REGULATION OF ACETYL-CoA CARBOXYLASE: IDENTITY OF SITES PHOSPHORYLATED IN INTACT CELLS TREATED WITH ADRENALINE AND IN VITRO BY CYCLIC AMP-DEPENDENT PROTEIN KINASE

Roger W. BROWNSEY and D. Grahame HARDIE*

Department of Biochemistry, University of Bristol Medical School, University Walk, Bristol BS8 1TD, England and **Department of Biochemistry, University of Dundee, Medical Sciences Institute, Dundee DD1 4HN, Scotland*

Received 3 September 1980

1. Introduction

The rate of fatty acid synthesis in mammals and birds is rapidly depressed by hormones which raise the concentration of cyclic AMP in target cells, i.e., adrenaline in adipose tissue [1] or glucagon in the liver [2–4]. This decrease correlates with a decrease in the activity of acetyl-CoA carboxylase measured in a freshly-prepared extract [4–9]. However there has been some controversy as to the mechanism by which an increase in cyclic AMP concentration leads to a decrease in the activity of acetyl-CoA carboxylase. This decrease in activity may be the result of a decrease in the cytosolic concentration of the allosteric activator, citrate [3]. According to this hypothesis, the decrease in the rate of fatty acid synthesis is secondary to the decrease in the rate of glycolysis produced by inhibition of phosphofructokinase [10]. An alternative hypothesis is that cyclic AMP brings about a direct inactivation of acetyl-CoA carboxylase through a phosphorylation mechanism. The phosphorylation of acetyl-CoA carboxylase in intact cells was first demonstrated in [11] and it has been found that the phosphorylation of the enzyme is increased by adrenaline in fat cells [5,7] or glucagon in hepatocytes [9]. In [12] it was reported that purified rabbit mammary gland acetyl-CoA carboxylase could be phosphorylated in vitro by cyclic AMP-dependent protein kinase and at least one cyclic AMP-independent protein kinase. In [13] it was shown that phosphorylation of rat mammary acetyl-CoA carboxylase by cyclic AMP-dependent protein kinase results in an inactivation of acetyl-CoA carboxylase which can be reversed on dephosphorylation. The activity of acetyl-CoA carboxylase correlates with the phosphorylation of a site located in a neutral tryptic peptide [13]. We now report that an apparently identical site is phosphorylated in intact rat fat cells treated with adrenaline. The techniques of immunoprecipitation and isoelectric focussing which we have used to compare sites phosphorylated in intact cells and in vitro should be of general application to studies of other proteins that are regulated by multiple covalent modification.

*To whom correspondence should be addressed

2. Materials and methods

Acetyl-CoA carboxylase was purified from lactating rat mammary gland and phosphorylated using [γ-^{32}P]-ATP and the catalytic subunit of skeletal muscle cyclic AMP-dependent protein kinase as in [13]. The preparation of fat cells, incubation with [^{32}P]phosphate, and preparation of acetyl-CoA carboxylase by immunoprecipitation have been described [5]. To remove contaminating phospholipids, the immunoprecipitates were suspended in 0.2 ml 10% (w/v) trichloroacetic acid and extracted with 0.8 ml chloroform/methanol (2:1, v/v). Trichloroacetic acid-insoluble pellets of acetyl-CoA carboxylase phosphorylated in vitro, or washed immunoprecipitates from intact cell experiments, were oxidised with performic acid, digested with trypsin, and analysed by isoelectric focussing as in [13]. The results were quantitated by densitometry of autoradiograms using a densitometer attachment on a Pye-Unicam SP8-100 spectrophoto-

meter. ^{32}P-Labelled tryptic peptides were also analysed by two-dimensional peptide mapping on thin layers (Kodak Eastman Chromagram 13255 cellulose) using electrophoresis in pyridine/acetic acid/water (1:10:189; pH 3.6) as the first dimension, and chromatography in butanol/pyridine/acetic acid/water (15:10:3:12) as the second dimension. Quantitative data are presented as mean ± standard error of the mean, with the number of experiments (each performed on separate batches of fat cells) in brackets. All effects of adrenaline quoted were statistically significant ($p < 0.02$).

