RAT MAMMARY GLAND ATP-CITRATE LYASE IS PHOSPHORYLATED BY CYCLIC AMP-DEPENDENT PROTEIN KINASE

Paul S. GUY, Philip COHEN and D. Grahame HARDIE
Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland

Received 16 November 1979

1. Introduction

Fatty acids and sterols are synthesized in the cytoplasm from acetyl-CoA, but acetyl-CoA is produced in the mitochondrion by the pyruvate dehydrogenase reaction. The carbon atoms of acetyl-CoA leave the mitochondrion as citrate, which is then reconverted to acetyl-CoA in the cytoplasm by the enzyme ATP citrate lyase [1]. ATP citrate lyase is therefore a key lipogenic enzyme in non-ruminants, and this conclusion is supported by the finding that dietary manipulation changes its activity in parallel with other lipogenic enzymes, such as acetyl-CoA carboxylase and fatty acid synthetase [2]. The activity of hepatic ATP-citrate lyase is depressed on starvation, and is elevated by refeeding a fat-free diet, and there is evidence that these changes are regulated by glucagon and insulin [3]. While these long-term effects on activity over a period of days probably represent alterations in the amount of the ATP-citrate lyase protein, the possibility that a mechanism exists for the short-term regulation of its activity cannot yet be excluded.

Linn and Srere [4] reported that ATP-citrate lyase purified from rat liver contained 0.5 mol acid-stable phosphate/subunit, which raised the possibility that it is regulated by reversible phosphorylation. ATP-citrate lyase has now been shown to become labelled in vivo in rat liver after injection of $^{32}P_i$ [4], and the incorporation of ^{32}P-radioactivity into the enzyme in rat hepatocytes is stimulated by both insulin and glucagon [5,6].

Here we report that ATP-citrate lyase purified to homogeneity from lactating rat mammary gland can be phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase at a rate sufficient to account for the effect of glucagon on the phosphorylation of the enzyme in hepatocytes.

2. Materials and methods

Phosphorylase kinase [7], protein phosphatase inhibitor-1 [8] and the catalytic subunit of cyclic AMP-dependent protein kinase [9] were isolated from rabbit skeletal muscle by standard procedures. The specific protein inhibitor of cyclic AMP-dependent protein kinase was purified as in [8]. Histone H2B was a gift from Dr E. W. Johns, Chester Beatty Res. Inst., London, SW3. ATP citrate lyase was purified from lactating rat mammary gland (14–16 days post partum) by a 0–35% ammonium sulphate precipitation, a 3–10% polyethylene glycol precipitation, chromatography on DEAE-cellulose and gel filtration on Sepharose 4B. The detailed procedure will be published elsewhere (P. G., P. C., D. G. H., in preparation). Coenzyme A, NADH and malate dehydrogenase were from Sigma, and [γ-^{32}P]ATP from the Radiochemical Centre, Amersham, Bucks.

ATP-citrate lyase was assayed as in [4] and 1 unit of activity was that amount which catalysed the oxidation of 1 μmol NADH/min at 25°C. Cyclic AMP-dependent protein kinase was assayed as in [10], and 1 unit activity was that amount which catalysed the incorporation of 1 nmol phosphate into mixed histone/min at 30°C [8]. Concentrations of phosphorylase kinase and ATP citrate lyase were determined using absorption coefficients ($A^{1\%}_{280}$) of 12.4 [7] and 11.9 ± 0.4, respectively, which were established by refractometric measurements in the analytical ultracentrifuge [7,11]. Alkali-labile phosphate bound covalently to protein was determined as in [12]. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) [13] was carried out as in [7]. Phosphorylation of ATP-citrate lyase by the catalytic subunit of cyclic AMP-dependent protein kinase was carried out at 30°C in 10 mM

sodium glycerophosphate, 0.4 mM EDTA, 0.1 mM EGTA, 2.0 mM magnesium acetate and 0.2 mM ATP (pH 7.0). The stoichiometry of phosphorylation was calculated using a subunit mol. wt 116 000 for ATP-citrate lyase.

3. Results

3.1. *Purity of ATP-citrate lyase*

ATP-citrate lyase was purified 30-fold from extracts of rat mammary gland and had final spec. act. 2 U/mg. The purified material gave a single protein-staining band when subjected to SDS–polyacrylamide gel electrophoresis (fig.1 A) and its mobility corresponded to app. mol. wt 116 000. This is in close agreement with values obtained for the subunit molecular weight of the rat liver enzyme [4,5]. The preparation also sedimented as a single symmetrical peak in the analytical ultracentrifuge with a sedimentation coefficient of 13.6 ± 0.2 S.

The purified enzyme contained 0.2 ± 0.1 mol alkali-labile phosphate/subunit (4 preps). A detailed characterization of the physicochemical properties of the mammary enzyme will be presented elsewhere (P. S. G., P. C., D. G. H., in preparation).

