Evidence for a protein regulator from rat liver which activates acetyl-CoA carboxylase

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1. A regulator of acetyl-CoA carboxylase has been identified in high-speed supernatant fractions from rat liver. The regulator was found to activate highly purified acetyl-CoA carboxylase 2–3-fold at physiological citrate concentrations (0.1–0.5 mM). The effects of the regulator on acetyl-CoA carboxylase activity were dose-dependent, and half-maximal activation occurred in 7–8 min at 30 °C. 2. The acetyl-CoA carboxylase regulator was non-dialysable and was inactivated by heating or by exposure to carboxypeptidase. The regulator was enriched from rat liver cytosol by first removing the endogenous acetyl-CoA carboxylase and then using a combination of purification steps, including $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography and size-exclusion chromatography. The regulator activity appeared to be a protein with a molecular mass of approx. 75 kDa, which could be eluted from mono-Q with approx. 0.35 M KCl as a single peak of activity. 3. Studies of the effects of the regulator on phosphorylation or subunit size of acetyl-CoA carboxylase indicated that the changes in enzyme activity are most unlikely to be explained by dephosphorylation or by proteolytic cleavage. 4. The regulator co-migrates with acetyl-CoA carboxylase through several purification steps, including ion-exchange chromatography and precipitation with $(NH_4)_2SO_4$; however, the proteins may be separated by Sepharose–avidin chromatography, and the association between the proteins is also disrupted by addition of avidin in solution. Furthermore, the binding of the regulator itself to DEAE-cellulose is altered by the presence of acetyl-CoA carboxylase. Taken together, these observations suggest that the effects of the regulator on acetyl-CoA carboxylase may be explained by direct protein–protein interaction *in vitro*.

INTRODUCTION

Acetyl-CoA carboxylase (EC 6.4.1.2) catalyses the ATP-dependent formation of malonyl-CoA, and thereby commits acetyl carbon towards the synthesis of long-chain fatty acids. Short-term control of the activity of acetyl-CoA carboxylase is critical for overall regulation of fatty acid synthesis *de novo* and involves complex allosteric regulation as well as covalent modification involving phosphorylation and dephosphorylation of as many as eight serine residues (for reviews see [1–3]). A number of low-molecular-mass compounds (aside from the reactants and products) have been found to affect acetyl-CoA carboxylase activity *in vitro*. The striking effects *in vitro* of physiological concentrations of citrate and fatty acyl-CoA esters appear to suggest an important cellular role for these regulators [3]. Very recently CoA has also been found to inhibit acetyl-CoA carboxylase potently *in vitro* [4], in contrast with previous reports [5,6]. The physiological significance of regulation by other potential regulators, such as guanine nucleotides [7,8] and phospholipids [9], remains to be established. In addition, potential regulatory roles for less well defined cellular components await further characterization, including those of low molecular mass [10,11], as well as an endogenous protein inhibitor [12] which appears to be a protein kinase [13].

The role of covalent regulation of acetyl-CoA carboxylase through phosphorylation and dephosphorylation is also complex. A convincing set of observations has provided strong support for the concept that the inhibition of acetyl-CoA carboxylase in response to hormones that increase the levels of intracellular cyclic AMP, for example adrenaline and glucagon, involves rapid phosphorylation at serine-79 by AMP-dependent protein kinase (followed by slower phosphorylation of serine-1200 and -1215 [2,14,15]). The activation of acetyl-CoA carboxylase in response to insulin is less well understood and, unlike the inactivation in response to adrenaline or glucagon, does not persist through Sepharose–avidin chromatography [11,16]. Nevertheless, in normal fat and liver cells insulin promotes the increased phosphorylation of acetyl-CoA carboxylase, probably at two different serine residues. One site (serine-29) which shows increased phosphorylation in response to insulin treatment of fat or liver cells is also phosphorylated *in vitro* by casein kinase II, although no corresponding change in catalytic activity of acetyl-CoA carboxylase was observed upon treatment with this kinase [16,17]. Another major site (or sites) of insulin-directed phosphorylation occur within a phosphopeptide originally described as the 'I-peptide' [18]. So far, the amino acid sequence of this peptide has yet to be determined, and the impact of phosphorylation on enzyme activity has not been unambiguously demonstrated. Phosphorylation of acetyl-CoA carboxylase, apparently within the 'I-peptide', by an insulin-activated protein serine kinase from rat epididymal adipose tissue leads to activation of acetyl-CoA carboxylase, especially if the enzyme is also exposed to a low-molecular-mass inhibitor [19]; subsequently, this inhibitor was shown to be CoA [4]. We have recently observed that a maturation-activated protein serine kinase from sea-star oocytes (initially detected by using myelin basic protein as a substrate and designated $p44^{mpk}$) is able to phosphorylate purified acetyl-CoA carboxylase. Phosphorylation by $p44^{mpk}$ occurred largely at a site within a phosphopeptide which, on the basis of chromatographic properties, was identical with the I-peptide observed after insulin treatment of adipose cells [20]. This represented the first report of the phosphorylation

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of purified acetyl-CoA carboxylase on a residue within the I-peptide by a highly purified protein kinase, but no evidence for a direct change in kinetic properties of acetyl-CoA carboxylase could be detected.

These observations prompted us to re-evaluate the possibility that additional factors may be important in the responses of acetyl-CoA carboxylase to insulin. We began with a re-assessment of the properties of acetyl-CoA carboxylase during its purification from rat liver, and this led to the identification of a cytosolic regulator which interacts with the enzyme and which may be involved in the mediation of the effects of hormones on the catalytic activity of this enzyme.

EXPERIMENTAL

Materials

Male Wistar rats (160–200 g) were maintained on a 12 h-light/12 h-dark cycle and were allowed free access to water and Purina rat chow up to the time of killing (09:00–10:00 h). Most laboratory chemicals and solvents were obtained from BDH Chemicals Canada Ltd. (Vancouver, B.C., Canada). Most biochemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), including proteinase inhibitors. Radioisotopes were from Amersham International (Oakville, Ont., Canada). Reagents for PAGE were from Bio-Rad Laboratories (Canada) Ltd. (Mississauga, Ont., Canada). Materials for chromatography, including f.p.l.c., were from Pharmacia–LKB (Canada) Inc. (Baie d'Urfe, Que., Canada), except Whatman DEAE-cellulose (DE-52), from Whatman International (Maidstone, Kent, U.K.). Avidin was kindly given by Canadian Lysozyme Co. (Abbotsford, B.C., Canada).

Determination of acetyl-CoA carboxylase activity

Assays were essentially as described previously [21], by using the method which monitors the incorporation of ^{14}C from [^{14}C]bicarbonate into acid-stable product. Briefly, after pre-incubation of acetyl-CoA carboxylase under the conditions indicated, assays were initiated by addition of 50 μl (1–2 m-units) of enzyme preparation to 0.45 ml of assay medium (pH 7.2), containing Mops (20 mM), $MgSO_4$ (10 mM), EDTA (0.5 mM), ATP (5 mM), 2-mercaptoethanol (5 mM), de-fatted BSA (10 mg/ml), acetyl-CoA (150 μM) and $KH^{14}CO_3$ (15 mM, approx. 600 d.p.m./nmol). Reactions were terminated after 2 min at 37 °C by addition of 200 μl of HCl (5 M). A sample of acidified reaction mixture (0.6 ml) was evaporated to dryness, and [^{14}C]malonyl-CoA was measured by liquid-scintillation counting. One unit of acetyl-CoA carboxylase is defined as the amount of enzyme which catalyses the formation of 1 μmol of malonyl-CoA/min under the conditions described.

