Immunological analysis of acetyl-CoA carboxylase mass, tissue distribution and subunit composition

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Changes in the mass and subunit structure of liver acetyl-CoA carboxylase (ACC) accompany altered nutrition *in vivo*. Enzyme activity in different tissues and cell lines is also, in part, determined by variations in both total mass and ACC isoenzyme composition. ACC isoenzyme mass and hetero-/homo-isoenzyme association were quantified by three sandwich e.l.i.s.a. assays, i.e. an avidin-based assay that measured total isoenzyme mass and two antibody-sandwich assays which measure polypeptide association. Results from the avidin-based assay reveal that the two major isoenzymes, of molecular mass 265 kDa (ACC 265) and 280 kDa (ACC 280), are present in markedly variable concentration in several rat and mouse tissues and in cell lines of rat and mouse origin. Hepatic ACC mass has been reported to be distributed between mitochondrial and cytosolic fractions and to undergo only a change in subcellular distribution without alteration in total mass on induction/repression of activity *in vivo* [Roman-Lopez, Shriver, Joseph & Allred (1989) *Biochem. J.* **260**, 927–930]. However, in the present study, immunoblotting and e.l.i.s.a. analysis reveals that, in rat liver, the mass of both isoenzymes is predominantly cytosolic in distribution, is markedly diminished on fasting and rises 6–8-fold on refeeding of a high-carbohydrate diet. These data support the results of several other investigations of hepatic ACC mass, and are consistent with known nutritionally altered changes in ACC mRNA content. By the two antibody-sandwich e.l.i.s.a. assays, isoenzyme complexes either composed of both ACC 280 and 265 or with multiple copies of ACC 265 are detectable in rat liver enzyme; their concentration varies independently of total ACC mass with the nutritional state of the rat, being lowest in fasting and highest on fasting/refeeding. E.l.i.s.a. analysis, applicable to crude tissue/cell extracts, provides a simple, sensitive and quantitative measurement of ACC mass and subunit composition. Its use may permit needed quantitative insight into the role of variable total ACC and isoenzyme mass and of alterations in ACC subunit composition that occur *in vivo* or in isolated cells in response to a variety of hormonal and nutritional influences.

INTRODUCTION

Acetyl-CoA carboxylase (ACC; EC 6.4.1.2) play a pivotal role in regulating the rate of fatty acid synthesis and fatty acid oxidation (through its product, malonyl-CoA). The total activity of ACC in several tissues, accounted for by one or more isoenzymes, is regulated over two different time frames [1–3]. Rapid regulation (minutes) involves changes in covalent phosphorylation, allosteric regulation and conversions between a relatively inactive protomeric species and more active oligomers/polymers [1–3]. Long-term (hours–days) regulation involves changes in enzyme mass, in part associated with changes in mRNA content [4,5], but also changes in enzyme specific activity owing to the superimposition of the aforementioned rapid regulatory mechanisms [6–8]. Thus the characterization in any system of changes in overall ACC activity necessarily involves multiple measurements, including phosphorylation state, enzyme size, total mass and relative isoenzyme content.

Early measurements of ACC mass relied heavily on immunotitration of enzyme activity measured during a period of enzyme preincubation [9,10]. However, this technique, which provides only an indirect measure of ACC mass and does not report actual enzyme protein, has several pitfalls. First, the enzyme is activated during the preincubation, especially in crude preparations; this is now recognized to be accompanied by enzyme dephosphorylation [11]. Thus, the ‘mass’ estimates reflect not only ACC protein content, but also its phosphorylation state and the activity/content of protein phosphatase(s). Second, ACC

is quite susceptible to proteolysis during such incubation, which leads to enzyme activation [12], and this has not been controlled for in immunotitration experiments. Third, it was not recognized in the cited studies that more than one isoenzymic species, which are distinguishable by citrate and acetyl-CoA kinetics, may be contributing to overall ACC activity in certain tissues [13]; thus the reactivity of antibody and the concentration of reactants in the enzyme assay become critical. Lastly, immunotitration is an insensitive technique, as it relies on activity measurement.

Measurements of enzyme size (protomer/polymer state) have relied on either sucrose-density-gradient centrifugation or molecular-sieve chromatography [8,14,15]; these techniques are laborious or limited in the number of samples that can be assessed conveniently. They have not been applied with recognition of the now-described isoenzymic heterogeneity, and employ relatively insensitive measurements of changes in molecular size as shifts in enzyme activity or A_{280} .

