Protein-serine kinase from rat epididymal adipose tissue which phosphorylates and activates acetyl-CoA carboxylase

Possible role in insulin action

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1. Most of the cyclic-nucleotide-independent acetyl-CoA carboxylase kinase activity in an extract of rat epididymal adipose tissue was eluted from a Mono Q column by 0.175 M-NaCl at pH 7.4. The activity of the kinase in this fraction (fraction 1) was increased after exposure of intact tissue to insulin. 2. Incubation of purified adipose-tissue acetyl-CoA carboxylase with [γ - 32 P]ATP and samples of fraction 1 led to the incorporation of up to 0.4 mol of 32 P/mol of enzyme subunit. Most of the phosphorylation was on serine residues within a single tryptic peptide. This peptide, on the basis of two-dimensional t.l.c. analysis, h.p.l.c. and Superose 12 chromatography, appeared to be the same as the acetyl-CoA carboxylase peptide ('I'-peptide) which exhibits increased phosphorylation in insulin-treated tissue. 3. Phosphorylation of purified acetyl-CoA carboxylase by the kinase in fraction 1 was found to be associated with a parallel 4-fold increase in activity. However, increases in both phosphorylation and activity were much diminished if fraction 1 was treated by Centricon centrifugation to remove low- M_r components. Among these components was a potent inhibitor of acetyl-CoA carboxylase activity which appeared to be necessary for the kinase in fraction 1 to be fully active. 4. The inhibitor remains to be identified, but inhibition requires MgATP, although the inhibitor itself does not cause any phosphorylation of the carboxylase. No effects of insulin were observed on the activity of the inhibitor. 5. It is concluded that the kinase probably plays an important role in the mechanism whereby insulin brings about the well-established increases in phosphorylation and activation of acetyl-CoA carboxylase in adipose tissue.

INTRODUCTION

Exposure of rat epididymal adipose tissue or isolated fat-cells to insulin leads within a few minutes to a 2–3-fold increase in the activity of acetyl-CoA carboxylase (Halestrap & Denton, 1973, 1974). Subsequently, evidence for activation has also been obtained from studies on brown adipose tissue (McCormack & Denton, 1977), liver (Witters *et al.*, 1979; Buechler *et al.*, 1984) and mammary tissue (Munday & Williamson, 1982). Activation, at least in rat epididymal adipose tissue, is characterized by an increase in the proportion of the enzyme in a polymeric form which exhibits activity in the absence of added citrate (Halestrap & Denton, 1973, 1974; Brownsey *et al.*, 1979; Brownsey & Denton, 1982; Borthwick *et al.*, 1986). After incubation of tissue or cell extracts with sufficient citrate to cause maximum activation and conversion of acetyl-CoA carboxylase into its polymeric forms, the effect of insulin is no longer evident (Halestrap & Denton, 1974; Brownsey & Denton, 1982; Borthwick *et al.*, 1987).

In rat epididymal fat-cells or liver cells, insulin activation of acetyl-CoA carboxylase is associated with an increase in the overall extent of phosphorylation of the enzyme (Brownsey *et al.*, 1977; Brownsey & Denton, 1982; Witters, 1981; Haystead & Hardie, 1986). This increased phosphorylation appears to occur on at least two distinct serine residues (Brownsey & Denton, 1982, 1987; Haystead *et al.*, 1988). One serine is within a tryptic peptide, which we have termed the 'I'-peptide (Brownsey & Denton, 1982). This peptide is well separated from all other phosphopeptides by two-dimensional t.l.c. analysis and barely binds to a C_{18} reverse-phase h.p.l.c. column (Brownsey & Denton, 1982; A. C. Borthwick, N. J. Edgell & R. M. Denton, unpub-

lished work). The other serine is within a tryptic peptide (designated T4A; Haystead *et al.*, 1988), which is eluted later than most other acetyl-CoA carboxylase phosphopeptides from reverse-phase h.p.l.c. and behaves similarly to dinitrophenol-lysine in the two-dimensional t.l.c. system (Haystead *et al.*, 1988; Haystead & Hardie, 1988). Whereas the serine within the 'I'-peptide remains to be identified, the serine in peptide T4A appears to be serine-29 of acetyl-CoA carboxylase (Haystead *et al.*, 1988; Haystead & Hardie, 1988), which is also phosphorylated *in vitro* by casein kinase 2.