Purification of acetyl-CoA carboxylase from rat liver

Generally, two or three male Wister rats (160–200 g) were stunned, killed by decapitation, and the livers were removed immediately and placed on ice. The livers were rinsed with distilled water, blotted and homogenized gently in 3 vol. of extraction buffer (pH 7.4) containing Tris/HCl (50 mM), sucrose (250 mM), EDTA (2 mM), EGTA (1 mM), citrate (5 mM), 2-mercaptoethanol (5 mM), phenylmethanesulphonyl fluoride (0.5 mM), pepstatin (2 μg/ml), leupeptin (2 μg/ml), benzamidine (2.5 mM) and NaN_3 (0.02%, w/v) with a Potter–Elvehjem homogenizer for 30 s at 4 °C (ensuring at least four passes of the probe). All subsequent operations were carried out at 4 °C, except where indicated. The homogenate was centrifuged at 3000 *g* for 10 min, and the supernatant was collected, filtered through eight layers of cheesecloth (to remove the floating fat layer) and then centrifuged at 25000 *g* for 25 min. The supernatant was decanted and filtered again as described above. This clarified 25000 *g* supernatant fraction was then centrifuged for 60 min at 125000 *g* and the supernatant (designated the cytosolic fraction) was collected. Solid $(NH_4)_2SO_4$ was added to the cytosolic fraction (pH 7.2) to give 40% saturation, and after incubation at 0 °C for 30 min the precipitated protein was pelleted by centrifugation (30 min, 25000 *g*). The $(NH_4)_2SO_4$ pellet was resuspended in a small volume (approx. 2–3 ml) of buffer (pH 7.5) containing Tris (50 mM), KCl (0.5 M), EDTA (2 mM), EGTA (2 mM), 2-mercaptoethanol (5 mM) and NaN_3 (0.02%) plus the proteinase inhibitors pepstatin (2 μg/ml), leupeptin (2 μg/ml) and benzamidine (2.5 mM), and after removal of insoluble material by centrifugation (12000 *g* for 10 min) was immediately applied to a Sepharose–avidin column [22]. After removal of unbound protein (the 'avidin void' fraction) by washing with the pellet resuspension buffer, acetyl-CoA carboxylase was eluted with the same buffer to which biotin (0.8 mM) was also added, and fractions containing acetyl-CoA carboxylase activity were pooled. This biotin-eluted fraction was concentrated by dialysis against flaked poly(ethylene glycol) ('Aquacide'), and the concentration of KCl was decreased to approx. 100 mM by dilution, before storage in small portions at −70 °C.

Partial purification of acetyl-CoA carboxylase by f.p.l.c.

Rat liver cytosol was prepared as described above through the $(NH_4)_2SO_4$-precipitation step. After desalting over a Sephadex G-50 spin column, a sample of the re-dissolved $(NH_4)_2SO_4$ pellet (0.5 ml) was applied to a Mono-Q ion-exchange column (in a Pharmacia f.p.l.c. system) equilibrated with buffer A (pH 7.5), containing Tris (50 mM), EDTA (2 mM), EGTA (1 mM), benzamidine (2.5 mM), pepstatin (2 μg/ml), leupeptin (2 μg/ml), 2-mercaptoethanol (5 mM) and NaN_3 (0.02%). The unbound protein was washed from the column with buffer A, and then the bound protein was eluted with the same buffer by application of a linear salt gradient (0–0.5 M KCl) in a total volume of 20 ml.

Assay for acetyl-CoA carboxylase regulator

Assessment of acetyl-CoA carboxylase regulator is indirect, since it is based on enhancement of activity of a fixed amount of acetyl-CoA carboxylase. A defined amount of affinity-purified acetyl-CoA carboxylase, usually 1–2 μg, was preincubated in a final volume of 100–150 μl (before the standard enzyme assay, in duplicate, described above) in buffer A for 20 min at 37 °C in the presence of an appropriate amount of regulator. Also included in the preincubation was de-fatted BSA (5 mg/ml) and various concentrations of citrate (over the range 0–10 mM) as indicated. One unit of regulator activity was defined as the amount required to give a 2-fold increase in the catalytic activity of 1 μg of affinity-purified acetyl-CoA carboxylase, measured after preincubation in the presence of 0.2 mM citrate as described above. Since the preincubated enzyme was diluted 10-fold into assay buffer, the citrate concentration in the assay itself was therefore 20 μM.

Partial purification of acetyl-CoA carboxylase regulator

The procedures adopted involve preparation of acetyl-CoA carboxylase regulator from the protein fraction which is eluted

Table 1 Partial purification of acetyl-CoA carboxylase regulator from rat liver

High-speed supernatant fractions were prepared from rat liver, and acetyl-CoA carboxylase was recovered by Sepharose–avidin chromatography. The protein fraction which was washed unbound from the affinity column ('avidin void') was used as the initial source for further purification of acetyl-CoA carboxylase regulator. The results presented are from a typical preparation starting with four rat livers. For full details and definition of units, see the Experimental section. For ion-exchange chromatography, the values (mM) indicate the range over the salt gradient from which fractions were pooled. For Sephacryl 200 chromatography, a single sharp peak of activity was eluted corresponding to the molecular-mass range 70–80 kDa.

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Avidin void	624	1400	2.33	100
DEAE-cellulose (200–400 mM)	22	576	26.2	42
$(NH_4)_2SO_4$ pellet (20–40%)	2.1	340	161.9	24
Mono-Q (320–360 mM)	0.36	268	744.4	19
Sephacryl 200	0.04	134	3350	9

unbound from the Sepharose–avidin column ('avidin-void'). The subsequent steps involve two ion-exchange chromatography steps [coupled with $(NH_4)_2SO_4$ precipitation], followed by size-exclusion chromatography. Rat liver cytosol was prepared, treated with $(NH_4)_2SO_4$ and subjected to Sepharose–avidin chromatography as described above for the purification of acetyl-CoA carboxylase. Protein which did not bind to Sepharose–avidin was desalted by gel filtration on a Sephadex G-50 column and subsequently applied to a DEAE-cellulose column (1.6 cm × 12 cm) equilibrated with buffer A. Unbound protein was washed from the column with buffer A, and then the bound protein eluted with the same buffer containing a linear salt gradient (0–500 mM KCl). Fractions containing acetyl-CoA carboxylase regulator (2 ml) were pooled and concentrated by addition of $(NH_4)_2SO_4$ (40% saturation). After incubation on ice for 30 min, the precipitated protein was recovered by centrifugation (20000 ***g***, 20 min). The pellet was resuspended in Tris buffer (50 mM, pH 7.5), centrifuged to clarify (12000 ***g***, 10 min), desalted by gel-filtration on a G-50 column and then applied to a Mono-Q ion-exchange column. Unbound protein was removed by washing the column with buffer A, and bound protein was eluted with buffer A containing a linear salt gradient (0–500 mM KCl). Fractions containing acetyl-CoA carboxylase regulator were pooled, concentrated and desalted by centrifugal ultrafiltration in Centricon tubes with a molecular-mass cut-off of 10 kDa (Amicon; W. R. Grace and Co., Beverley, MA, U.S.A.). This fraction was used in all further studies to characterize the regulator activity. A portion of this fraction (0.5 ml) was applied to a Sephacryl-200 gel-filtration column (1 cm × 38 cm) equilibrated with Mops buffer (50 mM, pH 7.4) containing KCl (50 mM), EDTA (2 mM), EGTA (1 mM) and 2-mercaptoethanol (5 mM), at a flow rate of 2 ml/h. Fractions containing acetyl-CoA carboxylase regulator were pooled and stored in small portions at −70 °C.

