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Enzymatically inactive forms of acetyl-CoA carboxylase in rat liver mitochondria

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Biotinyl proteins were labelled by incubation of SDS-denatured preparations of subcellular fractions of rat liver with [^{14}C]methylavidin before polyacrylamide-gel electrophoresis. Fluorographic analysis showed that mitochondria contained two forms of acetyl-CoA carboxylase [acetyl-CoA:carbon dioxide ligase (ADP-forming) EC 6.4.1.2], both of which were precipitated by antibody to the enzyme. When both forms were considered, almost three-quarters of the total liver acetyl-CoA carboxylase was found in the mitochondrial fraction of liver from fed rats while only 3.5% was associated with the microsomal fraction. The remainder was present in cytosol, either as the intact active enzyme or as a degradation product. The actual specific activity of the cytosolic enzyme was approx. 2 units/mg of acetyl-CoA carboxylase protein while that of the mitochondrial enzyme was about 20-fold lower, indicating that mitochondrial acetyl-CoA carboxylase was relatively inactive. Fractionation of mitochondria with digitonin showed that acetyl-CoA carboxylase was associated with the outer mitochondrial membrane. The available evidence suggests that mitochondrial acetyl-CoA carboxylase represents a reservoir of enzyme which can be released and activated under lipogenic conditions.

INTRODUCTION

Lipogenesis in rat liver rapidly responds to dietary alteration [1,2]. Several mechanisms have been reported by which diet might affect the activity of acetyl-CoA carboxylase [acetyl-CoA:carbon dioxide ligase (ADP-forming) EC 6.4.1.2], generally regarded as the regulatory enzyme in fatty acid biosynthesis [2]. These include metabolic effectors such as citrate [3], covalent modification [4] and changes in rates of synthesis and degradation of the enzyme [5–7]. By using a recently developed method to measure the quantity of acetyl-CoA carboxylase, based upon biotin content rather than enzyme activity [8,9], we found evidence that suggested that there is an additional, previously unrecognized, mechanism by which diet [10] and alloxan diabetes [11] alter the activity of acetyl-CoA carboxylase. The mechanism involves changing the subcellular distribution of the enzyme between active cytosolic and relatively inactive mitochondrial forms. In this report, we provide evidence that there are substantial amounts of two relatively inactive, immunologically precipitable, forms of acetyl-CoA carboxylase associated with the outer membrane of rat liver mitochondria.

MATERIALS AND METHODS

Animals

Sprague–Dawley rats (200–300 g) were fed a high carbohydrate, low fat diet [12]. Water was provided *ad libitum*. Lighting was controlled so that the rats were exposed to a light-dark cycle of 12 h each. Rats were killed by decapitation 2–3 h into the dark cycle. Liver was quickly removed, cooled in ice, and weighed.

Subcellular fractionation of liver

Liver was homogenized in 2 vol. of cold (0–4 °C) sucrose–mannitol buffer described by Greenawalt [13], using a loosely fitting Potter–Elvehjem homogenizer. Mitochondria were prepared [13] by differential centrifugation and washed three times. The resulting mitochondrial pellet was resuspended in a small volume of homogenizing media.

For the preparation of cytosolic and microsomal fractions, the homogenate was centrifuged at 27000 ***g*** for 20 min. This mitochondrial-free supernatant was then re-centrifuged at 105000 ***g*** for 60 min. Supernatant (cytosol) was removed and the microsomal pellet was resuspended in 0.5 vol. of homogenizing media. Microsomes were recovered by a second high-speed centrifugation and resuspended in a small volume of homogenizing media.

Cross-contamination of subcellular fractions was measured by assay of marker enzymes: lactate dehydrogenase (EC 1.1.1.27) for cytosol [14]; cytochrome *c* oxidase (EC 1.9.3.1) for inner mitochondrial membrane [15]; monoamine oxidase (EC 1.4.3.4) for outer mitochondrial membrane [16]; glucose 6-phosphatase (EC 3.1.3.9) for microsomes [17].