It has proved difficult to establish unequivocally the relationship between the increases in phosphorylation and the increases in activity of acetyl-CoA carboxylase in adipose tissue or fat-cells exposed to insulin (Haystead & Hardie, 1986; Brownsey & Denton, 1987; Witters *et al.*, 1988). Phosphorylation of the enzyme with casein kinase 2 does not appear to lead to any direct increase in enzyme activity (Tipper *et al.*, 1983; Munday & Hardie, 1984). On the other hand, the polymerized form of the enzyme, which is active in the absence of citrate, has been found to exhibit increased phosphorylation within the 'I'-peptide (Borthwick *et al.*, 1987). Definitive proof that phosphorylation of the 'I'-peptide brings about activation must include the characterization of the kinase involved and the demonstration that phosphorylation of the 'I'-peptide by the kinase leads to an increase in enzymic activity. Previous studies have provided some evidence that membranes prepared from fat-cells contain cyclic-nucleotide-independent protein kinase activity capable of phosphorylating added acetyl-CoA carboxylase, and that this phosphorylation was associated with an increase in activity of acetyl-CoA carboxylase (Brownsey *et al.*, 1981). Subsequently, it was found that brief exposure of rat epididymal adipose tissue to

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insulin led to an increase in the activity of an apparently similar acetyl-CoA carboxylase kinase in subsequently prepared high-speed-supernatant fractions (Brownsey *et al.*, 1984, 1988), associated with a small decrease in activity in the particulate fraction (Brownsey *et al.*, 1988).

In the present study, the insulin-stimulated acetyl-CoA carboxylase kinase activity has been partially purified from high-speed-supernatant fractions of adipose tissue by chromatography on Mono Q. In this system, we find that the kinase co-purifies with a potent inhibitor of acetyl-CoA carboxylase activity. In the presence of this inhibitor, parallel phosphorylation and activation of purified acetyl-CoA carboxylase was apparent, and much of the phosphorylation appeared to occur within the 'I'-peptide.

EXPERIMENTAL

Materials

Except as given below, sources of chemicals, radiochemicals and biochemicals were as described previously (Borthwick *et al.*, 1987). Male Wistar rats (180–220 g) were fed *ad libitum* up to the time of killing. Adenosine 5'-[γ -thio]triphosphate was from Boehringer Mannheim, Lewes, East Sussex, U.K. Kemptide, poly(Glu, Tyr) (4:1) and heparin were from Sigma Chemical Co., Poole, Dorset, U.K. Malantide was from Ocean Biologics, Edmonds, WA 98020, U.S.A.

Tissue incubation and preparation of extracts

Epididymal fat-pads were incubated in paired groups of 15 for 30 min at 37 °C in bicarbonate-buffered medium containing glucose (11 mM) and then for an additional 15 min in fresh medium of the same composition with or without insulin (0.5 μ g/ml) (Brownsey *et al.*, 1984). Pads were blotted and extracted at 0 °C in medium (pH 7.4; 4 ml/g) containing sucrose (0.25 M), Tris/Mops (20 mM), EGTA (2 mM), GSH (7.5 mM) and the proteinase inhibitors benzamidine (2 mM), pepstatin A (1 μ g/ml), antipain (1 μ g/ml) and leupeptin (1 μ g/ml), by using a Polytron PT20 homogenizer. Extracts were centrifuged successively at 3000 *g* for 90s, 25000 *g* for 10 min and 60000 *g* for 30 min to yield a high-speed-supernatant fraction; after the last two spins the supernatants were passed through muslin to remove all traces of fat.

