The role of acetyl-CoA carboxylase phosphorylation in the control of mammary gland fatty acid synthesis during the starvation and re-feeding of lactating rats

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1. Activation of acetyl-CoA carboxylase during incubation of crude extracts of lactating rat mammary gland with Mg^{2+} and citrate can be blocked by NaF, suggesting that it represents a dephosphorylation of the enzyme. The greater extent of activation in extracts from 24 h-starved rats (200%) compared with fed controls (70%) implies that the decrease in acetyl-CoA carboxylase activity in response to 24 h starvation may involve increased phosphorylation of the enzyme. 2. Acetyl-CoA carboxylase was purified from the mammary glands of lactating rats in the presence of protein phosphatase inhibitors by avidin–Sephadex chromatography. Starvation of the rats for 24 h increased the concentration of citrate giving half-maximal activation by 75%, and decreased the V_{max} of the purified enzyme by 73%. This was associated with an increase in the alkali-labile phosphate content from 3.3 ± 0.2 to 4.5 ± 0.4 mol/mol of enzyme subunit. 3. Starvation of lactating rats for 6 h, or short-term insulin deficiency induced by streptozotocin injection, did not effect the kinetic parameters or the phosphate content of acetyl-CoA carboxylase purified from mammary glands. 4. The effects of 24 h starvation on the kinetic parameters and phosphate content of the purified enzyme were completely reversed by re-feeding for only 2.5 h. This effect was blocked if the animals were injected with streptozotocin before re-feeding, suggesting that the increase in plasma insulin that occurs on re-feeding was responsible for the activation of the enzyme. 5. The effects of re-feeding 24 h-starved rats on the kinetic parameters and phosphate content of acetyl-CoA carboxylase could be mimicked by treating enzyme purified from 24 h-starved rats with protein phosphatase-2A *in vitro*. 6. Our results suggest that, in mammary glands of 24 h-starved lactating rats, insulin brings about a dephosphorylation of acetyl-CoA carboxylase *in vivo*, which may be at least partly responsible for the reactivation of mammary lipogenesis in response to re-feeding.

INTRODUCTION

The mammary gland of the lactating rat is extremely active in the synthesis of fatty acids *de novo* from glucose, providing some 50% of total milk fat under normal conditions (Williamson, 1980). This biosynthesis makes considerable demands for substrate and must be stringently regulated, according to dietary status, in order to maintain maternal carbohydrate balance (Williamson *et al.*, 1984). Withdrawal of food for 6 or 24 h results in dramatic decreases (87% and 98% respectively) in mammary-gland lipogenesis (Robinson *et al.*, 1978; Williamson *et al.*, 1983), and this inhibition is completely reversed within 2.5 h by re-feeding with chow diet (Robinson *et al.*, 1978; Munday & Williamson, 1981). These changes correlate with changes in the arterial plasma insulin concentration of lactating rats (Robinson *et al.*, 1978; Jones *et al.*, 1984), and acute insulin deficiency induced by streptozotocin treatment markedly inhibits mammary-gland lipogenesis in fed rats, and prevents the re-activation of lipogenesis observed in 24 h-starved rats in response to re-feeding (Robinson *et al.*, 1978).

There are many potential sites at which the pathway from glucose to fatty acid may be regulated in the lactating rat mammary gland. Glucose transport across the plasma membrane (Threadgold & Kuhn, 1984),

pyruvate dehydrogenase (Baxter & Coore, 1978) and acetyl-CoA carboxylase (McNeillie & Zammit, 1982; Munday & Williamson, 1982) all show decreased activity in response to starvation and re-activation upon re-feeding or insulin treatment *in vivo*. Furthermore, changes in the concentrations of the glycolytic metabolites under such conditions also indicate that hexokinase and 6-phosphofructo-1-kinase may be important in this regulation (Jones *et al.*, 1984). Acetyl-CoA carboxylase catalyses the step which is believed to control the rate of conversion of cytoplasmic acetyl-CoA into fatty acid. The activity of this enzyme can be regulated by allosteric effectors, e.g. citrate (activator) and long-chain fatty acyl-CoA (inhibitor), and by reversible phosphorylation (for reviews see Hardie, 1980; Hardie *et al.*, 1984).