Several issues about enzyme mass and distribution have not been resolved. For example, it has been suggested that, contrary to the results obtained in several laboratories using immunotitration, physical methods, enzyme isolation and immunoblotting [6–10,16], there is no change in total hepatic ACC mass during nutritional induction/repression *in vivo* [17–21]. Instead, it is claimed that there is a latent inactive pool of ACC in a mitochondrial fraction in fasted rats that translocates to the cytosol on nutritional induction, or conversely accumulates in the mitochondria during nutritional repression. These conclusions have relied exclusively on measurement of ACC mass by

Abbreviations used: ACC, acetyl-CoA carboxylase; PBS, phosphate-buffered saline.

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a technique employing [^3H]avidin binding coupled with SDS/polyacrylamide-gel electrophoresis and on assay of enzyme activity in cellular fractions after a prolonged period of pre-incubation [17,18].

The availability of ACC isoenzyme-specific antibodies has permitted the use of quantitative immunoblotting to address some of these issues and to circumvent some of the methodological problems [16]. However, this technique is laborious, slow, and subject to technical problems associated with enzyme transfer to nitrocellulose and the quality of the radioautography. In the present study, we have used novel sandwich e.l.i.s.a. assays that permit the ready measurement of total ACC mass, mass of individual isoenzymes and enzyme-subunit composition. These assays, together with immunoblotting, have been applied to the analysis of both crude tissue extracts and purified enzyme obtained from several rodent tissues and cell lines in order to delineate total ACC and isoenzyme mass, subcellular and tissue distribution, and isoenzyme hetero-/homo-association.

METHODS AND MATERIALS

Tissue/cell harvesting and subcellular fractionation

Tissues were harvested from male and female (non-pregnant and pregnant) Sprague-Dawley rats (150–250 g), lactating female Sprague-Dawley rats and male Balb/c3 mice. In selected experiments, rats were fasted for 48 h (fasted), maintained on normal rat chow (fed) or refed on a high-carbohydrate diet (Bioserv) for 48 h after a 48 h period of fasting. Tissues were removed and immediately homogenized in an ice-cold buffer containing Tris/HCl (50 mM; pH 7.5 at 4 °C), NaF (100 mM), β -mercaptoethanol (10 mM), EDTA (2 mM), sucrose (0.25 M) and seven protease inhibitors, as in [13]. Samples of whole-tissue homogenates were removed for immunoblot analysis, and the homogenate was then centrifuged at 160 000 *g* for 60 min; this high-speed supernatant (cytosol) was used for standard e.l.i.s.a. analysis. In some experiments, male-rat liver from all three nutritional states was homogenized in a low-ionic-strength sucrose/mannitol buffer that also contained the same protease inhibitors; subcellular fractions (cytosol, a mitochondrial fraction and microsomes) were then isolated by differential centrifugation and washing, as in [17].

The cytosolic contents of all cell lines were harvested by digitonin lysis, as in [22]. Fao and H356A-1 Reuber hepatoma cells were cultured as in [22]. 3T3-L1 adipocytes were grown and differentiated as previously detailed [23]. L6 myocytes and H9c2 embryonic cardiomyocytes were grown in Dulbecco's modified Eagle's medium with 10% (v/v) fetal-bovine serum and penicillin/streptomycin. RMo myoblasts were initially grown in Dulbecco's modified Eagle's medium with 10% (v/v) calf serum and penicillin/streptomycin, followed by differentiation/fusion in the same medium with 0.5% calf serum [24]. Other cell lines, as cultured in 100 mm-diam. dishes or six-well plates, were gifts from other investigators.

Antibodies and antibody purification: immunoblotting

Five different antibodies were used in various aspects of these studies. The production, purification and specificity of these antibodies are detailed in [13]. Where necessary, antibodies were biotinylated by a standard procedure [25]. For use in one of the sandwich e.l.i.s.a. assays, the *N*-terminal anti-peptide IgG was purified by peptide-affinity chromatography (*N*-terminal peptide immobilized on thiopropyl-Sepharose), by a standard procedure [26]. In this purification, ACC-reactive antibody was obtained in both the acidic and basic washes of the affinity column, which were collected into 0.1 vol. of BSA (10 mg/ml) to minimize

adsorptive loss; those antibodies eluted in the basic wash were used in the present study.

Immunoblotting was performed with these antibodies after electrophoresis on SDS/5% polyacrylamide gels, as previously detailed [13,16].