Use of Mono Q chromatography and Centricon filtration

Unless otherwise stated, high-speed-supernatant fraction equivalent to 6–8 g of adipose tissue was applied to a Mono Q HR 5/5 column (f.p.l.c. system; Pharmacia LKB) equilibrated in buffer A [potassium phosphate (10 mM), pH 7.4, containing EDTA (0.5 mM), citrate (1 mM), dithiothreitol (1 mM) and pepstatin A, antipain and leupeptin (each at 1 μ g/ml)] at 4 °C. After washing with buffer A until baseline values of A_{280} were obtained, the column was developed with a discontinuous gradient to buffer A plus 1 M-NaCl (see Fig. 1), with 0.5 ml fractions being collected. The flow rate throughout was 0.5 ml/min. In experiments in which the effect of insulin was being studied, equal amounts of high-speed-supernatant fractions prepared from paired tissue incubated in the absence and presence of insulin were applied to Mono Q columns. The A_{280} profiles of the proteins eluted were essentially identical. A typical example is shown in Fig. 1.

Tubes (usually 13–18) containing appreciable acetyl-CoA carboxylase kinase activity were combined (fraction 1). Where stated, fractions 1A (low M_r) and 1B (high M_r) were prepared from fraction 1 by using Centricon micro-concentrators (10000 M_r cut-off). Fraction 1A was the filtrate resulting from the initial treatment of 1 ml of fraction 1; the retained high- M_r fraction

(about 100 μ l) was diluted in buffer A plus 0.175 M-NaCl and re-filtered twice before being made up to its original volume with buffer A plus 0.175 M-NaCl to give fraction 1B.

Purification of acetyl-CoA carboxylase

Acetyl-CoA carboxylase was purified to homogeneity from rat epididymal adipose tissue by sequential use of Mono Q and Superose 6 chromatography. The tissue had previously been incubated in the absence of hormones to ensure minimal levels of phosphorylation. Preparation of extracts and the initial use of the Mono Q column were as described in the preceding sections. Acetyl-CoA carboxylase is eluted with 250–300 mM-NaCl. Fractions containing acetyl-CoA carboxylase were incubated with citrate (20 mM) for 20 min at 30 °C to ensure maximum polymerization. The polymeric form was then separated by gel permeation using a Superose 6 column (preparation grade, 1.6 cm $\times$ 45 cm) and then re-applied to a Mono Q column and eluted with NaCl (1 M). The final product of acetyl-CoA carboxylase had a specific activity of about 1 unit/mg and was stored at 0.5 mg/ml in buffer A plus 0.5 M-NaCl and glycerol (30%, w/v).

Assay of acetyl-CoA carboxylase activity

Acetyl-CoA carboxylase activity was assayed as described by Borthwick *et al.* (1987). Maximum activity was determined after preincubation of samples to be assayed with citrate (20 mM) for 20 min at 30 °C. One unit of activity is the amount catalysing the utilization of 1 μ mol of substrate/min at 30 °C.

General techniques

SDS/polyacrylamide-gel electrophoresis, radioautography and densitometric scanning of radioautographs were as described by Brownsey *et al.* (1984). Immunoprecipitation was carried out with sheep anti-(rabbit acetyl-CoA carboxylase) serum and subsequent mapping of tryptic peptides by two-dimensional t.l.c. analysis as in Brownsey & Denton (1982). Phosphoamino-acid analysis was as described by Cooper *et al.* (1983).

RESULTS AND DISCUSSION

Separation of acetyl-CoA carboxylase kinase activity by Mono Q chromatography

Preliminary studies indicated that acetyl-CoA carboxylase kinase activity present in the high-speed-supernatant fractions of rat epididymal adipose tissue was eluted from a Mono Q column by about 0.2 M-NaCl at pH 7.4. Optimal purification was achieved by using the discontinuous gradient indicated in Fig. 1, in which the column was developed isocratically with 0.175 M-NaCl between two linear gradients. Under these conditions, virtually all acetyl-CoA carboxylase kinase activity was eluted as a single peak. In agreement with earlier studies, using supernatant fractions (Brownsey *et al.*, 1984, 1988), this kinase activity was increased if the epididymal adipose tissue had previously been exposed to insulin.

