Use of rapid gel-permeation chromatography to explore the inter-relationships between polymerization, phosphorylation and activity of acetyl-CoA carboxylase

Effects of insulin and phosphorylation by cyclic AMP-dependent protein kinase

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1. Superose 6 chromatography was used to separate rapidly the polymeric and dimeric forms of acetyl-CoA carboxylase. 2. With preparations of acetyl-CoA carboxylase purified by Sepharose-avidin chromatography, it is shown that citrate promotes polymerization and that the extent of polymerization is diminished, but not eliminated, after phosphorylation by cyclic-AMP-dependent protein kinase. 3. After exposure of rat epididymal adipose tissue to insulin, evidence was obtained for a marked increase in polymerization. The polymeric form, which was active in the absence of citrate, exhibited increased phosphorylation, particularly on a tryptic peptide designated the I-peptide in an earlier study [Brownsey & Denton (1982) *Biochem. J.* **202**, 77–86]. In contrast, in tissue exposed to the β -agonist isoprenaline, most of the phosphorylated acetyl-CoA carboxylase appeared to be in the dimeric form if chromatography was carried out in the absence of citrate, whereas in the presence of citrate the degree of polymerization was diminished.

INTRODUCTION

Acetyl-CoA carboxylase (EC 6.4.1.2) catalyses the first committed step in fatty acid synthesis and is generally considered to have an important role in the regulation of this pathway. The activity of purified acetyl-CoA carboxylase from mammalian sources can be altered both by allosteric regulators and by reversible phosphorylation (for reviews see Hardie, 1980; Brownsey & Denton, 1985, 1986; Witters, 1985). The best studied examples of the former are citrate, which activates, and fatty acyl-CoA esters, which inhibit the activity of the enzyme. Such changes in activity are apparently associated with changes in the ratio of the inactive dimeric form of the enzyme and the active polymeric form, which can be composed of up to 20 monomers (Hardie, 1980; Numa & Tanabe, 1984; Brownsey & Denton, 1985). The enzyme can be phosphorylated by a number of different, well-characterized, protein kinases. Of these, phosphorylation by both cyclic-AMP-dependent protein kinase and protein kinase C has been shown to be associated with a decrease in activity (Hardie & Guy, 1980; Hardie *et al.*, 1986). A number of less well characterized cyclic-AMP-independent protein kinases which inactivate acetyl-CoA carboxylase have also been reported (Shiao *et al.*, 1981; Lent & Kim, 1982; Munday & Hardie, 1984). For the cyclic-AMP-dependent protein kinase, there is evidence obtained with a partially purified preparation of rat liver acetyl-CoA carboxylase and sucrose-density-gradient centrifugation that phosphorylation may be associated with a decreased ability to polymerize in the presence of citrate (Lent *et al.*, 1978).

Exposure of adipose tissue or liver cells to hormones which increase cyclic AMP leads to inhibition of enzyme activity. This can be largely accounted for by an increase in phosphorylation via cyclic-AMP-dependent protein kinase (Brownsey *et al.*, 1979; Brownsey & Hardie, 1980;

Holland *et al.*, 1984, 1985; Witters *et al.*, 1983). However, there is some disagreement as to whether these changes are associated with a change in the degree of polymerization. After exposure of rat hepatocytes to glucagon, or of fat-cells to adrenaline, and separation of the dimeric and polymeric forms of acetyl-CoA carboxylase by sucrose-density-gradient centrifugation, Swenson & Porter (1985) and Lee & Kim (1978) obtained evidence that polymerization may be diminished. In contrast, Buechler *et al.* (1984), using similar techniques with hepatocytes, found no such evidence for changes in polymerization with glucagon. Using digitonin to permeabilize the plasma membrane and avidin specifically to inactivate the dimeric form of acetyl-CoA carboxylase, Meredith & Lane (1978) proposed that a decrease in the polymer/dimer ratio may explain the inactivation of acetyl-CoA carboxylase by glucagon in chicken liver cells.

Exposure of rat epididymal adipose tissue to insulin leads to activation of acetyl-CoA carboxylase (Halestrap & Denton, 1973, 1974). Subsequently evidence for activation has also been obtained from studies on liver (Witters *et al.*, 1979; Buechler *et al.*, 1984), brown adipose tissue (McCormack & Denton, 1977) and mammary tissue (Munday & Williamson, 1982). In rat epididymal fat-cells and liver cells, activation is associated with an increase in the overall extent of phosphorylation (Brownsey *et al.*, 1977; Brownsey, 1981; Brownsey & Denton, 1982; Witters, 1981), which occurs on a tryptic peptide (I-peptide) which is not well phosphorylated by cyclic-AMP-dependent protein kinase (Brownsey & Denton, 1982; Witters *et al.*, 1983; Holland & Hardie, 1985). Although it seems likely that this phosphorylation is associated with the insulin activation of the enzyme, definitive proof is still required (Witters, 1985; Brownsey & Denton, 1986). After exposure of rat epididymal adipose tissue to insulin, there is a decrease in the proportion of the enzyme which remains in the

supernatant of extracts centrifuged at 260 000 *g* for 1 h, suggesting that activation may be associated with increases in the proportion of the enzyme in a polymeric form (Halestrap & Denton, 1974). However, Witters (1985) mentions unpublished studies, using both sucrose-gradient centrifugation and digitonin permeabilization, in which no evidence for increased polymerization of acetyl-CoA carboxylase was evident in rat liver cells exposed to insulin.

