Hepatic zonation of acetyl-CoA carboxylase activity

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The activities of several hepatic enzymes are preferentially zoned to the periportal or perivenous cells of the liver acinus. Employing dual-digitonin-pulse perfusion of rat liver in the study of acetyl-CoA carboxylase (ACC), we have identified a heretofore unrecognized feature of hepatic zonation, namely an intrahepatic gradient in enzyme specific activity. ACC activity shows a relative periportal localization in normally feeding rats, even when corrected for ACC protein mass. In contrast with results previously reported by us [Evans, Quistorff & Witters (1989) *Biochem. J.* **259**, 821–829], the total mass of both hepatic ACC isoenzymes was not found to differ between the two hepatic zones in the present study. In perfusion eluates from fed animals, periportal ACC displays enhanced citrate reactivity and two kinetic components of acetyl-CoA reactivity; the largest periportal/perivenous gradient (5-fold) is accounted for by a species with a lower K_m for acetyl-CoA. The zonal gradient in ACC maximal velocity, measured in eluates from fed rats, does not persist after ACC purification, although the isolated periportal enzyme, like dephosphorylated ACC, has a lower activation constant for citrate. Total ACC protein phosphatase activity is higher in periportal eluates, but no differences in the activities of either a 5'-AMP-activated ACC kinase or the cyclic-AMP-dependent protein kinase are noted between the hepatic zones. The induction of total hepatic ACC mass and specific activity, on fasting/refeeding with a high-carbohydrate diet, abolishes the periportal/perivenous activity gradient, largely owing to a selective activation of perivenous enzyme. Nutritional induction is also accompanied by a marked alteration in ACC acetyl-CoA kinetics and abolition of the gradient in total ACC phosphatase. These studies indicate that hepatic enzyme zonation, which is often attributed to differential expression of enzyme protein, may result from zonal variations in enzyme specific activity, owing to differences in allosteric regulation and/or covalent modification.

INTRODUCTION

The liver has a heterogeneous distribution of several metabolic pathways and enzymes. This phenomenon, termed hepatic zonation, involves the differential localization of several enzymes to either the periportal (adjacent to the portal triad) or perivenous (adjacent to the hepatic veins) zones of the liver acinus. Hepatic zonation has been noted for enzymes in many pathways, including gluconeogenesis, glycolysis, amino acid metabolism, fatty acid synthesis and cholesterol synthesis [1–3]. Several techniques have been employed to demonstrate this zonation, including micro-dissection, immunohistochemistry and isolation of perivenous and periportal hepatocytes [2]. A particularly important technique, termed dual-digitonin-pulse perfusion, has been developed that allows selective sampling of cytosolic contents of perivenous and periportal cells [4–7]. This technique possesses several advantages. First, sample collection occurs through the normal physiological hepatic circulation, resulting in very high zonal selectivity [6]. Second, the technique allows for rapid sampling, avoiding potential artifacts introduced by tedious micro-dissection or collagenase disruption. Lastly, it allows the simultaneous determination of the activity and mass of many enzymes in the same samples, thus increasing the information obtained and allowing for comparison of marker enzymes between different animals as important controls. In most instances, the information obtained from this technique has largely been confirmed, where possible by the other available estimates of zonation.

We have previously employed dual-digitonin-pulse perfusion to study the zonation of the cytosolic enzymes of fatty acid biosynthesis, ATP citrate-lyase, acetyl-CoA carboxylase (ACC)

and fatty acid synthase [7]. In this study, all three were found to have an increase in mass in periportal cells relative to perivenous cells in livers of fed or fasted male rats. Preliminary data also indicated an intrahepatic gradient in ACC specific activity, evident when total carboxylase activity was corrected for enzyme mass determined by immunoblotting. In the present study, we have undertaken to characterize further this latter gradient and to investigate factor(s) that might contribute to variable specific activity in the periportal and perivenous zones. The availability of newly-developed anti-ACC antibodies, the more recent recognition of the isoenzymic heterogeneity of ACC in the liver [8] and the opportunity to perform analyses on freshly isolated samples (previous activity analyses having been done on frozen and transported samples) prompted, in part, this present study. In this study, we report that the specific-activity gradient for ACC appears to be due largely to allosteric regulation, but may also be contributed to by zonal variation in enzyme phosphorylation. Unlike our previously reported observations [7], no intrahepatic gradient in ACC mass has been observed.

MATERIALS AND METHODS

Dual-digitonin-pulse perfusion and animals

All perfusions, initial enzyme assays and enzyme isolations were performed in Copenhagen by J. L. E. and B. Q. The methodology of the perfusion system and sample collection have been described previously in detail [4–7]. The initial direction of perfusion (performed at 4 °C) was reversed in consecutive experiments in the same sets of rats, so that an equal number of experiments were performed in which the initial direction of

Abbreviation used: ACC, acetyl-CoA carboxylase.