Sandwich e.l.i.s.a. assays

Three such assays were used in the present study. For the measurement of total ACC isoenzyme mass, egg avidin (3 μg /well; 100 μl) was added to micro-titre plates in phosphate-buffered saline (PBS; 150 mM-NaCl/5 mM-sodium phosphate, pH 7.4) and incubated for 2 h at 37 °C. The wells were then washed five times with PBS and incubated at 37 °C overnight after addition of 200 μl of a blocking solution [PBS containing 5% BSA (Fraction V) and 0.05% NaN_3]. These plates were then either used the next day or stored at –70 °C. For use, they were washed five times with PBS; tissue/cell extract or purified ACC [diluted as necessary in a buffer containing Tris/HCl (50 mM, pH 7.5), β -mercaptoethanol (10 mM) and fatty-acid-free BSA (1 mg/ml)] was then added in a volume of 50–100 μl , followed by a 2 h incubation at 37 °C. After additional washing with PBS, monospecific antibodies [rabbit anti-*N*-terminal IgG (0.8 μg /well) for ACC 265 or ACC 7AD3 monoclonal (0.3 μg /well) for ACC 280] were added (50–100 μl) diluted in blocking buffer, followed by a 2 h incubation at 37 °C. The wells were again washed, and anti-(rabbit Ig)-alkaline phosphatase (15 ng) or anti-(mouse Ig)-alkaline phosphatase (15 ng) conjugate diluted in blocking buffer was added for an additional 1 h of incubation at 37 °C. The wells were washed again, and 100 μl of *p*-nitrophenyl phosphate [0.6 mg/ml in Na_2CO_3 (50 mM; pH 9.8) and MgCl_2 (1 mM)] was added. The colour reaction, developed at room temperature, was terminated by the addition of an equal volume of 1 M-NaOH after 5–20 min, depending on the quantity of ACC antigen assayed and 1° antibody employed. All assays were linear with respect to time and antigen load. The A_{415} was then measured on a Dynatech ELISA reader (model MR 700). Blank reactions were performed in wells by omitting either antigen or anti-ACC antibody; preliminary experiments showed that these blanks yielded the same low A_{415} as observed with non-immune rabbit IgG and/or a control monoclonal antibody. In order to relate A_{415} to ACC mass, a standard curve of purified rat liver ACC was assayed in parallel (see the Results section). Results are expressed as ng of immunoreactive ACC 265 or ACC 280 per μg of cytosolic protein in tissue or cell extracts.

The other two assays were designed to measure complexes between ACC 280 and ACC 265 and to measure multiple copies of ACC 265 in total enzyme. These two assays, using either tissue/cell cytosol or purified enzyme, were performed similarly to the avidin-based assay, the major differences being that anti-ACC antibodies were initially plated in the wells and one of these assays employed a different detection system. For the assay measuring ACC 280/ACC 265, the ACC 7AD3 antibody (3 μg /well) was plated as above and the anti-*N*-terminal antibody (0.8 μg /well) used as the detecting antibody. For the third e.l.i.s.a. assay applied to the measurement of ACC 265 complexes, wells were coated with peptide-affinity-purified *N*-terminal IgG. The exact IgG mass employed could not be determined because of its dilution in BSA; the amount plated was determined by the highest dilution yielding a maximal signal in a titration curve in which antibody was titrated by e.l.i.s.a. against rat liver ACC (3 μg /well). In this assay of ACC 265/ACC 265 complexes, a different detection system was employed. After antigen binding and washing, the wells were probed with biotinylated anti-*N*-terminal IgG (0.3 μg /well), and the amount of this bound antibody was detected with avidin-alkaline phosphatase

(0.1 μg /well). Although the design of the ACC 280/ACC 265 sandwich is reasonably straightforward, the rationale of the design of the ACC 265/ACC 265 sandwich was somewhat different. It was elected to employ the same antibody as both the plating and detecting antibody in this assay, rather than use a second detecting antibody directed against another ACC 265 epitope, specifically to avoid reactivity with a different domain of the same immobilized polypeptide chain. The ACC 265-reacting anti-*N*-terminal peptide antibody was chosen because it recognizes a small, terminal, peptide epitope not known to be subject to covalent modification and likely to be exposed in the native enzyme; this should minimize potential problems with altered relative reactivity towards enzyme in different states of phosphorylation or aggregation. Biotinylation of the second detecting antibody in this assay was used, followed by avidin-alkaline phosphatase, because the use of alkaline phosphatase-anti-rabbit Ig is abrogated owing to its binding to the plated antibody. Despite the use of avidin-alkaline phosphatase, which would also react with ACC biotin, for detection, blanks without the addition of second antibody were acceptably low.