In this paper we report a new approach by which the relationships between activity, polymerization and phosphorylation of acetyl-CoA carboxylase can be studied. The technique employs Fast Protein Liquid Chromatography on Superose 6 gel-filtration columns, which allows the separation of the dimeric and polymeric forms of acetyl-CoA carboxylase in about 30 min. We have used this technique to investigate some aspects of (i) the effects of citrate and phosphorylation by cyclic-AMP-dependent protein kinase on purified preparations of rat mammary and adipose-tissue acetyl-CoA carboxylase, and (ii) the effects of insulin and isoprenaline on acetyl-CoA carboxylase in rat epididymal adipose tissue.

EXPERIMENTAL

Materials

Except as given below, sources of chemicals and biochemicals were as described previously (Brownsey *et al.*, 1977, 1979, 1984). Male Wistar rats (180–220 g) and lactating female Wistar rats (250–300 g) were fed *ad libitum* up to the time of killing. The proteinase inhibitors pepstatin A, antipain and leupeptin were obtained from Cambridge Research Biochemicals, Harston, Cambridge, U.K. Benzamidine, biotin, *Staphylococcus aureus* cell powder and avidin (approx. 14 units/mg of protein, where 1 unit binds 1 μ g of biotin) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Tosylphenylalanyl-chloromethane ('TPCK')-treated trypsin was from Worthington Diagnostic Systems, Freehold, NJ, U.S.A. Cyclic-AMP-dependent protein kinase (catalytic subunit; 10 units/ml) was kindly given by Dr. K. Murray (present address: Smith, Kline and French Research, Welwyn, Herts., U.K.). [γ - 32 P]ATP and [32 P]P_i were from The Radiochemical Centre, Amersham, Bucks., U.K.

Tissue incubation and preparation of concentrated tissue extracts

Epididymal fat-pads were incubated with shaking for 30 min at 37 °C in bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing glucose (11 mM) and then for an additional 15 min in fresh medium of the same composition with hormone additions as required. Pads were blotted and extracted at 0 °C in medium (pH 7.4; 4 ml/g) containing sucrose (0.25 M), Tris/HCl (20 mM), EGTA (2 mM), GSH (7.5 mM), NaN₃ (0.02%, w/v) and proteinase inhibitors pepstatin A, antipain and leupeptin (each at 1 μ g/ml) by using a Polytron (PT 20) homogenizer at setting 3 for 5–10 s. Defatted bovine serum albumin (30 mg/ml) was added to this extraction medium when indicated in the text. The extracts were centrifuged at 3000 *g* for 90 s (MSE Centaur) and the infranats then for 10 min at 25 000 *g* (MSE 21 centrifuge). The supernatants were made 40% saturated

with (NH₄)₂SO₄ at 0 °C, and the resulting precipitates collected by centrifugation at 30 000 *g* for 10 min and frozen at –20 °C.

In some experiments, pads were incubated with medium containing [32 P]P_i in order to examine hormonal effects on the phosphorylation of acetyl-CoA carboxylase and other proteins. In such experiments, the pads were incubated in bicarbonate-buffered medium containing glucose (11 mM) for 30 min as described above. At the end of this period the pads were transferred to fresh medium also containing glucose (11 mM) but with [32 P]P_i (100 c.p.m./pmol; 0.2 mM). The pads were incubated in this medium for 120 min and then for a further 15 min period with either no hormone, insulin (0.1 μ M) or the β -adrenergic agonist isoprenaline (10 μ M).

Purification of rat adipose or mammary tissue acetyl-CoA carboxylase

The procedures followed were essentially those described by Brownsey *et al.* (1984), involving the preparation of a 35%-satd.-(NH₄)₂SO₄ protein precipitate, which after dialysis was applied to a Sepharose-4B-CL-avidin affinity column. After biotin elution of acetyl-CoA carboxylase, the protein was concentrated by vacuum dialysis and then further concentrated by dialysis into Mops (20 mM), pH 7.4, containing 2 mM-benzamidine, 1 mM-citrate and glycerol (40%, w/v). The enzyme preparations were stored at –15 °C at about 3 units/ml for up to 4 weeks without appreciable loss of activity.