Results of a typical preparation (beginning with four rat livers) are shown in Table 1; the overall purification exceeded 1400-fold, with a yield close to 10%. Even at this point, the preparation was not homogeneous, as judged by the number of protein bands visible by Coomassie Blue staining after SDS/PAGE. Four major bands were detectable, with subunit molecular masses of 96, 72, 42 and 40 kDa. In addition, a number of minor bands were also apparent, indicating that further purification will be required to achieve a homogeneous preparation.

Phosphorylation of acetyl-CoA carboxylase and other proteins

Purified acetyl-CoA carboxylase (2–3 μg) was preincubated with $MgSO_4$ (5 mM) and the catalytic subunit of cyclic AMP-dependent protein kinase (bovine heart; Sigma) for 2 min at 37 °C in a sealed microcentrifuge tube in a final volume of 50 μl of Tris buffer (50 mM, pH 7.4) containing EDTA (2 mM), EGTA (1 mM) and protease inhibitors as used in buffer A. The reaction was initiated by addition of [γ-^{32}P]ATP (50 μM, approx. 1000 d.p.m./pmol) and allowed to proceed for up to 1 h at 37 °C. In order to monitor incorporation of ^{32}P into acetyl-CoA carboxylase, reactions were subsequently terminated by addition of an equal volume of SDS-sample digestion buffer (with immediate heating at 95 °C for 5 min) before analysis by SDS/PAGE. To assess the incorporation of ^{32}P into other proteins (casein and histone), reactions were carried out as for the phosphorylation of acetyl-CoA carboxylase. Reactions were stopped by spotting samples of the reaction mixture on to 1 cm squares of phosphocellulose paper (Whatman P81) and immersing in ice-cold H_3PO_4 (0.5%, w/v). Papers were washed with eight changes of acid over 60 min and then subjected to liquid-scintillation counting. Determination of background incorporation of ^{32}P involved removing samples at zero time after addition of [γ-^{32}P]ATP or by carrying out incubations in the absence of a substrate protein.

SDS/PAGE and analysis of phosphopeptides

Separation of proteins was achieved by SDS/PAGE in 0.6 cm lanes of slab gels containing acrylamide at 5% or 7% (w/v), by the discontinuous pH procedure [23]. Up to 20 μg of protein per sample was dissolved in digestion buffer, pH 6.8, containing Tris/HCl (65 mM), SDS (100 mg/ml), sucrose (200 mg/ml), Bromophenol Blue (0.2 mg/ml) and 2-mercaptoethanol (100 mM), by heating at 95 °C for 5 min. After electrophoresis, protein bands were detected by staining with Coomassie Blue or silver stain and the destained gels were sandwiched between Cellophane, clamped to a glass plate and left to dry in the air stream of a fume hood overnight.

For analysis of phosphopeptides, acetyl-CoA carboxylase was phosphorylated as described above, but the reaction was terminated by addition of a 10-fold excess of 'quench' buffer, containing β-glycerophosphate (80 mM, pH 7.2), EDTA (10 mM) and NaF (50 mM). Antiserum specific for rat liver acetyl-CoA carboxylase was then added (10 μl/ml), and mixtures were incubated for 30 min at 30 °C and finally for a further 20 min (with occasional vortex-mixing) after addition of protein A. Precipitates were collected by centrifugation (1 min, 12000 ***g*** in an Eppendorf centrifuge) and washed extensively (five times with quench buffer, followed by twice with 50 mM NH_4HCO_3 buffer, pH 8.2). Washed pellets were suspended in 200 μl of the same NH_4HCO_3 buffer and incubated with tosylphenylalanylchloromethane ('TPCK')-treated trypsin (0.1 mg/ml) for 6 h at 30 °C, with a second addition of trypsin after 3 h. Solubilized peptides were recovered after removal of insoluble material by centrifugation. Initial supernatants and three washes of the Protein A pellet were pooled and evaporated to dryness, and to ensure removal of inorganic salt the washing/drying cycle was repeated three times. Samples were then subjected to two-

dimensional thin-layer mapping as described in detail previously [18], using high-voltage electrophoresis in the first dimension (pH 3.6 for 3 h at 400 V) followed by ascending chromatography.

Immunodetection of acetyl-CoA carboxylase

After separation by SDS/PAGE (as described above), proteins were transferred to poly(vinylidene difluoride) membranes (Millipore Corp., Bedford, MA, U.S.A.) by electrophoresis (16 h at 0.2 A) in 10 mM Caps [3-(cyclohexylamino)-1-propanesulphonic acid] buffer, pH 11.0. After transfer, proteins were detected by briefly staining with Ponceau S (2%, w/v, in a mixture of trichloroacetic acid and salicylic acid, each 30%, w/v), to facilitate marking of lanes and molecular-mass standards. Membranes were then blocked (1 h, 20 °C) in KBS buffer, pH 9.0, containing NaCl (137 mM), KCl (2.7 mM), KH_2PO_4 (1.5 mM), Na_2HPO_4 (7.2 mM), NaN_3 (0.02%) and Tween-20 (0.05%, w/v), which was supplemented with dried milk powder (5%, w/v). The subsequent sequence of membrane treatments was as follows: 2 × 5 min in KBS, 2 h with primary antibody (raised in rabbits against affinity-purified acetyl-CoA carboxylase from rat liver) diluted 1:1000 with blocking solution, 5 × 5 min in KBS, 2 h with goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) diluted 1:1000 in blocking solution, 4 × 5 min in KBS, 1 × 5 min in water, 2–5 min with alkaline phosphatase buffer, pH 9.5, containing Tris (100 mM), NaCl (100 mM) and $MgCl_2$ (5 mM), into which was freshly diluted (1:100) stock solutions of Nitro Blue Tetrazolium [10 mg/ml in 70% (w/v) dimethylformamide] and bromochloroindolyl phosphate (5 mg/ml in 100% dimethylformamide).

After finally rinsing in water, membranes were blotted dry and scanned by reflectance using a Bio-Rad 600 video densitometer.

Protein determination

Protein concentration was determined by the dye-binding method [24], with BSA as standard.

RESULTS

Evidence for a regulator of acetyl-CoA carboxylase activity in rat liver cytosol

The kinetics properties of acetyl-CoA carboxylase purified to near homogeneity by Sepharose–avidin affinity chromatography were compared with those of a preparation partially purified by fractionation of a 100000 *g* supernatant by Mono-Q ion-exchange chromatography (see Figure 1a). The activity of these two preparations was determined after activation by pre-incubation (20 min at 37 °C) in the presence of BSA (5 mg/ml) and citrate (over the range 0–10 mM). Maximum activity (with 10 mM citrate) was in the range 0.2–0.5 unit/mg of protein or 1–2 units/mg of protein for the partially purified or affinity-purified preparations respectively. In the absence of citrate, the activity of both enzyme preparations was in the range 10–20% of activity measured in the presence of a maximally stimulating concentration of citrate. The affinity purified preparations of acetyl-CoA carboxylase exhibited a half-maximal activation with citrate concentrations ($K_{cit.}$) in the range 1.5–2.5 mM. Partially purified preparations of acetyl-CoA carboxylase, however, exhibited a markedly different response to citrate, with a $K_{cit.}$ of only 0.1–0.2 M. Values of $K_{cit.}$ calculated for several independent preparations were 2.11 ± 0.33 (4) and 0.12 ± 0.09 (3) mM for the affinity-purified and partially purified preparations respectively (means ± S.E.M. for the numbers of preparations in parentheses).