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perfusion was antegrade (portal vein to inferior vena cava) or retrograde (inferior vena cava to portal vein). No differences in the results obtained in the experiments reported here were seen as a function of the initial perfusion direction (results not shown). Eluates were fractionated and collected directly into different buffers (see below) by using a sample-splitting design [4–7]. The first four 10 s intervals of flow reversal after digitonin perfusion (5 mg/ml) of each hepatic zone were pooled for analysis, as previous experiments had indicated that these fractions contained virtually all of the eluted lipogenic-enzyme activity and mass [7]. Analysis of marker enzymes that indicate the reproducibility and selectivity of sample collection from the two zones (lactate dehydrogenase, alanine aminotransferase) revealed the same patterns previously ascertained and did not vary substantially between animals in these experiments ([7], and results not shown).

Experiments were performed on male Wistar rats (150–180 g) either maintained on normal rat chow (fed) or fasted for 48 h and then refed for 48 h with a high-carbohydrate (Bioserv) diet (fasted/refed).

Collection and processing of periportal and perivenous eluates

Periportal and perivenous eluates were collected directly into one of three ice-cold buffers, depending on the analyses to be performed. For analysis of ACC activity, the eluates were collected in tubes containing 0.4 vol. of a concentrated buffer yielding final concentrations of 50 mM-Tris/HCl (pH 7.5 at 4 °C), 2 mM-EDTA, 2 mM-dithiothreitol, 100 mM-NaF and 250 mM-sucrose (Buffer A), and protease inhibitors, as in [9]. These samples were then centrifuged (100 000 *g* for 60 min at 4 °C), and samples of the supernatant were taken for determination of total protein [10], ACC activity, and other enzyme activities that are markers of zonation [7]. Samples, matched for total eluate protein, were immediately prepared for subsequent gel electrophoresis by addition of an SDS/polyacrylamide gel solution, followed by boiling for 5 min, as in ref. [7]. The remainder of the supernatant was then snap-frozen and stored at –70 °C for subsequent transport to Dartmouth for e.l.i.s.a. analysis.

Eluates to be utilized for kinase assays were collected in tubes containing 1.0 vol. of a concentrated buffer, yielding buffer concentrations of 80 mM-Tris/HCl (pH 7.4 at 4 °C), 2 mM-dithiothreitol, 2 mM-EDTA, 2 mM-EGTA, 100 mM-NaF, 4 mM-sodium pyrophosphate, 250 mM-sucrose and 5% (v/v) glycerol (Buffer B), and protease inhibitors as above. After centrifugation (100 000 *g* for 60 min at 4 °C), the supernatant was removed and adjusted to 60% saturation with (NH₄)₂SO₄. The resulting (NH₄)₂SO₄ pellets were immediately frozen in liquid N₂ and transported on solid CO₂ to Dartmouth.

Eluates to be utilized for phosphatase assays were collected into tubes containing 0.4 vol. of concentrated buffer, yielding final concentrations of 50 mM-glycylglycine (pH 7.4), 100 mM-KCl, 2.5 mM-EDTA, 0.1 mM-dithiothreitol and 5% glycerol (Buffer C). Phosphatase activity was measured immediately after dilution of zonal eluates to equal protein concentration.

Purification of ACC

ACC was purified from the high-speed supernatants of eluates collected into Buffer A by avidin–Sepharose chromatography, as in [9]. ACC utilized as the substrate in kinase (fasted/refed) and phosphatase (fed) assays was purified from the livers of male Sprague–Dawley rats (130–150 g) by the same method.

Determination of ACC activity and kinetics

ACC was assayed by H¹⁴CO₃[–] incorporation into malonyl-CoA [11,12]. Citrate and acetyl-CoA kinetics were determined by measurement of activity at variable concentrations of each [0–10 mM-citrate (at 530 μM-acetyl-CoA); 5.3–530 μM-acetyl-

CoA (at 10 mM-citrate)]. The *K_s* for citrate and *K_m* for acetyl-CoA were determined from analysis of Eadie–Hofstee plots (*V* versus *V*/[citrate] or *V*/[acetyl-CoA]). Linear plots (for citrate) were analysed by Cricket Graph 1.2 on a Macintosh IIfx computer. For analysis of curvilinear plots (for acetyl-CoA), the experimental data, assuming a two-component model, were iterated in the NONLIN mode of SYSTAT, which employs piecewise linear-regression analysis [13]. In these analyses, the piecewise linear regression is accomplished by iterative analysis of two equations, where *V* (velocity) and *V*/[acetyl-CoA] are experimentally determined (where AcCoA = acetyl-CoA):

Low-*K_m* component:

$$V = B_0 + B_1(V/[AcCoA]) + B_2[(V/[AcCoA]) - XO] \quad [(V/[AcCoA]) \text{ values} > XO]$$

High-*K_m* component:

$$V = B_0 + B_1(V/[AcCoA]) + B_2[XO - (V/[AcCoA])] \quad [(V/[AcCoA]) \text{ values} < XO]$$

XO is entered as a 'guess' value for the break in the curve (assuming a two-component model); multiple guesses of XO are made (≥ 5 guesses above and below the perceived break with linearity in each curve) and analyses are rejected if the derived XO value is not the same for each guess. The iterative analysis then yields values for the constants *B* and for XO that give the best fit, along with standard errors. Analyses were also rejected if the same fitting parameters *B* were not obtained at each 'guess' value of XO. This program then reports *K_m* and *V_{max}* values for each linear component (with standard errors).

Determination of enzyme mass and isoenzyme distribution by immunoblotting and e.l.i.s.a.