The specific activity of the final product from both mammary and epididymal adipose tissue was approx. 1 unit/mg of protein, with an overall 20–50% recovery of activity compared with the initial extract. Analysis by SDS/polyacrylamide-gel electrophoresis of the purified enzymes showed that about 80% of the protein migrated with a subunit *M_r* of 230 000. In this purification procedure a minor contaminant (10–20%) is pyruvate carboxylase (subunit *M_r* approx. 140 000). The preparations were free of fatty acid synthase. The phosphate content of the preparations was not determined, but in an effort to keep this to minimum the adipose tissue was incubated in the absence of hormones for 30 min in the bicarbonate-buffered medium with glucose, whereas the mammary tissue was removed from animals which had been under deep anaesthesia for at least 15 min after injection of Sagatal (1 ml/kg body wt.).

Preparation of 32 P-labelled acetyl-CoA carboxylase

Samples of purified acetyl-CoA carboxylase were phosphorylated *in vitro* by using cyclic-AMP-dependent protein kinase and [γ - 32 P]ATP. The phosphorylation was carried out in Mops (25 mM) buffer, pH 7.4, containing MgCl₂ (5 mM), NaF (20 mM), cyclic-AMP-dependent protein kinase (catalytic subunit) (10 munits/ml) and [γ - 32 P]ATP (50 μ M; 1000–2000 d.p.m./pmol). The reaction was initiated by addition of ATP and incubated at 30 °C for 60 min. Unchanged ATP was then removed by gel filtration on a Sephadex G-25 column equilibrated in column buffer for Fast Protein Liquid Chromatography (see below) containing 20 mM-NaF.

The techniques of SDS/polyacrylamide-gel electrophoresis, radioautography and densitometric scanning of radioautographs were as described by Brownsey *et al.* (1984).

Gel filtration of acetyl-CoA carboxylase on a Superose 6 column attached to the Pharmacia Fast Protein Liquid Chromatography system

The Pharmacia system fitted with a Superose 6 HR (300 mm × 10 mm) gel-filtration column was kept at a constant 4 °C. Column equilibration and washing procedures were carried out as described by Pharmacia; all the solutions applied to the column were filtered with a 0.2 µm filter. The basic column buffer throughout this study was Mops (20 mM), pH 7.2, containing EDTA (2 mM), MgCl₂ (10 mM), dithiothreitol (1 mM), 2% (w/v) glycerol, 0.02% NaN₃, plus proteinase inhibitors (pepstatin A, antipain and leupeptin, each at 1 µg/ml).

The (NH₄)₂SO₄ precipitates prepared from tissue extracts as described above were resuspended in the basic column buffer without MgCl₂ (about 0.4 ml/g of original tissue), dialysed at 4 °C for 30 min against 20 vol. of the same buffer and centrifuged for 5 min at about 100 000 *g* in a Beckman Airfuge. Small pellets were evident, but no appreciable amounts of acetyl-CoA carboxylase activity were associated with them. Supernatants were stored for up to 30 min before application of samples to the Superose 6 column. Columns were developed at a flow rate of 0.4 ml/min with either basic column buffer or basic column buffer plus potassium citrate (20 mM), pH 7.2. In the latter case potassium citrate (20 mM) was also added to the supernatants, and these were then incubated in the absence of MgCl₂ for 30 min at about 30 °C to ensure maximum effect of citrate. Elution of protein from the columns was continuously monitored at 280 nm, and up to 20 fractions (each 1 ml) were collected. It should be noted that the addition of citrate to the basic column buffer caused a decrease in the free Mg²⁺ in the buffer from about 6 mM to 0.1 mM.

Enzyme assays

Acetyl-CoA carboxylase was assayed essentially as described by Brownsey *et al.* (1979). The assay was initiated by the addition of a sample (usually 50 or 100 µl) to 0.45 ml of 100 mM-Tris/HCl, pH 7.4, containing EDTA (0.5 mM), MgSO₄ (10 mM), ATP (2.5 mM), acetyl-CoA (150 µM), dithiothreitol (1 mM), albumin (10 mg/ml) and KH¹⁴CO₃ (15 mM; 0.5 µCi/µmol) at 30 °C. After 2 min, assays were terminated by precipitation of the protein with HCl (5 M). Unless otherwise stated, activity was determined after preincubation of the sample to be assayed with citrate (20 mM) at 30 °C for 20 min, giving a final concentration of citrate in the assay of 2–4 mM.

Fatty acid synthase was assayed as described by Halestrap & Denton (1973) and ATP citrate lyase as described by Martin & Denton (1970).