In most subsequent experiments, tubes containing appreciable acetyl-CoA carboxylase kinase activity (usually tubes 13–18) were combined. This fraction (referred to as fraction 1) contained 2–5% of the protein present in the original supernatant. It was also low in ATPase activity. Under the conditions of the acetyl-CoA carboxylase kinase assay carried out as in Fig. 1, only about 17% of ATP was hydrolysed in 15 min when 25 μ l of fraction 1 was incubated in a total volume of 50 μ l. This activity represents less than 1% of the ATPase activity in the original high-speed supernatant. Other protein kinases present in the supernatant fractions of rat epididymal adipose tissue, including cyclic AMP-dependent protein kinase (holoenzyme and free catalytic sub-

Fig. 1. Elution of acetyl-CoA carboxylase kinase activity during Mono Q chromatography

High-speed supernatant fractions prepared from epididymal adipose tissue incubated in the presence ($\blacktriangle$) and absence ($\bullet$) of insulin were applied to a Mono Q 5/5 column which was eluted with a NaCl gradient (----). Acetyl-CoA carboxylase kinase activity was assayed by incubating 25 μ l samples for 15 min at 30 $^{\circ}$ C in a total volume of 50 μ l containing (final concentrations) purified acetyl-CoA carboxylase (0.1 mg/ml), MgCl_2 (5 mM), $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (50 μM ; about 1000 d.p.m./pmol), potassium phosphate (5 mM), citrate (0.6 mM), EDTA (0.5 mM), dithiothreitol (0.5 mM), NaCl (86 mM), pH 7.4. Acetyl-CoA carboxylase was then separated by SDS/polyacrylamide-gel electrophoresis and ^{32}P incorporated determined by densitometric scanning of radioautographs. An arbitrary unit represents the incorporation of approx. 40 d.p.m. of ^{32}P into acetyl-CoA carboxylase. Further details are given in the Experimental section. Also shown are the A_{280} profiles for the extracts from control (—) and insulin-treated tissue (.....). Results are typical of at least three separate experiments.

unit), casein kinase 2, protein kinase C and the insulin-activated ATP citrate-lyase kinase activity are well separated from this acetyl-CoA carboxylase kinase activity. Acetyl-CoA carboxylase kinase activity in fraction 1 was not appreciably inhibited by EGTA (1 mM), the cyclic-AMP-dependent protein kinase inhibitor peptide (0.2 μM) (Cheng *et al.*, 1986), or heparin (1 μM). Moreover, no phosphorylation was detected of added ATP citrate-lyase (0.17 μM), Kemptide (LRRASLG) (16 μM) or Malantide (RTKRSGSVYYEPLKI) (20 μM).

Fig. 2(a) shows the typical time course of phosphorylation of acetyl-CoA carboxylase by using 25 μ l of fraction 1 from insulin-treated tissue (in a total volume of 50 μ l). The added acetyl-CoA carboxylase was the major protein phosphorylated, but small and variable amounts of phosphorylation of endogenous proteins present in the fraction was evident. The most consistently observed had a subunit M_r of about 40000. Rates of phosphorylation of acetyl-CoA carboxylase under the conditions used in Fig. 2(a) were linear for about 15 min and then declined, so that phosphorylation reached a maximum at about 30 min which was maintained for at least a further 90 min. From values of the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ specific radioactivity, amount of acetyl-CoA carboxylase protein, M_r of acetyl-CoA carboxylase and ^{32}P incorporated into acetyl-CoA carboxylase, it was calculated that the maximum extent of phosphorylation represented 0.39 ± 0.03 mol/mol of acetyl-CoA carboxylase (mean $\pm$ S.E.M. of five observations).