Immunoblotting was performed with four different antibodies that recognize the ACC isoenzymes and one polyclonal antibody that recognizes both fatty acid synthase and ATP citrate-lyase, as in refs. [7,8,14].

The content of each ACC isoenzyme in periportal and perivenous eluates was quantified by using an avidin-based sandwich e.l.i.s.a. [15]. In all instances, authentic ACC standards were included to allow expression of data as ng of ACC per μg of eluate protein. The content of the heteroisoenzyme complex between the two ACC hepatic isoenzymes was measured in a sandwich e.l.i.s.a. assay employing antibodies specific for each, as in [15].

Protein kinase assays: cyclic-AMP-dependent and 5'-AMP-activated kinase

Cyclic-AMP-dependent kinase activity was assayed with the synthetic peptide Kemptide (Sigma) as the substrate for phosphorylation. Frozen periportal and perivenous 60%-satd. (NH₄)₂SO₄ pellets were redissolved in 2 ml of 10 mM-potassium phosphate (pH 6.8)/150 mM-KCl/10 mM-EDTA/0.5 mM-isobutylmethylxanthine and matched for protein. Kinase reaction mixtures (70 μl total volume) contained sample (20 μl; 30–80 μg of protein), 10 mM-potassium phosphate (pH 6.8), 10 mM-NaF, 160 μM-Kemptide, 200 μM-[³²P]ATP (sp. radioactivity 500–1000 c.p.m./pmol) and 10 mM-magnesium acetate with or without cyclic AMP (1 μM). Blank assays were performed in the absence of substrate and in the absence of extract. After incubation at 30 °C, samples (20 μl) were removed at 6 and 12 min (to ensure linearity) and pipetted on to 1 cm² phosphocellulose filters (Whatman DE81), which were washed in 25% (v/v) H₃PO₄ and counted for ³²P radioactivity. Results are

expressed as pmol of ^{32}P incorporated/min per mg of eluate protein and as the activity ratio (–cyclic AMP/+cyclic AMP).

Initial attempts to detect 5'-AMP-activated kinase activity [16,17] in frozen $(\text{NH}_4)_2\text{SO}_4$ pellets after resuspension and dialysis were unsuccessful and necessitated the need for partial purification. Frozen pellets obtained from periportal and perivenous eluates were resuspended in 1 ml of Buffer B and dialysed against the same buffer. Equal amounts of periportal and perivenous protein (1–1.5 mg) were applied to 1 ml of DEAE-Sephacel columns equilibrated with Buffer B. After columns were washed with 12 vol. of buffer, kinase activity was eluted with 3 ml of Buffer B containing 200 mM-KCl directly into tubes containing 0.1 vol. of a 10 mg/ml solution of BSA. Preliminary experiments indicated that no 5'-AMP-activated kinase activity was present in the flow-through fractions and that all this activity was eluted by this KCl concentration. The eluates were concentrated by 50 %–satd. $(\text{NH}_4)_2\text{SO}_4$ fractionation and the pellets redissolved in 100 μl of Buffer B.

5'-AMP-activated kinase activity was measured by using ACC purified from the livers of fasted/refed rats as the substrate. Reactions were carried out in Buffer B (assay volume 60 μl) containing ACC (1 μM), [^{32}P]ATP (200 μM ; sp. radioactivity 1000 c.p.m./pmol), magnesium acetate (10 mM), bovine heart protein kinase inhibitor (type 2; Sigma, 27 inhibitory units) to inhibit the cyclic-AMP-dependent protein kinase, heparin (10 $\mu\text{g}/\text{ml}$; to inhibit casein kinase II) and 10 μl of DEAE-Sephacel eluate with or without 5'-AMP (200 μM). Blank reactions were performed in the absence of added ACC and in the absence of kinase fraction. Reactions, performed at 30 °C, were terminated after 5 min by the addition of an equal volume of a gel sample solution [7] that additionally contained unlabelled ATP (28 mM), EDTA (40 mM), NaF (50 mM) and sodium pyrophosphate (2 mM), followed by immediate boiling. These samples were subjected to gel electrophoresis (5.0 %–acrylamide gels) followed by autoradiography. The Coomassie-Blue-stained region corresponding to ACC was excised and counted for radioactivity in a scintillation fluor. 5'-AMP-activated kinase activity (activity in the presence of 5'-AMP minus that in its absence) is expressed as mol of ^{32}P incorporated/5 min per mol of ACC.

ACC phosphatase assay

Total ACC phosphatase activity was assayed in periportal and perivenous eluates by using a functional assay which measured the activation of ACC purified from the livers of normally fed male rats. The phosphatase reaction mixture contained (in 60 μl total volume) glycylglycine (50 mM; pH 7.4), KCl (100 mM), EDTA (2.5 mM), dithiothreitol (0.1 mM), glycerol (5 %), BSA (1 mg/ml), magnesium acetate (10 mM), MnCl_2 (2 mM) and ACC (1 μM). Assays were started by the addition of 30 μg of periportal or perivenous eluate protein. Reaction tubes were incubated at 30 °C, and samples (10 μl) were removed at specific time points (0, 5, 15, 30 min) and diluted (11-fold) into ice-cold stop buffer, consisting of Tris/acetate (25 mM; pH 7.4), NaF (50 mM), EDTA (2 mM), β -mercaptoethanol (5 mM) and fatty acid-free BSA (0.1 $\mu\text{g}/\text{ml}$). ACC activity was then measured with 50 μl samples (containing 1 μg of purified ACC) at a subsaturating concentration of citrate (0.05 mM). Control incubations included tubes lacking eluate protein and tubes lacking purified ACC. In the absence of added eluate protein, ACC activity remained constant during the 30 min incubation period.