This shift in apparent citrate sensitivity of acetyl-CoA carboxylase could conceivably be explained by a number of either reversible or irreversible effects, but we hypothesized that it may be due to the dissociation of a regulator of acetyl-CoA carboxylase which is eluted with unbound protein during Sepharose–avidin chromatography. This hypothesis was tested by experiments in which purified acetyl-CoA carboxylase was recombined with the fraction containing the unbound protein 'avidin void'. This recombination of the two protein fractions effectively reversed the changes in the kinetic properties of acetyl-CoA carboxylase observed after Sepharose–avidin

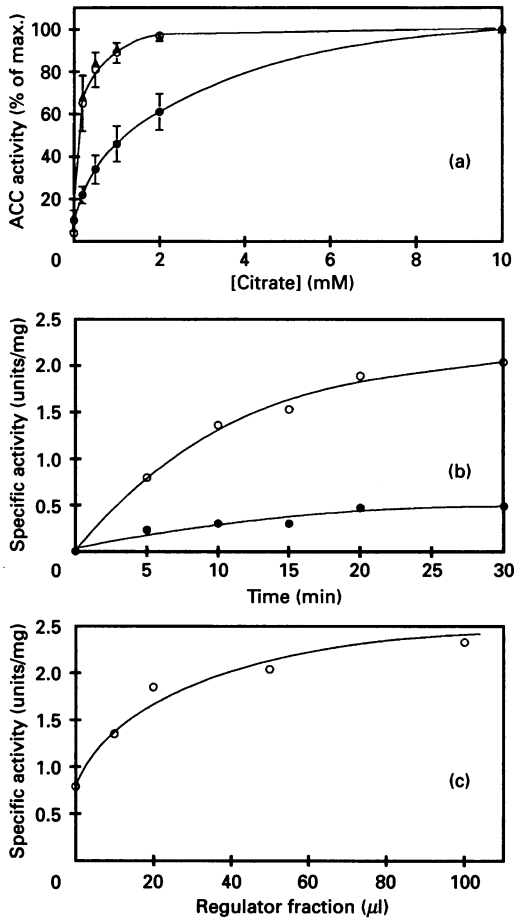

Figure 1 Identification of a regulator of acetyl-CoA carboxylase in rat liver which may be separated from the enzyme by Sepharose–avidin chromatography

The activity of acetyl-CoA carboxylase (ACC; **a**) was determined after partial purification by Mono-Q chromatography (○) or after Sepharose–avidin chromatography (●). Affinity-purified enzyme was also assayed after recombination with the void fraction of the affinity column (▲). The activity of acetyl-CoA carboxylase was measured after preincubation (20 min at 37 °C) in the presence of BSA (5 mg/ml) at the indicated citrate concentrations, and values given are means ± S.E.M. for four separate experiments with different preparations of acetyl-CoA carboxylase. The time course for activation of affinity-purified acetyl-CoA carboxylase (**b**) after preincubation (37 °C) for the indicated times, in the absence (○) or presence (●) of added regulator fraction is shown. (**c**) shows the activity of acetyl-CoA carboxylase after preincubation of enzyme (20 min at 37 °C) in the presence of the indicated amount of the regulator fraction before assay. For (**a**) and (**b**), the regulator fraction added to acetyl-CoA carboxylase in the recombination was equivalent to the 100 μl value in (**c**) and was sufficient to reproduce the concentration present in the fraction applied initially to the Sepharose–avidin column. Preincubations (**b** and **c**) were carried out in the presence of BSA (5 mg/ml) and citrate (0.2 mM), and results represent means of two experiments with different preparations of acetyl-CoA carboxylase and regulator.

chromatography (Figure 1a). The activation of acetyl-CoA carboxylase upon incubation with the regulator fraction was, as demonstrated in Figure 1, dependent on the presence of citrate. Further, the time-dependency for activation of acetyl-CoA carboxylase, measured upon incubation at 0.2 mM citrate, appeared to reflect the time course of activation with citrate alone (Figure 1b). Although the extent of activation was clearly enhanced, the rate of activation was similar in the absence or presence of regulator, with half-maximal activation being observed in 7–8 min and maximal activation within 20 min. Activation of acetyl-CoA carboxylase was also dose-dependent with respect to the amount of added regulator fraction (Figure 1c), rising to a maximum activation when the concentration of added regulator became equivalent to the estimated concentration present before affinity chromatography. In addition to altering the sensitivity of acetyl-CoA carboxylase to citrate, the regulator fraction also increased $V_{max.}$ by 50–60%. $V_{max.}$ for purified acetyl-CoA carboxylase was 2.2 ± 0.09 units/mg increasing to 3.72 ± 0.12 unit/mg after incubation with regulator fraction (means $\pm$ S.E.M. for four observations)

Characterization of acetyl-CoA carboxylase regulator from rat liver

Partial purification of the regulator of acetyl-CoA carboxylase has been carried out in order to achieve initial characterization and to minimize the possibility that enzyme activation may be an artefact of isolation. This was achieved by first inhibiting acetyl-CoA carboxylase by addition of avidin and then using a combination of purification techniques, for which full details are described in the Experimental section and briefly below.

The acetyl-CoA carboxylase regulator was precipitated by addition of $(NH_4)_2SO_4$ to concentrations in the range of 20–40% saturation. Further purification of acetyl-CoA carboxylase regulator was achieved by ion-exchange chromatography. Initial experiments using DEAE-cellulose chromatography indicated that the regulator was eluted as a broad peak between 200 and 400 mM KCl. Subsequently, with chromatography on a Mono-Q column and a linear salt gradient, the regulator was eluted as a single peak with KCl concentration in the range 320–360 mM (Figure 2a). After ion-exchange chromatography, the acetyl-CoA carboxylase regulator was further subjected to size-exclusion chromatography on Sephacryl-200. These results have provided an estimate of the molecular mass of the regulator under non-denaturing conditions of about 75 kDa (Figure 2b). Similar observations were made by using a Superose 6B column with an f.p.l.c. system (results not shown). Furthermore, on DEAE-cellulose, Mono-Q, S-200 and Superose-6B columns, single peaks of activity of acetyl-CoA carboxylase regulator were observed. No regulator activity could be detected in void fractions from the ion-exchange or size-exclusion columns, and recoveries within the single peaks at each step were greater than 70% of the regulator applied to the columns.