Acetyl-CoA carboxylase purified from the mammary glands of lactating rats fed *ad libitum* already contains approx. 3 mol of alkali-labile phosphate/mol of enzyme subunit (Hardie & Guy, 1980). However, this enzyme can be further phosphorylated and inactivated by incubation *in vitro* with Mg^{2+} -ATP and either cyclic AMP-dependent protein kinase or a cyclic nucleotide-independent kinase purified from the mammary gland, termed 'acetyl-CoA carboxylase kinase-2' (Hardie & Guy, 1980; Munday & Hardie, 1984). These effects are totally reversed by treatment of the enzyme with protein phosphatase-1, -2A or -2C purified from rat liver or rabbit skeletal muscle

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(Hardie & Guy, 1980; Ingebritsen *et al.*, 1983; Munday & Hardie, 1984). The phosphorylation and inactivation of acetyl-CoA carboxylase by cyclic AMP-dependent protein kinase has been shown to play a major role in the inhibition of hepatic lipogenesis by glucagon (Holland *et al.*, 1984) and the inhibition of lipogenesis in adipocytes by adrenaline and glucagon (Brownsey & Hardie, 1980; Witters *et al.*, 1983; Holland *et al.*, 1985). However, fatty acid synthesis in the lactating mammary gland is notable for its insensitivity to adrenaline and glucagon, hormones known to act by elevating intracellular cyclic AMP concentrations (Williamson *et al.*, 1983; Bussman *et al.*, 1984; Robson *et al.*, 1984). This may reflect an absence of receptors for these hormones in the lactating mammary gland. However, the presence in lactating mammary gland of cyclic AMP-independent protein kinases such as acetyl-CoA carboxylase kinase-2 (Munday & Hardie, 1984) suggests that the lipogenic enzymes, particularly acetyl-CoA carboxylase, may still be regulated by phosphorylation mechanisms in this tissue, despite its insensitivity to cyclic AMP-increasing hormones. Previous measurements in crude extracts of mammary gland have shown that acetyl-CoA carboxylase activity changes in parallel with the lipogenic rate *in vivo* according to the nutritional and physiological status of the rat (McNeillie & Zammit, 1982; Munday & Williamson, 1982). In the present study we have purified acetyl-CoA carboxylase from lactating rat mammary gland under these different physiological conditions, and have obtained direct evidence that these changes in activity are due to changes in phosphorylation state.

METHODS

Animals

Lactating Wistar rats (250–350 g) with litters of 9–12 pups were used at the peak of lactation (12–15 days *post partum*). Animals were maintained in artificial light from 06:00 to 18:00 h and in darkness from 18:00 to 06:00 h, and were fed *ad libitum* on a standard rat chow diet (BP Nutrition) unless stated otherwise. Streptozotocin (50 mg/kg body wt) was administered by injection into the tail vein in 0.01 M-sodium citrate, pH 4.5, 2.5 h before they were killed (Schein *et al.*, 1971).

Preparation and incubation of crude mammary-gland extracts

Dissected mammary glands were frozen and powdered under liquid N₂. The powder was homogenized in 10 vol. of 0.3 M-mannitol/100 mM-Tris/HCl buffer (pH 7.4)/2 mM-EDTA plus proteinase inhibitors as described by Hardie & Cohen (1979), and 50 mM-NaF where indicated. Homogenization was achieved with a single 30 s burst at top speed in a domestic blender (Kenwood Electronic). The homogenate was spun briefly in a bench centrifuge (12000 g, 1 min) and the supernatant was filtered through glass wool. The filtrate was diluted with an equal volume of incubation buffer to give (final concns.) 0.15 M-mannitol, 100 mM-Tris/HCl, pH 7.4, 1 mM-EDTA, 0.1% 2-mercaptoethanol, 20 mM-MgCl₂, 20 mM-sodium citrate, bovine serum albumin (10 mg/ml). This was incubated at 37 °C in the presence or absence of 50 mM-NaF as indicated. The time interval between homogenization and the start of the incubation never exceeded 3 min.

Purification of acetyl-CoA carboxylase

Dissected mammary glands were rinsed in ice-cold 'homogenization buffer' [0.25 M-sucrose, Tris/HCl buffer (*I* = 0.05, pH 8.4 at 4 °C), 2 mM-EDTA, 50 mM-NaF, plus proteinase inhibitors as described by Hardie & Cohen (1979)]. All subsequent steps were carried out at 4 °C. The glands were minced finely with scissors and homogenized in buffer (50 ml/rat) with a domestic blender (Kenwood Electronic; three 30 s bursts at top speed with 1 min in ice between each burst). The homogenate was centrifuged (100000 g; 60 min; 4 °C) and the clear supernatant was filtered through glass wool to remove floating fat. The supernatant was adjusted to 35% -satd. (NH₄)₂SO₄, and the pellet obtained after centrifugation (12000 g; 20 min; 4 °C) was resolubilized in 'column buffer' [0.1 M-Tris/HCl buffer (pH 7.5 at 4 °C), 0.5 M-NaCl, 0.02% NaN₃, 2 mM-EDTA, 50 mM-NaF, 10% (w/v) glycerol, 0.1% 2-mercaptoethanol, plus proteinase inhibitors as above]. The solution was centrifuged (12000 g; 10 min; 4 °C) to remove insoluble material, filtered through glass wool, and applied to an avidin-Sephadex affinity column. The column was washed extensively with column buffer until the *A*₂₈₀ was zero, and the acetyl-CoA carboxylase was eluted with column buffer containing 2 mM-biotin. The peak fraction of the eluate was assayed immediately for enzyme activity, and remaining fractions were pooled and concentrated by vacuum dialysis for determination of alkali-labile phosphate. This enzyme preparation gave a major 240 kDa Coomassie-Blue-staining band on polyacrylamide-gel electrophoresis, as described by Hardie & Guy (1980). However, this was sometimes accompanied by a second band at 140 kDa (possibly pyruvate carboxylase). This contamination did not exceed 5% of the total protein.

