Changes in the properties of cytosolic acetyl-CoA carboxylase studied in cold-clamped liver samples from fed, starved and starved–refed rats

Alison M. B. MOIR and Victor A. ZAMMIT

Hannah Research Institute, Ayr, Scotland KA6 5HL, U.K.

We have used the cold-clamping technique to study the changes in acetyl-CoA carboxylase activity that occur in the cytosolic and mitochondrial fractions of the liver of fed, starved and starved–refed rats. No evidence was found for a role of the mitochondrial enzyme as a pool from which cytosolic carboxylase could be replenished upon refeeding of starved rats. Starvation for 24 h or 48 h induced changes in the expressed (assayed at 20 mM-citrate), total (citrate- and phosphatase-treated) and citrate-independent activities of cytosolic carboxylase, as well as in its K_a for citrate. The expressed/total activity ratio was low even in the fed state and was depressed further by starvation. The effects of refeeding occurred in two phases: an acute phase (approx. 1 h) in which the starvation-induced changes in K_a and expressed/total activity ratio were rapidly reversed, and a prolonged slow phase in which the two parameters attained values that were lower and higher, respectively, than those in the normal fed state. Refeeding also resulted in a gradual increase in citrate-independent activity of acetyl-CoA carboxylase. An additional marked increase in this activity occurred only in 48 h-starved–refed rats between 24 h and 48 h of refeeding. These findings are discussed in terms of the observed time courses of changes in lipogenic rates that occur *in vivo* in starved–refed rats and of the possible molecular mechanisms involved.

INTRODUCTION

The product of the reaction catalysed by acetyl-CoA carboxylase, malonyl-CoA, plays a central role in the regulation of fatty acid metabolism in the liver [1,2]; the rate of fatty acid synthesis is directly related to its concentration [2,3]. Malonyl-CoA is also a potent inhibitor of the first reaction committed to fatty acid oxidation, catalysed by the overt mitochondrial carnitine palmitoyltransferase (CPT I) [4]. In previous publications [5,6] we showed that this role of malonyl-CoA may vary in importance, depending on the physiological state of the animal, e.g. during the early phases of refeeding of rats after starvation. These observations, coupled to ones that indicate that the rate of lipogenesis remains low during carbohydrate refeeding after starvation [7,8], raise the question as to whether the adaptations of CPT I that favour a delayed reversal of the starved state are complemented by parallel characteristics of acetyl-CoA carboxylase. It is already known that pyruvate dehydrogenase (PDH), which largely controls the rate of formation of substrate (acetyl-CoA) and activator (citrate) for carboxylase, remains phosphorylated and relatively inactive for several hours after the commencement of refeeding [7,9]. Although this hysteresis of the PDH inactivation–activation cycle would theoretically be sufficient, by itself, to control the rate of malonyl-CoA formation, it is evident from other metabolic systems that control of the flux of a pathway is seldom exerted exclusively at one reaction. Hence a role for acetyl-CoA carboxylase in the control of the rates of lipogenesis and fatty acid oxidation in the starved-to-fed transition cannot be ruled out. This is particularly true for the starved-to-refed transition, in which the complete recovery of hepatic PDH activity occurs before that of lipogenesis after refeeding of starved rats [7].

Acetyl-CoA carboxylase is a highly regulated enzyme. Its activity is affected allosterically by citrate and long-chain acyl-CoA, and is modulated by reversible phosphorylation (see [10]).

In particular, the control of the specific activity of the enzyme and of its sensitivity to citrate are under control by phosphorylation at multiple sites. Phosphorylation on serine-77 and -1200 by cyclic AMP-dependent protein kinase results in a decrease in the affinity of the enzyme for citrate and a relatively small decrease in specific activity, whereas phosphorylation on serine-79 and -1200 by the AMP-activated protein kinase [11,12] results in a large decrease in specific activity, with an additional effect on K_a for citrate. In addition, the enzyme undergoes polymerization to a form that is active in the absence of citrate.

In order to obtain a valid representation of the state of acetyl-CoA carboxylase *in vivo*, adequate sampling of the liver is a prerequisite. In previous studies, assessment of the phosphorylation state of acetyl-CoA carboxylase *in vivo* has been attempted through the rapid freezing of the tissue with subsequent assay of carboxylase activity, either in relatively crude preparations [13–15] or with preparations of the enzyme purified on avidin–Sephacrose (see, e.g., [16]). However, Allred and co-workers have reported [17–19] that mitochondria contain a substantial storage pool of inactive acetyl-CoA carboxylase that could be released into the cytosol to become metabolically active, depending on physiological state. These observations have raised the question as to whether freezing of whole tissue is an adequate technique with which to study the characteristics of metabolically relevant hepatic acetyl-CoA carboxylase. Freezing and disruption of subcellular structures would not allow the distinction between the changes in the characteristics of the largely inactive mitochondrial form from those of the metabolically active cytosolic carboxylase.

