Flux control exerted by overt carnitine palmitoyltransferase over palmitoyl-CoA oxidation and ketogenesis is lower in suckling than in adult rats

Stefan KRAUSS*§, Carol V. LASCELLES*†, Victor A. ZAMMIT‡ and Patti A. QUANT*†

*Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K., †Unit of Paediatric Surgery, Institute of Child Health and Great Ormond Street Hospital for Children NHS Trust, University of London, 30 Guilford Street, London WC1N 1EH, U.K., and ‡Department of Biochemistry and Molecular Biology, Hannah Research Institute, Ayr KA6 5HL, Scotland, U.K.

We examined the potential of overt carnitine palmitoyltransferase (CPT I) to control the hepatic catabolism of palmitoyl-CoA in suckling and adult rats, using a conceptually simplified model of fatty acid oxidation and ketogenesis. By applying top-down control analysis, we quantified the control exerted by CPT I over total carbon flux from palmitoyl-CoA to ketone bodies and carbon dioxide. Our results show that in both suckling and adult

rat, CPT I exerts very significant control over the pathways under investigation. However, under the sets of conditions we studied, less control is exerted by CPT I over total carbon flux in mitochondria isolated from suckling rats than in those isolated from adult rats. Furthermore the flux control coefficient of CPT I changes with malonyl-CoA concentration and ATP turnover rate.

INTRODUCTION

The pathways of fatty acid oxidation and ketogenesis are widely assumed to be controlled by two enzymes: overt carnitine palmitoyltransferase (CPT I) and mitochondrial 3-hydroxy-3-methylglutaryl-CoA (mHMG-CoA) synthase respectively [1–3]. As CPT I is a transmembrane protein [4] involved in the transport of long-chain fatty acids (LCFA) into the mitochondrial matrix, it is thought to play a pivotal role in controlling β -oxidation flux [2,3] and has been suggested as ‘rate-limiting’ for ketogenesis [1]. These suggestions arose from the discovery that malonyl-CoA (MCoA), an intermediate of the fatty acid synthesis pathway, is a potent inhibitor of CPT I catalytic activity [5] and therefore a potential effector of pathway flux. The postnatal onset of hepatic LCFA oxidation and ketogenesis during the suckling period in rat is closely correlated with the increase in the activities of CPT I and of mHMG-CoA synthase [1]. Recent work [1,6] interprets quantitative measurements of CPT I (CPT II and mHMG-CoA synthase) immunoreactive protein and mRNA in different physiological and/or developmental states in support of the key role of CPT I in controlling LCFA oxidation. On the basis of investigations of expression profiles of CPT I and mHMG-CoA synthase, Asins et al. [7] suggest that the intestine might contribute to the overall rate of ketogenesis in higher proportions than liver in suckling rats. However, this has not been analysed quantitatively in experimental rats, despite the fact that it has now become feasible to do so by using whole-body control analysis [8].

Although many investigations have focused on the effects of MCoA on CPT I activity, these effects are far from being fully understood. Current models describe CPT I sensitivity to MCoA inhibition as a function of membrane-dependent and MCoA concentration-dependent transitions between different conformations (reviewed in [3]). However, a quantitative, systemic as-

essment of how the pathways of fatty acid oxidation and ketogenesis might be regulated and controlled via the inhibition of CPT I by MCoA is still lacking.

Application of the top-down approach to metabolic control analysis (TDCA) overcomes the problem of incomplete information about the molecular basis of the MCoA–CPT I interactions, as it does not require any specific information of this kind. It offers the means to describe quantitatively the role of CPT I in controlling fatty acid oxidation and ketogenesis [9,10]. Therefore, an experimental application of TDCA would establish the importance of CPT I in controlling the carbon flow through the system, at steady state and under specific conditions, by assigning group flux control coefficients. These have numerical values that are usually between zero (signifying no control) and unity (signifying all the control); however, values outside this range do occur [9].