Specific immunoprecipitation and two-dimensional analysis of tryptic peptides of acetyl-CoA carboxylase

This was carried out by a modification of the method described by Brownsey & Denton (1982). Column fractions from Fast Protein Liquid Chromatography were incubated for 15 min at 25 °C in the presence of antiserum to acetyl-CoA carboxylase (3 µl of antiserum/ml) and *S. aureus* cells (1.5 mg/ml). After centrifugation at 12 000 *g* for 15 s, the immunoprecipitates were washed successively with 0.5 ml of 50 mM-potassium phosphate buffer, pH 7.2, 0.5 ml of chloroform/

Fig. 1. Separation of polymeric and dimeric forms of rat mammary-tissue acetyl-CoA carboxylase by rapid gel filtration on a Superose 6 column

Sephacrose-avidin-purified rat mammary-tissue acetyl-CoA carboxylase (100 µg) was applied in 200 µl of column buffer to a Superose 6 gel-filtration column which had been equilibrated in either (a) column buffer or (b) column buffer containing citrate (20 mM), where the sample in (b) had already been preincubated with citrate in the absence of added MgCl₂ for 30 min at 30 °C before application. —, *A*₂₈₀; ----, percentage of total recovered acetyl-CoA carboxylase activity. Overall recovery was about 40% in the absence of citrate and 80% in its presence. Elution of marker proteins (native *M_r* values given in parentheses) is also shown as follows: (1) pyruvate dehydrogenase complex (about 10 × 10⁶); (2) ferritin (450 000); (3) bovine serum albumin (67 000); (4) haemoglobin (64 000).

methanol (2:1 v/v) and 0.5 ml of 50 mM-NH₄HCO₃ (twice), and resuspended in 0.2 ml of 50 mM-NH₄HCO₃ containing trypsin (0.1 mg/ml). Digestion was carried out at 30 °C for 4 h, after which the *S. aureus* cells were removed by centrifugation, leaving 80–90% of the ³²P in the supernatant, the trypsin concentration was increased to 0.2 mg/ml and digestion was continued for a further 15 h. The samples were then boiled for 5 min and freeze-dried successively from water to remove all traces of NH₄HCO₃. The peptides were separated on thin-layer cellulose plates by high-voltage electrophoresis in the first dimension and then by ascending chromatography in the second dimension, as described by Brownsey & Denton (1982).

Expression of results

One unit of enzyme activity is the amount catalysing the utilization of 1 µmol of substrate/min at 30 °C.

Fig. 2. Effects of citrate on the polymerization of rat epididymal-adipose-tissue acetyl-CoA carboxylase

Sephacrose-avidin-purified rat epididymal-adipose-tissue acetyl-CoA carboxylase (100 μ g) was applied to a Superose 6 gel-filtration column in the presence (----) or absence (—) of citrate as described in Fig. 1; (1) and (2) show the elution of pyruvate dehydrogenase complex and ferritin respectively.

Results are given as means $\pm$ S.E.M. and statistical differences were assessed by Student's *t* test. All Figures are representative of experiments carried out on at least three different enzyme or tissue preparations.

RESULTS AND DISCUSSION

Gel filtration of purified preparations of acetyl-CoA carboxylase on Superose 6

Acetyl-CoA carboxylase that had been prepared from mammary tissue by Sepharose-avidin affinity chromatography was diluted with basic column buffer and either directly applied to the Superose 6 column or preincubated with citrate (20 mM) for 20 min before application.

As shown in Fig. 1(a), in the absence of citrate, most of the 280 nm-absorbing material and acetyl-CoA carboxylase activity was recovered in fractions 7–10. Calibration of the column with fatty acid synthase (native M_r 500 000), ATP citrate lyase (native M_r 520 000) and ferritin (native M_r 450 000) established that this peak corresponds to the dimeric form of acetyl-CoA carboxylase (expected M_r about 460 000). After chromatography in the presence of citrate, a marked increase in A_{280} was apparent in fractions 2–4, corresponding to a native M_r greater than 4×10^6 (Fig. 1b). Acetyl-CoA carboxylase activity was also predominantly recovered in these fractions, clearly demonstrating the citrate-induced polymerization of the enzyme. In fractions 2–4, acetyl-CoA carboxylase was fully active with or without further treatment with citrate. However, outside these fractions activity was negligible unless the fractions were first subjected to citrate treatment as described in the Experimental section. Pyruvate carboxylase (EC 6.4.1.1) was a minor contaminant (about 20%) of the preparations of purified acetyl-CoA carboxylase. Since this enzyme has a native M_r of about 560 000, the presence

of pyruvate carboxylase accounts for a proportion of material absorbing at 280 nm in fractions 7–10 still evident in enzyme treated with citrate. The remainder includes acetyl-CoA carboxylase, which is essentially inactive under all conditions (see below). It should be noted that, after treatment with citrate, the increase in A_{280} corresponding to the high- M_r form of acetyl-CoA carboxylase is greater than the loss of A_{280} in fractions 7–10, which include the dimeric form. The most likely reason is that the polymeric form causes an increase in light-scattering (Beatty & Lane, 1985).

Studies with preparations of acetyl-CoA carboxylase purified from rat epididymal adipose tissue gave broadly similar results (Fig. 2). However, although the preparations contained similar amounts of pyruvate carboxylase to the preparations from rat mammary tissue, there was markedly less material absorbing at 280 nm that was eluted in fractions 7–10 in the samples run in the presence of citrate.