In this study we have employed a technique (cold-clamping) of rapid liver sampling (without freezing) which we originally developed for the study of the phosphorylation state *in vivo* of another cytosolic (microsomal) enzyme and which avoids interference from mitochondrial activities [20]. This technique allowed us to obtain separate cytosolic, mitochondrial and

microsomal acetyl-CoA carboxylase activities with retention of their respective phosphorylation states *in vivo* during the starved-to-fed transition in rats.

MATERIALS AND METHODS

Animals

These were female Wistar rats (180–200 g) fed (*ad lib.*) on a standard laboratory rat diet (3% fat; Biosure, U.K.). Starved animals were placed in wire-bottomed cages and had their food removed for the number of hours indicated but had constant access to water. All animals were maintained on a 12 h-light/12 h-dark cycle. Animals referred to as 'fed' were used 1.5 h after the beginning of the light phase; the periods of starvation or refeeding were also started at this time.

Liver sampling and preparation of subcellular fractions

Cold-clamping. Rapid sampling, cooling and homogenization of approx. 1 g samples of liver (taken from the left lateral lobe) were performed exactly as described previously [20] in a medium containing 150 mM-sucrose, 20 mM-Tris (pH 7.4 at 0 °C), 10 mM-EGTA, 10 mM-EDTA, 100 mM-KF, 7.5 mM-GSH and leupeptin, pepstatin and antipain (all at 1 µg/ml). Fatty-acid-poor BSA (40 mg/ml) was added to the homogenates, which were centrifuged first at 500 g for 10 min to remove cell debris and then at 10000 g for 10 min to obtain the mitochondrial fraction. In some experiments, the 10000 g supernatant was centrifuged at 100000 g for 60 min to obtain microsomal and soluble fractions. As documented below, negligible amounts of acetyl-CoA carboxylase activity were found in microsomes. So, routinely, the whole 10000 g supernatant was used as a source of cytosolic (i.e. non-mitochondrial) enzyme. Samples were kept for assay of lactate dehydrogenase and citrate synthase activities. This fraction was then made 35% saturated with respect to $(\text{NH}_4)_2\text{SO}_4$, and the precipitated protein was collected by centrifugation. Preliminary experiments indicated that all cytosolic acetyl-CoA carboxylase was precipitated at this concentration of $(\text{NH}_4)_2\text{SO}_4$. The pellet was resuspended in medium containing 1 mM-EDTA, 20 mM-Tris, 100 mM-KF, 7.5 mM-GSH and protease inhibitors (see above). Part of the resulting solution was stored on ice, and the remainder was incubated with 20 mM-citrate, 5 mM-manganese acetate and 2 units of protein phosphatase [21,22] at 30 °C for 20–30 min. The latter conditions gave maximal activation of acetyl-CoA carboxylase. It was found that, if sufficient exogenous phosphatase was used, inclusion of Mn^{2+} at this concentration totally overcame the inhibition of phosphatase by fluoride, i.e. the activation induced by citrate, Mn^{2+} and phosphatase was the same in the presence or absence of fluoride. We were therefore able to measure acetyl-CoA carboxylase activity in fluoride-containing $(\text{NH}_4)_2\text{SO}_4$ fractions before and after phosphatase treatment, such that a direct comparison could be made between the citrate- and phosphatase-activated enzyme and the enzyme that had simply been extracted in fluoride. Preliminary experiments showed that the presence of manganese acetate did not affect carboxylase activity. It was also shown that extensive dialysis or Sephadex G-25 gel filtration of resuspended $(\text{NH}_4)_2\text{SO}_4$ pellets before assay did not affect citrate-independent activity, indicating that there was no interference from possible carry-over of citrate from the crude extracts into the assay (see also below).

Mitochondrial fractions were washed once in extraction medium and the resulting pellet was assayed without prior treatment with $(\text{NH}_4)_2\text{SO}_4$. Pellets were resuspended in a known volume of medium containing 150 mM-sucrose, 20 mM-Tris, 10 mM-EGTA, 10 mM-EDTA, 100 mM-KF, 20 mM-magnesium acetate, 7.5 mM-GSH and protease inhibitors (see above). They were either used

for carboxylase assay without further treatment, or incubated with 20 mM-citrate at 30 °C for 30 min. We were not able to examine the effects of exogenous phosphatase plus citrate on mitochondrial fractions, as the conditions described above for cytosolic fractions resulted in extremely high blank values. These probably resulted from damage to the mitochondria, but the effect was not studied further. Samples of mitochondrial suspensions were used for the assay of citrate synthase and lactate dehydrogenase activities after sonication. The absolute activities of lactate dehydrogenase and citrate synthase (per g wet wt.) were measured on extracts of a separate sample of liver.