Results of the study reported in this paper, where we have applied TDCA to fatty acid oxidation and ketogenesis in isolated rat liver mitochondria, taken together with recent theoretical refinements [11] of metabolic control analysis (MCA) [12–15], lead us to believe that it is no longer valid to assume that CPT I might be rate-limiting or even rate-controlling just because: (1) it can be considered to be at the beginning of the mitochondrial long-chain fatty acid oxidation pathway, (2) its level of expression changes markedly with developmental state and concomitantly with a change in substrate supply and changes in hormone levels, or (3) it is allosterically regulated.

Experimental application [16–18] of, and recent developments in, MCA [11] have considerably weakened the concept of the ‘rate-limiting step’ or ‘key regulatory step’ at the beginning of a metabolic pathway and have shown that it is not a general rule. Indeed, to achieve significant changes in flux through a pathway it is necessary for several steps to exert control in concert [11,19,20]. The purpose of this study is to assess, with TDCA, the

Abbreviations used: *C, group flux-control coefficient, defined by a fractional change in pathway flux caused by an infinitesimal fractional change in a system parameter when the system is allowed to relax to a new steady state; CPT, carnitine palmitoyltransferase; *e, group elasticity, defined as the fractional change in a local flux through a block of reactions caused by an infinitesimal fractional change in a system variable when everything else in the system is kept constant; ex-pc, external palmitoylcarnitine; J_{carbon} , total carbon flux through the pathway in units of ex-pc produced or consumed; LCFA, long-chain fatty acids; MCA, metabolic control analysis; MCoA, malonyl-CoA; mHMG-CoA, mitochondrial 3-hydroxy-3-methylglutaryl-CoA; PCoA, palmitoyl-CoA; TDCA, top-down control analysis.

§ To whom correspondence should be addressed.

potential of hepatic CPT I to control flux through external palmitoylcarnitine (ex-pc) in rats at two distinct developmental stages, and therefore to contribute to a greater understanding of control and regulation of LCFA metabolism.

MATERIALS AND METHODS

Materials

L-[methyl-³H]Carnitine hydrochloride was from Amersham International (Amersham, Bucks, U.K.). β -Hydroxybutyrate dehydrogenase (type V, impurities less than 0.05% lactate dehydrogenase and less than 0.1% malate dehydrogenase) and all other chemicals were from Sigma Chemical Co. (Poole, Dorset, U.K.).

Animals

Female Wistar rats were used. Two litters of newborn Wistar rats were combined and the number of individuals standardized to 10, with a minimum of 6 female rat pups. The age of the suckling rats was 11–15 days when used for mitochondrial preparations. Rat pups with opened eyes were not used. The animals were kept with their mothers under the same conditions as the adult rats, and allowed to suckle milk *ad lib*. The minimum body weight of the pups was 18 g and the maximum was 42 g; in a typical experiment this range was 20–30 g. For the experiments with adult rats, animals of 220–270 g body weight were chosen. They were kept under artificial light at 19–21 °C and fed a high-carbohydrate diet with access to water and food *ad lib*.

Isolation of mitochondria, and Western-blot analysis of CPT I

Mitochondria were isolated by a standard procedure [21]. In preparations from rat pup livers, the process was executed more quickly but less vigorously to preserve the integrity of the mitochondria. Protein was determined by a biuret method [22] with BSA as standard and using 5% (w/v) sodium deoxycholate.

Mitochondria were used for semi-quantification of immunoreactive CPT I by Western blot analysis. Before immunoblotting, mitochondrial proteins were solubilized in 8 M urea, 62 mM Tris/HCl, pH 6.8, 15 mM dithiothreitol, 10% (w/v) SDS, by heating (70 °C for 10 min, stirring continuously without boiling). The solubilized proteins were subjected to SDS/PAGE [9.5% (w/v) acrylamide]. The separated proteins were transferred on to a nitrocellulose membrane (Biomatra Ltd., Maidstone, Kent, U.K.) and the blots were incubated with sheep anti-(rat CPT I) polyclonal antiserum (1:1000, v/v) [23]. The blots were then washed and incubated with the secondary antibody (donkey anti-sheep-peroxidase conjugate). Finally, the blots were developed for 15 min in a solution containing 10 mM Tris/150 mM sodium chloride, pH 7.4, 0.3% methanolic 4-chloronaphthol solution and 0.1% (v/v) hydrogen peroxide.