Enzyme analysis

The activity of acetyl-CoA carboxylase was measured by the ¹⁴C-fixation assay as described previously (Holland *et al.*, 1984), except that the assays were carried out in a final volume of 100 µl for 4 min at 37 °C. Kinetic parameters were estimated with 0.1–0.2 µg of purified acetyl-CoA carboxylase, and the citrate concentrations were varied from 0 to 15 mM.

The *V*_{max} and *K*_a for citrate were calculated as described previously (Holland *et al.*, 1985), and the *A*_{0.5} for citrate (concentration of citrate giving half-maximal activation) was calculated by using $K_a = (A_{0.5})^h$, where *h* is the Hill coefficient. In these studies, the activity in the absence of added citrate was negligible.

Alkali-labile phosphate was determined as described by Guy *et al.* (1981), except that all volumes were halved to increase the sensitivity.

Materials

Streptozotocin, avidin and bovine serum albumin (essentially fatty acid-free) were from Sigma Chemical Co., Poole, Dorset, U.K. ATP and acetyl-CoA were from Boehringer Mannheim, Mannheim, Germany. NaH¹⁴CO₃ was from Amersham International, Amersham, Bucks, U.K. Avidin-Sephadex was synthesized as described by Tipper & Witters (1982). The catalytic subunit of protein phosphatase-2A was purified as far as the polylysine-Sephadex step as described by Resink *et al.* (1983).

Expression of results

Unless stated otherwise, results are expressed as means $\pm$ S.E.M., with the numbers of observations in parentheses, and statistical significance was determined by Student's *t* test.

RESULTS

Acetyl-CoA carboxylase activity in crude extracts of mammary gland

It has previously been shown that acetyl-CoA carboxylase activity in crude extracts of lactating rat mammary gland is decreased by 24 h starvation (McNeillie & Zammit, 1982; Munday & Williamson, 1982). This is confirmed in Fig. 1 where, before incubation ($t = 0$ min), acetyl-CoA carboxylase activity measured at 20 mM-citrate and expressed per g of tissue is 80% lower in extracts from 24 h-starved lactating rats than in fed controls. Such activities are often expressed as a percentage of the 'total enzyme activity', i.e. activity after a preincubation in high concentrations of Mg^{2+} and citrate (McNeillie & Zammit, 1982; Munday & Williamson, 1982). Fig. 1 shows a significant activation by preincubation with Mg^{2+} and citrate of acetyl-CoA carboxylase from both fed and 24 h-starved rats, but also demonstrates that this activation can be blocked by homogenization and incubation in the presence of 50 mM-NaF. This strongly suggests that the activation observed represents a dephosphorylation of the enzyme

by endogenous phosphatases in the extract. Evidence for the presence of such phosphatases in mammary-gland extracts has been previously reported (McNeillie *et al.*, 1981).

The residual difference in acetyl-CoA carboxylase activity in extracts from fed and 24 h-starved rats after activation by Mg^{2+} and citrate is likely to represent a decrease in enzyme concentration, as suggested previously (McNeillie & Zammit, 1982; Munday & Williamson, 1982). However, the extent of activation of acetyl-CoA carboxylase in extracts from 24 h-starved rats (200%) was much greater than that in extracts from fed rats (70%). Hence the 80% inhibition of mammary-gland acetyl-CoA carboxylase activity in response to 24 h starvation remained unchanged throughout incubation in the presence of NaF, but decreased to 60% after activation by Mg^{2+} and citrate (Fig. 1). Thus the decrease in acetyl-CoA carboxylase activity produced by 24 h starvation may be accounted for both by a decrease in enzyme content and by increased phosphorylation and inactivation of the enzyme.

Effect of physiological status on the kinetic parameters and phosphate content of purified acetyl-CoA carboxylase