Enzyme assays

Acetyl-CoA carboxylase was assayed at 30 °C as described previously [22]. The assay medium contained 100 mM-Tris/acetate (pH 7.4 at 30 °C), 0.5 mM-EDTA, 10 mM- MgSO_4 , 2.5 mM-ATP, 150 µM-acetyl-CoA, 1 mM-dithiothreitol, 10 mg of BSA/ml and 15 mM- $\text{NaH}^{14}\text{CO}_3$ (2.5 Ci/mol). Citrate concentrations were varied by adding increasing amounts of a stock solution containing 400 mM-citrate and 400 mM-magnesium acetate. Incubations (total volume 0.25 ml) were started by the addition of sample (20 µl) and terminated after 60 s by addition of 25 µl of 6 M-HCl. Incorporation of ^{14}C into non-volatile material was measured essentially as described previously [23]. Controls from which acetyl-CoA was omitted were run concurrently.

Calculation and expression of results

Acetyl-CoA carboxylase activities (nmol of HCO_3^- incorporated into acid-stable material/min) were obtained on the basis of the activities of lactate dehydrogenase or citrate synthase in the cytosolic or mitochondrial fractions, respectively, after correction for cross-contamination of the two fractions by using the marker enzyme activities. The absolute activities of lactate dehydrogenase and citrate synthase per unit liver weight were then used to convert carboxylase activities so that they could be expressed as units of activity/g wet wt. The following terms will be used in the text to denote individual parameters: 'citrate-independent activity' refers to the activity obtained when the enzyme was assayed in the absence of added citrate and without pre-activation; 'expressed activity' refers to that obtained at 20 mM-citrate in the assay, but without pre-activation; 'total activity' refers to that obtained at 20 mM-citrate in the assay after pre-activation with 20 mM-citrate and phosphatase (see above). The K_m for citrate was calculated as described previously [24], by using the values obtained by assaying enzyme activity at different citrate concentrations after subtraction of any citrate-independent activity.

Materials

Protein phosphatase was partially purified from rat liver as described previously [21]. It was dialysed before use against medium containing 20 mM-Tris/acetate (pH 7.4), 1.0 mM-EDTA, 7.5 mM-GSH and protease inhibitors (see above). $\text{NaH}^{14}\text{CO}_3$ was from Amersham International, Amersham, Bucks., U.K. Protease inhibitors were from Peptide Institute Inc. (Barnet, Herts., U.K.). Acetyl-CoA, ATP and Sephadex G-25 were from Sigma Chemical Co. (Poole, Dorset, U.K.). All other chemicals were of the highest purity available and were obtained from BDH Chemicals (Poole, Dorset, U.K.).

RESULTS AND DISCUSSION

The rationale for the experimental approach used in the present study arose from the consideration of several aspects of the properties of acetyl-CoA carboxylase described by other

Table 1. Activities of mitochondrial acetyl-CoA carboxylase from liver of fed, starved and starved-refed rats

The mitochondrial fraction was prepared as described in the Materials and methods section. Acetyl-CoA carboxylase activity was assayed in the presence of 20 mM-citrate, with or without prior incubation with 20 mM-citrate for 30 min at 30 °C. Values represent means $\pm$ S.E.M. for the numbers of determinations shown in parentheses.

Condition	Mitochondrial acetyl-CoA carboxylase activity (nmol/min per g wet wt.)	
	'Expressed' activity	'Total' activity
Fed (5)	1.62 $\pm$ 0.7	24.9 $\pm$ 2.0
24 h starved (6)	2.50 $\pm$ 0.7	31.1 $\pm$ 1.7
+ 1 h refed (6)	1.03 $\pm$ 0.4	26.9 $\pm$ 2.4
+ 3 h refed (6)	0.88 $\pm$ 0.3	21.6 $\pm$ 2.7
+ 24 h refed (2)	0.75	24.1
48 h starved (4)	0.68 $\pm$ 0.4	26.3 $\pm$ 2.5
+ 1 h refed (4)	1.95 $\pm$ 0.2	23.7 $\pm$ 1.9
+ 3 h refed (4)	1.10 $\pm$ 0.4	20.3 $\pm$ 1.9
+ 24 h refed (4)	1.89 $\pm$ 0.8	20.8 $\pm$ 2.3