The response of acetyl-CoA carboxylase to the allosteric activator citrate (as used here) is commonly employed in studies of the kinetic properties of this enzyme. We were also interested in investigating the effects of the regulator on the response of acetyl-CoA carboxylase to changes in concentration of one of the principal substrates. Acetyl-CoA was chosen for this study since intracellular concentrations of this substrate may vary considerably, whereas the concentrations of the other major substrates (ATP, Mg^{2+} and HCO_3^-) are more constant under normal cellular conditions. In fact, no effect of acetyl-CoA carboxylase regulator on the K_m for acetyl-CoA could be

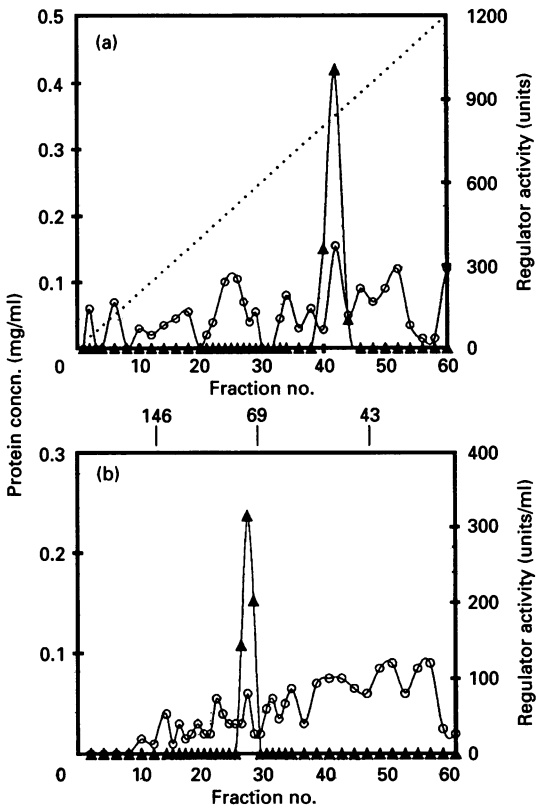

Figure 2 Acetyl-CoA carboxylase regulator migrates as a single symmetrical peak on ion-exchange and size-exclusion chromatography

High-speed supernatant fractions were prepared form four rat livers by centrifugation at 100000 *g* and endogenous acetyl-CoA carboxylase was completely inhibited by addition of avidin (0.1 mg/ml). After $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography, fractions containing acetyl-CoA carboxylase regulator were then subjected to Mono-Q anion-exchange (**a**) and further purified by Sephacryl S-200 size-exclusion (**b**) chromatography. (**a**) illustrates acetyl-CoA carboxylase regulator (▲), protein concentration (○) and salt gradient (· · · · · ·; 0–0.5 M KCl). (**b**) illustrates acetyl-CoA carboxylase (ACC) regulator (▲) and protein (○) eluted in 0.5 ml fractions from a Sephacryl S-200 column (1 cm × 38 cm). The migration of the standard proteins γ-globulin (146 kDa), BSA (69 kDa) and ovalbumin (43 kDa) is indicated along the top axis. Experiments in (**a**) and (**b**) were repeated three times with very similar results.

detected, the average values determined in two separate experiments being 65.4 μM and 64.9 μM in the absence and presence of regulator respectively.

In attempts to provide further general characterization, it was found that the acetyl-CoA carboxylase regulator was destroyed by acid precipitation and was heat-sensitive, being rendered completely inactive by heating at 90 °C for 10 min. At the more modest temperature of 60 °C, the activity of the acetyl-CoA carboxylase regulator was lost more slowly. The regulator was stable to incubation at 60 °C for up to 5 min (absolute activation of acetyl-CoA carboxylase was from 0.72 up to 2.00 units/mg), after which the ability of the regulator to enhance enzyme activity declined with further heating at 60 °C. The maximum activation of acetyl-CoA carboxylase declined to 82%, 39% and 29% after 10 min, 20 min and 30 min of heating of regulator at 60 °C respectively. (All values are means from two separate experiments, expressed as a percentage of the maximum activation of acetyl-CoA carboxylase observed after preincubation with 0.2 mM citrate with no prior heat treatment of regulator.)

The regulator was also found to be sensitive to mild treatment with carboxypeptidase Y, and to a lesser extent with proteinase K treatment. For these experiments acetyl-CoA carboxylase regulator was first dialysed to remove the proteinase inhibitors and then treated with proteinases (0.1% of total protein, by wt.) for 1 h at 37 °C. After incubation, the proteinase was inhibited by addition of phenylmethanesulphonyl fluoride (0.2 mM), and the regulator fraction was then tested for the ability to activate acetyl-CoA carboxylase. The full extent of activation of acetyl-CoA carboxylase with regulator in these experiments was from 0.46 m-units/mg to 1.83 units/mg. Treatment with carboxypeptidase Y led to almost complete abolition of regulator activity, acetyl-CoA carboxylase activity being increased by only 4% of the maximum activation achieved with untreated regulator. The regulator fraction was only 70% effective after treatment with proteinase K, and appeared to be almost insensitive to treatment with trypsin (under the conditions described), displaying 95% of the maximum effect of untreated regulator fraction.

Activation of acetyl-CoA carboxylase by the regulator does not involve phosphorylation or dephosphorylation of the enzyme

Firstly, it must be stressed that the actions of the acetyl-CoA carboxylase regulator were observed in the absence of Mg^{2+} and ATP, and therefore cannot be explained by phosphorylation of acetyl-CoA carboxylase during the preincubation before assay. However, the possible involvement of dephosphorylation of acetyl-CoA carboxylase in enzyme activation has required more detailed studies, as described below.

Purified rat liver acetyl-CoA carboxylase was phosphorylated (for 30 min at 37 °C) in the present of the catalytic subunit of cyclic-AMP-dependent protein kinase. Under these conditions, the incorporation of ^{32}P into acetyl-CoA carboxylase reached 0.67 ± 0.04 mol of P per mol of 265 kDa subunit (mean ± S.E.M. for three different preparations). Phosphorylation under these conditions resulted in inhibition of acetyl-CoA carboxylase by 87 ± 4% relative to control, where values are means ± S.E.M. for three separate preparations of enzyme and enzyme activities were determined after preincubation in the presence of 10 mM citrate. In the absence of added protein kinase, the phosphorylation of acetyl-CoA carboxylase reached values of less than 0.1 mol of P per mol of subunit, and resulted in less than 15% inhibition of catalytic activity. The ^{32}P-labelled acetyl-CoA carboxylase was then used as a substrate to test for protein phosphatase activity in the acetyl-CoA carboxylase regulator fraction. Acetyl-CoA carboxylase was incubated in the presence of the regulator fraction under conditions identical with those described for enzyme activation. Samples of the incubation mixture were removed at 5 min intervals for up to 20 min and then subjected to SDS/PAGE and autoradiography as described in the Experimental section. A typical autoradiograph (Figure 3a) shows that, under the conditions which lead to acetyl-CoA carboxylase activation, the regulator fraction does not cause detectable dephosphorylation of acetyl-CoA carboxylase. This conclusion was confirmed by densitometric scanning of autoradiographs, which indicated that after maximal acetyl-CoA carboxylase activation the extent of dephosphorylation represented removal of less than 10% of the initial ^{32}P. Identical incubations performed in a parallel experiment to that shown in Figure 3(a), and using the same preparation of acetyl-CoA carboxylase, revealed activation (measured after pretreatment with 0.2 mM citrate) from 0.62 to 1.74 units/mg after addition of the regulator fraction during the enzyme preincubation.