In further studies, acetyl-CoA carboxylase phosphorylated by fraction 1 was immunoprecipitated, digested with trypsin, and the released phosphopeptides were mapped by two-dimensional t.l.c. (Fig. 2b). The major phosphopeptide had the same R_f as dinitrophenyl-lysine in the electrophoretic dimension, but a markedly lower R_f in the chromatographic dimension. Phos-

phorylation of the peptide occurred exclusively on serine residues (Fig. 2d). These are characteristics of 'I'-peptide, which is the major peptide exhibiting increased phosphorylation after exposure of intact tissue to insulin. Fig. 2(c) illustrates the array of tryptic phosphopeptides released from acetyl-CoA carboxylase obtained from insulin-treated tissue incubated in the presence of $[\text{}^{32}\text{P}]\text{P}_i$ (Brownsey & Denton, 1982, 1987). The 'I'-peptide is indicated with an arrow. Further evidence that the major phosphopeptide in Fig. 2(b) is the same as the 'I'-peptide observed on the exposure of intact tissue to insulin was that both peptides barely bound to a C_{18} reversed-phase h.p.l.c. column (Lichrosorb 5RP18) equilibrated with 0.1% trifluoroacetic acid (results not shown). Both peptides also show very similar elution characteristics during gel-permeation chromatography on a Superose-12 column (Fig. 2e).

Effects of fractions separated by Mono Q chromatography on acetyl-CoA carboxylase activity

The effects of incubating purified acetyl-CoA carboxylase with various volumes of Mono Q fractions in the presence of ATP resulted in more complex changes in activity than expected from the phosphorylation studies, as biphasic dose-response curves were obtained (Fig. 3a). When small amounts of fraction 1 (2–5 μ l) were incubated with acetyl-CoA carboxylase for 15 min a marked diminution in activity was observed, but these inhibitory effects were less with larger volumes, until with 25 μ l the activity of acetyl-CoA carboxylase was greater than that observed when the enzyme was incubated with ATP alone. Moreover, although the maximum amount of inhibition seen with 2 μ l appeared to be similar with fractions from control and insulin-treated tissue, the extent of activation with 25 μ l samples was clearly greater when the fraction was derived from insulin-treated tissue (Figs. 3a and 3b). It should be noted that in these incubations of acetyl-CoA carboxylase with fraction 1 the concentration of citrate was kept constant at 0.6 mM. This concentration of citrate was used in all the studies reported in this paper. We have also carried out separate studies in which fraction 1 has been prepared in the absence of citrate, which allows the concentration of citrate in the incubations to be lowered to 0.1 mM. Essentially similar results to those reported in Fig. 3 were obtained, except that the activity of acetyl-CoA carboxylase tended to be somewhat lower when it was incubated in the absence of fraction 1.

The biphasic effects of fraction 1 on acetyl-CoA carboxylase activity suggested that the fraction may contain a mixture of inhibitory and activatory components. This view was supported by the finding that after boiling for 2 min fraction 1 only exhibited inhibitory effects (results not shown). Subsequent studies showed that Centricon filtration using filters with a 10000- M_r cut-off was a convenient and rapid means of separating the two components (Fig. 4).

The low- M_r fraction (fraction 1A) contained the inhibitory component (Fig. 4a). Maximum inhibition was observed with between 2 and 5 μ l and half-maximum effects at about 0.5 μ l under the standard conditions used in the present study (Fig. 5). No appreciable differences were found between fractions prepared from control or insulin-treated tissue (Fig. 5). Further studies showed that inhibition of acetyl-CoA carboxylase activity required MgATP, since little or no inhibition was evident in the absence of ATP (Table 1), with ATP alone, with MnATP or with Mg-adenosine 5'-[γ -thio]triphosphate. It should be emphasized that, despite this requirement for MgATP, there was virtually no phosphorylation of acetyl-CoA carboxylase (Figs. 4b and 6b). The inhibitory effects of fraction 1A could not be completely reversed by subsequent incubation with potassium citrate (Table 1). The possibility was explored that the apparent reversal of the inhibitory effects when higher volumes of fraction 1 were used