In both antibody sandwich assays, the times of incubation, blocking and diluent buffers and *p*-nitrophenyl phosphate mixture were all identical with those in the avidin-based assay. In both these assays, because standards are not available, the data are expressed as A_{415} per μg of protein load. With isolated rat liver or mammary enzyme, the A_{415} is linear with respect to load between 0 and 500 ng of enzyme antigen; the final phosphatase reaction was terminated after 5–20 min at absorbances which were within this range.

Miscellaneous methods

ACC was isolated from male and female rat livers in the three nutritional states or from other tissues/cells by monomeric-avidin-Sepharose chromatography, as in [27]. SDS/polyacrylamide-gel electrophoresis was performed by the method of Laemmli [28]. Protein content was determined by the method of Bradford [29] or by the bicinchoninic acid method (BCA protein assay reagent; Pierce).

Materials

Rats and mice were purchased from Charles River Breeding Laboratories. ^{125}I -labelled goat anti-(mouse Ig) and goat anti-

(rabbit Ig) were obtained from Dupont/New England Nuclear. Alkaline-phosphatase-labelled antibodies were purchased from Southern Biotechnology (Birmingham, AL, U.S.A.). Avidin and avidin-alkaline phosphatase were obtained from Sigma. Cell media were purchased from Gibco; calf and fetal-bovine serum were obtained from Hazelton (Leuka, KS, U.S.A.). L6 and H9c2 cells were purchased from American Type Culture Collection. Other cell lines were gifts from several investigators (see the acknowledgement). Nitrocellulose was obtained from Schleicher and Schuell. The micro-titre wells employed were Falcon type 3912. All chemicals and gel reagents were purchased from Sigma.

RESULTS

Qualitative analysis of ACC tissue and subcellular distribution by immunoblotting

The choice and specificity of antibodies to be used in e.l.i.s.a. analysis to study the distribution/presence of 265 kDa ACC (ACC 265) and 280 kDa ACC (ACC 280) has been previously established by immunoblotting with monospecific antibodies [13]. These previous results have shown that both isoenzymes are present in rat liver, whereas rat skeletal muscle (diaphragm and leg muscle) and heart contain exclusively ACC 280, and ACC 265 is the only recognized isoform in rat white adipose tissue. Isoenzyme distribution has also been qualitatively estimated in a number of cultured cell lines representative of rat liver, heart and skeletal muscle by immunoblotting. Reuber hepatoma cells (Fao and H356A-1), H9c2 embryonic cardiomyocytes and differentiated/fused RMo myocytes (but not L6 myocytes) all contain both isoforms. In mouse-derived cell lines (3T3-L1 adipocytes, 23A2 myocytes, C2C12 myocytes, G8 myocytes and BC3H1 myocytes), we have only been able to detect ACC 265. Mouse tissues display the same distribution of ACC as in the rat (absent from heart and skeletal muscle; present in liver and adipose tissue), but no ACC 280 is detectable. The failure to identify a mouse homologue of the rat ACC 280 enzyme may reflect its lack of reaction with the ACC 7AD3 monoclonal antibody raised against rat liver enzyme. On direct streptavidin-alkaline phosphatase blotting, a biotin-containing protein of molecular mass 280 kDa is shown to be present in mouse liver, heart and skeletal muscle (N. Louis & L. A. Witters, unpublished work).

Fig. 1. Subcellular distribution of ACC isoenzymes in rat liver with altered nutrition

Immunoblot analysis is shown of liver subcellular fractions, prepared as in the Methods and materials section, with a sheep polyclonal antibody that recognizes both ACC 265 and ACC 280 (SPC), ACC 280-specific 7AD3 monoclonal antibody, and ACC 265-specific *N*-terminal IgG (N). On the left in each instance is shown isolated rat liver ACC (40 ng). Liver cytosol (high-speed supernatant; HSS) lanes were loaded with 50 μg of protein. The microsomal (high-speed pellet; HSP) and washed mitochondrial (Mito) fractions were resuspended in a volume of sample buffer equivalent to that of total HSS volume; a sample volume equal to that of HSS was then loaded in each lane. In this way, the blot directly reflects the absolute distribution of immunostainable proteins. Abbreviation: F/R, fasted/refed.