Purified mammary tissue acetyl-CoA carboxylase which had been phosphorylated by the catalytic subunit of cyclic-AMP-dependent protein kinase to the extent of about 0.6 mol/mol of acetyl-CoA carboxylase subunit was applied to the Superose 6 column run in the presence of citrate (Fig. 3). Comparison of the A_{280} profiles of phosphorylated and untreated enzyme indicated that phosphorylation may markedly diminish the ability of citrate to promote full polymerization (Fig. 3a). This conclusion was confirmed by examining the proteins present in each fraction by SDS/polyacrylamide-gel electrophoresis (Fig. 3b). Although the phosphorylated enzyme exhibited some polymerization in the presence of citrate, the extent of polymerization was markedly less than that of the unphosphorylated sample. It should be noted that about 25% of the protein in the unphosphorylated samples appears to behave as a dimeric form and to be eluted in fractions 8–10 in the presence of citrate. This may represent purified enzyme which is unable to respond to citrate activation, perhaps through being already phosphorylated or inactivated by some other means, such as limited proteolysis.

The elution of 32 P-labelled acetyl-CoA carboxylase formed by the phosphorylation of the mammary tissue enzyme by cyclic-AMP-dependent protein kinase and [γ - 32 P]ATP was also studied (Fig. 3c). In the absence of citrate, the labelled enzyme was eluted predominantly in fractions 8–10, whereas in the presence of citrate there was a shift towards higher- M_r forms, which were eluted in a broad peak centred around fraction 6. This is in general agreement with the results of Figs. 3(a) and 3(b). Altogether, it is evident that phosphorylation of acetyl-CoA carboxylase by cyclic-AMP-dependent protein kinase caused a marked decrease in the ability of the enzyme to polymerize in the presence of citrate. However, at the levels of phosphorylation attained in the present study, some polymerization to forms of apparent M_r in the range 1×10^6 – 3×10^6 occurs.

Effect of insulin on the activity and polymerization of rat epididymal-adipose-tissue acetyl-CoA carboxylase

Concentrated tissue extracts were prepared by $(\text{NH}_4)_2\text{SO}_4$ precipitation, dialysed and separated on the Superose 6 column in the presence and absence of citrate (Fig. 4). These extracts contained essentially all the acetyl-CoA carboxylase, fatty acid synthase and pyruvate carboxylase activity in the original tissue extract and

Fig. 3. Rapid gel filtration of mammary-tissue ^{32}P -labelled acetyl-CoA carboxylase after phosphorylation by cyclic-AMP-dependent protein kinase [γ - ^{32}P]ATP

Details of phosphorylation are given in the Experimental section. (a) Continuous A_{280} profile of acetyl-CoA carboxylase before (—) and after (-----) phosphoryl-

more than 60% of the ATP citrate lyase activity (results not shown).

In the absence of citrate there was a marked increase in both 280 nm-absorbing material and acetyl-CoA carboxylase activity in fractions 2–4 (peak 1), corresponding to a native M_r of greater than 4×10^6 , in samples from insulin-treated tissue (Figs. 4a and 4c). There were no appreciable differences in the remainder of the A_{280} profiles. Enzyme eluted in fractions 2–4 exhibited the same activity before and after incubation of samples of the fractions with citrate.

The peak corresponding to fraction 10 (peak 2) includes not only the dimeric form of acetyl-CoA carboxylase, which was inactive until exposed to citrate, but also fatty acid synthase, ATP citrate lyase and pyruvate carboxylase (see Fig. 5). The third peak (fractions 11–12) is mainly albumin, which was a component of the original tissue extraction buffer in these experiments.

In contrast, if chromatography was carried out in the presence of citrate, there were no consistent differences in the elution of acetyl-CoA carboxylase activity or A_{280} profiles of samples from control and insulin-treated tissue. In both cases, there was a large peak in the A_{280} profile corresponding to fractions 2–4, which also contained most of the acetyl-CoA carboxylase activity (Figs. 4b and 4d). The overall recovery of acetyl-CoA carboxylase activity through the chromatography of concentrated tissue extracts in either the presence or the absence of citrate was in the range 50–75%.

Results of 11 experiments carried out as that shown in Fig. 4 are summarized in Table 1. To allow for differences in column loading between experiments, the height of peak 1 is expressed either as a fraction of that of peak 2 (which was unaltered by insulin) or as a fraction of the height of peak 1 in the same sample when the column was run with citrate present. Overall, a 3–4-fold increase in peak 1 of the A_{280} profile in the absence of citrate was evident after insulin treatment. In the presence of citrate, peak 1 was increased, especially in control samples, and the effect of insulin was no longer apparent.