groups as well as from constraints imposed by cellular compartmentation and interference from pyruvate carboxylase activity. The use of avidin-Sephadex chromatography of tissue extracts to obtain pure acetyl-CoA carboxylase on which kinetic studies can be performed has facilitated greatly the study of this enzyme. However, we were concerned that, whereas a form that displays citrate-independent activity was isolated, by this technique, by at least one group [16,25], the enzyme is completely citrate-dependent for activity after avidin-Sephadex chromatography of extracts prepared from animals in the same physiological state, by another group of workers [26]. We were also conscious of the suggestion by Denton and co-workers [27] that such chromatography may discriminate in favour of the dimeric form of the enzyme. In addition, we wanted to obtain absolute activities of carboxylase expressed on a unit-liver-weight basis. Our choice of technique was also influenced by reports from Allred and co-workers ([17-19]; see the Introduction) that an inactive form of acetyl-CoA carboxylase may be associated with the mitochondrial outer membrane. Consequently, in whole extracts of frozen tissue the overall characteristics of carboxylase may be distorted by the presence of this inactive form. Extracts prepared from freeze-clamped samples would also have been susceptible to errors in the determination of acetyl-CoA carboxylase introduced by interference from pyruvate carboxylase activity owing to breakage of the mitochondria. The use of cold-clamping [20] in which the liver is sampled, cooled (not frozen) and homogenized very rapidly (~ 5 s) allowed us to avoid these problems and to obtain kinetic data on carboxylase in different cell fractions. In addition, the use of cold-clamping also obviated the need for avidin-Sephadex purification of acetyl-CoA carboxylase before assay, as it leaves the mitochondria intact [20], and thus avoids the risk of serious interference from pyruvate carboxylase activity [28,29]. Validation of the cold-clamping technique has been performed previously [21]. In the present study we performed pilot experiments in which livers from animals in the same physiological state were either freeze-clamped or cold-clamped. The values for K_m and expressed/total activity ratios for acetyl-CoA carboxylase in extracts of freeze-clamped liver samples from normal fed rats were not significantly different from the values obtained on cold-clamped samples (results not shown).

Acetyl-CoA carboxylase activity in mitochondrial and microsomal fractions

Pilot experiments established that microsomal fractions contained negligible activities of acetyl-CoA carboxylase even after incubation with citrate and exogenous phosphatase. All the carboxylase activity in post-mitochondrial supernatants was recovered in the respective 100 000 g supernatants (results not shown). By contrast, some acetyl-CoA carboxylase activity was always associated with the mitochondrial fraction, over and above that which could be accounted for by contamination with cytosol. However, this form of the enzyme was always very inactive unless the mitochondrial fractions were preincubated with citrate. As mentioned in the Materials and methods section, we were unable to study the additional effects of phosphatase treatment on mitochondria. Citrate treatment of the mitochondrial fraction always resulted in a 10-15-fold activation. Consequently, this form of the enzyme appears to be physiologically inert in freshly prepared mitochondria. Even

Fig. 1. Cytosolic acetyl-CoA carboxylase activity of liver extracts from fed, starved and starved/refed rats, measured as a function of the citrate concentration

Acetyl-CoA carboxylase activity was assayed in the presence of increasing citrate concentrations in $(\text{NH}_4)_2\text{SO}_4$ pellets prepared from cytosolic fractions from rats (a) fed (■), starved for 24 h (○) and then refed for 1 h (△) and 48 h (■), and (b) fed (■), starved for 48 h (□) and then refed for 1 h (▲), 24 h (◆) and 48 h (●). Values are means $\pm$ S.E.M. for 4-11 separate determinations.

after preincubation with citrate, its activity constituted a minor proportion (approx. 15%) of total carboxylase (Table 1). We found no evidence to support the suggestion by Allred and co-workers that mitochondrial carboxylase constitutes a pool of enzyme from which cytosolic carboxylase is replenished on refeeding of starved rats [19]. Thus the activity of the mitochondrial enzyme did not change with physiological condition (Table 1), and no reciprocal changes in activity between the mitochondrial and cytosolic forms were detected upon refeeding. Because of these findings and of its very low activity before incubation with citrate, we did not pursue a more detailed study of the kinetics of the mitochondrial form.

Cytosolic acetyl-CoA carboxylase

The above data strongly suggested that only cytosolic acetyl-CoA carboxylase is actively involved in malonyl-CoA synthesis under physiological conditions. Our experimental protocol allowed us to perform detailed kinetic studies on the cytosolic enzyme with retention of its phosphorylation state *in vivo*.

After concentration by precipitation with $(\text{NH}_4)_2\text{SO}_4$, we determined several characteristics of the cytosolic enzyme. These were (i) the citrate-independent activity, (ii) the activities in the presence of different concentrations of citrate, and (iii) the activity measured at 20 mM-citrate after preincubation of the resuspended protein pellet with citrate, phosphatase and Mn^{2+} (see the Materials and methods section). Fig. 1 shows the data obtained for selected physiological states. Not all the data are shown, in the interests of clarity.

Effects of different periods of starvation

Starvation for either 24 h or 48 h resulted in an increase in the K_a of acetyl-CoA carboxylase for citrate, from about 5 mM to about 8 mM (Fig. 2). This was accompanied by a decrease in total activity of the enzyme, which was more pronounced after 48 h (Figs. 3 and 4). The data suggest that the half-life of liver carboxylase upon starvation is approx. 48 h. The decrease in expressed activity after starvation was proportionately greater than that in total activity, such that the expressed/total activity ratios declined from approx. 0.21 in the fed state to 0.10 and 0.08 for 24 h- and 48 h-starved rats respectively (see Figs. 3 and 4). Starvation also resulted in the virtual elimination of citrate-independent activity (Fig. 5), which in the fed state amounted to 10 nmol/min per g wet wt.