Sub-mitochondrial fractionation

The location of acetyl-CoA carboxylase within mitochondria was determined using various concentrations of digitonin to solubilize membranes [18]. Isolated, washed mitochondria were resuspended in Greenawalt's homogenization buffer [13] to a concentration of 77 mg of protein/ml. An equal volume of various concentrations of digitonin (recrystallized from ethanol) dissolved in the same buffer was added and the mixture was maintained

Abbreviation used: PAGE, polyacrylamide-gel electrophoresis.

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in an ice bath for 10 min before centrifugation (2 min) in an Eppendorf table-top centrifuge. The supernatant was assayed for marker enzymes: adenylate kinase (EC 2.7.4.3) (soluble between the inner and outer mitochondrial membrane) [19]; monoamine oxidase (outer mitochondrial membrane) [16]; malate dehydrogenase (EC 1.1.1.37) (soluble matrix enzyme) [20]; and cytochrome *c* oxidase (mitochondrial matrix) [15]. Activity and quantity of acetyl-CoA carboxylase was also measured as described below.

Acetyl-CoA carboxylase activity and quantity

Acetyl-CoA carboxylase activity was measured by fixation of radioactive bicarbonate in acid-stable form in 1 min at 37 °C [12] after preincubation of either cytosol, mitochondria or sub-mitochondrial fractions with 10 mM-citrate for 30 min at 37 °C. Enzyme preparations were maintained in an isotonic media during preincubation but were assayed in a hypotonic media. Radioactive products from this assay have been shown to comigrate with malonate and malonyl-CoA [10] during t.l.c. [21]. Enzyme activity (1 unit) is defined as the fixation of 1 μmol of product in acid-stable form in 1 min.

Acetyl-CoA carboxylase and other biotinyl proteins were detected by incubation of SDS-denatured preparations with [^{14}C]methylavidin (528 d.p.m./pmol) before SDS/PAGE and fluorography [9]. For quantitative determination, radioactive bands were cut from the gel, resolubilized [22] and assayed for radioactivity. After correcting for quenching using an internal standard, the quantity of radioactive avidin bound was calculated and related to the quantity of protein subjected to SDS/PAGE as previously described [9]. Protein was determined by dye binding [23] with bovine serum albumin as a standard.

Antibody preparation

Acetyl-CoA carboxylase (1 mg), purified from rat liver by polyethylene glycol precipitation and Sepharose 4B column chromatography [24], was injected under the skin of the necks of sheep along with Freund's Complete Adjuvant. Purified enzyme (1 mg) was again injected 3 weeks later but with Freund's Incomplete Adjuvant. Blood was collected 4 weeks later, serum was prepared and heated for 30 min at 56 °C. Sodium azide (0.01%) was added as a preservative and serum was stored at 0–4 °C. The titre of the antibody against purified acetyl-CoA carboxylase (7.5 units/mg of protein) was about 10 units/ml.

Materials

Scintillation solution for fluorography (Enlightning), [^{14}C]methylavidin and [^{14}C]bicarbonate (New England Nuclear, Boston, MA, U.S.A.), SDS and electrophoresis chemicals (Bio-Rad, Richmond, CA, U.S.A.), biochemicals (Sigma Chemical Co., St. Louis, MO, U.S.A.) and X-ray film (Eastman–Kodak, Rochester, NY, U.S.A.) were obtained from the indicated sources.