High-speed supernatants from insulin-treated tissue was applied to a Mono Q column as in Fig. 1. Tubes (13–18) containing acetyl-CoA carboxylase kinase activity were combined (fraction 11). (a) Radioautograph showing the time course of phosphorylation of acetyl-CoA carboxylase when 25 μ l of fraction 1 was incubated with acetyl-CoA carboxylase and [γ - 32 P]ATP under conditions as given in Fig. 1 for the time indicated. Tracks labelled E and K show phosphorylation of acetyl-CoA carboxylase occurring over 30 min in the absence of fraction 1 and with the fraction in the absence of added acetyl-CoA carboxylase respectively. The positions of M_r markers are indicated as follows: A, acetyl-CoA carboxylase (M_r 265 000); B, ATP citrate-lyase (M_r 121 000); C, BSA (M_r 67 000). (b) Two-dimensional analysis of [32 P]phosphopeptides released by digestion with trypsin of acetyl-CoA carboxylase immunoprecipitated after incubation of purified acetyl-CoA carboxylase with 25 μ l of fraction 1 for 15 min. Hatched area indicates migration of dimethylphenyl-lysine. (c) Two-dimensional analysis of [32 P]phosphopeptides released by digestion with trypsin of acetyl-CoA carboxylase immunoprecipitated from a crude homogenate previously prepared from tissue that had been preincubated in medium containing [32 P]P, then incubated with insulin as described by Brownsey & Denton (1982). The migration of the '1'-peptide (arrowed) is indicated. (d) Phosphoamino acid analysis of the '1'-peptide obtained from a two-dimensional analysis as in (b). Separation was by electrophoresis at pH 3.5 with 1 μ g each of phosphoserine (P-S), phosphothreonine (P-T) and phosphotyrosine (P-Y) as markers (O, origin). (e) Elution of the '1'-peptide separated by two-dimensional analysis in (b) (—) and (c) (---) from a Superose 12 column. A and B indicate the elution of positions of glucagon (M_r 3522) and bacitracin (M_r 1411). Results are typical of at least two separate experiments.

Fig. 3. Effects of Mono Q fractions on acetyl-CoA carboxylase activity

Purified acetyl-CoA carboxylase was incubated with 0–25 μ l of Mono Q fractions in a total volume of 50 μ l for 15 min at 30 °C as given in the legend to Fig. 1, except that unlabelled ATP was used. The final concentration of citrate was 0.6 mM in all cases. (a) Effects of different volumes of fraction 1 (tubes 13–18 combined) from control (▲) and insulin-treated (●) tissue. (b) Effects of 2 μ l samples (○, △) and 25 μ l samples (●, ▲) of individual tubes obtained from the chromatography of high-speed supernatants from control (○, ●) and insulin-treated tissue (△, ▲). Results are typical of three separate experiments.

may have been the result of some breakdown of the inhibitory component or of ATP, perhaps by some heat-labile enzyme in the fraction. However, no loss of any inhibitory effects was evident if fraction 1 was preincubated at 30 °C for 15–30 min before addition of the acetyl-CoA carboxylase. Moreover, if the incubations (15 min) of acetyl-CoA carboxylase, ATP and fraction 1 were treated at 100 °C for 10 min, the expected amounts of inhibitory activity then became evident when re-incubated with further additions of acetyl-CoA carboxylase and ATP.

Incubation of acetyl-CoA carboxylase with the high- M_r fraction (fraction 1B) resulted in little change in activity of the enzyme (Fig. 4a). This was associated with some phosphorylation of acetyl-CoA carboxylase, but less than that found with the corresponding volume of fraction 1 (Fig. 4b). In contrast, incubation of acetyl-CoA carboxylase with fraction 1A plus fraction 1B resulted in changes in activity and phosphorylation that were very similar to those observed with the original fraction 1 (Figs. 4a and b).