Incubations of mitochondria

Mitochondria (3 mg of mitochondrial protein per ml, final concentration) were incubated at 30 °C in a medium containing 120 mM potassium chloride, 5 mM Hepes, 1 mM EGTA and 5 mM KH₂PO₄. Endogenous substrate was depleted by the initial addition of 300 μ M ADP and 8.75 μ M L-carnitine. After stirring to reoxygenate the medium, the following were added: glucose (500 μ M); MCoA (0, 1, 3 or 5 μ M); defatted BSA (0.6%); L-[methyl-³H]carnitine hydrochloride (1.5 μ Ci/ μ mol; 175 μ M); palmitoyl-CoA (PCoA; 60 μ M) and ADP (100 μ M) to induce state 3. When state 4 had been re-established, hexokinase (4 units/ml) plus ADP (187.5 μ M) or hexokinase (2 units/ml)

plus ADP (50 μ M) or oligomycin (2 μ g/ml) were added to impose state 3, state 3.5 or state 4 respectively. As soon as steady states had been established (as assessed by linear oxygen consumption traces), two samples were taken at an interval of 3.5 min to determine intermediate and ketone body formation rates. The maximum rate of ketone body formation under the conditions studied was 20 nmol/min per mg of mitochondrial protein. The incubation time with MCoA was standardized to 10 min in all incubations before the first of the two samples was taken for analysis of ketone bodies and intermediate.

Experiments were conducted for all possible combinations of respiratory state (3, 3.5 or 4) and MCoA concentration (0, 1, 3 or 5 μ M), leading to a set of 12 incubation conditions for both suckling and adult rat.

Measurements of oxygen consumption

Mitochondrial respiration rates were determined polarographically, with a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) which allowed incubation of the mitochondrial suspension thermostatically at 30 °C.

Determination of intermediate

Intermediate levels were determined by radioactive assay of external palmitoyl-L-[methyl-³H]carnitine. Samples of 1 ml were taken from the mitochondrial incubations (for incubation conditions, see above), transferred into microtubes and centrifuged for 2 min at 10000 g in an Eppendorf bench centrifuge. Supernatant (0.9 ml) was transferred into microtubes containing 0.2 ml of 6 M HCl for deproteinization. The intermediate was then extracted with butanol from the supernatant in a procedure modified from [24]. The primary butanolic extracts were washed twice with water saturated with butan-1-ol.

Ketone body determination

Ketone bodies were determined as described in [25] following the modified version for fluorimetric assay with an initial-rate method [26]. The latter was adapted for use with a manual fluorimeter (Perkin-Elmer MPF-44E fluorescence spectrophotometer). The assay is based on the interconversion of ketone bodies by β -hydroxybutyrate dehydrogenase (EC 1.1.1.30) with fluorimetrically detectable changes in NADH concentrations at 340 nm excitation and 460 nm emission wavelengths. Standards up to 10 μ M were prepared in 3% (w/v) perchloric acid and kept on ice until assayed. The standard curve, determined by linear regression, had a correlation coefficient of 0.98.

Theory and approach

Defining the system

Our system consisted of isolated rat liver mitochondria oxidizing PCoA to ketone bodies and carbon dioxide (Scheme 1). We divided the system conceptually into two blocks connected by a single pool of metabolite, ex-pc. The ex-pc-producers block of reactions consists of a single step, CPT I and the consumers block incorporates all the other steps involved in the conversion of ex-pc to ketone bodies and carbon dioxide.