In order to examine more directly the role of phosphorylation in the regulation of mammary-gland acetyl-CoA carboxylase, we purified the enzyme from rats in different physiological states by using avidin-Sephadex chromatography in the presence of protein phosphatase inhibitors. Table 1 shows that the effect of 24 h starvation on acetyl-CoA carboxylase purified from lactating mammary gland is a large and significant decrease in the V_{max} (73%) and a similar increase (75%) in the concentration of citrate required for half-maximal activation of the enzyme [$A_{0.5}(\text{citrate})$]. These changes are accompanied by an increase in the alkali-labile phosphate content, of approx. 1 mol/mol of 240 kDa subunit. This inhibition of purified acetyl-CoA carboxylase activity induced by 24 h starvation is totally reversed by re-feeding the animal for 2.5 h. Both the V_{max} and the $A_{0.5}(\text{citrate})$ return to control values, and the phosphate content is decreased by approx. 0.75 mol/mol of subunit (Table 1). This dephosphorylation and activation of acetyl-CoA carboxylase in response to re-feeding is blocked if the 24 h-starved animals are made insulin-deficient by streptozotocin administration immediately before re-feeding. Neither the kinetic parameters nor the phosphate content of the purified enzyme differ significantly between 24 h-starved and 24 h-starved/2.5 h-streptozotocin-treated/2.5 h-re-fed animals (Table 1). Streptozotocin treatment does not affect the eating behaviour of the rats, since 24 h-starved/2.5 h-re-fed and 24 h-starved/2.5 h-streptozotocin-treated/2.5 h-re-fed animals ate 17.9 ± 1.0 (11) and 15.8 ± 1.1 (6) g respectively of chow during the 2.5 h re-feeding period.

The effectiveness of streptozotocin in producing short-term insulin deficiency was assessed by its induction of hyperglycaemia. Chow-fed, chow-fed/2.5 h-streptozotocin-treated, 24 h-starved/2.5 h-re-fed and 24 h-starved/2.5 h-streptozotocin-treated/2.5 h-re-fed animals had arterial glucose concentrations of 5.2 ± 0.2 (5), 17.0 ± 2.0 (9), 5.2 ± 0.3 (3) and 19.9 ± 2.1 (7) mmol/litre of whole blood respectively. The 3-fold increase in blood glucose concentration in chow-fed animals treated with streptozotocin for 2.5 h indicated that these rats were insulin-deficient. However, neither

Fig. 1. Activation of acetyl-CoA carboxylase in crude extracts of lactating rat mammary gland by incubation with Mg^{2+} and citrate

Crude extracts from fed control rats (○, ●) or 24 h-starved rats (□, ■) were prepared and incubated in the presence (○, □) or absence (●, ■) of 50 mM-NaF as described in the Methods section. Samples (10 μ l) were removed from each incubation at the time points shown, and acetyl-CoA carboxylase activity was assayed for 1 min at 37 °C in the presence of 20 mM- $MgCl_2$, 20 mM-sodium citrate and bovine serum albumin (10 mg/ml) as described in the Methods section. Each point represents the mean of six experiments for fed controls and three experiments for the 24 h-starved rats, and vertical lines represent S.E.M.

Table 1. Kinetic parameters and alkali-labile phosphate content of acetyl-CoA carboxylase purified from lactating rat mammary gland under different physiological conditions

Animals were killed by stunning and cervical dislocation at the individual times shown. Acetyl-CoA carboxylase was purified, and its kinetic parameters and phosphate content were determined, as described in the Methods section. Results are means $\pm$ S.E.M. for the numbers of observations shown. Values significantly different from their respective controls (by Student's *t* test) are indicated: **P* < 0.05; ***P* < 0.005; ****P* < 0.0005.

Treatment	No. of observations	$V_{\max}$ ($\mu\text{mol/min per mg}$)	$A_{0.5}(\text{citrate})$ (mM)	Phosphate content (mol/mol of subunit)
(a) Killed at 10:00 h				
(1) Fed control	26	2.55 ± 0.24	2.10 ± 0.13	3.31 ± 0.21
(2) 24 h starved	19	$0.70 \pm 0.09^{***}$	$3.67 \pm 0.54^{**}$	$4.46 \pm 0.42^*$
(3) 24 h starved/2.5 h re-fed	8	2.12 ± 0.23	2.40 ± 0.22	3.72 ± 0.17
(4) 24 h starved/2.5 h streptozotocin-treated/2.5 h re-fed	10	$0.78 \pm 0.10^{***}$	$4.13 \pm 0.35^{***}$	$4.05 \pm 0.27^*$
(5) Fed/2.5 h streptozotocin-treated	7	2.56 ± 0.21	2.54 ± 0.36	3.44 ± 0.20
(b) Killed at 14:30 h				
(6) Fed control	6	1.92 ± 0.26	3.04 ± 0.41	3.64 ± 0.41
(7) 6 h starved	8	1.96 ± 0.13	2.94 ± 0.42	3.84 ± 0.29

the kinetic parameters nor the phosphate content of purified acetyl-CoA carboxylase in these insulin-deficient rats differed significantly from those of chow-fed controls. This is in contrast with previous reports that streptozotocin treatment of fed lactating rats leads to 40% inhibition of acetyl-CoA carboxylase activity measured in crude extracts of mammary gland (McNeillie & Zammit, 1982; Munday & Williamson, 1982). The response of mammary-gland lipogenesis to food withdrawal is very rapid, exhibiting some 80% inhibition within the first 6 h (Williamson *et al.*, 1983; Jones & Williamson, 1984; Jones *et al.*, 1984). However, this did not correlate with acetyl-CoA carboxylase activity in crude mammary extracts, where no inhibition was observed (Williamson *et al.*, 1983). Similarly, in the present study (Table 1) 6 h starvation did not significantly alter either the kinetic parameters or the phosphate content of purified acetyl-CoA carboxylase compared with fed control animals killed at the same point in the diurnal cycle.