Phosphopeptide analysis revealed that most of the ^{32}P incorporated into acetyl-CoA carboxylase in the presence of cyclic-AMP-dependent protein kinase was accounted for by phosphorylation of two major and two or more minor tryptic phosphopeptides (Figure 3). The two major phosphopeptides (also the two most basic peptides) phosphorylated by cyclic-AMP-dependent protein kinase accounted for 72 ± 5% of total incorporated ^{32}P (mean ± S.E.M. for five separate preparations). Phosphopeptide analysis (Figures 3b–e) also revealed that incubation with the regulator did not induce detectable dephosphorylation of these major ^{32}P-labelled phosphopeptides. This was confirmed by densitometric scanning of autoradiograms and by subsequently scraping and counting individual phosphopeptide spots. Some dephosphorylation of the minor peptides was evident after treatment of acetyl-CoA carboxylase with the regulator, but this amounted to less than 10% of the initial ^{32}P incorporated. The possibility that the acetyl-CoA carboxylase regulator fraction may contain protein phosphatase activity was further tested with phosphorylated casein and histone III-S as potential phosphatase substrates. Protein phosphatase activity could not be detected under conditions used to activate acetyl-CoA carboxylase, even with these alternative protein phosphatase substrates (results not shown). These results indicate that the activation of acetyl-CoA carboxylase in response to the regulator does not occur as a result of a phosphorylation or of dephosphorylation of the major sites phosphorylated in the presence of cyclic-AMP-dependent protein kinase. Indeed, under the conditions used for expression of activation of acetyl-CoA carboxylase it is unlikely there is any significant protein phosphatase activity present.

Activation of acetyl-CoA carboxylase by the regulator does not involve proteolysis

Although the buffers used for the preparation and assay of regulator contained a cocktail of proteinase inhibitors, to minimize proteolytic effects, the inhibitors may not be completely effective. However, removal of the regulator from acetyl-CoA carboxylase during purification resulted in isolation of an inhibited form of acetyl-CoA carboxylase that could be re-activated upon re-addition of the regulator. This demonstrates that the activation is a reversible phenomenon and therefore unlikely to be caused by proteolysis. Furthermore, there was no decrease in apparent ^{32}P labelling, nor any change in apparent molecular mass of the major protein subunit (265 kDa) of acetyl-CoA carboxylase after incubation with the regulator for up to 20 min (Figure 3a). This indicates that most of the protein remains intact upon exposure to the regulator. This conclusion is also supported by the lack of any evidence for the appearance of any ^{32}P-labelled polypeptides of lower molecular mass on the autoradiogram (Figure 3a).

Evidence for association between acetyl-CoA carboxylase and the regulator

The activation of acetyl-CoA carboxylase by the regulator could not be accounted for by either of the two well-established mechanisms involving covalent modification by which this enzyme activity is known to be altered, namely by dephosphorylation or by proteolysis. Subsequent experiments were carried out to explore the hypothesis that a physical association (possibly direct binding) occurs between acetyl-CoA carboxylase and the regulator. The interaction between acetyl-CoA carboxylase and the regulator persisted through several purification steps, being disrupted during Sepharose–avidin chromatography (Figure 1). Further support for an interaction between acetyl-CoA carboxylase and the regulator has been

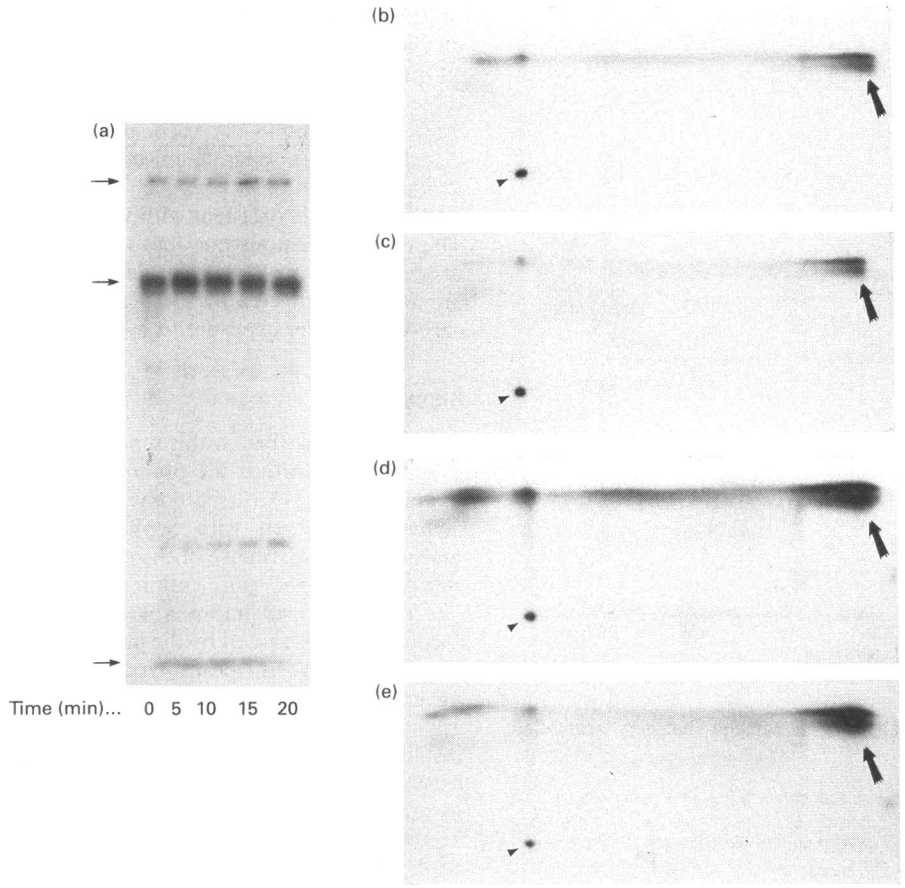

Figure 3 Activation of acetyl-CoA carboxylase by regulator is not associated with dephosphorylation of the enzyme

Sepharose–avidin-purified acetyl-CoA carboxylase (25 μg) was incubated (30 min at 37 °C) with the catalytic subunit of cyclic-AMP-dependent protein kinase (5 units) in the presence of [γ-^{32}P]ATP and $MgSO_4$ (5 mM). The reaction was stopped by addition of EDTA (10 mM), and the incubation continued (37 °C) after addition of acetyl-CoA carboxylase regulator. Samples of the reaction mixture containing identical amounts (approx. 3 μg) of acetyl-CoA carboxylase were quenched in SDS-sample buffer (95 °C for 5 min) at the indicated times and subjected to SDS/PAGE. The autoradiogram shown (**a**) is representative of three separate experiments which gave very similar results. The arrows in the left-hand margin indicate the origin of the separating gel (top), the positions of migration of acetyl-CoA carboxylase and the tracking dye (bottom). Panels (**b**)–(**e**) are autoradiograms indicating ^{32}P-labelled peptides released by trypsin digestion of acetyl-CoA carboxylase, followed by two-dimensional phosphopeptide analysis. Acetyl-CoA carboxylase was incubated with [γ-^{32}P]ATP in the absence (**b**, **c**) or presence (**d**, **e**) of cyclic-AMP-dependent protein kinase as described for (**a**), the reaction was quenched with EDTA and incubation continued (30 min, 37 °C) in the absence (**b**, **d**) or presence (**c**, **e**) of regulator fraction. The two-dimensional separation of phosphopeptides involved high-voltage electrophoresis (horizontal, with the anode to the left) and chromatography (vertical). The origin is indicated by the arrowheads (lower left) and the two closely migrating major peptides are indicated by large arrows (upper right).

provided by studying the elution of these proteins during anion-exchange chromatography.