3. Results

Isolated fat cells were incubated for 60 min at 37°C in medium containing [^{32}P]phosphate, by which time the labelling of acetyl-CoA carboxylase and other cytosolic phosphoproteins has reached a steady state [11]. Adrenaline (1 μM) was then added to some of the incubations and after a further 15 min the cells were homogenised and acetyl-CoA carboxylase was prepared by immunoprecipitation. As reported in [5], adrenaline increases the amount of ^{32}P-radioactivity associated with acetyl-CoA carboxylase in intact fat cells. Here, adrenaline increased the amount of radioactivity associated with the immunoprecipitates by 181 ± 40% (5). This is somewhat larger than the effect reported previously, where the ^{32}P content of acetyl-CoA carboxylase was estimated by densitometry of autoradiograms of soluble proteins separated by SDS–polyacrylamide gel electrophoresis [5]. The nature and number of the sites phosphorylated was investigated by thin-layer isoelectric focussing of tryptic peptides prepared from the immunoprecipitates. In control cells (fig.1B) acetyl-CoA carboxylase was phosphorylated predominantly in two sites located in tryptic peptides of isoelectric point (pI) ~7 and ~4.3. In adrenaline-treated cells (fig.1C) there was a large increase in the labelling of the pI 7 peptide, and there was also increased labelling of several acidic peptides which were scarcely detectable in control cells. The increase in the radioactivity associated with the pI 7 peptide was 544 ± 111% (5). The labelling of the pI 4.3 peptide was not significantly affected by adrenaline.

The sites phosphorylated in adrenaline-treated fat cells were compared with the sites phosphorylated on purified rat mammary acetyl-CoA carboxylase by

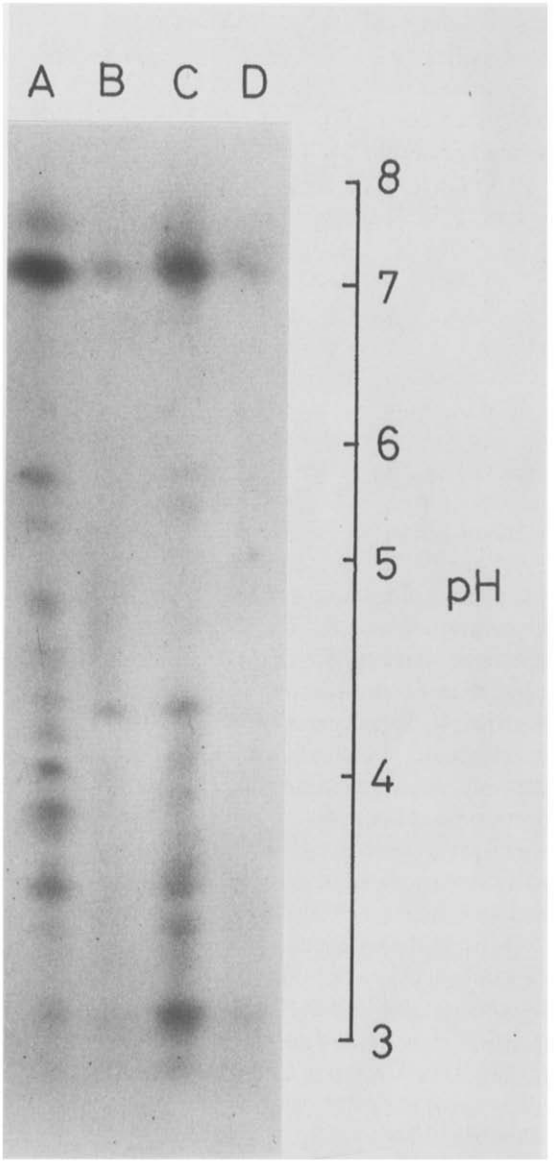

Fig.1. Autoradiogram of tryptic peptides of ^{32}P-labelled acetyl-CoA carboxylase analysed by polyacrylamide thin-layer isoelectric focussing. The pH gradient was determined using a surface electrode on completion of isoelectric focussing and should be regarded as an approximate guide only. (A) Purified rat mammary acetyl-CoA carboxylase phosphorylated to 1.2 phosphate molecules/subunit using skeletal muscle cyclic AMP-dependent protein kinase; (B) acetyl-CoA carboxylase from rat fat cells treated without hormone; (C) acetyl-CoA carboxylase from rat fat cells treated for 15 min with adrenaline; (D) as (C); extract incubated for 30 min with $MgCl_2$ (5 mM) at 37°C prior to isolation of acetyl-CoA carboxylase. Acetyl-CoA carboxylase from rat fat cells was isolated by immunoprecipitation in all cases.

