# Purification and characterisation of an insulin-stimulated protein-serine kinase which phosphorylates acetyl-CoA carboxylase

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**Abstract An insulin-stimulated protein kinase specific for acetyl-CoA carboxylase has been purified from rat epididymal adipose tissue using Mono-Q chromatography. The kinase binds to (and phosphorylates) the relatively inactive, dimeric form of acetyl-CoA carboxylase, but not to its active, polymeric form, and this property has been used to purify the kinase. Under the conditions used, phosphorylation by the purified kinase did not result in a detectable increase in acetyl-CoA carboxylase activity. These studies also led to the recognition of an 'activator' protein which is capable of increasing the activity of acetyl-CoA carboxylase without changing its phosphorylation state. It is suggested that this 'activator' protein, together with the insulin-activated acetyl-CoA carboxylase kinase, may play a role in the activation of acetyl-CoA carboxylase by insulin.**



*Key words:* Insulin; Acetyl-CoA carboxylase; Serine phosphorylation; Protein activator; Rat epididymal adipose tissue

## 1. Introduction

Treatment of rat epididymal adipose tissue or isolated fat cells with insulin leads to a substantial increase in the rate of fatty acid synthesis, which is explained in part by the rapid activation of acetyl-CoA carboxylase [1–5]. This enzyme exists as both an inactive dimer and an active polymer, with interconversion between these two forms depending on both allosteric factors (e.g. citrate, fatty acyl-CoA and CoA [6–8]) and also on the phosphorylation state of the enzyme [9,10]. The activation of acetyl-CoA carboxylase in response to insulin is characterised by an increase in the proportion of the enzyme in the active, polymeric form and by an increase in the phosphorylation of the enzyme on at least two serine residues [9–12]. One has been identified as serine-29, and is phosphorylated in vitro by casein kinase-2 [12,13] with no apparent change in carboxylase activity. The other serine is within a tryptic peptide termed the 'I-peptide' which has not yet been sequenced [9–11].

An insulin-stimulated protein kinase which appears to phosphorylate this site has been separated by Mono-Q anion exchange chromatography of fat cell extracts and initial evidence was obtained which suggested that phosphorylation of acetyl-CoA carboxylase by this kinase led to an activation of the enzyme [14]. The present paper describes the purification of this kinase from rat epididymal adipose tissue using a novel affinity purification step.

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## 2. Materials and methods

### 2.1. Materials

Except as given below, chemicals were from BDH, Lutterworth, Leicester, UK and biochemicals were from Sigma, Poole, Dorset, UK. Adenosine 5′-[$^{32}$P]triphosphate and [$^{14}$C]bicarbonate were from Amersham International, Buckinghamshire, UK. Trypsin was from Worthington Biochemical Corporation, Freehold, NJ, USA. Acetyl-CoA carboxylase was purified from rat epididymal adipose tissue which had been incubated in the absence of insulin for 15 min at 37°C, as described previously [14].

### 2.2. Tissue incubation and preparation of extracts

Epididymal fat pads from male Wistar rats (150–200 g), fed ad libitum until killing by decapitation, were incubated at 37°C in pre-gassed ($O_2$/$CO_2$, 19:1) Krebs bicarbonate buffered medium, pH 7.4 [15] containing 10 mM HEPES and 11 mM glucose. After pre-incubation for 15 min, pads were transferred to fresh medium of the same composition and incubated for a further 15 min in the presence of 83 nM insulin. Control pads had no additions. Pads were extracted and a high-speed supernatant fraction prepared as described previously [14].

### 2.3. Chromatographic procedures

The high-speed supernatant fraction, equivalent to 6–8 g of tissue (approximately 25 ml in volume), was applied to a FPLC Mono-Q HR 5/5 column equilibrated in buffer A (10 mM $KH_2PO_4$, pH 7.4, containing 0.5 mM EDTA, 1 mM DTT, pepstatin A, antipain and leupeptin, each at 1 μg/ml and with or without citrate 1 mM). The column was developed with a discontinuous gradient of buffer A containing 1 M NaCl (see Fig. 1).