These results indicate that, although phosphorylation and inhibition of acetyl-CoA carboxylase may not occur rapidly in response to starvation or insulin deficiency, the enzyme does become phosphorylated after a period of prolonged starvation, and this has profound effects on its kinetic parameters.

Treatment of acetyl-CoA carboxylase with protein phosphatase-2A

To confirm that the inactivation of acetyl-CoA carboxylase after 24 h starvation was due to increased phosphorylation, we examined the reversal of this effect by treatment of the purified enzyme with purified protein phosphatase *in vitro*. Fig. 2 illustrates a set of experiments in which acetyl-CoA carboxylase purified from the mammary glands of fed or 24 h-starved lactating rats was incubated at 37 °C in the presence or absence of the catalytic subunit of protein phosphatase 2A from rabbit skeletal muscle.

In control incubations, in the absence of protein phosphatase-2A, the activity of acetyl-CoA carboxylase purified from either fed or 24 h-starved rats did not

Fig. 2. Activation of acetyl-CoA carboxylase purified from the mammary glands of fed or starved lactating rats by incubation with the catalytic subunit of protein phosphatase-2A in the presence of citrate

Acetyl-CoA carboxylase (0.1 mg/ml) purified from fed (○, ●) or 24 h-starved rats (STV; □, ■) was incubated at 37 °C in 25 mM-NaHepes buffer, pH 7.0, 10 mM-sodium citrate, 10 mM-MgCl₂, bovine serum albumin (1 mg/ml), 1 mM-EDTA, in the presence of catalytic subunit of protein phosphatase-2A (6 units/ml) and 1 mM-MnCl₂ (P2A; ■, ●) or the absence of phosphatase and MnCl₂ and the presence of 1 mM-EGTA (control; □, ○). Samples were removed at various times and diluted 10-fold in 50 mM-NaHepes buffer/1 mM-EDTA, pH 7.0, and assayed for acetyl-CoA carboxylase activity at 10 mM-citrate. Each point represents the mean of experiments with six separate preparations of acetyl-CoA carboxylase, and vertical lines represent S.E.M.

Fig. 3. Bar charts showing the phosphate content, V_{max} , and $A_{0.5}(\text{citrate})$ of acetyl-CoA carboxylase from fed (F) and 24 h-starved (S) lactating rats treated with (+P2A) or without (control) protein phosphatase-2A

The purified enzyme was incubated for 90 min as described in Fig. 1, and the alkali-labile phosphate and kinetic parameters were determined as described in the Methods section. The heights of the bars represent means from four observations, and vertical lines within the bars represent S.E.M.

change significantly over 90 min. In the presence of protein phosphatase-2A, the activity of acetyl-CoA carboxylase purified from fed rats increased by 1.8-fold, whereas that from 24 h-starved rats increased some 5.5-fold over the 90 min incubation. The significant difference between the specific activities of acetyl-CoA carboxylase purified from fed and 24 h-starved rats rapidly disappeared within the first 10 min of incubation with protein phosphatase-2A, and by 90 min these enzyme activities were identical (Fig. 2). In these experiments, unlike those shown in Fig. 1, the results are expressed as units/mg of purified enzyme protein, and therefore are not affected by any loss of enzyme protein that may occur during 24 h starvation. The results show that the specific activity of acetyl-CoA carboxylase purified from the mammary glands of 24 h-starved lactating rats can be restored to values for fed controls by dephosphorylation *in vitro*.

To strengthen this conclusion, we incubated purified acetyl-CoA carboxylase for 90 min as in Fig. 2, and subsequently measured the kinetic parameters and phosphate contents. Measurements of alkali-labile phosphate showed that incubation with protein phosphatase-2A for 90 min removed approx. 1 mol of phosphate/mol of acetyl-CoA carboxylase purified from mammary glands of fed lactating rats, and approx. 2 mol of phosphate/mol of enzyme from 24 h-starved rats (Fig. 3). The phosphate contents of the acetyl-CoA carboxylase from fed and 24 h-starved rats did not significantly differ after treatment with protein phosphatase-2A. Fig. 3 shows that this dephosphorylation produced a modest increase in the V_{max} of mammary-gland acetyl-CoA carboxylase purified from fed rats, but a very substantial increase in the V_{max} of the enzyme from 24 h-starved rats (comparable with the increase in activity shown in Fig. 2). Hence the V_{max} of the acetyl-CoA carboxylase from fed and 24 h-starved rats did not significantly differ after protein phosphatase-2A treatment. The $A_{0.5}(\text{citrate})$ for the acetyl-CoA carboxylase from fed rats in this series of experiments was slightly lower than that quoted in Table

