DEPHOSPHORYLATION AND ACTIVATION OF ACETYL-CoA CARBOXYLASE FROM LACTATING RABBIT MAMMARY GLAND

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1. Introduction

Acetyl-CoA carboxylase, the rate limiting enzyme in fatty acid synthesis, is completely dependent on citrate for activity and is inhibited by long chain fatty acyl CoA [1]. Kim and coworkers [2–5] partially purified the enzyme from rat liver and showed that the activity decreased following incubation with Mg-ATP and a protein fraction isolated from the same tissue. They also showed that the inactivation could be reversed by incubation with a protein fraction from hen oviduct. These results therefore suggested that acetyl-CoA carboxylase might be regulated by a phosphorylation–dephosphorylation mechanism, as well as by the concentration of allosteric effectors.

The phosphorylation of a homogeneous preparation of acetyl-CoA carboxylase was first demonstrated [6]. The enzyme isolated from lactating mammary gland was found to be contaminated by traces of two different protein kinases that could phosphorylate the enzyme. One was cyclic AMP-dependent protein kinase, while the other was a cyclic AMP-independent protein kinase, which was termed acetyl-CoA carboxylase kinase-2.

The phosphorylation of acetyl-CoA carboxylase by the purified catalytic subunit of cyclic AMP-dependent protein kinase was found to take place at a very similar rate to that of other well established physiological substrates of this enzyme, suggesting that the reaction was likely to occur in vivo [6]. Evidence supporting this idea was recently obtained using isolated adipose tissue and hepatocyte preparations. Acetyl-CoA carboxylase was phosphorylated in both cell types and the degree of phosphorylation was increased 1.5–2-fold by hormones which elevated the intracellular level of cyclic AMP (adrenaline in adipose tissue and glucagon in hepatocytes) [7–9]. The increased phosphorylation was accompanied by a 40–50% decrease in the activity, measured in the cell extracts in the presence of saturating concentrations of citrate.

Although these results suggested that hormones regulate the activity of acetyl-CoA carboxylase in vivo by altering the degree of phosphorylation of the enzyme, a change in the activity of the purified enzyme as a result of either phosphorylation or dephosphorylation has not yet been reported. In this paper we demonstrate that acetyl-CoA carboxylase can be isolated in a highly phosphorylated form which has a low specific activity, and which can be converted to a form of high specific activity by a dephosphorylation reaction.

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2. Materials and methods

2.1. *Materials*

Protein phosphatase-1, an enzyme which catalyses several functionally related dephosphorylation reactions involved in the control of glycogen metabolism, was purified 1000-fold from rabbit skeletal muscle through the DEAE–Sephadex step [10] and was provided by Dr Ann Burchell in this laboratory. The enzyme purified in this manner is stimulated several fold by Mn^{2+} when phosphorylase *a* is used as a substrate [10] and shows a much higher dependence

on Mn^{2+} when other substrates, such as glycogen synthase or inhibitor-1, are employed (J. G. Foulkes, unpublished work). Inhibitor-2, a heat stable protein which inhibits protein phosphatase-1 specifically [10], was partially purified from rabbit muscle as a byproduct of the purification of protein phosphatase inhibitor-1 [11] and further purified by gel filtration on Sephadex G-150. This preparation, which was purified 5000-fold, was provided by Mr Gordon Foulkes in this laboratory. One unit of protein phosphatase-1 was that amount which dephosphorylated 1.0 nmol phosphorylase *a*/min [12] and one unit of inhibitor-2 was that amount which inhibited 0.02 U protein phosphatase-1 by 50% in the standard assay [13]. Phosphorylase *b* was purified from rabbit muscle according to [14] and converted to phosphorylase *a* using purified phosphorylase kinase [12]. Fatty acid synthase was isolated from lactating rabbit mammary glands as a byproduct of the purification of acetyl-CoA carboxylase [15].

The proteinase inhibitors leupeptin, antipain and pepstatin were purchased from the Peptide Research Institute, 476 Ina, Minoh-Shi, Osaka 562; phenylmethanesulphonyl fluoride (PMSF), tosyl-phenylchloromethylketone (TPCK), tosyl-lysyl-chloromethylketone (TLCK) and soybean trypsin inhibitor were obtained from Sigma Chemical Co., Poole, Dorset. The sources of other materials have been given [6,15].