Mono-Q fractions containing kinase activity were pooled and incubated with 20 mM citrate at 30°C for 20 min and then applied to a Superose 6 column (preparation grade, 1.6×45 cm) equilibrated in buffer B (20 mM MOPS, pH 7.2, containing 2 mM EDTA, 10 mM $MgCl_2$, 20 mM citrate, 1.85% (v/v) glycerol, 1 mM DTT, pepstatin, antipain and leupeptin, each at 1 μg/ml).

Superose 6 fractions containing kinase activity were dialysed overnight against citrate-free buffer A to remove citrate and then applied to a Pharmacia SMART system Mono-Q column PC 1.6/5 previously equilibrated in the same buffer. A linear gradient of citrate-free buffer A containing 1 M NaCl was developed over 3 ml at a flow rate of 100 μl/min, and 80 μl fractions were collected. Fractions containing kinase activity were then concentrated using a speedvac (Savant) and separated by gel filtration using the SMART system Superose 12 PC 3.2/30 column equilibrated in citrate-free buffer A containing 175 mM NaCl. A flow rate of 40 μl/min was used and 80 μl fractions were collected.

### 2.4. Assay of acetyl-CoA carboxylase activity

The ability of column fractions to activate acetyl-CoA carboxylase was assessed essentially as described by Borthwick et al. [16] (using 20 μl of sample in a total assay volume of 50 μl), but with the inclusion of 0.8 μM CoA in the reaction incubation to ensure the acetyl-CoA carboxylase was present in the de-polymerised form [8]. The maximum activity of the added acetyl-CoA carboxylase was determined by pre-incubation with citrate (20 mM) for 20 min at 30°C and the ability of column fractions to activate acetyl-CoA carboxylase expressed as a percentage of this maximum activity. To correct for endogenous acetyl-CoA carboxylase in certain fractions, including fractions 36–42 from FPLC Mono-Q chromatography, assays were also conducted without added acetyl-CoA carboxylase.


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*2.5. Analysis of acetyl-CoA carboxylase phosphorylation*

Column fractions (20 μl) were incubated in a total volume of 50 μl for 15 min at 30°C with purified acetyl-CoA carboxylase (0.1 mg/ml), 0.8 μM CoA, 8 mM $KH_2PO_4$, 0.4 mM EDTA, 0.8 mM DTT, 5 mM $MgCl_2$, 175 mM NaCl, pepstatin A, antipain and leupeptin, each at 0.8 μg/ml and [γ-$^{32}$P]ATP (50 μM). SDS/polyacrylamide-gel electrophoresis and radioautography were as described by Brownsey et al. [17]. Phosphorylation was essentially linear up to 15 min and the extent of phosphorylation of acetyl-CoA carboxylase was less than 50% of the maximum incorporation (attained after 60 min or more).

## 3. Results and discussion

*3.1. The effect of citrate on acetyl-CoA carboxylase kinase elution during Mono-Q chromatography*

In initial experiments, an acetyl-CoA carboxylase kinase was separated from extracts of rat epididymal fat pads using Mono-Q chromatography in the presence of citrate (1 mM) (Fig. 1A). Fractions containing kinase activity eluted from the anion exchange column at 175 mM NaCl and stimulated added purified acetyl-CoA carboxylase. These results confirmed the earlier observations of Borthwick et al. [14], although the present study included some modifications to the elution profile and all assays were carried out in the presence of 0.8 μM acetyl-CoA to ensure that the added acetyl-CoA carboxylase was in its relatively inactive, dimeric form [8]. However, if the separation of extracts by Mono-Q chromatography was performed in the absence of citrate, the kinase activity eluted at a much higher salt concentration (280–380 mM NaCl) (Fig. 1B). When the position of elution of the endogenous fat pad acetyl-CoA carboxylase was analysed using Western blotting, it was noted that this acetyl-CoA carboxylase and the kinase activity co-eluted (fraction K1) when chromatography was performed in the absence of citrate. The elution of endogenous acetyl-CoA carboxylase was essentially unaffected by the presence of citrate.