The increase in A_{280} absorption in peak 1 of samples from insulin-treated tissue was associated with increases not only in the activity of acetyl-CoA carboxylase in these fractions (Figs. 4a and 4c) but also in the amount of acetyl-CoA carboxylase protein (Figs. 5a and 5b). The major protein component in fractions 2 and 3, with an

ation. In each case samples of acetyl-CoA carboxylase (100 μg) were pretreated with citrate (20 mM) in the absence of added Mg^{2+} and run in standard column buffer containing citrate (20 mM). (b) Acetyl-CoA carboxylase protein in fractions separated as in (a) before ($\bullet$) and after (Δ) phosphorylation. The fractions were treated with 10% (w/v) trichloroacetic acid, and proteins were separated by SDS/polyacrylamide-gel electrophoresis (6% gels). The amount of acetyl-CoA carboxylase protein was determined from densitometric traces of the Coomassie-Blue-staining band of subunit M_r 230000, and is expressed in arbitrary units. Immunoprecipitation with antiserum raised against acetyl-CoA carboxylase confirmed that over 90% of this protein was acetyl-CoA carboxylase. (c) Distribution of ^{32}P -labelled acetyl-CoA carboxylase in fractions carried out in standard column buffer either containing no citrate ($\circ$) or with citrate (20 mM) ($\blacktriangle$).

Fig. 4. Rapid gel filtration on Superose 6 of concentrated extracts prepared from control and insulin-treated epididymal adipose tissue

Paired groups of ten pads were incubated for 15 min with (c, d) or without (a, b) insulin (10 munits/ml), and concentrated tissue extracts were prepared as described in the Experimental section. Samples (200 μ l) (equivalent to about 0.5 g of original tissue) were applied to a Superose 6 column equilibrated in either basic column buffer (a, c) or in buffer containing citrate (20 mM) (b, d). In (b) and (d) the samples were also preincubated with citrate (20 mM) in the absence of added $MgCl_2$ for 20 min at 30 °C before chromatography. In each case the eluate was monitored at 280 nm (—) and acetyl-CoA carboxylase activity was assayed in each fraction (●).

Table 1. Effect of exposure of rat epididymal adipose tissue to insulin in the polymerization of acetyl-CoA carboxylase

The procedure was as described in the Experimental section and Fig. 4 legend. Results are given as means $\pm$ S.E.M. for 11 observations on separate groups of fat-pads, and were calculated from A_{280} profiles. Peak 1 corresponds to the polymeric form of acetyl-CoA carboxylase and peak 2 to fatty acid synthase, pyruvate carboxylase, ATP citrate lyase, the dimeric form of acetyl-CoA carboxylase and other proteins.

Parameter	Value for control tissue	Effect of insulin (% of control)
Peak 1 height (as fraction of that of peak 2) in:		
Samples run in absence of citrate	0.47 ± 0.15	368 ± 88
Samples run in presence of citrate	1.57 ± 0.33	98 ± 7
Peak 1 height in samples run in absence of citrate (as a fraction of that in samples run in the presence of citrate)	0.30 ± 0.05	350 ± 73

apparent subunit M_r of 230000, was shown by specific immunoprecipitation with antiserum to be acetyl-CoA carboxylase (results not shown). Fig. 5 also indicates the migration of fatty acid synthase, ATP citrate lyase and pyruvate carboxylase in the SDS/polyacrylamide gels. Assays for the activity of these enzymes in the column fractions confirmed that the enzymes were very largely eluted in fractions 8–10, corresponding to a native M_r of about 500000. However, with ATP citrate lyase significant amounts of activity were present in other fractions, including fractions 2–4. The presence of a high- M_r form of ATP citrate lyase in tissue extracts has been previously reported (Corrigan & Rider, 1981). There were no consistent differences in the elution of ATP citrate lyase, fatty acid synthase or pyruvate carboxylase from Superose 6 columns in samples from control and insulin-treated tissue under the conditions of the present study. Further studies were carried out as in Fig. 5, but in the presence of citrate. Under these conditions, the protein composition of the fractions remained essentially unaltered, except that the amount of acetyl-CoA carboxylase protein in fractions 2–3 was increased and the effect of insulin treatment was no longer apparent (results not shown). The presence of citrate tended to diminish the amount of ATP citrate lyase in fractions 2 and 3.

Fig. 5. Effect of insulin and citrate on the polymerization of acetyl-CoA carboxylase

Samples of extract prepared from (a) control and (b) insulin-treated tissue were separated on a Superose 6 column in the absence of citrate essentially as described in Fig. 3. The protein present in each fraction was recovered by precipitation with 10% trichloroacetic acid and electrophoresed on a SDS/6%-polyacrylamide gel. Migration of standard proteins is indicated as follows: 1, fatty acid synthase and acetyl-CoA carboxylase; 2, pyruvate carboxylase; 3, ATP citrate lyase.