Initial studies were carried out in which freshly prepared cytosolic fractions from rat liver were subjected directly to DEAE-cellulose chromatography; a single peak of acetyl-CoA carboxylase activity was eluted with a salt gradient, emerging when the applied salt concentration was in the range 50–150 mM. As noted above, the acetyl-CoA carboxylase activity eluted from DEAE-cellulose displayed high sensitivity to citrate, exhibiting a $K_{cit.}$ of 0.25 ± 0.08 mM (mean $\pm$ S.E.M. for four separate preparations), confirming the coincident elution of regulator activity in the same column fractions. It is important to point out that all column fractions which did not contain acetyl-CoA carboxylase activity itself, including the protein fraction which did not bind to the column, were tested for regulator activity by the ability to activate exogenous acetyl-CoA carboxylase which had been highly purified by Sepharose–avidin chromatography (and which therefore exhibited very low sensitivity to citrate). No acetyl-CoA carboxylase regulator activity was detected in any of these other column fractions tested, suggesting that regulator was eluted only in the fractions which contained acetyl-CoA carboxylase. Identical chromatography was performed with a similar amount of liver cytosol protein which had been pre-incubated with sufficient purified avidin (0.1 mg/ml) to inhibit completely the endogenous acetyl-CoA carboxylase present. Column fractions were assayed for regulator activity with purified acetyl-CoA carboxylase. The acetyl-CoA carboxylase regulator activity was eluted at markedly higher salt concentration (range 200–300 mM) if the sample was incubated with avidin before DEAE-cellulose chromatography. The elution of acetyl-CoA carboxylase regulator at the higher salt concentration (200–300 mM) during DEAE-cellulose chromatography was also observed if the endogenous acetyl-CoA carboxylase was instead removed by routine Sepharose–avidin chromatography. These initial experiments prompted us to carry out a set of anion-exchange fractionations using an f.p.l.c. Mono-Q column. Acetyl-CoA carboxylase was purified through $(NH_4)_2SO_4$ precipitation or further purified to remove activator protein by Sepharose–avidin chromatography. These two preparations were applied separately to a Mono-Q column, eluted with a salt gradient, and column fractions were assayed for acetyl-CoA carboxylase activity. As shown in Figure 4(a), enzyme activity emerged as a

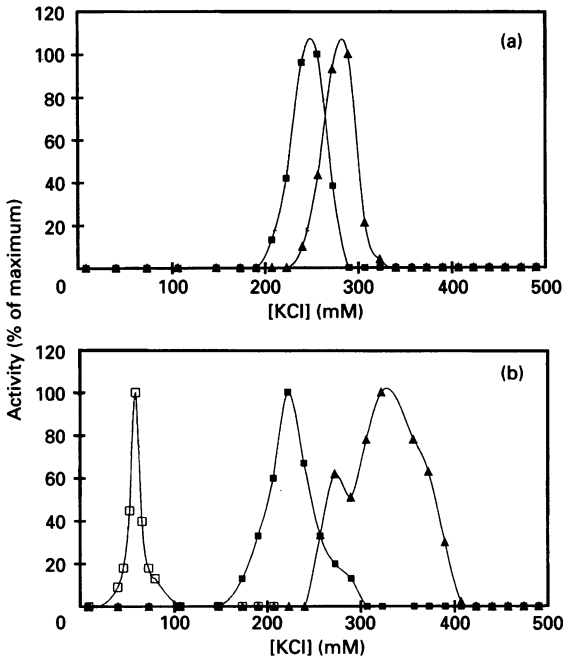

Figure 4 Evidence for formation of a complex between acetyl-CoA carboxylase and regulator

Preparations of acetyl-CoA carboxylase were subjected to Mono-Q chromatography (**a**) after partial purification through $(NH_4)_2SO_4$ precipitation (■) and purification by Sepharose–avidin chromatography (▲). Acetyl-CoA carboxylase activities in column fractions (1 ml) through the indicated salt gradient were determined after maximal activation with citrate (20 mM) and BSA (5 mg/ml) for 20 min (37 °C) before assay. Results are expressed as percentages of activity in the peak fractions, which were 17 and 36 m-units/ml respectively for the partially purified and affinity-purified preparations. A sample of the same partially purified preparation of acetyl-CoA carboxylase was completely inactivated by incubation (10 min, 20 °C) with avidin (0.1 mg/ml) and subjected to Mono-Q chromatography (**b**) under conditions identical with those in (**a**). Column fractions were analysed for immunoreactive acetyl-CoA carboxylase (■) or acetyl-CoA carboxylase regulator (▲). The migration of free avidin (□) was confirmed in a separate gradient fractionation. Maximum values for the peaks were: 0.15 O.D. unit (immunoreactive acetyl-CoA carboxylase by densitometric scanning of immunoblots), 0.055 A_{280} unit (avidin) and 36 units/ml (acetyl-CoA carboxylase regulator).

single symmetrical peak in each case, and was eluted slightly earlier when the regulator was still present (peak fractions corresponding to KCl concentration of 240–260 mM) than after removal of the regulator by Sepharose–avidin chromatography (peak fractions 270–290 mM KCl). As with the previous DEAE-cellulose chromatography, column fractions not containing acetyl-CoA carboxylase itself were tested for regulator by incubation with exogenous affinity-purified acetyl-CoA carboxylase. No evidence for regulator could be found in fractions eluted over the range 50–200 mM nor above 300 mM KCl, including a final 1 M KCl wash step.

The relative elution of free activator protein was subsequently confirmed after inactivation of acetyl-CoA carboxylase by treatment with avidin. The $(NH_4)_2SO_4$-purified acetyl-CoA carboxylase was incubated (10 min, 20 °C) with avidin (0.1 mg/ml) and then applied to a Mono-Q column. Column fractions were analysed for regulator activity by incubation with additional affinity-purified acetyl-CoA carboxylase. In addition, inactive acetyl-CoA carboxylase–avidin complex was detected by immunoblot analysis using anti-(acetyl-CoA carboxylase) antiserum, after SDS/PAGE and transfer to poly(vinylidene difluoride) membranes. The results shown in Figure 4(b) demonstrate that the elution of regulator was shifted considerably in the salt gradient, giving peak fractions over the range 320–390 mM KCl. In contrast, the inactive acetyl-CoA carboxylase–avidin complex emerged earlier (200–240 mM KCl) than native acetyl-CoA carboxylase. Free avidin, which was detected after a separate injection of purified protein under identical conditions, emerged as a sharp protein peak at 40–80 mM KCl consistent with the basic nature of this protein. These results demonstrate that the chromatographic properties of acetyl-CoA carboxylase regulator on anion-exchange columns are markedly affected by specific removal of active acetyl-CoA carboxylase after exposure to avidin.