1, and the dephosphorylation did not appear to affect this value. However, the $A_{0.5}(\text{citrate})$ of the acetyl-CoA carboxylase from 24 h-starved rats was dramatically decreased by the dephosphorylation (Fig. 3), such that the $A_{0.5}(\text{citrate})$ of enzyme from fed and 24 h-starved rats did not significantly differ after treatment with protein phosphatase-2A. It is clear from these results that, although removal of 1 mol of phosphate/mol of enzyme subunit from fed rats does slightly increase the enzyme activity, the additional mol of phosphate/mol of subunit incorporated as a result of 24 h starvation has much more profound effects in terms of both decreased V_{max} and increased $A_{0.5}(\text{citrate})$. These effects can be completely reversed by dephosphorylation of enzyme with protein phosphatase-2A *in vitro*.

DISCUSSION

Our results provide direct evidence that the effect of prolonged starvation on acetyl-CoA carboxylase activity in lactating rat mammary gland is due to increased phosphorylation of the enzyme. Previous investigations of this problem involved measurement of enzyme activity in crude extracts both before ('initial activity') and after ('total activity') incubation at 37 °C in the presence of Mg^{2+} and citrate (Munday & Williamson, 1982; McNeillie & Zammit, 1982), and found that starvation (24 h) significantly decreased the 'initial'/'total' activity ratio. The results in Fig. 1 confirm this finding and also provide evidence that the effect of the incubation with Mg^{2+} and citrate is to promote dephosphorylation by endogenous protein phosphatases. Two lines of evidence support this contention: (1) activation in crude extracts in the presence of Mg^{2+} and citrate is prevented by NaF, a protein phosphatase inhibitor; (2) we also showed that activation in crude extracts from 24 h-starved rats by incubation with Mg^{2+} and citrate persisted during purification on avidin-Sepharose. Enzyme purified after incubation in the absence and presence of 50 mM-NaF had kinetic parameters indistinguishable from those of

enzyme purified from the mammary glands of 24 h-starved rats and then incubated in the presence and the absence respectively of protein phosphatase-2A (M. R. Munday, unpublished work).

These results suggest that the inhibition of acetyl-CoA carboxylase activity brought about by 24 h starvation is due to an increase in phosphorylation of the enzyme rather than a change in concentration of an allosteric effector. The further evidence in favour of this hypothesis is as follows.

(1) The extent of activation on incubation of extracts with Mg^{2+} and citrate is considerably greater (200%) for enzyme from 24 h-starved rats than for enzyme from fed rats (70%).

(2) Starvation causes a significant and substantial increase (≈ 1.2 mol/mol of subunit) in the phosphate content of the purified enzyme.

(3) The effect of starvation is preserved during extensive washing of the enzyme on the avidin-Sepharose column in high ionic strength, i.e. conditions that would be expected to remove even a tightly bound allosteric effector.

(4) The effects of starvation on both the kinetic parameters and the phosphate content of the enzyme are completely reversed on treatment of the purified enzyme with protein phosphatase.

If the activity of acetyl-CoA carboxylase measured in crude extracts of mammary gland after incubation with Mg^{2+} and citrate is taken to be a reliable measure of total enzyme protein, then data from this study (Fig. 1) and previous studies (Munday & Williamson, 1982; McNeillie & Zammit, 1982) suggest that there may be a loss of 50–60% of total enzyme protein during 24 h starvation. However, a re-feeding of 24 h-starved lactating rats for only 2.5 h completely restores the rate of lipogenesis (Munday & Williamson, 1981) without increasing the total enzyme concentration, as judged by the same criterion (Munday & Williamson, 1982). Thus short-term regulation of existing enzyme protein by phosphorylation may be more important in the regulation of mammary-gland lipogenesis than are changes in enzyme content during starving and re-feeding.

Our data firmly establish for the first time the important role of phosphorylation of acetyl-CoA carboxylase in the regulation of mammary-gland lipogenesis. The role of phosphorylation is already well established for the two other main lipogenic tissues, i.e. liver and adipose tissue. In both these tissues there is good evidence that hormones which increase cyclic AMP (glucagon in hepatocytes, glucagon and adrenaline in adipocytes) inhibit fatty acid synthesis in part via phosphorylation of acetyl-CoA carboxylase by cyclic AMP-dependent protein kinase (Brownsey & Hardie, 1980; Holland *et al.*, 1984, 1985). However, it seems unlikely that phosphorylation by cyclic AMP-dependent protein kinase underlies the effect of starvation in lactating mammary gland. Neither adrenaline nor glucagon produces any significant changes in rates of fatty acid synthesis in lactating rat mammary gland *in vivo* (Bussman *et al.*, 1984) or in mammary-gland acini *in vitro* (Williamson *et al.*, 1983; Robson *et al.*, 1984). Mammary acinar cells appear to lack glucagon receptors (Robson *et al.*, 1984) and, although they have been reported to contain β_2 -adrenergic receptors, β -agonists do not increase cyclic AMP significantly in the absence of phosphodiesterase inhibitors (Clegg & Mullaney, 1985).