RESULTS AND DISCUSSION

Fluorographic analysis has shown [9–11] that SDS-denatured preparations of rat liver mitochondria contained two biotinyl proteins with subunit molecular masses much larger than those of known mitochondrial biotinyl enzymes. The largest of these, which has a molecular mass of about 280 kDa, was tentatively identified as a form of acetyl-CoA carboxylase on the basis of similarity of subunit molecular mass to that of the cytoplasmic enzyme and the detection of acetyl-CoA carboxylase activity in mitochondria [10]. [The molecular mass values given in the text and Figures include the mass (16 kDa) of monomeric avidin unless otherwise

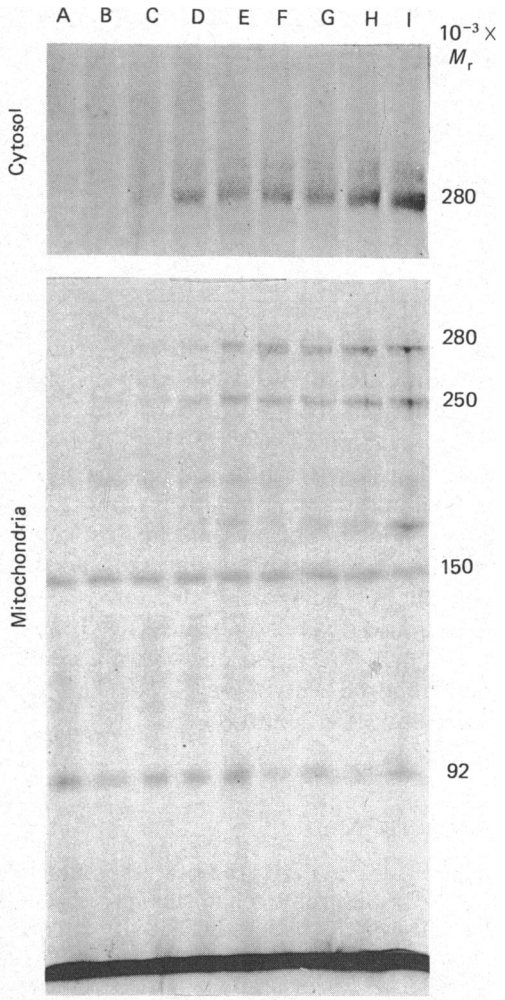

Fig. 1. Fluorograph of biotinyl proteins remaining in the supernatant after antibody precipitation of acetyl-CoA carboxylase

Various dilutions of sheep serum antibody to acetyl-CoA carboxylase were added to cytosol and mitochondrial fractions of rat liver after Triton X-100 (0.1% final concn.) and the preparations were incubated for 1 h at 37 °C and then for 16 h at 0–4 °C. The supernatant was separated by centrifugation and analysed for biotinyl proteins. Mass, shown in kDa, was determined by comparison with migration of proteins of known molecular mass [8] and includes the mass of avidin subunit (16 kDa). The smaller radioactive proteins have been identified on the basis of molecular mass [9,10] as known biotinyl proteins: 150 kDa, pyruvate carboxylase (EC 6.4.1.1); and 92 kDa, doublet which includes 3-methylcrotonyl-CoA carboxylase (EC 6.4.1.4) and propionyl-CoA carboxylase (EC 6.4.1.3). Final dilutions of antibody for lanes A–H, respectively, were 4, 10, 20, 40, 80, 160, 320 and 640. Lane I was incubated without antibody.

Table 1. Subcellular distribution of marker enzymes in rat liver homogenates

Marker enzyme activities were measured and the percentage of the summation of activities in each subcellular fraction was calculated. The formation or loss of 1 μmol of product or reactant per min is defined as 1 unit of activity. A portion of each of these same preparations was used for analysis of biotinyl enzymes shown in Fig. 2.

Enzyme	Activity (units/g of liver)	Distribution (%)		
		Cytoplasm	Mitochondria	Microsomes
Lactate dehydrogenase	334	93.6	3.3	3.0
Cytochrome *c* oxidase	15	0	99.0	1.0
Monoamine oxidase	69	0	97.0	2.4
Glucose 6-phosphatase	9.4	2.1	13.3	84.5

stated.] The fluorograph shown in Fig. 1 confirmed this identification because antibody to acetyl-CoA carboxylase precipitated the 280 kDa mitochondrial protein as well as the subunits of the active form of acetyl-CoA carboxylase in cytosol.