Effects of insulin and isoprenaline on the phosphorylation of the polymeric and dimeric forms of acetyl-CoA carboxylase in rat epididymal adipose tissue

It seemed important to compare the extent of phosphorylation of the various molecular forms of acetyl-CoA carboxylase separated by Superose 6 chromatography from control and insulin-treated adipose tissue. Tissue was therefore preincubated with [^{32}P] P_i for 120 min to ensure steady-state incorporation of ^{32}P into all intracellular proteins, incubations were then continued for a further 15 min in the presence or absence of insulin, and extracts prepared and applied to the Superose 6

column in the absence of citrate. Fig. 6 shows radioautographs locating the ^{32}P -labelled proteins in the fractions separated by SDS/polyacrylamide-gel electrophoresis. The labelled proteins of subunit M_r 230 000 and 123 000 were shown by specific immunoprecipitation to be acetyl-CoA carboxylase (1) and ATP citrate lyase (2) respectively. With acetyl-CoA carboxylase, there was a marked increase in the amount of labelled protein in the high- M_r form eluted in fractions 2–4, together with a decrease in the amount in fractions 8–10, which correspond to the dimeric form (Fig. 6c). Overall there was a modest increase in total ^{32}P associated with acetyl-CoA carboxylase, as found in earlier studies (Brownsey *et al.*, 1977; Brownsey, 1981; Brownsey & Denton, 1982). In contrast, as found in many previous studies (see Denton *et al.*, 1984), there was a much more marked increase in the phosphorylation of ATP citrate lyase. However, the pattern of elution of ^{32}P -labelled ATP citrate lyase from the Superose 6 column was essentially unaltered by prior exposure of the tissue to insulin (Fig. 6). If the column was run in the presence of citrate, most of the ^{32}P -labelled acetyl-CoA carboxylase was eluted in the polymeric form (i.e. in fractions 2–4) for samples from both control and insulin-treated tissue.

In three experiments carried out as in Fig. 6, ^{32}P -labelled acetyl-CoA carboxylase in fractions 2–4 was recovered by specific immunoprecipitation, digested with trypsin, and the resulting ^{32}P -labelled peptides were separated by two-dimensional analysis on thin-layer cellulose plates. Fig. 7 shows radioautographs from a typical experiment illustrating the pattern of labelled peptides obtained with samples from control and insulin-treated tissue. The major difference is the relative amount of a ^{32}P -labelled peptide which has a similar mobility to dinitrophenyl-lysine in the electrophoretic dimension and about half the mobility in the chromatographic dimension. This corresponds to that designated the I-peptide by Brownsey & Denton (1982).

Experiments were also carried out to explore the effect of the β -adrenergic agonist isoprenaline (Fig. 8). In this case, as in earlier studies with adrenaline (Brownsey *et al.*, 1979; Holland *et al.*, 1985), there was an overall increase in the phosphorylation of acetyl-CoA carboxylase. However, in contrast with the effect of insulin, the ^{32}P -labelled acetyl-CoA carboxylase eluted in the absence of citrate was largely in the dimeric form (Fig. 8a). In the presence of citrate, the ratio of ^{32}P -labelled acetyl-CoA carboxylase in fractions 2–4 to that in fractions 5–9 was 1.68 in control tissue, whereas for tissue exposed to isoprenaline this ratio was only 0.53.

General conclusions

The use of rapid gel filtration on Superose 6 columns would appear to be a useful technique for the separation of the polymeric and dimeric forms of acetyl-CoA carboxylase both in purified preparations and in tissue extracts. In terms of speed, flexibility, convenience and completeness of separation, it appears to represent a distinct improvement on techniques used previously, including differential centrifugation and digitonin permeabilization of cells. One area of possible weakness (in common with other techniques) is the possibility of changes in the degree of polymerization occurring during the procedure. Nevertheless, it seems reasonable to assume that the substantial increase in the apparent degree of polymerization found after exposure of

Fig. 6. Effects of insulin on the ^{32}P -labelled proteins separated by SDS/polyacrylamide-gel electrophoresis in fractions eluted from the Superose 6 gel-filtration column

Extracts were prepared from control (a) and insulin-treated (b) epididymal fat-pads incubated in medium containing ^{32}P . Samples ($200\ \mu\text{l}$) equivalent to 0.5 g of tissue were separated by gel filtration on a Superose 6 column equilibrated in buffer containing no citrate, and the ^{32}P -labelled proteins were recovered by trichloroacetic acid precipitation of each fraction. The pellets were resuspended and analysed by SDS/polyacrylamide-gel electrophoresis, then radioautographed for 3 days. Markers: 1, acetyl-CoA

Fig. 7. Two-dimensional analysis of ^{32}P phosphopeptides released by trypsin digestion of the polymeric ^{32}P -labelled acetyl-CoA carboxylase from (a) control and (b) insulin-treated tissue

Tissue extracts were prepared and Superose 6 chromatography was carried out as in Fig. 6. ^{32}P -labelled acetyl-CoA carboxylase present in fractions 2–4 was then specifically immunoprecipitated, digested with trypsin and peptides were analysed as in the Experimental section. Origin (X), migration of dinitrophenyl-lysine (hatched area) and I-peptide (arrowed) are indicated. Electrophoresis was carried out in direction 1 and chromatography in direction 2.

adipose tissue to insulin is a reflection of an increase in polymerization within the intact tissue. It is also possible that some dephosphorylation may occur if protein phosphatase activity co-purified with acetyl-CoA carboxylase.