The primary hormone regulating lipogenesis in lactating mammary gland appears to be insulin. The 98% decrease in mammary fatty acid synthesis produced by 24 h starvation correlates with a 50–60% decrease in the arterial plasma insulin concentration (Robinson *et al.*, 1978; Jones *et al.*, 1984). Short-term insulin deficiency (2.5 h) induced by streptozotocin also inhibits lipogenesis in lactating rat mammary gland by 87% (Robinson *et al.*, 1978). Starvation of lactating rats for 6 h, by which time arterial plasma insulin concentrations are decreased by 48% (Jones *et al.*, 1984), causes a similar degree of inhibition of lipogenesis (85%). Somewhat surprisingly, neither 2.5 h streptozotocin treatment nor 6 h starvation has any effect on either the phosphate content or the kinetic parameters of purified acetyl-CoA carboxylase (Table 1), although these treatments have been reported to decrease the 'initial' activity of acetyl-CoA carboxylase measured in crude extracts of mammary gland (Munday & Williamson, 1982; McNeillie & Zammit, 1982). These observations demonstrate that acetyl-CoA carboxylase is not the sole rate-limiting enzyme for lipogenesis in mammary gland. Short-term insulin deficiency induced by either streptozotocin or 6 h starvation may cause inhibition of other controlling steps, e.g. glucose transport and/or hexokinase, phosphofructo-1-kinase or pyruvate dehydrogenase (Threadgold & Kuhn, 1984; Jones *et al.*, 1984; Baxter & Coore, 1978). Inhibition of earlier steps would decrease flux through acetyl-CoA carboxylase, either by limitation of substrate supply, or by decreases in concentrations of the allosteric activator, citrate. Such non-covalent effects on acetyl-CoA carboxylase may still be measurable in crude extracts, but would be lost on purification. The inhibition of acetyl-CoA carboxylase produced by increased phosphorylation that occurs between 6 and 24 h after withdrawal of food (Table 1) is, however, likely to play an important part in the further dramatic inhibition of lipogenesis (from 85 to 98%) that occurs during this period.

The reason for the slow onset of increased phosphorylation of acetyl-CoA carboxylase after food withdrawal is not clear. It certainly lags behind the fall in arterial plasma insulin concentration, and it is possible that changes in protein synthesis are required, e.g. induction of a protein kinase or repression of a protein phosphatase. We have previously reported the existence in mammary gland of fed lactating rats of a cyclic AMP-independent protein kinase (acetyl-CoA carboxylase kinase-2) which inactivates acetyl-CoA carboxylase (Munday & Hardie, 1984). However, further investigation is required to establish whether this kinase is involved in increased phosphorylation of acetyl-CoA carboxylase during starvation, and also its mechanism of regulation.

Whatever the underlying mechanism for the slow increase in phosphorylation of acetyl-CoA carboxylase during starvation, it is clear that the enzyme can be rapidly dephosphorylated and activated when food is available again. The inhibition of lipogenesis (Robinson *et al.*, 1978; Munday & Williamson, 1981) and the phosphorylation and inhibition of acetyl-CoA carboxylase (Munday & Williamson, 1982; the present paper) are both completely reversed within 2.5 h of re-feeding. During this time the rat eats approx. 30% of its normal daily intake of food (Munday & Williamson, 1983) and the arterial plasma insulin concentration is rapidly restored (Robinson *et al.*, 1978). It seems clear that the increase in plasma insulin is responsible for reversal of the

effects of starvation, since administration of streptozotocin immediately before re-feeding prevents the re-activation of lipogenesis (Robinson *et al.*, 1978) and the dephosphorylation and re-activation of acetyl-CoA carboxylase (Table 1).

In mammary gland of starved lactating rats, therefore, insulin brings about an activation of acetyl-CoA carboxylase by causing a dephosphorylation of the enzyme. This stands in contrast with effects reported in isolated adipocytes and hepatocytes, where activation of acetyl-CoA carboxylase is associated with a site-specific increase in phosphorylation of the enzyme (Brownsey & Denton, 1982; Witters *et al.*, 1983; Holland & Hardie, 1985). However, in these cases it is not clear that the increased phosphorylation is the cause of enzyme activation, since, unlike the effects reported in the present paper, the effects of insulin on enzyme activity in adipocytes and hepatocytes are lost on purification of the enzyme on avidin-Sepharose (Witters *et al.*, 1983; Holland & Hardie, 1985). It is also worth pointing out that the effects of insulin on isolated adipocytes and hepatocytes, and the effects of insulin manipulation *in vivo* described in the present paper, are not directly comparable. The increased phosphorylation in isolated adipocytes or hepatocytes is observed within 10–15 min of insulin administration, whereas the effects observed in the present paper were measured after 2.5 h of re-feeding. It is also not yet certain that the effects observed *in vivo* in the present study result from direct action of insulin on the mammary cells themselves.