Materials

Male Wistar rats, obtained from the University of Copenhagen breeding colony, were used for hepatic perfusion; male Sprague–

Dawley rats, purchased from Charles River Breeding Laboratories, were used for ACC isolation. Chemicals were of analytical grade or higher, and were purchased from Sigma, Fisher, Boehringer, Merck or Mallinckrodt. Electrophoresis supplies were purchased from Bio-Rad, and nitrocellulose (BA83) was from Schleicher and Schull. $\text{NaH}^{14}\text{CO}_3$ was purchased from Amersham, and [γ - ^{32}P]ATP from ICN. ^{125}I -labelled goat anti-mouse and goat anti-rabbit antibodies and ^{125}I -Protein A were purchased from Du Pont/New England Nuclear. Affinity-purified goat anti-mouse and goat anti-rabbit antibodies conjugated to alkaline phosphatase were purchased from Southern Biotechnology.

RESULTS

Zonation of ACC activity

Total ACC activity in fed male rats, expressed as m-units/mg of eluate protein, shows a marked gradient across the liver, with the highest activity being present in periportal eluates (Table 1). In fed animals, virtually all ACC activity is citrate-dependent. At maximal velocity (10 mM-citrate), a 3-fold gradient (periportal/perivenous) is noted, whereas at sub-saturating citrate (0.5 mM) the observed gradient is larger (5-fold). A striking difference between these eluates is noted in the ratio of activities at sub-saturating (0.5 mM) and saturating (10 mM) citrate. In periportal samples, this ratio averages 0.70, whereas in perivenous samples it averages 0.39, implying a difference in citrate-sensitivity between the enzyme in these two hepatic zones. In rats that had been fasted and refed on a high-carbohydrate diet, the periportal/perivenous gradient measured at all citrate concentrations is abolished, principally owing to the 5–6-fold induction of ACC activity in perivenous samples (Table 1). The citrate activity ratio (0.5 mM/10 mM) is identical in the two zonal eluates (approx. 0.50) after fasting/refeeding.

Table 1. Zonation of ACC activity in fed and fasted/refed rat liver

Shown are data obtained in four separate perfusions of the livers of normally feeding rats and two separate perfusions of fasted/refed animals. Similar data were obtained in three other experiments in the latter group, but these experiments were not included, as all other analyses were not completed on these samples. The data are expressed both as the means ($\pm$ S.D.) of ACC activity (m-units) per mg of total eluate protein, as measured in the absence of citrate, at a sub-saturating citrate concentration (0.5 mM) and at maximal velocity (10 mM-citrate), and as actual ACC specific activities [units/mg of total ACC protein; the latter determined by e.l.s.a. (see the text)] in the same eluate samples. Significant differences, as determined by a two-tailed *t* test, are shown for comparison of periportal and perivenous data in the fed-rate eluates: **P* < 0.02, ***P* < 0.01 and †*P* < 0.005.

ACC	[Citrate] (mM)	Periportal		Perivenous	
		Fed	Fasted/ refed	Fed	Fasted/ refed
m-units of eluate protein	0	0.11 $\pm$ 0.03*	1.5	0.05 $\pm$ 0.01	0.96
	0.5	6.5 $\pm$ 2.3**	8.1	1.2 $\pm$ 0.3	6.7
	10	9.3 $\pm$ 3.1†	17.1	3.1 $\pm$ 0.4	15.9
units/mg of total enzyme protein	0	0.06 $\pm$ 0.04	0.65	0.05 $\pm$ 0.01	0.41
	0.5	3.0 $\pm$ 0.9†	2.2	0.79 $\pm$ 0.25	1.5
	10	3.4 $\pm$ 0.8†	4.1	1.3 $\pm$ 0.12	3.1

Table 2. ACC mass in periportal and perivenous eluates as determined by e.l.i.s.a.

Results are shown from the e.l.i.s.a. analyses of the same eluates shown in Table 1. The results are expressed as ng of ACC [M_r 265 000 isoenzyme (ACC 265); M_r 280 000 isoenzyme (ACC 280)] and total ACC (ACC 265 + ACC 280) per μ g of total eluate protein ($\pm$ S.D.). The actual mass of each isoenzyme was determined by reference to a standard curve for fasted/refed-liver ACC in each e.l.i.s.a. Since the two isoenzymes are both present in this mixture, and since the ACC 280 represents $\sim 10\%$ of total Coomassie-Blue-stainable protein [8], it was assumed in this standard curve that 90% of the protein mass was ACC 265 and 10% was ACC 280. This assumption, if in error, would alter only the absolute values and not the relative values for the various eluates.