Taken together, these observations indicated that fraction 1B contained acetyl-CoA carboxylase kinase activity, which required the presence of the inhibitory component in fraction 1A. Under these conditions, phosphorylation resulted in the reversal of the

Fig. 4. Effects of fractions 1A and 1B on acetyl-CoA carboxylase activity and phosphorylation

Fractions 1A and 1B were obtained by Centricon filtration of fraction 1 from insulin-treated tissue. Changes in acetyl-CoA carboxylase activity and phosphorylation were studied after incubation for 15 min at 30 °C as in Figs. 1–3. In all cases, the final concentration of citrate was 0.6 mM. (a) Effects of increasing volumes of fractions 1 (●), 1A (■), 1B (▲) and 1A plus 1B (○) on activity. Results are the means of more than three independent observations (S.E.M. values were less than 5% of the means in all cases). (b) Effects of increasing volumes of fractions 1, 1A, 1B and 1A plus 1B on phosphorylation as indicated.

inhibitory effects. Fig. 6 illustrates the dose relationship between changes in phosphorylation and activity of acetyl-CoA carboxylase. Fig. 6(a) shows the effects of incubating acetyl-CoA carboxylase for 15 min with increasing volumes of fraction 1 from insulin-treated tissue. It is evident that there is a reasonably

Fig. 5. Effects of fraction 1A from control (●) and insulin-treated (■) tissue on the activity of acetyl-CoA carboxylase

Acetyl-CoA carboxylase was incubated with increasing volumes of fraction 1A in the presence of ATP as in Fig. 4(a).

Table 1. Effects of incubation with fractions 1 and 1A in the presence and absence of ATP on the initial and maximum activity of acetyl-CoA carboxylase

Incubations were carried out as in Fig. 3(b), except that ATP was absent where indicated. Acetyl-CoA carboxylase activity was then assayed either immediately (initial activity) or after treatment with potassium citrate (20 mM) for 20 min (maximum activity; value given in parentheses). Results are expressed as % of maximum activity, i.e. activity of untreated acetyl-CoA carboxylase after incubation with citrate (*) and are the mean of three separate experiments.

Incubation time (min)	Fraction	ATP	Vol. (μ l) of fraction ...	Acetyl-CoA carboxylase activity (% of max.)		
				0	2	25
0	—	—		87 (100*)	—	—
15	—	—		66 (100)	—	—
15	—	+		55 (97)	—	—
15	1	—		—	73	79
15	1	+		—	24 (99)	69 (99)
15	1A	—		—	95	80
15	1A	+		—	15 (69)	9 (27)

good correlation above 2 μ l of the fraction. Fig. 6(b) shows the time course of the effects of incubating the enzyme with 2 μ l of fraction 1A for 10 min and then adding 10 μ l of fraction 1B. Parallel increases in phosphorylation and activity are again evident.

It might be argued that the activation observed in these studies is due to the dephosphorylation of inhibitory sites on acetyl-CoA carboxylase brought about by some protein phosphatase activity in fraction 1 (or fraction 1B), which occurs with the same time course as the observed phosphorylation. However, this is most unlikely for the following reasons. (i) No acetyl-CoA carboxylase phosphatase activity was detectable in fraction 1 by using 32 P-labelled acetyl-CoA carboxylase (prepared by incubating the purified enzyme with the catalytic subunit of cyclic AMP-dependent protein kinase and then removing all [32 P]ATP by gel filtration). (ii) Addition of the general protein phosphatase inhibitor, 50 mM-2-glycerolphosphate, had no appreciable effects on the extent of reactivation in experiments carried out as in Figs. 3 and 4. (iii) The acetyl-CoA carboxylase used in the present studies was prepared from tissue which had been incubated in the absence of hormones, and thus contained only basal levels of phosphorylation; moreover, the enzyme was purified in the absence of phosphatase inhibitors, which we know from other studies (A. C. Borthwick, unpublished work) results in further dephosphorylation. Thus not only does fraction 1 appear to be free of phosphatase activity, but, even if it were to be present, it is unlikely to have any major effect on acetyl-CoA carboxylase activity. In agreement with this view, incubation of acetyl-CoA carboxylase with phosphatase 2A in the presence of 2 μ l of fraction 1B did not lead to any activation.