In a few preparations of ATP-citrate lyase, SDS–polyacrylamide gel electrophoresis showed two further protein staining bands of mol. wt 59 000 and 52 000 (fig.1C). Since the combined molecular weights of these components is almost equivalent to that of the ATP-citrate lyase subunit, and preparations showing predominantly the 59 000 and 52 000 mol. wt species had the same specific activity as preparations containing only the 116 000 mol. wt species, it is concluded that the smaller components are generated by limited proteolysis. A similar phenomenon has been observed with the rat liver enzyme, and the smaller subfragments can also be generated by incubation with trypsin [4,5].

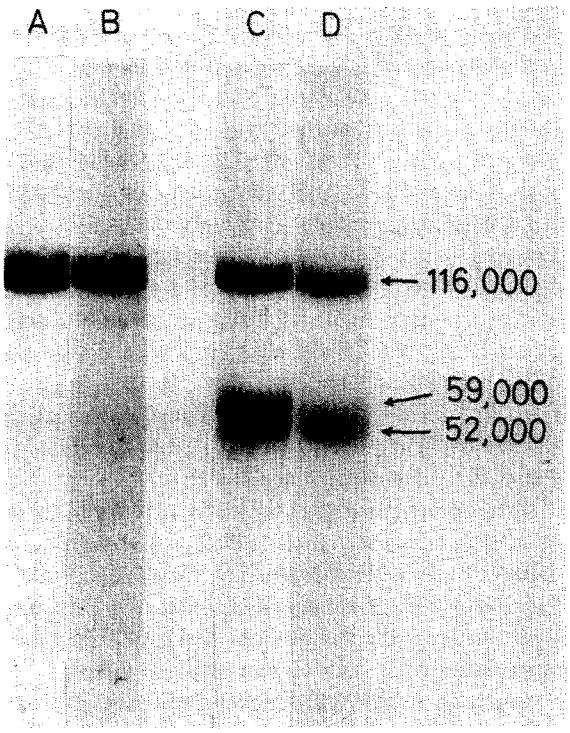

Fig.1. Analysis of ATP-citrate lyase by SDS–polyacrylamide gel electrophoresis. ATP-citrate lyase (0.6 mg/ml) was phosphorylated with the catalytic subunit of cyclic AMP-dependent protein kinase (1.1 U/ml) and [γ-^{32}P]ATP as in section 2. After incubation at 30°C for 60 min an aliquot was denatured in 1% SDS and analysed by electrophoresis on 5% polyacrylamide gels. (A) and (B) show an undegraded preparation stained for protein and autoradiographed, respectively. (C) and (D) show a partially degraded preparation stained for protein and autoradiographed, respectively. The migration is from top to bottom.

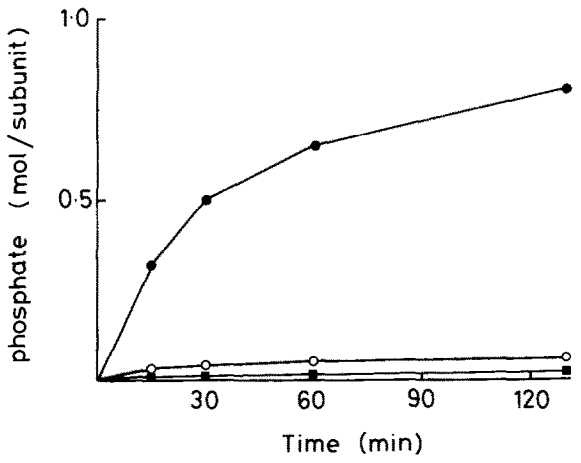

Fig.2. Phosphorylation of ATP-citrate lyase by the catalytic subunit of cyclic AMP-dependent protein kinase. ATP-citrate lyase was incubated with [γ-^{32}P]ATP as in section 2 with no additions (open circles), the catalytic subunit of cyclic AMP-dependent protein kinase (1.1 U/ml) (closed circles), and catalytic subunit of cyclic AMP-dependent protein kinase (1.1 U/ml) plus an excess of the specific protein inhibitor of cyclic AMP-dependent protein kinase (closed squares).

3.2. *Phosphorylation of ATP-citrate lyase by cyclic AMP-dependent protein kinase*

When ATP-citrate lyase was incubated with the catalytic subunit of cyclic AMP-dependent protein kinase there was a rapid phosphorylation of the enzyme which reached 0.7 ± 0.2 molecules of phosphate incorporated/subunit (5 preps). The phosphorylation was dependent on the addition of protein kinase and could be blocked by addition of the specific protein kinase inhibitor (fig.2).

The initial rate of phosphorylation of ATP-citrate lyase was compared to those for other proteins which are the most effective substrates for cyclic AMP-dependent protein kinase in vitro. In the standard assay described in section 2, the relative rates of phosphorylation at 5 μM protein substrate concentration were found to be: phosphorylase kinase 100%; histone H2B 33%; protein phosphatase inhibitor-1 20%; ATP-citrate lyase 2%. The relative rates of phosphorylation of phosphorylase kinase, histone H2B and inhibitor-1 are similar to those in [14].