In five separate experiments, the effect of insulin on this kinase activity was determined by measuring the total incorporation of $^{32}$P into acetyl-CoA carboxylase by peak fractions (36–42) from control and insulin-treated tissue. In this way, the kinase in fraction K1 was found to be stimulated by insulin 2.4 ± 0.7-fold (mean ± S.E.M.); i.e. to a similar extent as the kinase activity in fraction K prepared in the presence of citrate [14].

These results suggest two important points. Firstly, it now appears that the phosphorylation of acetyl-CoA carboxylase by this kinase may not be solely responsible for the activation of acetyl-CoA carboxylase seen in the initial studies in the presence of citrate, since in the absence of citrate, the kinase and activating activities are separated by Mono-Q chromatography (Fig. 1B). It seems likely that a protein is responsible for the activation of acetyl-CoA carboxylase seen in fraction K, since this activity was found to be non-dialysable, destroyed by boiling and had a molecular weight of approximately 130 kDa (as determined by size exclusion chromatography). It should be noted however, that although the kinase activity in fraction K1 was stimulated by insulin, the ability of the 'activator' in fraction K to activate acetyl-CoA carboxylase was not significantly affected by insulin under the conditions used in the present study (data not shown).

The second important point suggested by these studies is that since the kinase elutes with acetyl-CoA carboxylase during Mono-Q chromatography conducted in the absence, but not the presence of citrate, the kinase may bind to dimeric, but not polymeric, acetyl-CoA carboxylase. This result was confirmed by incubating fractions containing endogenous acetyl-CoA carboxylase and co-eluted kinase (fraction K1 in Fig. 1B) with citrate and then separating the pooled fraction using size exclusion chromatography. Incubation of the fractions with citrate causes polymerisation of the acetyl-CoA carboxylase present, resulting in the early elution of this protein from the Superose 6 column due to its large size (again determined by Western blotting, Fig. 2A, insert). Polymerisation of the acetyl-CoA carboxylase also appeared to result in release of the bound kinase, since this elutes much later from the Superose 6 column and was completely separated from the acetyl-CoA carboxylase (Fig. 2A). In contrast, application of fraction K1 which had not been treated with citrate resulted in the continued co-elution of acetyl-CoA carboxylase and the kin-