2.2. *Methods*

Acetyl-CoA carboxylase was purified from lactating rabbit mammary glands as described [6,15] with the following minor modifications. All buffers contained 0.02% sodium azide and proteinase inhibitors at the concentrations specified below: leupeptin, antipain and pepstatin (all at 0.4 μg/ml). TPCK and TLCK (both at 0.01 mM), PMSF (0.1 mM) and trypsin inhibitor (0.1 μg/ml). In some preparations NaF (50 mM) was included in the homogenisation buffer and at all subsequent steps. Acetyl-CoA carboxylase was stored at room temperature (20°C) in 100 mM sodium phosphate buffer (pH 7.0) containing 1.0 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, 25 mM sodium citrate, proteinase inhibitors at the concentrations specified above, and in the presence or absence of 50 mM sodium fluoride. Under these conditions, the activity was stable for several days, and all experiments were carried out within 2 days of completing the preparations. For studies of the activation of acetyl-CoA carboxylase, the enzyme was dialysed at 20°C against 0.05*I* Tris/HCl (pH 7.5), containing 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.1 mM sodium citrate and 0.02% sodium azide. This dialysis did not affect the activity of the enzyme.

The activity of acetyl-CoA carboxylase was monitored during the enzyme preparation as in [6]. For studies of the activation of the enzyme, a modified assay was used. The assays (1.0 ml) comprised 0.1*I* Tris/HCl (pH 7.2) (at 37°C), 0.3 mM acetyl-CoA, 0.24 mM NADPH, 1.0 mg/ml bovine serum albumin, acetyl CoA carboxylase, 0.2 mg/ml fatty acid synthase, 50 mM sodium bicarbonate, magnesium citrate as specified, 4 mM ATP and 4 mM $MgCl_2$. All the components except the Mg-ATP were incubated for 10 min at 37°C in 0.95 ml, this period being sufficient to maximally activate acetyl-CoA carboxylase at all concentrations of citrate employed. The reaction was then initiated with 0.05 ml of 80 mM $MgCl_2$–80 mM ATP and monitored at 340 nm. One unit of activity was that amount which catalysed the formation of 1.0 μmol malonyl-CoA/min (equivalent to 2.0 μmol NADPH oxidised in the coupled assay).

The content of alkali-labile phosphate bound covalently to acetyl-CoA carboxylase was measured as in [16] using phosphorylase *b* and phosphorylase *a* as internal standards. Phosphorylase *b* always contained < 0.02 phosphate molecules and phosphorylase *a*, 1.0 ± 0.1 phosphate molecules/mol. wt 100 000. In this method, 5.0 nmol phosphate yield an A_{820} 0.14. In view of the high phosphate content of acetyl-CoA carboxylase, it was therefore only necessary to analyse 1–2 nmol enzyme (0.25–0.5 mg) in order to obtain reliable values. The concentration of acetyl-CoA carboxylase was measured by the methods of Lowry [17] and Bradford [18] using bovine serum albumin ($A^{1\%}_{280}$ = 6.5) as a standard. These two methods agreed to within 5%.

3. Results

3.1. *Purification of acetyl-CoA carboxylase in the presence and absence of sodium fluoride*

Acetyl-CoA carboxylase isolated by the procedure described [6] shows one major band (mol. wt 250 000) and two faint minor bands (mol. wt 240 000 and

Table 1
Purification of acetyl-CoA carboxylase from lactating rabbit mammary gland prepared in the absence (A) or presence (B) of 50 mM sodium fluoride

Step	(A) Prepared without sodium fluoride				(B) Prepared with sodium fluoride			
	Activity (Units)	Protein (mg)	Spec. act. (Units/mg)	Yield (%)	Activity (Units)	Protein (mg)	Spec. act. (Units/mg)	Yield (%)
1. 90 000 × *g* supernatant	15.7	3371	0.0046	100	11.2	2906	0.0038	100
2a. 35% ammonium sulphate precipitate (before dialysis)	10.0	880	0.011	64	5.7	934	0.006	51
2b. 35% ammonium sulphate precipitate (after dialysis)	19.5	880	0.022	124	7.3	934	0.008	65
3. 1st polyethylene glycol precipitate	15.5	13.8	1.1	99	6.0	12.8	0.47	54
4. 2nd polyethylene glycol precipitate	10.9	3.6	3.0	69	4.3	3.6	1.2	38

The glands from 2 rabbits (240 g) were finely chopped, rinsed and divided into two equal portions. Acetyl-CoA carboxylase was purified from each portion either in the absence (A) or presence (B) of 50 mM NaF

230 000) when examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. In the present work, inclusion of the proteinase inhibitors listed under section 2.2 minimized, and occasionally eliminated, the appearance of the minor bands from the final product. This confirmed our previous suggestion that these components are generated from the species of mol. wt 250 000 by limited proteolysis.

Homogeneous preparations of acetyl-CoA carboxylase were obtained whether the preparations were carried out in the presence or absence of 50 mM NaF. However the specific activity of preparations prepared in the presence of NaF were 2.5-fold lower, when the activities were measured under optimal conditions using saturating concentrations of citrate (table 1). The difference in specific activity was largely generated during dialysis of the redissolved ammonium sulphate precipitate (step 2b). In the presence of NaF this dialysis produced only a slight activation (1.3-fold) whereas 2-fold activation took place if NaF was excluded.