On immunoblotting analysis of whole-tissue homogenates derived from fasted, normally feeding and fasted/refed rats, the mass of both isoenzymes is diminished markedly in the liver of fasted animals, and both are 'superinduced' on fasting/refeeding, whereas ACC 280 mass in both heart and skeletal muscle is unchanged by varying nutrition *in vivo* [13]. Analysis of subcellular fractions of rat liver from these three nutritional states indicates that most of the mass of both isoenzymes (as defined both by monospecific antibodies for each and by an antibody that recognizes both) is confined to the cytosol, regardless of prior nutrition, and that the marked induction/repression of the mass of both isoenzymes with varying nutrition observed in whole liver homogenates is precisely mirrored in the cytosol fraction (Fig. 1). No significant mass of either isoenzyme is observed in the mitochondrial fraction in any nutritional state; a very small amount of ACC 280 is detectable in extracts from fasted/refed rats only. The isoenzymes detectable in the high-speed (microsomal) pellet likely reflect trapping of cytosolic enzyme or sedimentation of enzyme polymer, although we have previously reported the presence of microsomal ACC not accounted for by either of these events [30]. On the basis of all the results obtained with qualitative immunoblotting, all e.l.i.s.a. analyses of crude tissue/cell extracts in the present study were performed on the cytosolic fraction, employing the appropriate antibodies dictated by the isoenzymes present in individual tissues/cells.

Avidin-antibody sandwich e.l.i.s.a. analysis of purified ACC and crude tissue/cell fractions

For quantification of ACC isoenzyme mass in various tissues and cells, the avidin-antibody sandwich e.l.i.s.a. has been used. Enzyme mass is estimated by comparison with a standard curve constructed with fasted/refed-rat liver enzyme. As shown in Fig. 2, as measured with all ACC 265- or ACC 280-specific antibodies,

Fig. 2. Avidin e.l.i.s.a. standard curves

Variable quantities of total rat liver ACC, isolated by avidin-Sepharose chromatography from fasted/refed rats, were assayed in the avidin-based e.l.i.s.a., by using four different anti-ACC antibodies. Results are shown obtained by probing avidin-immobilized ACC with ACC 265-specific anti-N-terminal IgG (●), ACC 265-specific anti-C-terminal IgG (□), ACC 265-specific rabbit polyclonal IgG (○) and ACC 280-specific 7AD3 monoclonal antibody (■). The amounts of ACC 265 and ACC 280 in the measured ACC protein of the isolate were assumed to be 90% ACC 265 and 10% ACC 280, as judged by Coomassie Blue staining [13]. Development time with *p*-nitrophenyl phosphate was 10–20 min, depending on the antibody employed.

Table 1. Avidin e.l.i.s.a. analysis of cytosolic proteins from tissues and cell lines

Cytosolic ACC 265 and ACC 280 from tissues and cells were quantified by avidin-based e.l.i.s.a. analysis using as the probing antibody either ACC-265-specific anti-N-terminal IgG or ACC-280-specific 7AD3 monoclonal antibody with parallel standard curves, as in Fig. 2. Variable loads of total cytosolic protein were employed. Total protein loads for ACC 265 measurement were 4 µg for liver samples, 1 µg for white adipose tissue, 20 µg for L6, H9c2 and Rmo cells, 3 µg for Fao and H356A-1 cells, and 2.5 µg for 3T3-L1 adipocytes; total protein loads for ACC 280 measurement were 40 µg for liver samples, 20 µg for diaphragm muscle and heart, and 30 µg for all cell extracts. Results are expressed as ng of ACC isoenzyme per µg of cytosolic protein and represent the means of duplicate analyses of each sample: ND, none detected.

Tissue/cell	ACC 265 (ng/µg of protein)	ACC 280 (ng/µg of protein)
Liver		
Fed	0.87	0.06
Fasted	0.35	0.01
Fasted/refed	1.73	0.08
White adipose tissue		
Fed	2.38	ND
Fasted	0.98	ND
Fasted/refed	1.57	ND
Skeletal muscle (diaphragm)		
Fed	ND	0.08
Fasted	ND	0.08
Fasted/refed	ND	0.10
Heart		
Fed	ND	0.10
Fasted	ND	0.08
Fasted/refed	ND	0.10
Cell lines		
Fao	1.17	0.03
H356A-1	1.64	0.01
H9c2	0.14	0.01
Rmo	0.12	0.01
L6	0.14	ND
3T3-L1 adipocytes	2.40	ND

this assay is linear between 0 and 6–9 ng of each isoenzyme; all tissue samples were analysed within this linear range of absorbance. The amounts of tissue/cell cytosolic extract protein assayed in each e.l.i.s.a. vary between tissues and cell lines and for each isoenzyme (see Table 1 legend). These amounts were determined in preliminary experiments with multiple dilutions of each extract, such that the estimates of mass were within the linear range of the standard curve and were the same at varying loads. Because limited quantities of purified ACC 280 were available from heart and skeletal muscle, we have used the rat liver enzyme (containing both isoenzymes) as the standard for both the ACC 265 and ACC 280 assays, assuming that 90% of the purified protein is ACC 265 and 10% ACC 280 [13]. Errors in this assumption would only change the absolute, rather than relative, mass estimation.