In addition, antibody to acetyl-CoA carboxylase precipitated the smaller, 250 kDa biotinyl protein but did not precipitate the three known mitochondrial biotinyl proteins (Fig. 1). This suggests that the 250 kDa biotinyl protein is another form of acetyl-CoA carboxylase although such an interpretation must be made with caution since biotinyl enzymes may exhibit considerable homology [25]. An alternative but implausible explanation for the 250 kDa protein is that it represents a biotinyl protein not previously described since this protein is clearly heavier than the subunits of any known biotinyl protein other than acetyl-CoA carboxylase. Thus, our data suggest that rat liver mitochondria contain two different forms of acetyl-CoA carboxylase (264 and 234 kDa after subtraction of 16 kDa for monomeric avidin).

The finding of a substantial quantity of acetyl-CoA carboxylase in mitochondria [9–11] (Fig. 1) was unexpected because, since its original discovery as a soluble enzyme [26], it has generally been considered to be associated with the cytosolic compartment [2]. However, there have been reports that some acetyl-CoA carboxylase activity was associated with subcellular particles, the two latest of which indicated that activity of the enzyme was found in high-speed precipitates of homogenates from liver [27] and adipose tissue [28]. This was interpreted as evidence of an association of the enzyme with microsomes.

Previous work on subcellular distribution of acetyl-CoA carboxylase relied, by necessity, on measurement of enzyme activity. Therefore the distribution of the enzyme among mitochondria, microsomes and cytosol was re-examined on the basis of enzyme quantity. Differential centrifugation of carefully homogenized rat liver provided subcellular fractions with minimal cross-contamination as evidenced by the distribution of marker enzymes (Table 1). Fluorographic analysis (Fig. 2) showed that the intact subunit form of acetyl-CoA carboxylase in cytosol occurred as a doublet with an average mass of about 280 kDa. We have repeatedly observed that the cytosolic enzyme occurs as a doublet in both purified [9,24] and crude [8–11] preparations subjected to SDS/PAGE. Cytosol also contained a biotinyl protein with a mass of about 150 kDa which has been identified [8] as a biotin-containing peptide produced when the intact enzyme undergoes proteolysis [29]. The 150 kDa cytosolic biotinyl protein has a mass almost identical to that of mitochondrial pyruvate carboxylase and therefore could not be distinguished from pyruvate carboxylase which leaked out of the mitochondria. However, marker enzyme assays (Table 1) indicated that there was minimal contamination of cytosol with mitochondrial enzymes.

Fluorographic analysis showed that microsomes contained biotinyl proteins with similar subunit molecular masses to those in cytosol except that microsomes contained substantially less of them (Fig. 2). In contrast, both of the mitochondrial forms of acetyl-CoA

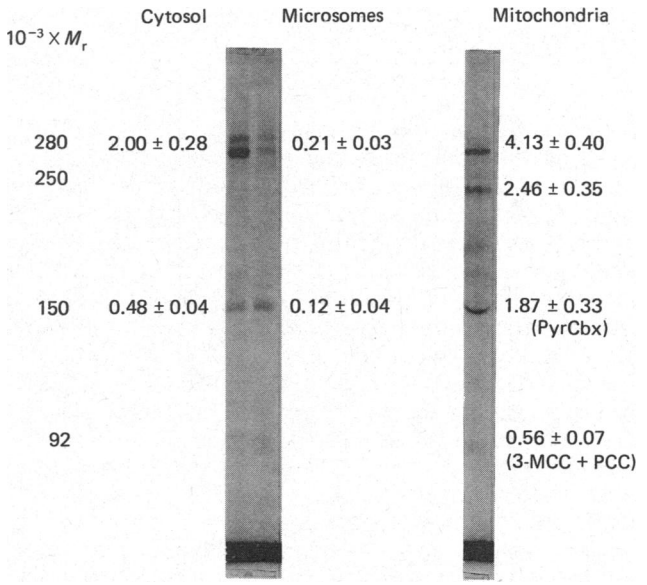

Fig. 2. Distribution of biotinyl proteins among subcellular fractions of rat liver