Since NaF is a powerful inhibitor of many protein phosphatases, the results suggested that this compound might have prevented the activation of acetyl-CoA carboxylase by inhibiting the dephosphorylation of the enzyme which was catalysed by an endogenous protein phosphatase(s). The amount of phosphate bound covalently to the purified enzyme was therefore measured, and these experiments showed that preparations prepared in the presence of NaF contained 6.2 ± 0.2 phosphate molecules/subunit (mean ± SD of 4 different preparations) whereas preparations prepared in the absence of NaF contained only 4.8 ± 0.3 phosphate molecules (mean ± SD of 4 different preparations). The phosphate content of preparations purified in the absence of NaF was a little higher than in a previous series of preparations [15]. The reasons for this discrepancy are not clear. However, it should be noted that in the present work the phosphate content was measured within 2 days of the completion of the preparation.

3.2. *Dephosphorylation and activation of acetyl-CoA carboxylase by protein phosphatase-1*

Preparations of acetyl-CoA carboxylase were incubated with highly purified protein phosphatase-1 from rabbit skeletal muscle and the results are shown in fig.1 and table 2.

If the preparation isolated in the absence of NaF was incubated with protein phosphatase-1, a slight (1.4-fold) activation was observed after 2 h when the assays were carried out at 1.0 mM citrate. In contrast, the activity of the preparation isolated in the presence of NaF increased considerably following incubation with protein phosphatase-1. After 2 h the activity at 1.0 mM citrate had increased 4-fold and was approaching the activity of the preparation prepared in the absence

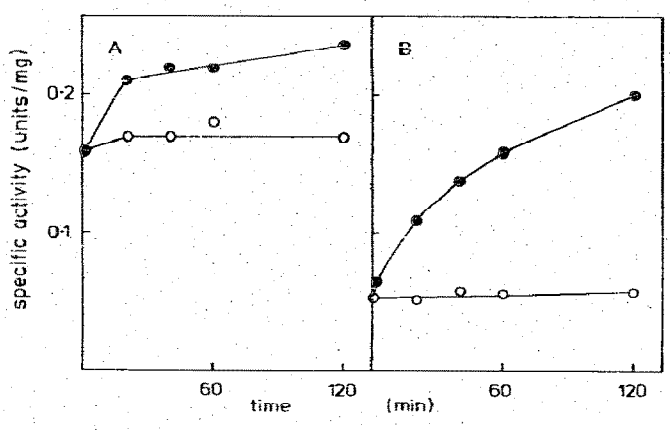

Fig.1. Activation of acetyl-CoA carboxylase by protein phosphatase-1. Acetyl-CoA carboxylase was dialysed at 20°C into 0.05*I* Tris/HCl (pH 7.5) containing 1.0 mM EDTA, 1.0 mM dithiothreitol, 0.02 mM sodium azide and 0.1 mM sodium citrate. Acetyl-CoA carboxylase (0.75 mg/ml) 0.25 ml, was incubated at 25°C with 0.025 ml $MnCl_2$ (24 mM) and either 0.025 ml protein phosphatase-1 (20 U/ml) (closed circles) or dialysis buffer (open circles). At the times indicated by the circles 0.05 ml aliquots were withdrawn and assayed for activity at 1.0 mM citrate as described in section 2. (A) Preparation isolated in the absence of NaF; (B) preparation isolated in the presence of NaF.

Table 2
Activation of acetyl-CoA carboxylase by protein phosphatase-1

Additions to incubation	Relative activity (% of control)
None	100
$MnCl_2$ + phosphatase-1	282
$MgCl_2$ + phosphatase-1	99
Phosphatase-1	116
$MnCl_2$ + phosphatase-1 + inhibitor-2	113
$MnCl_2$ + inhibitor-2	94
$MnCl_2$	96

Acetyl-CoA carboxylase prepared in the presence of NaF was dialysed overnight and then incubated at 20°C for 60 min, as described in the legend to fig.1. The concentration of acetyl-CoA carboxylase was 0.28 mg/ml and the final concentrations of protein phosphatase-1, $MnCl_2$, $MgCl_2$ and inhibitor-2, where added, were 2.0 U/ml, 2.0 mM, 5.0 mM and 3000 U/ml, respectively. The assays were carried out in duplicate in the presence of 1.0 mM citrate

of NaF. Control experiments showed that the activation was completely dependent on protein phosphatase-1 and that the activity of the enzyme was completely stable over the time period of the incubation (fig.1).