cyclic AMP-dependent protein kinase (fig.1A). The pI 7 tryptic peptide containing the major phosphorylation site which is increased 5–6-fold by adrenaline comigrated exactly with a tryptic peptide containing the major phosphorylation site for cyclic AMP-dependent protein kinase. These peptides also comigrated on thin-layer chromatography and on thin-layer electrophoresis at pH 3.6 with R_F values of 0.63 (relative to the solvent front) and 0.54 (relative to lysine), respectively. Several of the other sites which were labelled to a greater extent in adrenaline-treated cells also corresponded to minor sites phosphorylated in vitro by cyclic AMP-dependent protein kinase (fig.1). The pI 4.3 peptide which did not show increased labelling in adrenaline-treated cells did not correspond to any sites labelled in vitro.

In [2] it was shown that the decrease in the activity of acetyl-CoA carboxylase produced by adrenaline treatment of fat cells could be completely reversed by incubation of the extract with divalent metal ions. This treatment would be expected to favour dephosphorylation by endogenous phosphatases, and indeed there was a decrease in the ^{32}P-radioactivity associated with acetyl-CoA carboxylase as judged by SDS–polyacrylamide gel electrophoresis [2]. Since it had already been shown that the activity of purified rat mammary acetyl-CoA carboxylase correlated with the state of phosphorylation of the site located in the pI 7 peptide [13], we anticipated that the treatment of the fat cell extracts with Mg^{2+} would lead to dephosphorylation of the pI 7 peptide in the fat cell acetyl-CoA carboxylase. This prediction was confirmed by isoelectric focussing (fig.1D). The ^{32}P-radioactivity associated with the immunoprecipitate was decreased by 85% and the labelling of the pI 7 peptide was reduced by 87%.

4. Discussion

These results confirm the hypothesis that the inhibition of fatty acid synthesis by adrenaline involves the phosphorylation of acetyl-CoA carboxylase by cyclic AMP-dependent protein kinase. Four criteria have been listed which should be met before an effect mediated by cyclic AMP can be said to occur through phosphorylation of a protein [14]. These criteria have now been met for the effect of adrenaline on acetyl-CoA carboxylase in adipose tissue:

(1) Purified acetyl-CoA carboxylase is phosphorylated at a rate sufficient to account for the speed at which inhibition of fatty acid synthesis occurs in intact cells [12,13];
(2) The activity of purified acetyl-CoA carboxylase undergoes a reversible alteration by phosphorylation and dephosphorylation, catalysed by cyclic AMP-dependent protein kinase and protein phosphatase-1 [13];
(3) A reversible change in the activity of acetyl-CoA carboxylase occurs in intact fat cells in response to adrenaline [5–7], which is known to increase cyclic AMP concentrations in adipose tissue [15];
(4) Phosphorylation of acetyl-CoA carboxylase occurs in intact fat cells in response to adrenaline at a site apparently identical to that phosphorylated by cyclic AMP-dependent protein kinase in vitro [this work].

The identity of these sites is indicated by the fact that the tryptic peptides containing them comigrate in 3 different analytical systems which separate according to isoelectric point, charge at pH 3.6, and polarity. The only other systems where the role of protein phosphorylation has been so rigorously demonstrated are the activation of phosphorylase kinase by adrenaline in rabbit skeletal muscle [16], the inactivation of pyruvate kinase by glucagon in rat liver [17], and the activation of pyruvate dehydrogenase by insulin in adipose tissue [18].

The correlation between the phosphorylation of the site located in the pI 7 tryptic peptide and the activity of acetyl-CoA carboxylase which was established in vitro [13] also holds in the present study of intact cells. Adrenaline causes a 5–6-fold increase in [^{32}P]phosphate content at this site [this work] and this is associated with a 60% decrease in enzyme activity measured at 1 mM citrate [5]. Incubation of extracts from adrenaline-treated cells with divalent cations causes an 87% reduction in ^{32}P-radioactivity at this site [this work] and this is associated with an almost complete reactivation of the enzyme activity to control values [5]. Unfortunately we do not have an accurate estimate of the stoichiometry of phosphorylation at this site after adrenaline treatment of fat pads. However the effect of phosphorylation in intact fat pads treated with adrenaline and stoichiometric phosphorylation in vitro by cyclic AMP-dependent protein kinase on the kinetic properties of the enzyme are almost identical. In each case there is a 1.5–2-fold decrease in V_{max} and a 1.5–2-

fold increase in the $K_{0.5}$ for citrate [5,13].