General conclusions

Our previous attempts to purify and characterize the acetyl-CoA carboxylase kinase responsible for the increased phosphorylation of the 'I'-peptide in rat epididymal adipose tissue or cells have been frustrated by a very rapid loss in kinase activity. The results of the present study offer an explanation for this problem. The kinase apparently requires the presence of a low- M_r component which is a potent inhibitor of acetyl-CoA carboxylase.

Fig. 6. Correlation between phosphorylation and increases in activity of acetyl-CoA carboxylase on incubation with fraction 1 or fraction 1B in the presence of fraction 1A

Acetyl-CoA carboxylase phosphorylation (■, ○) and activity (●, ○) were determined as in Figs. 1 and 3(b) respectively. (a) Effects of increasing volumes of fraction 1. (b) Time course of the effects of the addition at 10 min of the equivalent of 10 μ l of fraction 1B per 50 μ l total volume added (■, ●). The equivalent of 2 μ l of fraction 1A per 50 μ l total volume was present from zero time. Also shown are the effects of incubation in the absence of added fraction 1B (□, ○). Results are the means of three separate experiments (S.E.M. values are not shown, but were all less than 10% of the means). All fractions were prepared from insulin-treated tissue.

Removal of this component during conventional purification techniques involving dialysis or gel filtration probably accounted for much of the observed loss of kinase activity.

The application of Mono Q chromatography results in the copurification of the insulin-sensitive acetyl-CoA carboxylase kinase with the low- M_r component. For the first time this has allowed the demonstration of parallel increases in phosphorylation and activity of acetyl-CoA carboxylase under conditions where phosphorylation up to about 0.4 mol/mol of acetyl-CoA carboxylase subunit appears to be occurring mainly into serine residues of the 'I'-peptide.

The acetyl-CoA carboxylase kinase appears to be distinct from other insulin-stimulated protein kinases. The apparent requirement for acetyl-CoA carboxylase in an inhibited state should be viewed in the context of the intact fat-cell, where the basal activity of the enzyme (as measured in fresh extracts of tissue incubated in the absence of insulin) is low (typically 10–20% of maximal activity; Halestrap & Denton, 1974; Brownsey & Denton, 1982). Further studies are required to re-examine the extent to which the increase in kinase activity in tissue extracts

may be the result of translocation of the kinase, perhaps from the plasma membrane (Denton *et al.*, 1981; Brownsey *et al.*, 1984, 1988), and/or changes in its phosphorylation. In preliminary studies, little or no changes in kinase activity were apparent if tissue or cell extractions were performed in the presence of phosphatase inhibitors such as glycerol phosphate or fluoride.

The low- M_r component in fraction 1A remains to be identified. Its inhibitory effects on acetyl-CoA carboxylase exhibit a number of rather striking characteristics, including a requirement for MgATP yet the absence of any phosphorylation of the enzyme. Moreover, at higher concentrations of the inhibitor, its effects appear to be only partially reversed on the subsequent incubation of the enzyme with citrate (Table 1), but reversal was observed after phosphorylation by the kinase activity in fraction 1B (Fig. 6). Fatty acyl-CoA esters do not appear to be the inhibitory component. Their effects do not require the presence of MgATP; [14 C]palmitoyl-CoA when added to tissue extracts is not eluted from the Mono Q column in fraction 1; and palmitoyl-CoA does not stimulate the kinase activity in fraction 1B (A. C. Borthwick, N. J. Edgell & R. M. Denton, unpublished work).

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