Rats		ACC (ng/ μ g)	
		Periportal	Perivenous
Fed	ACC 265	1.23 ± 0.24	1.31 ± 0.18
	ACC 280	0.20 ± 0.04	0.22 ± 0.03
	Total ACC	1.43 ± 0.28	1.53 ± 0.19
Fasted/refed	ACC 265	1.77	2.26
	ACC 280	0.26	0.31
	Total ACC	2.03	2.61

Measurement of ACC mass

The gradient in total ACC activity could be due to an increase in ACC mass in the periportal zone, an increase in specific activity, or both. In addition, since it is now known that hepatic ACC activity is contributed to by two discrete isoenzymes [8], it was possible that varying isoenzyme zonation might also be contributing to the observed gradient. In order to distinguish these possibilities, the contents of the two isoenzymes of ACC (ACC 280 and ACC 265, of M_r 280 000 and 265 000 respectively) were measured in periportal and perivenous eluates (matched for total protein) by using an avidin-based sandwich e.l.i.s.a. The content of each isoenzyme is virtually identical in the periportal and perivenous eluates, with increases in each noted in samples obtained from fasted/refed animals (Table 2). This isoenzyme distribution was additionally confirmed on immunoblotting with four different antibodies that recognize one or both of the isoenzymes (Fig. 1). In contrast with the ACC isoenzymes, the mass of fatty acid synthase is greater in periportal than in perivenous eluates from normally fed animals (Fig. 1), confirming our previous observation [7]. After fasting and refeeding, fatty acid synthase mass increases in both hepatic zones. However, induction appears greater in the perivenous zone, resulting in the abolition of the mass gradient. The content of ATP citrate-lyase is below the limits of detection in fed animals, and is not zoned after fasting and refeeding.

Quantification of ACC mass allows a calculation of the actual specific activity of ACC [units/mg of total ACC (ACC 280 + ACC 265) protein] in the eluates (Table 1). This correction assumes that the specific activities of the two isoenzymes are the same; we have been unable to date to isolate each of the species independently to test this assumption. However, even if this assumption is in error, the bulk of ACC mass is present as the M_r -265 000 isoenzyme in all samples [8], and the estimate of relative specific activities in periportal and perivenous samples is not affected, only the actual specific activities. After correction for enzyme mass, a periportal preponderance of ACC activity is still evident in normally feeding males; this gradient is markedly diminished on fasting/refeeding (Table 1). Thus the difference in total ACC activity between periportal and perivenous samples in

Fig. 1. Lipogenic-enzyme masses determined by immunoblotting

Shown is a montage of representative immunoblots of periportal (PP) and perivenous (PV) eluates from fed and fasted/refed (F/R) animals. After SDS/polyacrylamide-gel electrophoresis (5% gels) of 42 μ g of eluate protein in each lane and transfer to nitrocellulose, the individual pieces of nitrocellulose were probed with polyclonal antibodies specific for the M_r -265 000 ACC isoenzyme ACC 265 (antibodies N, C and RPC), a monoclonal antibody specific for the M_r -280 000 isoenzyme ACC 280 (antibody 7AD3) and a sheep polyclonal antibody that recognizes both fatty acid synthase and ATP citrate-lyase (FAS/ACL), as in [8,14]. Also shown flanking the middle four lanes are purified enzyme standards [fasted/refed liver ACC 40 ng (top four panels) or fasted/refed liver FAS 200 ng (bottom panel)]. The apparent enhanced mobility of ACC 265 in the fasted/refed samples is an artifact of its co-migration with fatty acid synthase, which is markedly induced in content.

fed animals is due to a gradient in enzyme specific activity, and not in enzyme mass. The increase in total ACC activity in perivenous samples from refed animals is largely due to an increase in actual specific activity, though small increases in mass are noted in both the perivenous and periportal zones (Table 2). The mass of heteroenzyme complex containing the two isoenzymes did not vary between periportal and perivenous zones [mass expressed as e.l.i.s.a. A_{415} units per mg of eluate protein ($\pm$ S.E.M.): fed periportal 0.12 ± 0.06 ; fed perivenous 0.14 ± 0.04 ($n = 4$ fed); fasted/refed periportal 0.38, fasted/refed perivenous 0.59 ($n = 2$ fasted/refed)]. Its content is higher in samples from fasted/refed animals, confirming results previously obtained with purified liver enzymes [8].

Acetyl-CoA kinetics of periportal and perivenous ACC

On analysis of ACC acetyl-CoA kinetics in zonal eluates, curvilinear kinetic plots are observed for both periportal and perivenous samples obtained from fed animals, implying the

Fig. 2. Eadie-Hofstee plots of ACC acetyl-CoA kinetics

Shown are Eadie-Hofstee plots (velocity versus velocity/[acetyl-CoA]) based on mean activity data ($\pm$ S.D.) obtained in six perfusions in fed animals (a) and three perfusions in fasted/refed animals (b). The perivenous recovery in one of the latter experiments was too low to permit accurate data assessment. $\bullet$, Periportal eluates; $\circ$, perivenous eluates. The activity data in each instance were corrected for the mass of total ACC protein in the eluate as determined by avidin-based e.l.i.s.a. For purposes of graphic representation only, the curves/lines were fitted with Cricket Graph 1.2; in other analyses (see Table 3) fitting was done with the MGLH (linear) and NONLIN (curvilinear) modes of SYSTAT [13].