Subcellular fractions of rat liver were prepared and analysed for biotinyl proteins. The extent of cross-contamination was determined on the same fractions by assay of marker enzyme activities as shown in Table 1. Values are expressed as nmol of biotin enzyme/g of liver ± S.E.M. ($n = 6$). Except for mitochondrial pyruvate carboxylase (PyrCbx), and the doublet including 3-methylcrotonyl-CoA carboxylase (3-MCC) and propionyl-CoA carboxylase (PCC), the values are for various forms of acetyl-CoA carboxylase as identified in the text.

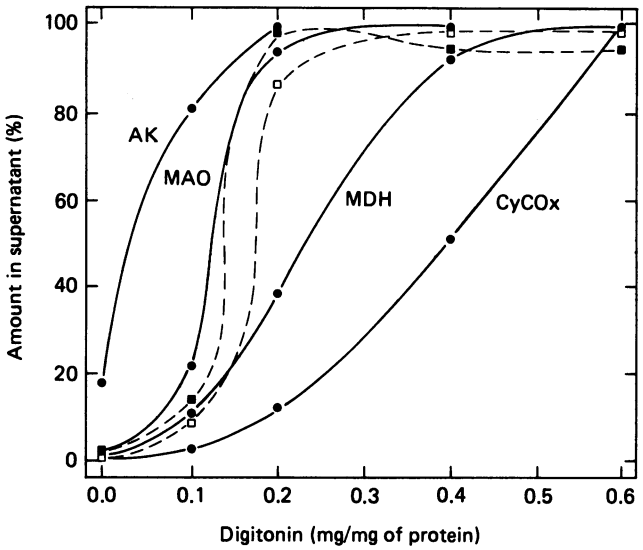

Fig. 3. Activity and quantity of acetyl-CoA carboxylase and activity of marker enzymes solubilized by various concentrations of digitonin

Results are shown as the percentage of total activity of marker enzymes recovered in the supernatant. Marker enzymes (solid lines) include: adenylate kinase (AK; soluble between outer and inner membranes); monoamine oxidase (MAO; outer membrane); malate dehydrogenase (MDH; soluble matrix); and cytochrome *c* oxidase (CyCOx; matrix). Broken lines show the percentage of the total acetyl-CoA carboxylase activity (open squares) and quantity (closed squares) of the sum of both forms of mitochondrial acetyl-CoA carboxylase solubilized by digitonin. The quantity of the larger (280 kDa) and smaller (250 kDa) biotinyl proteins followed identical curves.

carboxylase were present in larger amounts than either cytosolic or microsomal forms of the enzyme. In terms of percentage distribution, the mitochondrial fraction contained almost three-quarters of the total acetyl-CoA carboxylase in liver from fed rats while the microsomes contained only 3.5%. This small amount of enzyme associated with microsomes could be attributed to contamination with enzyme from cytosol and, perhaps, mitochondria. Thus, our results show that rat liver contains particulate forms of acetyl-CoA carboxylase but these are associated with mitochondria, not microsomes, as previously suggested [27,28].