Throughout the study we have monitored the column eluate at 280 nm. This appears to provide a most convenient guide to the elution of the polymeric forms of acetyl-CoA carboxylase, even in tissue extracts. The signal is probably largely the result of light-scattering by the high- M_r forms. It should be noted that the height of peak 1 in tissue extracts chromatographed on Superose 6 in the presence of citrate is considerably greater than expected from studies on purified adipose-tissue enzyme.

carboxylase (subunit M_r 230000); 2, ATP citrate lyase (subunit M_r 123000). (c) The amount of ^{32}P -labelled acetyl-CoA carboxylase was determined from densitometric scans of the M_r -230000-subunit band (1) and peak area was expressed in arbitrary units: ●, control tissue; △, insulin-treated tissue.

Fig. 8. Behaviour of ^{32}P -labelled acetyl-CoA carboxylase from control and isoprenaline-treated tissue during chromatography on Superose 6 in (a) the absence and (b) the presence of citrate

Extracts were prepared from control (●) or isoprenaline-treated (□) tissue. Superose 6 chromatography, electrophoresis, radioautography and densitometric scanning were carried out as for Fig. 6(c). Panels (a) and (b) represent two separate experiments.

The total activity of the purified enzyme applied to the column in Fig. 2 is similar to the total activity in the tissue extracts applied in Fig. 4. Thus in the presence of citrate the A_{280} of the polymeric form in the tissue extract was some 10 times that seen with the purified enzyme. Further studies are required to establish the reason for the difference, but a likely explanation is that the enzyme in tissue extracts is able to form larger polymers. The increased A_{280} of tissue extracts is not likely to be due to other components in the fractions, since in extracts from control tissue there is little A_{280} in these fractions in the absence of citrate (Fig. 4a).

The present studies have confirmed previous reports (Lee & Kim, 1978; Swenson & Porter, 1985) that phosphorylation of acetyl-CoA carboxylase by cyclic-

AMP-dependent protein kinase results in a decrease in polymerization of the enzyme, especially in the presence of citrate. It is noteworthy that some polymerization was still evident, but the predominant forms had apparent M_r values of 1×10^6 – 3×10^6 (i.e. 4–12 subunits), whereas in the absence of phosphorylation the predominant forms had M_r values above 3×10^6 (i.e. above 12 subunits). Treatment of intact tissue with isoprenaline appeared to result in the formation of acetyl-CoA carboxylase with a similar but perhaps less clear-cut loss in the ability to polymerize fully.

A main objective of these studies was to employ Superose 6 chromatography to explore the effects of insulin on the polymerization of acetyl-CoA carboxylase in rat epididymal adipose tissue. Evidence for a striking increase in the degree of polymerization in the absence of citrate was obtained not only from the A_{280} profiles but also from measurements of acetyl-CoA carboxylase activity and protein in fractions eluted from the Superose 6 column. The polymeric form was active in the absence of citrate. Thus the original suggestion by Halestrap & Denton (1974) that the increase in acetyl-CoA carboxylase in adipose tissue exposed to insulin is associated with an increase in polymerization has been confirmed. It has been suggested that the polymeric form of acetyl-CoA carboxylase might act as a 'scaffold' to which proteins involved in fatty acid synthesis might bind (Gregolin *et al.*, 1966; Hansen *et al.*, 1971). However, we have seen no evidence for any specific association between the proteins in the present study. A large form of ATP citrate lyase was detected, as previously reported by Corrigan & Rider (1981), but the amount of this form did not increase with increases in the amount of the polymeric form of acetyl-CoA carboxylase after either exposure of adipose tissue to insulin or treatment of extracts with citrate.

It should be emphasized that, in the present studies, the effects of insulin on activity and polymerization survived at least 100-fold dilution in medium containing a high concentration of albumin on tissue extraction, storage at -15°C as an $(\text{NH}_4)_2\text{SO}_4$ precipitate, resuspension in column buffer, dialysis and then chromatography on Superose 6. This persistence suggests that the changes in activity and polymerization induced by insulin are more likely to be the result of covalent modification, such as phosphorylation, than of changes in a tightly bound regulator, such as citrate or fatty acyl-CoA esters. However, the effect of insulin on acetyl-CoA carboxylase activity is not evident after Sepharose-avidin chromatography (Witters *et al.*, 1983; Holland & Hardie, 1985; A. C. Borthwick, unpublished work). This may be because polymeric forms of acetyl-CoA carboxylase bind less well to avidin [see Brownsey & Denton (1986) for fuller discussion], and thus the enzyme purified by this technique is enriched in forms which polymerize poorly (for example, after phosphorylation by cyclic-AMP-dependent protein kinase).

The increased phosphorylation of the polymeric form from insulin-treated tissue on a specific peptide (I-peptide) further supports the contention that increased phosphorylation of a site (or sites) within this peptide may be the means whereby the effects of insulin on acetyl-CoA carboxylase are brought about. However, further studies are still required to obtain definitive proof. These must include the characterization of the

kinase involved and the demonstration in a purified system that phosphorylation of the appropriate sites in the enzyme can lead to activation and polymerization.

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