DISCUSSION

The studies described in this report were initiated in an attempt to investigate further the observed loss of insulin-induced activation of acetyl-CoA carboxylase (as well as the loss of total activity) associated with Sepharose–avidin chromatography. Owing to the persistence of the effect of insulin during the early stages of enzyme purification observed in previous studies (see [3]), it seemed improbable that activation of acetyl-CoA carboxylase was caused by the interaction with a low-molecular-mass ligand. We therefore hypothesized that a non-dialysable ligand, able to interact with acetyl-CoA carboxylase with high affinity, may be involved in the activation seen in response to insulin. The studies presented here provide direct evidence for the existence of an endogenous regulator of acetyl-CoA carboxylase in rat liver. Initial observations indicated that deactivation of acetyl-CoA carboxylase did indeed occur as a result of Sepharose–avidin affinity chromatography and that this was not due to loss of intrinsic activity, but rather could be reversed upon re-addition of the protein fraction removed during chromatography. This re-activation of acetyl-CoA carboxylase at physiological citrate concentrations was rapid, was dependent on the concentration of added regulator fractions and occurred in the absence of MgATP. The inactivation of the acetyl-CoA carboxylase regulator by exposure to acid, high temperature or carboxypeptidase, together with further purification involving ion-exchange and size-exclusion chromatography, suggests it is a protein with molecular mass of approx. 75 kDa. It is worth emphasizing that the time course for the citrate-dependent activation of acetyl-CoA carboxylase before assay appears very similar in the absence or presence of regulator. It seems reasonable to suggest that the activation may be a multi-step process involving interactions between acetyl-CoA carboxylase regulator and citrate as well as polymerization of enzyme dimers. The rate-limiting step in this complex process would appear to occur after the interaction between regulator and acetyl-CoA carboxylase. It is worth noting that the time course for activation of acetyl-CoA carboxylase in the presence of citrate is similar to that reported previously for enzyme from rat adipose tissue, which displayed a half-time of approx. 5 min [21]. Similar values have been observed by us for acetyl-CoA carboxylase in adipose or liver extracts, as well as for purified enzyme from these sources. These values are in marked contrast with extremely rapid activation (half-time < 1 s) observed with concentrated preparations of enzyme purified from chick liver [25]. At present, we are unable to conclude if the differences reflect species differences in the enzymes, the effect of enzyme concentration during pre-incubation, or other factors.

In order to investigate the mechanism by which the regulator may lead to the activation of acetyl-CoA carboxylase, several possibilities have been considered. Arguably, the most likely possibility is that the regulator may act through de-

phosphorylating acetyl-CoA carboxylase. A number of different studies have demonstrated that dephosphorylation of acetyl-CoA carboxylase *in vitro* does indeed lead to enzyme activation [26–29] and that the effects of adrenaline induced within intact fat-cells can be reversed upon subsequent dephosphorylation of the enzyme after cell homogenization [11,30]. Although the ability reversibly to remove and add back regulator would argue against a dephosphorylation mechanism, we nevertheless prepared ^{32}P-labelled acetyl-CoA carboxylase by incubation with the catalytic subunit of cyclic-AMP-dependent protein kinase and then investigated the phosphorylation state of acetyl-CoA carboxylase during incubations in the presence of the regulator which led to enzyme activation. Little or no evidence for significant total dephosphorylation of acetyl-CoA carboxylase could be detected. Phosphopeptide analysis confirmed that the phosphorylation of the major site on acetyl-CoA carboxylase phosphorylated by cyclic-AMP-dependent protein kinase (presumably the peptide containing serine-77) was completely unaffected by incubation with regulator. Some dephosphorylation of minor peptides was observed, but this accounted for removal of less than 0.05 mol of P/mol of subunit. In addition, the phosphorylation of these minor sites is not associated with any inactivation of acetyl-CoA carboxylase (see [3,18,30]). Experiments with other phosphoprotein substrates (histone and casein) also confirmed that little or no protein phosphatase activity could be detected in the most purified preparations of acetyl-CoA carboxylase regulator. The small activation in the absence of regulator (Figure 1b) might be explained by the action of endogenous protein phosphatase activity on sites of the enzyme already phosphorylated before purification. It is possible that the regulator may lead to dephosphorylation of sites on acetyl-CoA carboxylase other than those phosphorylated by cyclic-AMP-dependent protein kinase (used in this study). However, of the multiple phosphorylation sites on acetyl-CoA carboxylase, only the phosphorylation of serine residues 77 (phosphorylated by cyclic-AMP-dependent protein kinase) and 79 have been demonstrated to occur rapidly, with parallel and marked decreases in enzyme activity [3]. To explain the results presented here, it would be necessary to invoke specific dephosphorylation of serine-79, with no parallel dephosphorylation of serine-77. No protein phosphatase with such properties has been identified. Since acetyl-CoA carboxylase is readily activated by mild proteolysis [31], it is important to ensure that no such modification occurred under the conditions described for activation by the regulator fraction. This possibility seems very unlikely, in view of the reversible deactivation and subsequent re-activation of acetyl-CoA carboxylase upon removal and re-addition of the regulator fraction. Furthermore, there is no apparent change in the subunit size of ^{32}P-labelled acetyl-CoA carboxylase after incubation with the regulator fraction, nor any appearance of low-molecular-mass ^{32}P-labelled fragments.

Considering the above evidence against a role for dephosphorylation or proteolysis in enzyme activation, we next explored the possibility that the regulator may directly associate with acetyl-CoA carboxylase. It was already apparent that the regulator co-purified with acetyl-CoA carboxylase through $(NH_4)_2SO_4$ precipitation and ion-exchange chromatography. We also observed that the regulator migrated differently on DEAE-cellulose (or Mono-Q) chromatography when applied in the presence or absence of active acetyl-CoA carboxylase. The effect of removal or inactivation of acetyl-CoA carboxylase on the binding of the regulator to these anion-exchange columns strongly suggests that close association in solution, perhaps in a strictly stoichiometric relationship, occurs. That this association is not simply a non-specific interaction is suggested by two further observations. Firstly, the effects of the regulator are observed in the presence of a large number of other endogenous liver proteins as well as with the further addition of BSA (5 mg/ml). Secondly and more specifically, the interaction between acetyl-CoA carboxylase and the regulator is disrupted by the biotin-binding protein avidin, which is known to bind to the active site biotinyl prosthetic group of the enzyme. The effects of avidin are observed during Sepharose–avidin chromatography (as shown in Figure 1) and also by the addition of free avidin. After this latter treatment acetyl-CoA carboxylase is irreversibly inactivated, and regulator activity may be recovered for subsequent addition to affinity-purified enzyme.

The physiological significance of the acetyl-CoA carboxylase regulator identified in these studies remains to be resolved. Further investigation will be required to define the nature of interaction and the mechanism of activation. Kinetically, the regulator influences the sensitivity of acetyl-CoA carboxylase to citrate as well as increasing $V_{max.}$. Effects on other regulatory factors is, however, also possible. For example, the regulator may remove a feedback or other regulatory inhibitor. The most likely candidates may be fatty acyl-CoA, malonyl-CoA or CoA. The first of these possibilities is unlikely, as inclusion of BSA in all incubations and assays should immobilize any free long-chain fatty acids or esters. Malonyl-CoA might eventually accumulate in enzyme assays to μM levels, but all assays were carried out under conditions where reaction rates were linear and initial rates were calculated. The significance of CoA availability is more difficult to refute, since it may be an extremely potent inhibitor, although inhibition appears to be dependent on Mg^{2+} and ATP, which are omitted from incubations with regulator fractions.

Even considering the possible factors discussed above, the regulator described here seems to differ appreciably from any other regulator of acetyl-CoA carboxylase described, based on molecular size and mechanism of action. The affinity with which the regulator interacts with acetyl-CoA carboxylase, though not determined quantitatively, must be appreciable to allow the observed co-purification. Although the evidence so far described does not directly address the possible role of this regulator in insulin-induced activation of acetyl-CoA carboxylase, the studies suggest that it will be important to obtain a fuller understanding of the properties of the regulator and of its interaction with this key hormone-sensitive lipogenic enzyme.

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