presence of more than one kinetic component (Fig. 2a). In contrast, in eluates from fasted/refed animals, linear plots are observed for both periportal and perivenous samples (Fig. 2b). The curvilinear plots were analysed by using piecewise linear regression (assuming a two-component model), revealing the presence of two kinetic species, designated the high- K_m and low- K_m components, in both periportal and perivenous eluates from fed animals (Table 3). The actual values for the high and low K_m for acetyl-CoA do not differ between periportal and perivenous samples from fed animals (Table 3). However, a striking difference is evident upon comparison of the relative individual V_{max} values. The V_{max} of the high- K_m species is 1.8 (± 0.2)-fold greater in the periportal samples, and the V_{max} of the low- K_m species is 4.9 (± 1.6)-fold greater in periportal compared to perivenous samples. Total V_{max} (high- K_m + low- K_m components) derived from this analysis is about 3-fold higher in the periportal eluates [periportal 4.30 ± 0.41 , perivenous 1.44 ± 0.16 units/mg of ACC protein ($\pm$ S.D.; $P < 0.0005$)], in agreement with the

measured ACC activity gradient (Table 1). The percentage of the total V_{max} contributed by the high- K_m species is significantly higher in perivenous samples [$64 \pm 5\%$ (perivenous) versus $40 \pm 5\%$ (periportal); $P < 0.006$].

In fasted/refed animals, there is only a single kinetic component, which has similar K_m and V_{max} values in periportal and perivenous eluates (Fig. 2b; Table 3). The K_m value in these eluates from fasted/refed animals approximates to that seen in the high- K_m component of fed-liver ACC from either hepatic zone. The most dramatic effect of fasting and refeeding is a 2.5-fold increase in the V_{max} of perivenous eluates. Therefore, the abolition of the ACC activity gradient with fasting/refeeding is due to a marked increase in perivenous ACC specific activity to the activity observed in the periportal zone of fed animals, whereas periportal specific activity does not change appreciably with this nutritional manipulation. Fasting/refeeding also increases hepatic isoenzyme mass in both zones. In the limited number of experiments analysed here, this mass induction appears to be slightly greater in the perivenous cells (Fig. 1, Table 2).

Isolation of periportal and perivenous ACC by avidin-Sephadex chromatography

Changes in ACC activity due to altered enzyme phosphorylation will persist after isolation of the enzyme to homogeneity on monomeric avidin-Sephadex, whereas those due to allosteric regulation do not, likely owing to the removal of allosteric regulators on dialysis and/or high-salt washing of the columns before enzyme elution with biotin [12,18–22]. ACC was isolated to homogeneity by this technique from both periportal and perivenous eluates of normally feeding rats. We found that, although the enzyme isolated from the periportal zone possesses a slightly lower K_a for citrate, the periportal/perivenous gradient in specific activity, measured at maximal velocity, observed in eluates does not persist through enzyme isolation (Table 4). Recovery of enzyme mass of ACC (both isoenzymes), as measured by immunoblotting, is identical for periportal and perivenous isolates. However, there is an enhanced recovery of perivenous activity, as measured at maximal velocity (25.6% perivenous versus 13% periportal), consistent with the differential removal of an allosteric effector.

ACC protein kinases and ACC protein phosphatase

The small persistent zonal difference in the K_a for citrate after ACC isolation could reflect a difference in the phosphorylation state of ACC in the periportal and perivenous zones, since this kinetic difference is a feature of an alteration in ACC phosphorylation [12,19–22]. We were unable to isolate enough ACC from eluates to determine alkali-labile phosphate content. Indirect evidence for possible altered phosphorylation was sought in the measurement of ACC protein kinase and phosphatase activities in the eluates. Cyclic-AMP-dependent kinase activity (measured against Kemptide) is identical in periportal or perivenous eluates, whether measured in the absence (free catalytic subunit) or presence (total catalytic subunit) of cyclic AMP (cAMP) [periportal: +cAMP, 91 ± 27 pmol/min per mg of protein; -cAMP, 52 ± 11 ; activity ratio 0.60 ± 0.07 ; perivenous: +cAMP, 109 ± 33 ; -cAMP 73 ± 22 ; activity ratio 0.67 ± 0.02 ($\pm$ S.E.M., $n = 3$ experiments)]. The activity of the 5'-AMP-activated ACC kinase also does not vary between the perivenous and periportal zones in three separate assays [periportal 0.019 ± 0.003 (S.E.M.) mol of 32 P/mol of ACC; perivenous 0.021 ± 0.010]. Additionally, no difference in kinase activity measured in the absence of 5'-AMP (and in the presence of the protein kinase inhibitor protein and heparin) is observed (results not shown).

Total ACC phosphatase activity, however, does differ between

Table 3. Acetyl-CoA kinetics in periportal (PP) and perivenous (PV) eluates

Results shown are the calculated values obtained by non-linear or linear SYSTAT analysis of data obtained from six independent perfusions in fed rats and two perfusions in fasted/refed rats. Mean values are shown in each instance ($\pm$ S.D.). $V_{\max}$ values represent enzyme velocity (as units/mg of total ACC protein); K_m values are indicated as μ M-acetyl-CoA. As determined by a two-tailed *t*-test, statistical significance is indicated: * $P < 0.0005$, PP components versus PV components, and † $P < 0.002$, high- K_m versus low- K_m (PP and PV).