Effects of refeeding on K_a for citrate

The decrease in K_a for citrate observed upon refeeding of either 24 h- or 48 h-starved rats occurred in two distinct phases. The first was extremely rapid and resulted in the complete reversal of the starvation-induced increase in K_a within 1 h of commencement of refeeding (Fig. 2). No further change in this parameter occurred over the next 2 h. This initial phase was followed by a gradual further decrease in K_a for citrate to values even lower than those obtained in the fed state, by 12 h of refeeding. Preliminary experiments indicated that this was not due simply to diurnal effects on K_a . Carboxylase in extracts of livers from fed animals sampled at the start of the light phase (i.e. normal, fed) shown in Fig. 2 had the same K_a values as those observed for animals sampled at the start of the dark phase (i.e. equivalent to 12 h-refed animals; results not shown). Surprisingly, K_a values stayed low after 24 h and 48 h of refeeding. Although they approached the value for fed animals in 24 h-starved-refed rats, in 48 h-starved-refed animals the K_a values were still approximately half those of fed rats even after 48 h of refeeding.

Both cyclic AMP-dependent and AMP-activated protein kinase-mediated phosphorylation of acetyl-CoA carboxylase result in sizeable decreases in the affinity of the enzyme for citrate, although AMP-activated kinase appears to have a greater effect on K_a (at least when phosphorylation of the avidin-Sepharose-purified totally dephosphorylated rat mammary-gland carboxylase is studied [12]). Consequently, it is not possible to distinguish which residues on carboxylase are being preferentially dephosphorylated to yield the two phases observed. In this context it is worth noting that the purified enzyme used in the studies reported in [12] was always devoid of citrate-independent activity, whereas in our preparations (Fig. 5) and in the studies reported on avidin-Sepharose-purified carboxylase [16,22] there was substantial citrate-independent activity, especially after 48 h refeeding of 48 h-starved rats. Because of the presence of citrate-independent activity, the present K_a values have had to be determined only for the portion of the activity that was citrate-dependent (see the Materials and methods section). Consequently the K_a values shown in Fig. 2 are not directly comparable with those reported for the purified citrate-dependent enzyme [12]. Much lower (if less informative) values would be obtained if the citrate-independent component is included in the calculations.

The data on the expressed/total activity ratio (see above) indicated that, even at an optimal citrate concentration, only

Fig. 2. Effect of starvation and subsequent refeeding on the K_a for citrate of acetyl-CoA carboxylase

Rats were starved for (a) 24 h or (b) 48 h before refeeding for the indicated periods. Values are means $\pm$ S.E.M. for 4–9 separate determinations.

Fig. 3. Effect of 24 h starvation and subsequent refeeding on expressed and total acetyl-CoA carboxylase activity in liver cytosol

Activities are denoted by (○) total and (■) expressed. The time course for mean values of expressed/total ratio is denoted by (◆). Values are means $\pm$ S.E.M. for 4–15 separate determinations.

Fig. 4. Effect of 48 h starvation and subsequent refeeding on expressed and total acetyl-CoA carboxylase activity in liver cytosol

Activities are denoted by (○) total and (■) expressed. The time course for mean values of expressed/total ratio is denoted by (◆). Values are means $\pm$ S.E.M. for 4–15 separate determinations.

about 20% of the total enzyme activity was expressed. The kinase most likely to be responsible for such a high background level of phosphorylation is the AMP-activated kinase, as its effect on the specific activity of carboxylase is very marked [11,12].

Effects of refeeding on enzyme activity

There was no effect of refeeding during the first 24 h on the total carboxylase activity in either 24 h- or 48 h-starved rats. This was unexpected, but may represent part of the mechanism whereby the rates of lipogenesis are kept relatively low, especially during the early phases of refeeding [7,8]. Indeed, there was evidence that during this initial period the total activity of the enzyme continued to decline, on a liver-weight basis, rather than increase (Figs. 3 and 4). When refeeding was continued beyond 24 h there was a very marked increase in total activity, such that by 48 h refeeding the total activity was equal to (24 h-starved rats) or greater than (48 h-starved rats) that in the fed state. This effect is presumed to have been due to synthesis of new protein. It was not accompanied by any change in the mitochondrially associated carboxylase activity (Table 1).