Application of TDCA

To apply TDCA to the system it was necessary to know the rates of oxygen consumption, ketone body formation and carbon dioxide formation to determine the total carbon flux through the

Scheme 1 Definition of the conceptually simplified pathway used for the analysis in the model system of isolated rat liver mitochondria

(a) PCoA is translocated across the inner mitochondrial membrane in a carnitine-dependent process, mediated by CPT I, the acylcarnitine transferase and latent CPT (CPT II). Block 1 comprises solely the first step in the translocation process, CPT I. The intermediate, ex-pc, is converted to acetyl-CoA by the remaining steps of the translocation process and the β -oxidation spiral. Acetyl-CoA enters either the Krebs or the ketogenic HMG-CoA cycle. Block 2 encapsulates all the reactions beyond CPT I involved in PCoA oxidation, i.e. acylcarnitine transferase, CPT II, the enzymes of the β -oxidation pathway, the ketogenic HMG-CoA cycle and the Krebs cycle, the electron transport chain, ATPases and the adenine nucleotide and P_i carriers. (b) Final carbon products are ketone bodies and/or carbon dioxide. The fluxes (or rates) through the two sub-branches of ketogenesis ($J_{\text{ketogenesis}}$) and Krebs cycle (J_{Krebs}) sum to the total carbon flux, which is equivalent to the flux through Block 1 leading to acetyl-CoA formation within Block 2. Note therefore that the flux through the system (J_{carbon}) is a total carbon flux. (c) This shows some of the stoichiometric details of the pathway depicted in (a) that underlie the derivation of eqns. (1), (2) and (3) in the text.

system (in units of rates of ex-pc production or consumption) (Scheme 1b) and the levels of the system intermediate, ex-pc.

Calculation of carbon dioxide production rates

The rate of total oxygen consumption in the system depicted in Scheme 1 (see Scheme 1c for details) can be expressed in terms of the ketone body and carbon dioxide production rates according to:

$$\{O\} = [14(2y + x/2)]/8 + 2x - z \quad (1)$$

and therefore the amount of carbon dioxide produced is given by:

$$x = (8/23)\{O\} + (8/23)z - (28/23)y \quad (2)$$

where x is the carbon dioxide formation rate, $\{O\}$ is the (atomic) oxygen consumption rate, z is the β -hydroxybutyrate formation rate and y is the ketone body (acetoacetate plus β -hydroxy-

Figure 1 Titration of producer and consumer block in the system depicted in Scheme 1

(a) Results plotted as J_{carbon} against $\Delta[\text{ex-pc}]$ [$4 \leq n \leq 6$ independent mitochondrial preparations; x/y errors on the state 3 titration with MCoA (■) have been omitted for clarity]. The system was titrated with increasing MCoA concentrations (0, 1, 3 and 5 μM) in state 3 (■), state 3.5 (◇) and state 4 (○). The change in intermediate ($\Delta[\text{ex-pc}]$) is expressed as a percentage. This change can be positive or negative when expressed relative to the steady-state intermediate level in an experiment with full flux conditions, where CPT I is not inhibited and an ADP regenerating system allows continuous oxidation of substrate (PCoA). Similarly, the flux values (J_{carbon}) represent a percentage of the highest flux determined for the system (i.e. for the state 3, 0 μM MCoA condition). The point labelled 0/100 in (a) is the reference point for the full set of conditions (see also Table 1). For each given [MCoA], lines connecting points from bottom right to top left [e.g. the line with slope x in (b)] represent a titration of the consumers block by imposing state 3, state 3.5 or 4 as described in the Materials and methods section. Therefore the slopes of these lines represent the reciprocals of the elasticity of the producer block (CPT I) to changes in intermediate concentration. Similarly, the slopes of lines connecting points of the same symbol, e.g. slope y , for a given respiratory state, represent the reciprocals of the elasticity of the consumers to changes in intermediate. The two sets of results – both adult and suckling condition – consist of a net of twelve points each, through which seven lines can be drawn: four at constant MCoA and three at constant respiratory state. Three of these lines are exemplified in (a) and (b): the line with slope x connects the points where [MCoA] = 0 μM ; the line with slope y connects the points representing state 3.5 (□); also shown in (a) is the line representing MCoA titrations in state 4 (○). The control coefficients at any of the 12 points can be determined from parameters A and B (see text and Appendix for derivation), which in turn are defined by the titration curves that intersect at the point of interest. In (b) is exemplified the determination of the pair of group flux-control coefficients $*C_{\text{CPT I}}^{\text{carbon}}$ and $*C_{\text{ex-pc}}^{\text{carbon}}$ for the state 3.5, 0 μM MCoA condition, which can be defined as the ratio of the slopes of the lines x and y that intersect at that point and also therefore by the ratio of A and B [see Appendix, eqns. (A5), (A9) and (A10)]. Figure 3 shows all control coefficients determined for the full range of conditions in adult and suckling rats.