Fed liver	PP				PV			
	High		Low		High		Low	
	K_m	$V_{\max}$	K_m	$V_{\max}$	K_m	$V_{\max}$	K_m	$V_{\max}$
	38 $\pm$ 8.2	1.70 $\pm$ 0.16*†	8.3 $\pm$ 2.1	2.59 $\pm$ 0.42*	44 $\pm$ 2.5	0.91 $\pm$ 0.18†	5.0 $\pm$ 2.4	0.50 $\pm$ 0.10
Fasted/refed liver	PP				PV			
	K_m	$V_{\max}$	K_m	$V_{\max}$	K_m	$V_{\max}$	K_m	$V_{\max}$
			30.8	4.28	38.3	3.54		

Table 4. ACC recovery on enzyme isolation

Shown are recovery data ($\pm$ S.D.) derived from (*n*) isolations of ACC from periportal (PP) and perivenous (PV) eluates of fed animals. Except where indicated, ACC activity is maximal velocity (10 mM-citrate). The K_a for citrate was determined by linear-regression analysis of Eadie-Hofstee plots. ACC mass was measured by avidin-based e.l.i.s.a. in column loads and isolates; the specific-activity data uses total mass (ACC 265 + ACC 280) to derive actual specific activity (see the text). * indicates $P < 0.01$, PP versus PV (citrate K_a) and ** $P < 0.001$, PP versus PV (activity recovery).

	Periportal	Perivenous
ACC specific activity (units/mg of total ACC)		
10 mM-Citrate	2.49 $\pm$ 0.60 (6)	2.36 $\pm$ 0.33 (5)
0.5 mM-Citrate	1.09 $\pm$ 0.28 (6)	0.92 $\pm$ 0.17 (5)
0 Citrate	0.20 $\pm$ 0.09 (6)	0.15 $\pm$ 0.09 (5)
K_a for citrate (mM)	0.84 $\pm$ 0.28 (7)	1.20 $\pm$ 0.14 (6)*
ACC recoveries		
Activity		
Load (m-units)	60.3 $\pm$ 5.0 (6)	11.6 $\pm$ 2.0 (5)
Isolates (m-units)	7.9 $\pm$ 1.1 (6)	3.1 $\pm$ 0.7 (5)
% recovery	13 $\pm$ 1.1	25.6 $\pm$ 2.3**
Mass (ACC 280 + ACC 265)		
Load (μ g)	26.5 $\pm$ 3.1 (6)	10.4 $\pm$ 2.2 (5)
Isolates (μ g)	3.5 $\pm$ 0.4 (6)	1.5 $\pm$ 0.4 (5)
% recovery	14.1 $\pm$ 1.4	15.2 $\pm$ 1.8

the periportal- and perivenous-zonal eluates (Fig. 3). In normally feeding animals, incubation of purified ACC from fed animals (a relatively phosphorylated species [19–22]) with periportal fractions causes a 2-fold increase in ACC specific activity by 15 min, and a 4-fold increase by 30 min. During the same incubation interval, the activity of ACC incubated with perivenous fractions rises about 1.5-fold and 2.8-fold respectively. In fasted/refed animals, a condition in which no zonation of ACC specific activity is observed, there is no detectable difference in total phosphatase activity between perivenous and periportal eluates. The abolition of the difference is due principally to an increase in perivenous phosphatase activity, and not to a decrease in

Fig. 3. ACC phosphatase activity in perivenous and periportal eluates

Shown are the results of ACC phosphatase assays performed, as described in the Materials and methods section, with periportal (○) or perivenous (●) eluates from seven fed (a) and four fasted/refed (b) liver perfusions. The data are displayed as the fold increase in ACC activity on addition of the fraction ($\pm$ S.E.M.) over time. No change in ACC activity over the 30 min assay period was seen in the absence of eluate fractions (not shown). * indicates periportal/perivenous difference at $P < 0.03$ (Student's two-tailed *t*-test).

periportal phosphatase activity. This latter observation is consistent with the selective activation of perivenous ACC specific activity seen on refeeding.

DISCUSSION

In fed rats, there is a unique type of intrahepatic gradient not previously noted in the study of other liver enzymes. The preferential zonation of ACC total activity to the periportal hepatocytes appears to be due to a gradient of ACC specific activity, not a zonation of enzyme mass. Such a gradient would not be detectable by some other methods, such as immunohistochemistry, that have been employed to estimate intrahepatic gradients for other enzymes. Current models of hepatic zonation of enzyme activity have largely focused on zonal gradients of enzyme mass. Since many hepatic enzymes are subject to allosteric regulation and/or regulation by covalent modification, we suggest that zonal variation of enzyme specific activity, as with ACC, could be a more common phenomenon.