Even though our results (Fig. 2) show that there was more than 3 times as much acetyl-CoA carboxylase associated with mitochondria than there was in cytosol, previous investigations which relied upon enzyme activity measurements did not detect this large mitochondrial pool of enzyme because it is relatively inactive. For example, in a typical experiment, we found that the apparent specific activity of acetyl-CoA carboxylase in cytosolic and mitochondrial fractions of liver from fed rats was 16.4 ± 1.1 and 1.81 ± 0.11 munits/mg of protein (mean $\pm$ S.E.M., $n = 3$), respectively. This suggested that most of the enzyme was cytosolic, in agreement with numerous previous observations [1–7]. However, when the quantity of enzyme was determined in each of these fractions from the same rats, it was found that cytosol contained only 3.09 ± 0.23 nmol/g of liver while the quantity of the 280 and 250 kDa subunits in mitochondria was 5.74 ± 0.45 and 4.30 ± 0.24 nmol/g, respectively. Thus, in contrast with the apparent distribution of the enzyme on the basis of specific enzyme activity, quantitative determination showed that most of the acetyl-CoA carboxylase in liver of fed rats was associated with mitochondria.

Since both the activity and quantity of acetyl-CoA carboxylase was determined in cytosol and mitochondria, the actual specific activity of the enzyme could be calculated. The results showed that the actual specific activity of the cytosolic enzyme was 2.05 ± 0.05 units/mg of acetyl-CoA carboxylase, in close agreement with reported actual specific activity values of crude [10] and rapidly purified [30] enzyme. In contrast, the actual specific activity of the mitochondrial forms of the enzyme was 0.09 ± 0.01 units/mg of enzyme protein. Thus, cytosolic acetyl CoA carboxylase was more than 20 times as active as the mitochondrial forms of the enzyme.

Previous work has shown that fasting (48 h) [10] and acute alloxan diabetes (3 days) [11] resulted in a decrease in activity and amount of rat liver cytosolic acetyl-CoA carboxylase with an apparent compensatory increase in the quantity of mitochondrial forms of the enzyme when compared with the distribution of enzyme in liver of fed control rats. On the other hand, refeeding (48 h) of previously fasted (48 h) rats [10] resulted in an increase in activity and amount of cytosolic enzyme with again an apparent compensatory decrease in the quantity of mitochondrial forms. These results suggested that mitochondrial acetyl-CoA carboxylase represents a reservoir of relatively inactive enzyme which can be released and activated under lipogenic conditions. Such a mechanism would obviously be facilitated if acetyl-CoA carboxylase were associated with the outer mitochondrial membrane.

When isolated mitochondria were fractionated by incubation with various amounts of digitonin, the results (Fig. 3) indicated that both forms of mitochondrial acetyl-CoA carboxylase were indeed associated with the outer mitochondrial membrane. That is, activity of mitochondrial acetyl-CoA carboxylase was released into the supernatant at about the same digitonin concentration required for the solubilization of an outer mitochondrial membrane marker enzyme, monoamine oxidase. Furthermore, both of the mitochondrial forms of acetyl-CoA carboxylase were shown by quantitative determination to be solubilized at about the same digitonin concentration required to release monoamine oxidase. Lower concentrations of digitonin resulted in the release of a soluble enzyme located between the inner and outer membrane (adenylate kinase) while higher concentrations were required to release either soluble (malate dehydrogenase) or particulate (cytochrome *c* oxidase) matrix enzymes.

In confirmation of our results, K.-H. Kim (personal communication) has found that clone-specific antibody to acetyl-CoA carboxylase, when attached to an electron dense adduct, can be shown by electron microscopy to be bound to mitochondria isolated from liver of fasted rats.

Our results do not contradict the long-held view that fatty acid biosynthesis *de novo* is a cytosolic process. Our evidence indicates, however, that, in addition to an active cytosolic form of acetyl-CoA carboxylase, rat liver contains substantial amounts of the enzyme associated with the outer mitochondrial membrane. Previous results

[10,11] indicate that acetyl-CoA carboxylase, like hexokinase [31], can be considered an ambiquitous enzyme since the subcellular distribution of it depends upon the physiological state of the animal. The available evidence suggests that relatively inactive mitochondrial forms of acetyl-CoA carboxylase can be released/activated under lipogenic conditions. The mechanism(s) by which this might occur has not been established.

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