Quantification of tissue/cellular cytosolic contents of ACC 280 and ACC 265 in selected tissues/cells is shown in Table 1. These quantitative analyses are entirely consistent with previous qualitative estimates made by immunoblotting [13]. ACC 265 content, depending on the tissue examined, varies between 0.002 and 0.24% of total cytosolic protein, while that of ACC 280 varies between 0.0001 and 0.011%. There is a marked decrease in rat liver ACC 265 (60%) and ACC 280 (80%) content on fasting, and a 6–8-fold increase on subsequent refeeding with a high-carbohydrate diet. In adipose tissue, there is 60% decrease

Table 2. Determination of isoenzyme-complex content by e.l.i.s.a.

(a) The amount of ACC 280/ACC 265 complex (expressed as A_{415} unit/ μg of total ACC) was quantified in total ACC isolated by avidin-Sepharose chromatography from the livers of male and female rats in three nutritional states. In these assays, equal amounts of total ACC protein (200 ng) were loaded into all wells; analyses were performed in duplicate, which agreed within 5%. (b) In a separate experiment, the mass of ACC 280/ACC 265 complex and a complex with multiple copies of ACC 265 (ACC 265/ACC 265) was determined in parallel assays with ACC isolated from male-rat livers. No complexes were detectable above the blank reaction in isolates from fasted rats in the latter assay. The results of these analyses, performed in quadruplicate on 300 ng loads, are expressed as A_{415} unit/ μg of ACC (mean $\pm$ S.D.), since no standards are available.

(a) Comparison of male- and female-rat liver ACC with varying nutrition

Tissue	ACC 280/ACC 265 (A_{415} /μg of ACC)
Male liver	
Fed	0.011
Fasted	0.002
Fasted/refed	0.042
Female liver	
Fed	0.017
Fasted	0.007
Fasted/refed	0.037

(b) Nutritional changes in ACC 280/ACC 265 and ACC 265/ACC 265

	ACC 280/ACC 265 (A_{415} /μg of ACC)	ACC 265/ACC 265 (A_{415} /μg of ACC)
Fed	0.024 $\pm$ 0.001	0.058 $\pm$ 0.012
Fasted/refed	0.059 $\pm$ 0.005	0.170 $\pm$ 0.040

in ACC 265 on fasting, but no 'superinduction' on refeeding. In contrast, the ACC 280 content in heart and diaphragm is not significantly diminished on fasting or 'superinduced' on refeeding. Rat white adipose tissue and mouse 3T3-L1 adipocytes contain the highest quantities of ACC 265, whereas the amounts of ACC 280 in rat muscle, heart and fasted/refed liver are comparable. Marked variation in isoenzyme content is encountered in cell lines tested, with the hepatoma cells having a greater content of ACC 280 than the muscle cells, in contrast with the relative amounts of these isoenzymes in rat liver, heart and skeletal-muscle extracts. Furthermore, all muscle cell lines (rat or mouse) examined contain ACC 265, unlike the exclusive presence of ACC 280 in skeletal and cardiac muscle *in vivo*.

Sandwich e.l.i.s.a. analysis of ACC subunit composition

Previous investigations, employing either sucrose-density-gradient centrifugation or f.p.l.c. molecular-sieve chromatography, have documented changes in enzyme association between protomeric and oligomeric/polymeric forms that vary depending on the nutritional state or hormonal modulation [8,15]. For example, the enzyme from fasted/refed rats is highly polymerized, whereas that from fasting animals is largely protomeric [8]. We have used sandwich e.l.i.s.a. analysis to examine total ACC in either tissue/cell cytosol or in purified enzyme for the presence of either association between the two isoenzymes or multiple copies of a single isoenzyme as a function of the prior nutritional state *in vivo*. Using the ACC 280/ACC 265 sandwich in an analysis of

