at 10 mM citrate, completely prevented the large (~70%) inactivation that was produced by the AMP-activated protein kinase in a control preincubated in the absence of cyclic AMP-dependent protein kinase (cf. fig.1B and D).

(ii) Phosphorylation by cyclic AMP-dependent protein kinase and the AMP-activated protein kinase was not additive (figs 1 and 2) and there was no increase in labelling of peptides containing sites 2 and 3 (TC1 or T1) when acetyl-CoA carboxylase labelled with one kinase was incubated further with the second kinase.

(iii) Isoelectric focussing gives no evidence that doubly phosphorylated forms of TC1 or T1 were produced when acetyl-CoA carboxylase was incubated with both protein kinases (fig.3). TC1 is a rather acidic peptide, and it is conceivable that a doubly phosphorylated form of TC1 could have run off the end of the isofocussing gel and have been missed. However this is certainly not the case with T1.

It has been demonstrated using synthetic peptides [14] that basic residues (usually two adjacent arginines) on the N-terminal side of the phosphorylated serine are important specificity determinants for cyclic AMP-dependent protein kinase. Although comparable studies have not yet been carried out for the AMP-activated protein kinase, all sites so far sequenced ([7] and unpublished) contain at least one arginine residue on the N-terminal side. Our results suggest that the introduction of a negatively charged phosphate group almost adjacent to the site of phosphorylation is a negative specificity determinant for both protein kinases.

Various types of positive and negative interaction between phosphorylation sites have been reported previously in other systems. For example, phosphorylation of glycogen synthase by casein kinase-2, which does not affect the kinetic properties of the enzyme, creates a recognition site for phosphorylation by glycogen synthase kinase-3, which inactivates the enzyme [15]. On the other hand, phosphorylation of second and third serine residues on the E1 α -subunit of pyruvate

dehydrogenase by its specific kinase, inhibits dephosphorylation at the first, regulatory serine residue [16].

Our results show that prior phosphorylation by cyclic AMP-dependent protein kinase prevents the larger inactivation normally produced by the AMP-activated protein kinase (fig.1). This confirms our previous suggestion [7] that it is site 3 phosphorylation that is responsible for the large decrease in V_{\max} produced by the AMP-activated protein kinase. However this finding is at first sight paradoxical since cyclic AMP-elevating hormones inhibit fatty acid synthesis in hepatocytes and adipocytes [1,2]. Recent studies on sequencing of peptides TC1 and T1 from isolated hepatocytes [17] have shown that site 3, but not site 2, is phosphorylated in basal hepatocytes, and that increased phosphorylation in response to the cyclic AMP-elevating hormone, glucagon, also occurs exclusively at site 3. The physiological significance of the effect of site 2 phosphorylation described in this paper is therefore unclear, at least for hepatocytes.

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