The results illustrated in table 2 showed that the activation of acetyl-CoA carboxylase was completely dependent on the presence of Mn^{2+} as well as protein phosphatase-1, and that Mg^{2+} could not substitute for Mn^{2+}. The activation of acetyl-CoA carboxylase by protein phosphatase-1 in the presence of Mn^{2+} could be blocked completely by inhibitor-2, and control experiments showed that inhibitor-2 did not affect the activity of acetyl-CoA carboxylase itself. The addition of Mn^{2+} in the absence of protein phosphatase-1 did not affect the activity, showing that the purified acetyl-CoA carboxylase was not contaminated with endogenous protein phosphatase activity.

At the end of the incubation in fig.1, the various samples of acetyl-CoA carboxylase were analysed in duplicate for their content of covalently bound phosphate. The preparation isolated in the absence of NaF contained 4.9 ± 0.1 phosphate molecules/subunit, and this decreased to 4.3 ± 1.0 molecules/subunit, following the 2 h incubation with protein phosphatase-1. The preparation isolated in the presence of NaF contained 5.9 ± 0.4 molecules/subunit, and this decreased to 4.4 ± 0.1 phosphate molecules/subunit, after the 2 h incubation with protein phosphatase-1.

Some preliminary investigations were made of the effect of citrate concentration on the activity of acetyl-CoA carboxylase before and after treatment with protein phosphatase-1, using the preparation isolated in the presence of NaF. At 1.0 mM citrate (fig.1) or 0.3 mM citrate, the phosphatase-treated enzyme containing 4.4 phosphate molecules/subunit had a 4-fold higher activity than the control preparation containing 5.9 phosphate molecules. However, at saturating concentrations of citrate (10 mM), the activity of the phosphatase treated preparation was only 2.3-fold higher. This suggests that dephosphorylation increases the V_{max} of the enzyme and decreases the concentration of citrate required for activation.

4. Discussion

The idea that the activity of acetyl-CoA carboxylase

is regulated by a phosphorylation–dephosphorylation mechanism has been disputed for several years [19,20]. However, the results presented in this paper show conclusively that the activity of the enzyme can be altered by changing its state of phosphorylation. The isolation of the enzyme in the presence of a protein phosphatase inhibitor (NaF) produced a form of the enzyme which had a lower specific activity and a higher content of covalently bound phosphate than the enzyme prepared in the absence of NaF. Furthermore, the difference in phosphate content and activity of acetyl-CoA carboxylase prepared in the presence and absence of NaF could be eliminated by treating the preparation with protein phosphatase-1 (fig.1). The finding that the activation was completely dependent on Mn^{2+} (see section 2.1) and prevented by inhibitor-2 (a specific inhibitor of protein phosphatase-1) provided conclusive evidence that the effect was due to dephosphorylation by protein phosphatase-1 rather than to some other mechanism, e.g., limited proteolysis. The latter possibility was also excluded by the finding that the migration of the enzyme on polyacrylamide gels in the presence of sodium dodecyl sulphate was unaffected by incubation with protein phosphatase-1.

The results presented in table 1 show that the enzyme is activated at early stages of the preparation by an endogenous protein phosphatase. A similar activation during the purification of rat mammary acetyl-CoA carboxylase was observed [21], although this was attributed to the removal of inhibitors of the enzyme during its isolation. Dephosphorylation and activation of acetyl CoA carboxylase could also explain, at least in part, the time-dependent activation of acetyl-CoA carboxylase observed when rat liver extracts were incubated at 37°C [22]. Although these workers observed an 8-fold activation at saturating concentrations of citrate as compared with ~2.5-fold activation in the present work, it is possible that acetyl-CoA carboxylase isolated from mammary gland in the presence of NaF, does not represent fully phosphorylated enzyme. In addition, the activation of the enzyme had not reached a plateau at the end of the 2 h incubation with protein phosphatase-1 (fig.1).

Although the activation of acetyl-CoA carboxylase can be catalysed by protein phosphatase-1 in vitro, it is not known whether this is identical to the endogenous protein phosphatase in mammary gland which catalyses the dephosphorylation reaction at step 2b in the purification (table 1). However it should be noted that protein phosphatase-1 is present in mammary gland, liver and adipose tissue at very similar concentrations to that which is present in skeletal muscle [23].

The present results raise the question of which protein kinase can reverse the dephosphorylation reactions investigated in this paper. It has been established that acetyl-CoA carboxylase can be phosphorylated by at least two different protein kinases, namely cyclic AMP-dependent protein kinase and acetyl-CoA carboxylase kinase 2 [6]. Experiments are in progress to determine whether either of these two protein kinases is the enzyme involved in reversing the dephosphorylation reactions described in this paper.

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