rat liver, heteroisoenzyme association can be readily detected and, like the physical measurements of enzyme size, this association varies as a function of nutrition *in vivo* (Table 2a), as assayed at identical total enzyme mass and relative isoenzyme composition (the latter remains the same in all nutritional states; [13]). As shown with ACC isolated from rat liver from all three nutritional states in both males and females, there is a substantial decrease in ACC 280/ACC 265 association in fasting that markedly increases on subsequent induction with a high-carbohydrate diet. Results of identical magnitude can be seen on analysis of liver cytosol (not shown). No apparent gender variation in this phenomenon is evident. As analysed with the male enzyme, the increase in ACC 280/ACC 265 association noted with fasting/refeeding is paralleled by an increase in a species with multiple copies of ACC 265, detectable in the ACC 265/ACC 265 sandwich assay (Table 2b); these latter associations could not be detected in the fasting enzyme (results not shown). Taken together, these results are entirely consistent with enzyme oligo-/poly-merization that occurs on induction or activation of ACC activity and with a protomerization during enzyme repression, as measured by physical techniques [8,15]. In support of this conclusion, preliminary results using the ACC 280/ACC 265 sandwich with isolated rat liver enzyme indicate that citrate (a polymerizing ligand) increases ACC 280/ACC 265 association, whereas malonyl-CoA (a protomerizing ligand) diminishes it. In addition, phosphorylation of the enzyme *in vitro* with protein kinases that inactivate total ACC also diminishes the mass of this complex, whereas dephosphorylation increases it, again consistent with results observed by others on the relationship between the relative phosphorylation state and the protomer/oligomer/polymer state of the enzyme (results not shown) [6-8].

DISCUSSION

Analysis of ACC mass and subunit composition with monospecific antibodies confirms and extends information gained from other techniques of analysis, and provides direct quantification of relative tissue and cell ACC isoenzyme mass and hetero-/homo-isoenzyme association. Total mass can be measured by using the avidin-based sandwich e.l.i.s.a. Tetrameric avidin has a high affinity for biotin-containing proteins (K_d 10^{-14} – 10^{-15} M) and thus proves to be a useful ligand to bind specifically all biotinylated ACC species in the micro-titre wells, even in the analysis of crude tissue fractions. Since avidin, in contrast with immobilized antibodies, does not discriminate between isoforms, all ACC is immobilized and total mass can then be estimated with antibodies that recognize all isoforms and/or isoform-specific antibodies. Avidin binding apparently does not interfere with the absolute reactivity of ACC with any of the ACC-specific antibodies, permitting quantification of amounts as low as 0.5 ng of ACC protein. This sensitivity exceeds that of other techniques by at least an order of magnitude; in our experience with immunoblotting using the same antibodies, the detection limit is generally 5 ng, being limited by background radioactivity on the nitrocellulose. Furthermore, quantification by immunoblotting is tedious and time-consuming, and is often compromised by imperfections in protein transfer, extraneous spots on the radioautograph and inherent errors either in densitometric scanning of radioautographs or in band alignment for excision of labelled protein to determine ^{125}I content. Techniques of mass measurement that require estimation of enzyme activity, like immunotitration, are even less sensitive, requiring up to 100–500 times (depending on enzyme specific activity) the mass of ACC for accurate measurement by either isotopic or enzyme-linked assays. Immunotitration, which does not report actual mass, is also limited by the specificity of the

antibodies employed and their ability to recognize all isoenzymes of ACC.

This assessment of ACC isoenzyme mass and tissue/cell distribution by using immunoblotting and e.l.i.s.a. analysis provides new information about several tissues and cell lines, and is in qualitative agreement with results obtained previously by ourselves and others in the study of the liver enzyme, using immunotitration, enzyme isolation, separation by molecular size or immunoblotting [6–10,13,16]. By direct quantitative measurement, striking differences in ACC isoenzyme distribution, relative tissue mass and response to nutritional induction/repression are evident. Particularly intriguing is the repression/induction of ACC 280 in the liver, with 6–8-fold changes in total isoenzyme mass, whereas ACC 280 in both heart and skeletal muscle is unchanged during variation in nutrition, suggesting strongly that ACC 280 is under differential control at either a transcriptional or translational level in these different tissues. It is also of interest that, except for the H9c2 and RMo cell lines, we have not been able to identify ACC 280 in any cultured muscle cell line and that ACC 265, not normally expressed in adult muscle *in vivo*, is present in all. This could represent some property of the transformed subcloned cells or the culture conditions employed; alternatively, such distribution might denote a 'fetal' phenotype. Studies of the developmental aspects of ACC isoenzyme expression are needed.

Results of the present analysis are in direct disagreement with data derived in Allred's laboratory, indicating that the total mass of liver ACC does not change during starvation or on high-carbohydrate refeeding and that there is a sequestered form of mitochondrial ACC accumulating during fasting (up to 5–6 times the mass in the cytosol) that translocates to the cytosol on refeeding [17–21]. Our data agree with all other estimates of changing hepatic ACC mass (though the degree of change reported varies) accompanying these nutritional alterations as measured by immunotitration, immunoblotting or enzyme isolation [6–10,13,16] and as predicted by changes in ACC mRNA content with nutritional change [4,5]. The technique of assessment of ACC mass, used by Allred, employing [³H]avidin binding followed by SDS/polyacrylamide-gel electrophoresis, has not been employed in parallel in nutritional studies with any other physical method or immunological estimate of mass. Furthermore, no explanations have been offered for their markedly discrepant results. Extrapolation of activity data to actual enzyme mass, as also used in these studies, relies on measurement of activity in crude fractions incubated without protease or phosphatase inhibitors. The enzyme under these conditions is subject to variable activation, depending on both phosphatase activity and protease content, leading to potential misinterpretation of the data.