The recovery of the expressed/total activity ratio upon refeeding showed a similar two-phase response to that noted for the changes in K_a (see above). There were rapid (1–3 h) increases in ratio equivalent to approx. 10% and 15% of total enzyme activity for 24 h- and 48 h-starved rats respectively. These were followed over the next 21 h of refeeding by further increases in the value of the ratio equivalent to 20% and 10% of total enzyme activity, in 24 h- and 48 h- starved rats respectively. However, whereas in 24 h-starved rats the ratio returned to the normal fed value between 24 h and 48 h of refeeding, that in 48 h-starved–48 h-refed rats continued to increase. This difference between 24 h-starved and 48 h-starved rats in their response to refeeding beyond 24 h was accompanied by another divergence in their responses. Throughout the first 24 h of refeeding, the citrate-independent activity increased as a proportion of total acetyl-CoA carboxylase activity. Thus, after 24 h or 48 h starvation, the citrate-independent activity was negligible (Fig. 5), whereas after 24 h refeeding it represented 10% of total activity. However, whereas in 48 h starved–48 h-refed rats it increased further (to 15% of total), in 24 h-starved–48 h-refed rats it declined to approx. 5% (as found in the normal fed animal). The results obtained with 48 h-starved–48 h-refed rats

Fig. 5. Effect of starvation and subsequent refeeding on the citrate-independent and citrate-dependent acetyl-CoA carboxylase activity

Rats were starved for (a) 24 h or (b) 48 h before refeeding for the indicated periods. Activity was measured in the absence of citrate (■) or in the presence of 1 mM- (●) or 2 mM- (▲) citrate. Values are means $\pm$ S.E.M. for 4–11 separate determinations.

are in agreement with the data of Thamby & Wakil [16], who found that a form of carboxylase that is largely citrate-independent for activity can be purified from such livers by avidin-Sephadex chromatography. It is possible that in livers of 48 h-starved-refed animals the sustained elevation of the expressed/total activity ratio between 24 h and 48 h refeeding and the marked increase in citrate-independent activity may be causally related, i.e. decreased phosphorylation giving rise to increased polymerization (the polymerized enzyme is active in the absence of citrate [27]). Such a large difference in response between the two groups of animals at a relatively late stage of refeeding raises the question as to whether the length of the period of starvation affects the subsequent expression of individual protein kinases and/or phosphatases in the liver upon refeeding (e.g. of the AMP-activated kinase). It is less likely that differences in acute changes in the concentration of effector ligands (e.g. cyclic AMP or AMP) would occur at this stage of refeeding.

The present data provide evidence that control at the level of acetyl-CoA carboxylase may be equally important as that exerted at PDH [7,9] during the starved-to-refed transition. It is clear from Fig. 5 that, although within the first hour of refeeding there was a modest decrease in phosphorylation state of the enzyme (i.e. the expressed/total ratio increased), the effective activity of the enzyme at physiologically relevant citrate concentrations [30] was still about 50 % of that in the fed state. This agrees with data from experiments on measurements of rates of lipogenesis *in vivo*, in which it was found that the rate of lipogenesis increased from approx. 25 % to 60 % of the 'fed' rate in going from the 48 h-starved to the 48 h-starved-3 h-refed state [7]. This suppression of activity of carboxylase is evidently due to the large decreases in total activity of the enzyme that occur upon starvation, such that for the same expressed/total activity ratio (compare fed with 48 h-starved-1 h-refed rats in Figs. 3 and 4) the activity at 1 mM-citrate after 1 h refeeding is only half that observed in the fed state (Fig. 5). Furthermore, the recovery of the lipogenic rate after 6-12 h refeeding [7] to that observed in the fed state is due not to increased enzyme synthesis (which would have been expected to result in increased total activity) but to a further phase of dephosphorylation to attain levels of expressed/total activity ratio beyond those observed in the fed state. The initial phase is likely to be associated with an acute change in the concentration of an effector of protein kinases

and/or phosphatases. It is noteworthy that the hepatic concentrations of both cyclic AMP [31] and AMP [32] are elevated in starvation, but that, whereas cyclic AMP concentration is rapidly restored to 'fed' values by glucose refeeding (< 1 h), AMP concentrations are still elevated after 2 h of refeeding [33]. Similarly, an acute decrease in the intracellular concentration of long-chain acyl-CoA (owing to a rapid fall in the rate of delivery of fatty acids to the liver upon refeeding [34]) would be expected to result in a decrease in the activity of the acyl-CoA-stimulated kinase that phosphorylates and activates the AMP-activated kinase [35]. There is compelling evidence [10,36] that rat liver acetyl-CoA carboxylase *in situ* is almost totally phosphorylated on serines that are acted on by the AMP-activated kinase and not by cyclic AMP-dependent protein kinase. Our data tend to support this contention, primarily because the enzyme is so heavily phosphorylated and inactivated (i.e. low expressed/total activity ratio) even in the fed state. However, it is not inconceivable that, *in vivo*, part of the effects of starvation (which only induces a further decrease in ratio equivalent to approx. 12 % of total activity) could be mediated through the action of cyclic AMP-dependent protein kinase. This would still leave the enzyme largely phosphorylated at the serine residues that are the targets for AMP-activated kinase, but would enable the direct effect of any lowering of hepatic cyclic AMP concentrations that occur upon refeeding [33] to achieve the acute phase of dephosphorylation observed within the first hour of refeeding. The concomitant sharp decrease in the K_s for citrate would also result from such dephosphorylation. We would like to suggest that acute changes in the phosphorylation state of carboxylase upon refeeding could affect only about 10 % of the total enzyme, but could be proportionately more important because of the high level of basal phosphorylation in which the enzyme normally exists. As discussed above, the slower (second phase) changes in expressed/total activity ratio and in K_s for citrate are likely to be due to changes in the expression of individual kinases. (Protein phosphatase 2A is reported to account for > 90 % of the phosphatase activity when acetyl-CoA carboxylase is the substrate in liver extracts [37]. We are not aware of any work showing that the activity of phosphatase 2A is modulated *in vivo*.)