3.3. *Identification of ATP-citrate lyase as the labelled product*

In order to confirm that ATP-citrate lyase, and not a trace contaminant in the preparation, was being phosphorylated, the ^{32}P-labelled enzyme was analysed by polyacrylamide gel electrophoresis. It can be seen that the ^{32}P-radioactivity coincided exactly with the ATP-citrate lyase subunit (fig.1A,B).

Preparations which contained the 59 000 and 52 000 mol. wt subfragments, as well as the undegraded 116 000 mol. wt subunit, were phosphorylated by cyclic AMP-dependent protein kinase to a similar extent as undegraded preparations. When these preparations were analysed by polyacrylamide gel electrophoresis, radioactivity was associated with the 52 000 mol. wt subfragment as well as the 116 000 mol. wt species (fig.1C,D).

4. Discussion

This work represents the first direct demonstration that ATP-citrate lyase can be phosphorylated in vitro, and that stoichiometric quantities of phosphate can be incorporated into the protein. It is also the first report that the enzyme is phosphorylated by cyclic AMP-dependent protein kinase.

Although the rate of phosphorylation is not as fast as that of phosphorylase kinase, histone H2B and inhibitor-1, it is 2–3 orders of magnitude faster than the rate of phosphorylation of many other enzymes tested, such as the glycolytic enzymes in muscle [14] or fatty acid synthetase [15]. The ATP-citrate lyase used in the phosphorylation experiments (fig.2) was 5 μM, which is only slightly lower than the intracellular concentration of the enzyme in lactating rat mammary gland, or in rat liver after starvation and refeeding (10–20 μM). However the concentration of cyclic AMP-dependent protein kinase (1.1 U/ml) was much lower than the intracellular concentration in rat liver (17 U/ml) or rat mammary gland (18 U/ml) (D. G. H., unpublished) and the incubations were only at 30°C. ATP-citrate lyase is therefore theoretically capable of being phosphorylated in vivo with a half-time of <1 min, which is sufficient to account for the effect of glucagon, which increased the ^{32}P-radioactivity associated with ATP-citrate lyase in isolated hepatocytes by 50% after a 10 min incubation at 38°C. Of course such a calculation ignores many other factors which could alter the rate of phosphorylation of ATP-citrate lyase in vivo. The degree of phosphorylation depends on the relative activities of cyclic AMP-dependent protein kinase and ATP-citrate lyase phosphatase, and substrates and products of the reaction may also affect the rate of phosphorylation and/or dephosphorylation. It will clearly be essential to demonstrate that glucagon stimulates the phosphorylation of ATP-citrate lyase at the same site phosphorylated in vitro by cyclic AMP-dependent protein kinase. It may however be significant that the acid stable ^{32}P-radioactivity found in ATP-citrate lyase after administration of $^{32}P_i$ in vivo [4] or after incubation of hepatocytes with $^{32}P_i$ [5] is located in the 52 000 mol. wt subfragment, as was observed here after phosphorylation in vitro with cyclic AMP-dependent protein kinase.

The effect of phosphorylation on the activity of ATP-citrate lyase is currently under investigation in this laboratory.

Acknowledgements

This work was supported by grants from the Medical Research Council and the British Diabetic Association. The inhibitor-1 and phosphorylase kinase used in this work were provided by Dr Alastair Aitken and Dr Colin Picton, respectively, in this laboratory.

References

[1] Srere, P. A. (1972) Curr. Top. Cell. Reg. 5, 229–283.
[2] Romsos, D. A. and Leveille, G. A. (1974) Adv. Lipid Res. 12, 97–146.
[3] Nepokroeff, C. M., Lakshmanan, M. R., Ness, G. L., Muesing, R. A., Kleinsek, D. A. and Porter, J. W. (1974) Arch. Biochem. Biophys. 162, 340–344.
[4] Linn, T. C. and Srere, P. A. (1979) J. Biol. Chem. 254, 1691–1698.
[5] Alexander, M. C., Kowaloff, E. M., Witters, L. A., Dennihy, D. T. and Avruch, J. (1979) J. Biol. Chem. 254, 8052–8056.
[6] Janski, A. M., Srere, P. A., Cornell, N. W. and Veech, R. L. (1979) J. Biol. Chem. 254, 9365–9368.
[7] Cohen, P. (1973) Eur. J. Biochem. 34, 1–14.
[8] Nimmo, G. A. and Cohen, P. (1978) Eur. J. Biochem. 87, 341–351.
[9] Beavo, J. A., Bechtel, P. J. and Krebs, E. G. (1974) Methods Enzymol. 38, 299–308.
[10] Witt, J. J. and Roskowski, R. (1975) Anal. Biochem. 66, 253–258.
[11] Babul, J. and Stellwagen, E. (1969) Anal. Biochem. 28, 216–221.
[12] Nimmo, H. G. and Cohen, P. (1976) Eur. J. Biochem. 68, 21–30.
[13] Shapiro, A. L. and Maizel, J. V. (1969) Anal. Biochem. 29, 505–514.
[14] Cohen, P. (1978) Curr. Top. Cell. Reg. 14, 117–196.
[15] Hardie, D. G. and Cohen, P. (1979) Eur. J. Biochem. 92, 25–34.