The finding that acetyl-CoA carboxylase is phosphorylated at multiple sites in intact cells is not unexpected, because the enzyme purified from rat liver and rat mammary gland has been reported to contain from 2–6 phosphate molecules/subunit [13,19,20]. While we cannot be certain that each labelled peptide represents a unique site of phosphorylation, the pattern of labelled peptides does not change if the time of tryptic digestion is varied from 0.5–6 h (not shown). Although the large number of sites phosphorylated on the purified mammary gland acetyl-CoA carboxylase may appear to demonstrate a lack of specificity in the proton kinase reaction, it is worth noting that there are around 250 serine and threonine residues in the enzyme which could be phosphorylated [19,21]. In addition, many of the minor phosphorylated peptides comigrate with peptides phosphorylated in adrenaline-treated fat cells (fig.1). Acetyl-CoA carboxylase from rat adipose tissue and mammary gland have closely related molecular weights [11,13,20]. The marked similarities in the patterns of labelled peptides in fig.1A and 1C further suggest that the primary structures of the two enzymes are closely related, if not identical.

On the basis of these findings, we propose that the inactivation of acetyl-CoA carboxylase produced by glucagon treatment of rat hepatocytes [4,9] is also the result of phosphorylation by cyclic AMP-dependent protein kinase. Application of the techniques described in this paper should resolve this question.

Acknowledgements

This study was supported by grants from the Medical Research Council and the British Diabetic Association. We are indebted to Dr John Mayer of Nottingham University who provided the antibody to acetyl-CoA carboxylase, and to Dr Richard Denton for his help in preparing the manuscript.

References

[1] Cahill, G. F., LeBoeuf, B. and Flinn, R. B. (1960) J. Biol. Chem. 235, 1246–1250.
[2] Cook, G. A., Nielsen, R. C., Hawkins, R. A., Mehlman, M. A., Lakshmanan, M. R. and Veech, R. L. (1977) J. Biol. Chem. 252, 4421–4424.
[3] Watkins, P. A., Tarlow, D. M. and Lane, M. D. (1977) Proc. Natl. Acad. Sci. USA 74, 1497–1501.
[4] Geelen, M. J. H., Beynen, A. C., Christiansen, R. Z., Lepreau-Jose, M. J. and Gibson, D. M. (1978) FEBS Lett. 95, 326–330.
[5] Brownsey, R. W., Hughes, W. A. and Denton, R. M. (1979) Biochem. J. 184, 23–32.
[6] Halestrap, A. P. and Denton, R. M. (1974) Biochem. J. 142, 365–377.
[7] Lee, K. H. and Kim, K. H. (1979) J. Biol. Chem. 254, 1450–1453.
[8] Klain, G. J. and Weiser, P. C. (1973) Biochem. Biophys. Res. Commun. 55, 76–83.
[9] Witters, L. A., Kowaloff, E. M. and Avruch, J. (1979) J. Biol. Chem. 254, 245–248.
[10] Clarke, S. D., Watkins, P. A. and Lane, M. D. (1979) J. Lipid Res. 20, 974–985.
[11] Brownsey, R. W., Hughes, W. A., Denton, R. M. and Mayer, R. J. (1977) Biochem. J. 168, 441–445.
[12] Hardie, D. G. and Cohen, P. (1978) FEBS Lett. 91, 1–7.
[13] Hardie, D. G. and Guy, P. S. (1980) Eur. J. Biochem. in press.
[14] Nimmo, H. G. and Cohen, P. (1977) Adv. Cyclic Nucl. Res. 8, 145–266.
[15] Soderling, T. R., Corbin, J. D. and Park, C. R. (1973) J. Biol. Chem. 248, 1822–1829.
[16] Cohen, P. (1978) Curr. Top. Cell. Regul. 14, 117–196.
[17] Engstrom, L. (1980) in: Molecular Aspects of Cellular Regulation (Cohen, P.) vol. 1, pp. 11–31, Elsevier/North-Holland, Amsterdam, New York.
[18] Hughes, W. A., Brownsey, R. W. and Denton, R. M. (1980) Biochem. J. in press.
[19] Inoue, H. and Lowenstein, J. M. (1973) J. Biol. Chem. 247, 4825–4832.
[20] Ahmad, F., Ahmad, P. M., Pieretti, L. and Watters, G. T. (1978) J. Biol. Chem. 253, 1733–1737.
[21] Hardie, D. G. and Cohen, P. (1978) Eur. J. Biochem. 92, 25–34.