The late increase in expressed/total activity ratio that occurs after 12 h refeeding, and which takes the ratio beyond the values observed in the normal fed state, demonstrates that achievement

Fig. 6. Proposed regulatory cascade through which the effects of changes in cytosolic malonyl-CoA concentration are transmitted to the mitochondrial matrix via acetyl-CoA

Solid arrows indicate direction of flux, heavy arrows indicating greater flux. The hollow arrow in (c) indicates that the potential capacity of the CPT I reaction is still high, although the actual flux is substantially below maximal. Dashed lines indicate regulatory effects of malonyl-CoA and acetyl-CoA. Abbreviations: PEP, phosphoenolpyruvate; OXA, oxaloacetate; KB, ketone bodies; LC, long-chain.

of a much lower state of phosphorylation is not impossible for acetyl-CoA carboxylase *in vivo*. If the high degree of phosphorylation observed in the fed state is attributed to the action of the AMP-activated kinase, it would appear likely that this overshoot of dephosphorylation during the later stages of refeeding (particularly in 48 h-starved rats) is due to a decline in AMP-activated kinase activity.

Physiological considerations

We have suggested previously [5,6] that there could be substantial interplay between the regulation of the rate of formation of malonyl-CoA and its twin roles (i) directly to regulate the rate of hepatic fatty acid oxidation, and (ii) indirectly to control the partitioning of pyruvate between carboxylation (which diverts pyruvate to gluconeogenesis) and oxidative decarboxylation (Fig. 6). Other authors have supported this interpretation [38,39]. The present data suggest that the delayed reversal, upon refeeding, of the effects of starvation on CPT I activity and kinetics [5] are complemented by a delayed re-establishment of acetyl-CoA carboxylase activity (to an even more pronounced degree than the delay shown by re-activation of PDH [7]). As shown in Fig. 6, these interactions will result in a regulatory cascade through which changes in malonyl-CoA concentration (as determined by acetyl-CoA carboxylase activity) coupled to changes in CPT I sensitivity to malonyl-CoA (see [40–42]) would effectively determine the level of intramitochondrial acetyl-CoA, especially since PDH remains inactive for up to 3 h after refeeding [7]. Acetyl-CoA is required for pyruvate carboxylase activity [43] and activates PDH kinase [44], i.e. favours PDH inactivation. Consequently the concerted action of the responses of acetyl-CoA carboxylase and CPT I may result in a degree of continued flux of fatty acids through β -oxidation which would enable the maintenance of a sufficiently high intramitochondrial acetyl-CoA concentration to ensure continued diversion of pyruvate away from lipogenesis and towards gluconeogenesis. It is suggested that the rate of fatty acid oxidation (i.e. the availability of fatty acids) would not need to be especially high. This is because utilization of acetyl-CoA during this period would be minimized by (i) inhibition of ketone-body formation through succinyl-CoA inhibition of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase [45–48] and (ii) the continued low rate of utilization of acetyl-CoA for lipogenesis necessitated by the low activity of acetyl-CoA carboxylase.