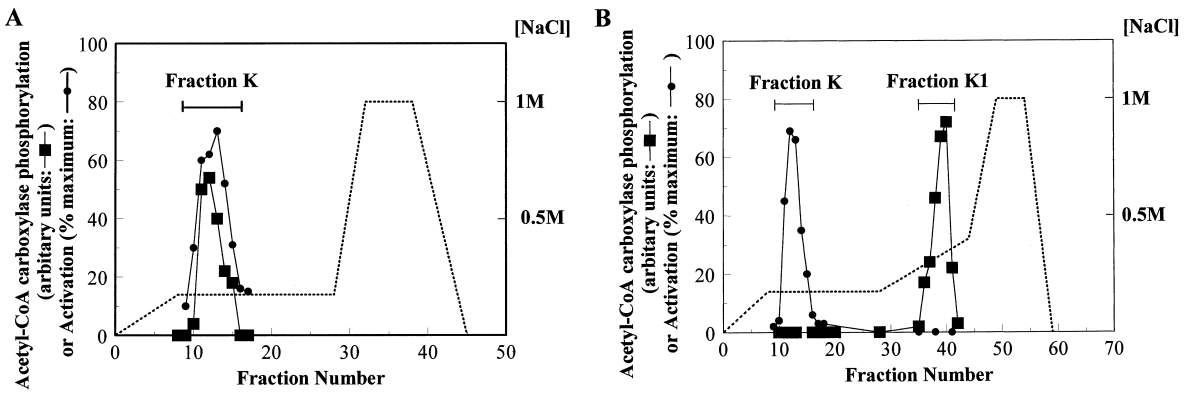


Fig. 1. Elution of acetyl-CoA carboxylase kinase activity during Mono-Q chromatography in the presence (A) and absence (B) of citrate. Rat epididymal fat pads were treated with insulin (0.5 μg/ml) prior to extraction. Following extraction, high-speed supernatant fractions were separated by Mono-Q chromatography in the presence (A) or absence (B) of 1 mM citrate using the discontinuous salt gradient shown in the figure. A sample (20 μl) of the resulting fractions was assayed for kinase activity (■) by incubating with purified acetyl-CoA carboxylase (0.1 mg/ml) in the presence of [γ-$^{32}$P]ATP (50 μM) and CoA (0.8 μM). After incubation for 15 min at 30°C the proteins were separated by SDS-PAGE. Gels were stained with Coomassie blue and labelled proteins revealed by radioautography. Results are expressed in arbitrary units resulting from densitometric scanning of radioautographs with a Joyce-Loebl Chromoscan 3 instrument. A further sample (20 μl) of the Mono-Q fractions was assayed for the ability to activate purified acetyl-CoA carboxylase (0.1 mg/ml) in the presence of CoA (0.8 μM) (●). Results are expressed as a percentage of the maximum activation achieved when the purified acetyl-CoA carboxylase was incubated for 20 min at 30°C in the presence of 20 mM citrate. The figures show the typical results from five separate preparations.