The sandwich e.l.i.s.a. analysis using isoenzyme-specific antibodies that we have employed is a simple and rapid way of measuring ACC isoenzyme association with sub-microgram amounts of ACC and can be directly applied to crude extracts. Results from two other laboratories indicate that, in addition to changes in enzyme mass, there are changes in ACC specific activity, phosphorylation and the protomer/oligomer/polymer subunit association accompanying altered nutrition [6–8]. The liver enzyme in fasted animals has a low specific activity and a higher phosphate content, and is largely protomeric (15S; ~ 500 kDa consisting of two polypeptide chains). On refeeding with a high-carbohydrate diet, the specific activity increases coincident with dephosphorylation and the assumption of an oligomeric/polymeric state of subunit association (50S; ≥ 4000 kDa). These same changes are also observed after insulin stimulation of ACC in rat adipocytes [15]. Our data in nutritionally manipulated animals are consonant with these

changes in molecular size seen with either sucrose-density-gradient or molecular-sieve fractionation. The e.l.i.s.a. sandwich assay, employing isoenzyme-specific antibodies, indicates a decrease in isoenzyme association with fasting and a marked increase in the mass of this complex on refeeding that is not a function of the absolute mass or relative amount of either isoenzyme. These changes must therefore represent some other property of the polypeptide chains, perhaps altered phosphorylation. Unlike data derived from other techniques, these data yield direct evidence for changes in both hetero- and homo-isoenzyme association based on the mass of individual polypeptides, rather than merely a shift in molecular size of enzyme activity.

It should be stressed that neither of these isoenzyme-complex assays necessarily detects a discrete species composed just of ACC 280 and ACC 265 or just ACC 265. Although such species could exist, it seems more likely that a single species composed of variable amounts of ACC 280 and 265 polypeptide chains is being measured. Immunoprecipitation of rat liver enzyme with either monospecific antibody has been shown to lead to nearly quantitative precipitation of both isoenzymes, consistent with this view [13]. The spatial orientation of the polypeptide chains in the enzyme protomer/polymer could affect antibody reactivity in either of these complex assays. For example, in the ACC 265/ACC 265 assay, if only one of the polypeptide chains in the protomer is bound to the plating antibody, the parallel or anti-parallel orientation (not known) of these two ACC 265 chains could affect the ability of the antibody to recognize the *N*-terminus in the second non-bound polypeptide chain, owing to steric hindrance. An anti-parallel orientation of the two chains might also permit bridging of one ACC 265 protomer between adjacent molecules of immobilized *N*-terminal specific antibody, negating a signal. Our working model is that the detecting antibody in this assay is reacting with *N*-termini in other protomeric units linked in the oligomer/polymer by protein-protein association rather than with the immobilized protomeric unit, and that this oligomeric/polymeric complex is composed of both ACC 280 and ACC 265 chains (in different proportions as homo- or hetero-protomers) detectable with either antibody sandwich assay. Analysis of various species of enzyme protomers/oligomers/polymers separated by physical techniques should give insight into the nature of these associations and by using e.l.i.s.a. analysis, the reconstitution of isolated ACC 265 and ACC 280 chains can be studied to determine the factors that influence it.

The application of e.l.i.s.a. analysis to the study of the regulation of ACC *in vivo* and *in vitro* by a variety of factors that regulate enzyme/isoenzyme mass, specific activity and phosphorylation state should permit an accurate quantitative assessment of the relative role(s) of variable mass and/or of altered isoenzyme association in determining total ACC activity. In addition, use of the sensitive avidin-based assay employing any monospecific anti-ACC antibody, coupled with measurement of enzyme activity, can provide information about the actual enzyme specific activity in unpurified fractions (units/mass of ACC) or, coupled with ³²P labelling of intact cells, allow expression of ³²P incorporation into ACC as ³²P content per unit mass. This avidin-based e.l.i.s.a. might also prove useful, in combination with other reagents, to measure easily the mass of any biotinylated molecule, such as a protein or oligonucleotide biotinylated *in vitro*.

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