In the present study, we have not confirmed our previously reported observation of a small intrahepatic gradient to the periportal side in ACC mass [7]. The reasons for this discrepancy are probably contributed to by several factors. The previous study was performed on freeze-dried shipped samples, and it is possible that degradation of ACC protein during transit could have altered the results. In addition, since that report, we have developed superior methods (e.l.i.s.a.) for the measurement of ACC mass, which give much more reproducible estimates than the technique of immunoblotting. The previously observed mass gradient to the periportal side for fatty acid synthase, however, has been confirmed; we were unable to estimate the potential gradient for ATP citrate-lyase in fed animals in the present study.

Two parameters of ACC activity are especially notable in the analysis of the specific-activity gradient in fed rats. First, there is a marked difference in citrate reactivity, with the periportal eluate displaying nearly maximal velocity at a citrate concentration that only activates the perivenous one to a limited extent. Second, two components that differ in the K_m for acetyl-CoA are also evident, with the largest periportal/perivenous difference being in the low- K_m component. Both of these differences are abolished on fasting/refeeding, with only a single component of acetyl-CoA reactivity being present. We initially hypothesized that these differences in the fed animals might reflect either zonal differences in isoenzyme distribution or a zonal variation in phosphorylation state of ACC. Citrate reactivity varies as a function of the phosphorylation state [19–22], and the M_r -265000 and M_r -280000 ACC isoenzymes have different citrate reactivity and K_m for acetyl-CoA [8]. Furthermore, fasting/refeeding is associated with ACC dephosphorylation [19–22], and in our study with abolition of the ACC gradient. By direct measurement, the mass of both ACC isoenzymes does not, however, differ between the hepatic zones. Proteolysis may activate ACC [23]; however, on immunoblotting, no substantive ACC degradation is noted in either zonal eluate. Except for a small persistent difference in the K_a for citrate, the differences in specific activity evident in the periportal and perivenous eluates (at maximal velocity) are eliminated after ACC isolation to homogeneity. Taken together, these data argue that covalent modification and/or variable ratios of the two isoenzymes do not account for the majority of the activity gradient seen in eluates, and that the presence of other allosteric factors in the periportal and/or perivenous eluates is the most likely explanation for zonal variation in ACC activity. Although we have noted periportal zonation of total ACC phosphatase activity (only in fed animals and abolished in fasted/refed animals), the significance of this finding with respect to the state

of ACC phosphorylation is uncertain, since we were unable to measure ACC phosphate content directly. It should also be noted that this functional phosphatase assay does not discriminate between several protein phosphatases that are active on ACC [24]; a selective and larger increment in the zonation of the activity of any single phosphatase species is quite possible.

The zonal gradient in ACC specific activity is particularly striking when ACC is measured at variable concentrations of acetyl-CoA. The determinants of this varying acetyl-CoA reactivity of ACC are not apparent from our results. The M_r -265000 isoenzyme has a lower K_m for acetyl-CoA than does the M_r -280000 isoenzyme, and mixtures of the two isoenzymes may show non-linear kinetics ([8]; A. Bianchi, A.-C. Nordlund & L. A. Witters, unpublished work). Phosphorylation of ACC by the cyclic-AMP-dependent protein kinase does not affect acetyl-CoA reactivity [25]; however, we have observed changes in acetyl-CoA kinetics on phosphorylation of both ACC isoenzymes by the 5'-AMP-activated protein kinase (L. A. Witters & A.-C. Nordlund, unpublished work). Such phosphorylation, not directly measurable in these experiments, could affect one or the other of the isoenzymes to a different extent, altering its individual substrate reactivity, its association with the other isoenzyme and/or its modulation by other intracellular effectors.

Changes in total ACC mass, specific activity and citrate-dependence accompanied by enzyme dephosphorylation on nutritional induction have been noted in several previous investigations [14,19–22]. The present study reveals two other features of this induction of hepatic ACC not previously recognized. First, as discussed above, there is a pronounced change in acetyl-CoA kinetics on such induction. Second, there is a selective increase in the specific activity of perivenous ACC, coincident with increases in the mass of both hepatic isoenzymes in each liver zone (perhaps to a slightly greater extent in perivenous cells). This increase, accompanied by an increase in perivenous total ACC phosphatase activity, is responsible for the abolition of intrahepatic gradient on nutritional induction.

The zonation of ACC has also been studied by others in microdissected rat liver tissue and in isolated periportal and perivenous hepatocytes [26,27]. These results differ from our own in that a net gradient of ACC activity to the perivenous side has been observed. There are, however, several technical differences that could account for the results of these investigations. First, neither of these techniques possesses the demonstrated zonal selectivity of the dual-digitonin-pulse perfusion technique. Second, there are considerable time lags in other techniques between the handling of the intact liver and the enzyme measurement; it is quite conceivable that there could be considerable alterations in ACC activity during these time periods. Third, ACC activity in these experiments was measured only at a single citrate concentration, and no estimate of ACC mass was made (allowing for determination of actual specific activity). The results in the isolated hepatocytes additionally indicate that fatty acid synthesis is zonated to the perivenous zone [27]; however, it is not clear that the techniques of cellular isolation and incubation before measurement do not also significantly alter the prior synthetic rate *in vivo*. Indeed, lipogenic rates are depressed after cell isolation, and all measurements are made after a period of cell preincubation. We have at present no estimate of fatty acid synthesis rates under the conditions of our liver perfusion. Quite obviously, it is dependent on factors other than ACC specific activity and fatty acid synthase mass zonation.

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