In this study we have shown that the catalytic subunit of protein phosphatase-2A will mimic the dephosphorylation and re-activation of mammary acetyl-CoA carboxylase that occurs on re-feeding of a starved lactating rat. It remains to ascertain whether this protein phosphatase is responsible for the effect of re-feeding *in vivo*, and to identify the cyclic AMP-independent protein kinase responsible for phosphorylation of the enzyme in response to starvation. Studies of these questions, and of the mechanism of regulation of the relevant protein kinases and phosphatases, will increase our understanding of the regulation of this important metabolic pathway by insulin.

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REFERENCES

- Baxter, M. A. & Coore, H. G. (1978) *Biochem. J.* **174**, 553–561
- Brownsey, R. W. & Denton, R. M. (1982) *Biochem. J.* **202**, 77–86
- Brownsey, R. W. & Hardie, D. G. (1980) *FEBS Lett.* **120**, 67–70
- Bussman, L. E., Ward, S. & Kuhn, N. J. (1984) *Biochem. J.* **219**, 173–180
- Clegg, R. A. & Mullaney, I. (1985) *Biochem. J.* **230**, 239–246
- Guy, P. S., Cohen, P. & Hardie, D. G. (1981) *Eur. J. Biochem.* **114**, 399–405
- Hardie, D. G. (1980) in *Molecular Aspects of Cellular Regulation* (Cohen, P., ed), vol. 1, pp. 33–62, Elsevier, Amsterdam
- Hardie, D. G. & Cohen, P. (1979) *FEBS Lett.* **103**, 333–338
- Hardie, D. G. & Guy, P. S. (1980) *Eur. J. Biochem.* **110**, 167–177
- Hardie, D. G., Holland, R. & Munday, M. R. (1984) *Horm. Cell Regul.* **8**, 117–138
- Holland, R. & Hardie, D. G. (1985) *FEBS Lett.* **181**, 308–312
- Holland, R., Witters, L. A. & Hardie, D. G. (1984) *Eur. J. Biochem.* **140**, 325–333
- Holland, R., Hardie, D. G., Clegg, R. A. & Zammit, V. A. (1985) *Biochem. J.* **226**, 139–145
- Ingebritsen, T. S., Blair, J., Guy, P., Witters, L. & Hardie, D. G. (1983) *Eur. J. Biochem.* **132**, 275–281
- Jones, R. G. & Williamson, D. H. (1984) *Biosci. Rep.* **4**, 421–426
- Jones, R. G., Ilic, V. & Williamson, D. H. (1984) *Biochem. J.* **223**, 345–351
- McNeillie, E. M. & Zammit, V. A. (1982) *Biochem. J.* **204**, 273–280
- McNeillie, E. M., Clegg, R. A. & Zammit, V. A. (1981) *Biochem. J.* **200**, 639–644
- Munday, M. R. & Hardie, D. G. (1984) *Eur. J. Biochem.* **141**, 617–627
- Munday, M. R. & Williamson, D. H. (1981) *Biochem. J.* **196**, 831–837
- Munday, M. R. & Williamson, D. H. (1982) *FEBS Lett.* **138**, 285–288
- Munday, M. R. & Williamson, D. H. (1983) *Biochem. J.* **214**, 183–187
- Resink, T. J., Hemmings, B. A., Lim Tung, H. Y. & Cohen, P. (1983) *Eur. J. Biochem.* **131**, 455–461
- Robinson, A. M., Girard, J. R. & Williamson, D. H. (1978) *Biochem. J.* **176**, 343–346
- Robson, N. A., Clegg, R. A. & Zammit, V. A. (1984) *Biochem. J.* **217**, 743–749
- Schein, P. S., Alberti, K. G. M. M. & Williamson, D. H. (1971) *Endocrinology (Baltimore)* **89**, 827–834
- Threadgold, L. C. & Kuhn, N. J. (1984) *Biochem. J.* **218**, 213–219
- Tipper, J. P. & Witters, L. A. (1982) *Biochim. Biophys. Acta* **715**, 162–169
- Williamson, D. H. (1980) *FEBS Lett.* **117**, K93–K105
- Williamson, D. H., Munday, M. R., Jones, R. G., Roberts, A. F. C. & Ramsey, A. J. (1983) *Adv. Enzyme Regul.* **21**, 135–145
- Williamson, D. H., Munday, M. R. & Jones, R. G. (1984) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43**, 2443–2447
- Witters, L. A., Tipper, J. P. & Bacon, G. W. (1983) *J. Biol. Chem.* **258**, 5643–5648

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