REFERENCES

- McGarry, J. D., Mannaerts, G. P. & Foster, D. W. (1977) *J. Clin. Invest.* **60**, 265–270
- McGarry, J. D., Takabayashi, Y. & Foster, D. W. (1978) *J. Biol. Chem.* **253**, 8294–8300
- Guynn, R. W., Veloso, D. & Veech, R. L. (1972) *J. Biol. Chem.* **247**, 7325–7331
- McGarry, J. D., Leatherman, G. F. & Foster, D. W. (1978) *J. Biol. Chem.* **253**, 4128–4136
- Grantham, B. D. & Zammit, V. A. (1986) *Biochem. J.* **239**, 485–488
- Grantham, B. D. & Zammit, V. A. (1988) *Biochem. J.* **249**, 409–414
- Holness, M. J., MacLennan, P. A., Palmer, T. N. & Sugden, M. C. (1988) *Biochem. J.* **252**, 325–330
- Agius, L. & Williamson, D. H. (1981) *Biochim. Biophys. Acta* **666**, 127–132
- Holness, M. J. & Sugden, M. C. (1989) *Biochem. J.* **258**, 529–533
- Hardie, D. G. (1989) *Prog. Lipid Res.* **28**, 117–146
- Munday, M. R., Campbell, D. G., Carling, D. & Hardie, D. G. (1988) *Eur. J. Biochem.* **175**, 331–338
- Davies, S. P., Sim, A. T. R. & Hardie, D. G. (1990) *Eur. J. Biochem.* **187**, 183–190
- Stansbie, D., Brownsey, R. W., Crettaz, M. & Denton, R. M. (1976) *Biochem. J.* **160**, 413–416
- Zammit, V. A. & Corstorphine, C. G. (1982) *Biochem. J.* **204**, 757–764
- Jamil, H. & Madsen, N. B. (1987) *J. Biol. Chem.* **262**, 638–642
- Thampy, K. G. & Wakil, S. J. (1988) *J. Biol. Chem.* **263**, 6447–6453
- Allred, J. B., Roman-Lopez, C. R., Pope, T. S. & Goodson, J. (1985) *Biochem. Biophys. Res. Commun.* **129**, 453–460
- Allred, J. B. & Roman-Lopez, C. R. (1988) *Biochem. J.* **251**, 881–885
- Roman-Lopez, C. R., Shriver, B. J., Joseph, C. R. & Allred, J. B. (1989) *Biochem. J.* **260**, 927–930
- Easom, R. A. & Zammit, V. A. (1984a) *Biochem. J.* **220**, 733–738
- Easom, R. A. & Zammit, V. A. (1984b) *Biochem. J.* **220**, 739–745
- Brandt, H., Capulong, Z. L. & Lee, E. Y. C. (1975) *J. Biol. Chem.* **250**, 8038–8044
- Halestrap, A. P. & Denton, R. M. (1973) *Biochem. J.* **132**, 509–517
- Holland, R., Hardie, D. G., Clegg, R. A. & Zammit, V. A. (1985) *Biochem. J.* **226**, 139–145
- Thampy, K. G. & Wakil, S. J. (1988) *J. Biol. Chem.* **263**, 6454–6458
- Sim, A. T. R. & Hardie, D. G. (1988) *FEBS Lett.* **233**, 294–298
- Borthwick, A. C., Edgell, N. J. & Denton, R. M. (1987) *Biochem. J.* **241**, 773–782
- Davies, D. R., Van Schaftingen, E. & Hers, H.-G. (1982) *Biochem. J.* **202**, 559–560
- Buechler, K. F., Beynen, A. C. & Geelen, M. J. H. (1984) *Biochem. J.* **221**, 869–874
- Siess, E. A., Brocks, D. G. & Wieland, O. H. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 785–798
- Selawry, H., Gutman, R., Fink, G. & Recant, L. (1973) *Biochem. Biophys. Res. Commun.* **51**, 198–204
- Bois-Joyeux, B., Chanez, M., Azzout, B., Delhomme, B. & Peret, J. (1986) *Diabetes Metab.* **12**, 239–245
- Seitz, H. J., Müller, M. J., Krone, W. & Tarnawski, W. (1977) *Arch. Biochem. Biophys.* **183**, 647–663
- McGarry, J. D., Meier, J. M. & Foster, D. W. (1973) *J. Biol. Chem.* **248**, 270–278
- Carling, D., Zammit, V. A. & Hardie, D. G. (1987) *FEBS Lett.* **223**, 217–222
- Sim, A. T. R. & Hardie, D. G. (1988) *FEBS Lett.* **233**, 294–298
- Haystead, T. A. J., Moore, F., Cohen, P. & Hardie, D. G. (1990) *Eur. J. Biochem.* **187**, 199–205
- McGarry, J. D., Woeltje, K. F., Kuwajima, M. & Foster, D. W. (1989) *Diabetes/Metab. Rev.* **5**, 271–284
- Sugden, M. C., Holness, M. J. & Palmer, T. N. (1989) *Biochem. J.* **263**, 313–323
- Robinson, I. N. & Zammit, V. A. (1982) *Biochem. J.* **206**, 177–179
- Zammit, V. A. (1983) *Biochem. J.* **210**, 953–956
- Zammit, V. A. (1984) *Biochem. J.* **218**, 379–386
- Scrutton, M. C. (1971) *Metab. Clin. Exp.* **20**, 168–173
- Randle, P. J., Sale, G. J., Kerbey, A. L. & Kerns, A. (1981) *Cold Spring Harbor Conf. Cell Proliferation: Protein Phosphorylation, Book A* (Rosen, O. M. & Krebs, E. G., eds.), vol. 8, pp. 687–799
- Quant, P. A. (1990) *Biochem. Soc. Trans.* **18**, 994–995
- Lowe, D. M. & Tubbs, P. K. (1985) *Biochem. J.* **232**, 37–42
- Quant, P. A., Tubbs, P. K. & Brand, M. D. (1989) *Biochem. J.* **262**, 159–164
- Quant, P. A., Tubbs, P. K. & Brand, M. D. (1990) *Eur. J. Biochem.* **187**, 169–174

Received 4 June 1990/14 August 1990; accepted 23 August 1990