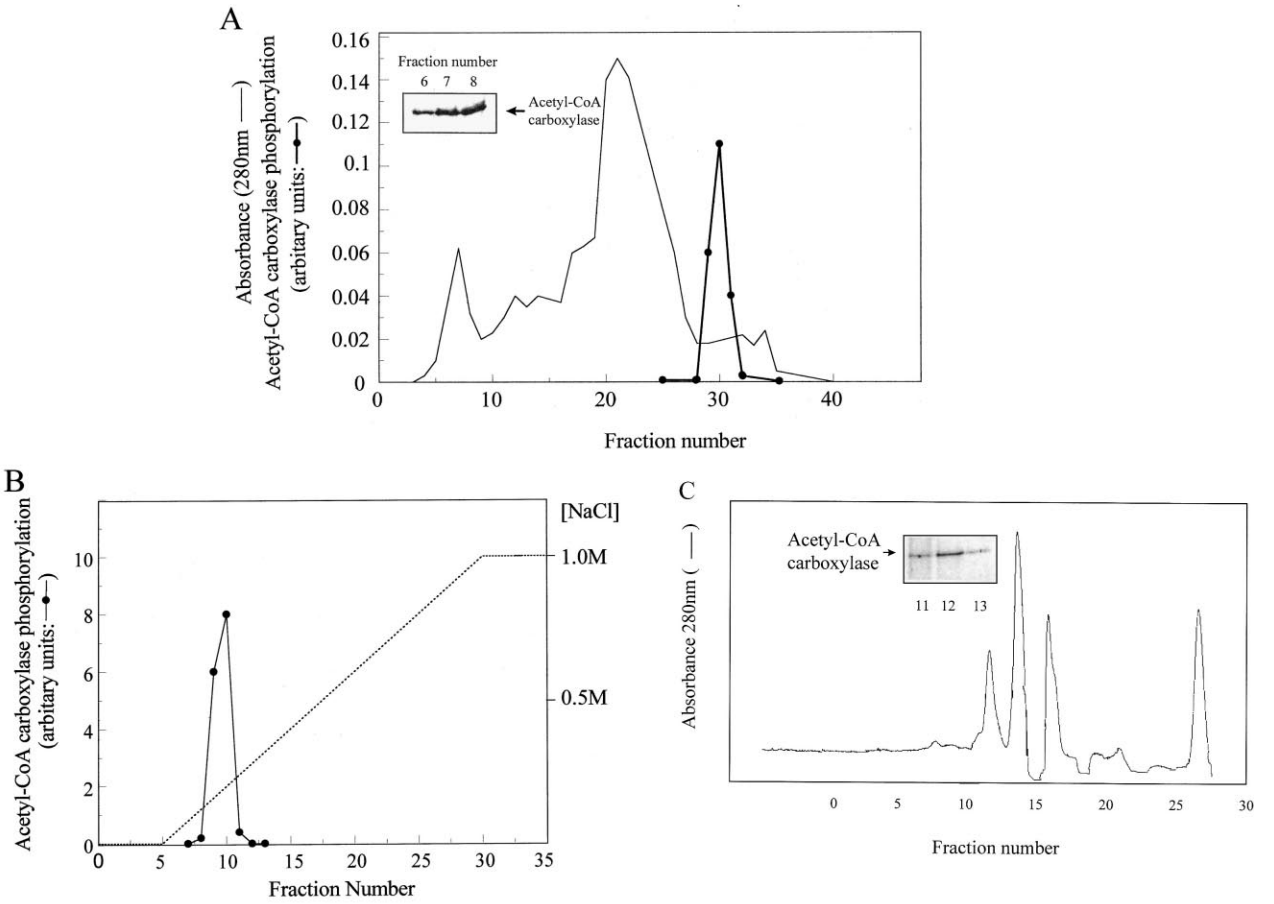


Fig. 2. Further purification of acetyl-CoA carboxylase kinase. A: Fraction K1 was incubated in the presence of citrate (20 mM) for 30 min at 30°C and then applied to a Superose 6 column previously equilibrated in buffer B. The solid line indicates the absorbance at 280 nm. Fractions were assayed for their ability to phosphorylate purified acetyl-CoA carboxylase as described for Fig. 1 (●). The presence in the fractions of acetyl-CoA carboxylase was investigated by Western blotting (insert). B: Superose 6 fractions (6 ml in total) containing kinase activity were dialysed overnight at 4°C against 5 l of citrate-free buffer A and then applied to a SMART system Mono-Q PC 1.6/5 column previously equilibrated in the same buffer. The column was developed with a gradient of NaCl (dotted line). Fractions were assayed for their ability to phosphorylate purified acetyl-CoA carboxylase (●). C: SMART Mono-Q fractions (200 μl in total) containing kinase activity were concentrated to 50 μl using a speedvac (Savant) and then applied to a SMART Superose 12 PC 3.2/30 column previously equilibrated in citrate-free buffer A containing 175 mM NaCl. The solid line indicates the absorbance at 280 nm. Fractions were assayed for their ability to phosphorylate purified acetyl-CoA carboxylase (insert). The results in A–C are typical of five preparations.

ase during Superose 6 chromatography performed in the absence of citrate.

In further confirmation of the specific binding of the kinase to dimeric, but not polymeric acetyl-CoA carboxylase, the kinase was found to phosphorylate dimeric acetyl-CoA carboxylase at approximately 10 times the rate of its ability to phosphorylate acetyl-CoA carboxylase which had been previously polymerised by incubation in the presence of citrate.

### *3.2. Further purification of the acetyl-CoA carboxylase kinase*

Fractions containing kinase activity from the Superose 6 column were pooled, dialysed to remove any citrate present, and re-applied to a SMART system Mono-Q column. At this stage, the kinase reverted to its *original* elution position of about 175 mM NaCl (even in the absence of citrate), since the acetyl-CoA carboxylase which was responsible for its later elution had been removed (Fig. 2B). However, the majority of other proteins which eluted at the higher salt concentration from the original Mono-Q column continued to do so and thus this step provided a considerable degree of purification of the kinase from other proteins present in the fat pad extract.

After a final purification step using a SMART Superose 12 column (Fig. 2C), the kinase containing fraction 12 was separated by SDS-PAGE and visualised by silver staining. Only two protein bands (probably representing less than 1 μg of protein) could be seen, of apparent molecular weights 53 kDa and 65 kDa but the identity of these proteins has not been established.

### *3.3. Properties of the purified acetyl-CoA carboxylase kinase*

The purified kinase phosphorylated acetyl-CoA carboxylase prepared from rat epididymal adipose tissue on serine residue(s) and within the 'I-peptide' in agreement with earlier studies [14]. Although phosphorylation occurred to a high stoichiometry (typically 0.6 mol of phosphate were incorporated per mol acetyl-CoA carboxylase subunit following incubation for 60 min at 30°C), no changes in acetyl-CoA carboxylase activity were detected under the conditions used.

The activity of the kinase towards acetyl-CoA carboxylase was shown to be unaffected by EGTA, $Ca^{2+}$, cyclic-AMP and

cyclic-GMP in earlier studies [14]. In the present study the kinase was found to be inhibited by α-glycerophosphate, β-glycerophosphate and glucose 6-phosphate with $K_i$ values of 20 mM, 10 mM and 30 mM, respectively.

As emphasised above, the kinase is more active towards the dimeric form of acetyl-CoA carboxylase than the polymeric form. No other substrates for the kinase have been found. The kinase does not phosphorylate myelin basic protein, ribosomal protein S6-derived peptides (RRLSSLRA and KEAKEKRQEQIAKKRRLSSLRASTSKSESSQK), and the casein kinase 2 substrate peptide (RRREEETEEE). It also fails to phosphorylate a number of synthetic peptides derived from the amino-terminus of the rat acetyl-CoA carboxylase sequence (residues 1–9, 10–21, 18–33, 24–38, 38–57, 46–58, 56–69, 60–85, 91–100 and 99–107) including those containing the sites phosphorylated by the calmodulin-dependent multiprotein kinase and casein kinase II (serines 25 and 29, see [18,19]); protein kinase C (serines 77 and 95, see [12,19]); the AMP-activated protein kinase and the cAMP-dependent protein kinase (serines 79 and 77 [20]). In addition, synthetic peptides derived from the centre of the protein (residues 1181–1190, 1194–1206 and 1207–1222) which include additional sites phosphorylated by the AMP-activated protein kinase and the cAMP-dependent protein kinase (serines 1200 and 1215 [20]) were also not phosphorylated by the kinase.

### *3.4. General conclusions*

The insulin-activated kinase purified from rat epididymal adipose tissue in this study is apparently highly specific for the dimeric, inactive form of acetyl-CoA carboxylase and phosphorylates this protein within the 'I-peptide' (which exhibits increased phosphorylation when acetyl-CoA carboxylase is activated by insulin). A novel affinity purification step has been employed in the purification of this kinase. This step is based on the binding of the kinase to acetyl-CoA carboxylase in its depolymerised form and its subsequent release when the acetyl-CoA carboxylase is polymerised. This technique is presumably highly specific for the purification of this kinase, as it is dependent on the ability of citrate to cause conformational changes in the kinase's substrate, acetyl-CoA carboxylase.

The activation of acetyl-CoA carboxylase seen in the presence of Mono-Q fractions containing acetyl-CoA carboxylase kinase activity in earlier work [14] now appears to be due to the presence in these fractions of an 'activator' protein which can increase the activity of acetyl-CoA carboxylase without phosphorylating it. This 'activator' does not appear to be significantly stimulated by insulin and is separated from the kinase when Mono-Q chromatography is performed in the absence of citrate, i.e. under conditions where the kinase is largely bound to dimeric acetyl-CoA carboxylase. An acetyl-CoA carboxylase 'activator' protein has also been found in rat liver [21], but further studies are required to establish the relationship between the liver and adipose tissue proteins.

Despite the lack of activation of acetyl-CoA carboxylase on phosphorylation by the kinase purified in the present studies, it is still possible that this insulin-activated kinase is important in bringing about the activation of acetyl-CoA carboxylase seen in insulin-treated fat cells, since it is difficult to be certain that the conditions used to assay acetyl-CoA carboxylase activity reflect those occurring within the cell. For example, concentrations of regulatory metabolites (CoA, citrate, etc.) may be critical, or phosphorylation may sensitise acetyl-CoA carboxylase to the effects of the 'activator' protein and in this way both the 'activator' protein and the kinase may be involved in bringing about the effects of insulin on lipogenesis within the intact cell.

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