butyrate) formation rate. All rates are given in nmol of produced or consumed species/min per mg of mitochondrial protein.

Calculating J_{carbon}

Substituting values for x , the absolute values for the total carbon flux to and from the intermediate (in units of ex-pc production or consumption) shown in Scheme 1 can then be calculated as:

$$J_{\text{carbon}} [\text{nmol C16/min per mg}] = x/16 + y/4 \quad (3)$$

Table 1 J_o , J_{carbon} , [ex-pc] and group flux control coefficients (state 4)

Liver mitochondria isolated from suckling or adult rats were incubated (3 mg ml^{-1}) at 30°C in the medium described in the Materials and methods section. Results are shown for experiments where a range of MCoA concentrations (0, 1, 3 and $5 \mu\text{M}$) and respiratory state 4 conditions were imposed. State 3 (0 μM MCoA) results are shown for reference. J_o (in nmol of atomic oxygen $\cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) is the oxygen consumption measured in an oxygen electrode; ex-pc was measured by radioactivity assay (see the Materials and methods section). J_{carbon} and the group flux-control coefficients $*C_{\text{CPT I}}^{\text{carbon}}$ and $*C_{\text{cons}}^{\text{carbon}}$ were calculated as described in the Theory and approach subsection (see also Figure 1). All values are means $\pm$ S.E.M. (except for the control coefficients) for $4 \leq n \leq 6$ independent preparations.

	J_o (nmol of atomic oxygen/min per mg)	(% of State 3, with no MCoA)	J_{carbon} (% of State 3, with no MCoA)	$\Delta[\text{ex-pc}]$ (% change relative to State 3, with no MCoA)	$*C_{\text{CPT I}}^{\text{carbon}}$	$*C_{\text{cons}}^{\text{carbon}}$
Suckling:						
State 3	28.48 ± 2.22	100.00 ± 7.79	100.00 ± 0	0 ± 0	0.98	0.02
0 μM MCoA	8.54 ± 1.22	29.98 ± 4.88	83.36 ± 10.16	77.48 ± 24.74	0.73	0.27
1 μM MCoA	7.94 ± 0.90	27.88 ± 3.84	81.26 ± 5.96	69.81 ± 24.94	0.85	0.15
3 μM MCoA	7.82 ± 0.86	27.46 ± 3.70	80.41 ± 5.16	61.17 ± 18.60	0.93	0.07
5 μM MCoA	7.76 ± 0.83	27.25 ± 3.61	69.15 ± 7.66	50.09 ± 16.56	0.82	0.18
Adult:						
State 3	20.41 ± 1.14	100.00 ± 5.59	100.00 ± 0	0 ± 0	0.97	0.03
0 μM MCoA	9.04 ± 0.32	44.29 ± 2.93	86.54 ± 12.83	121 ± 11.29	0.95	0.05
1 μM MCoA	8.92 ± 0.26	43.70 ± 2.75	68.01 ± 11.96	105.42 ± 7.16	0.87	0.13
3 μM MCoA	8.69 ± 0.36	42.58 ± 2.96	62.27 ± 11.29	84.90 ± 12.24	≈ 1	≈ 0
5 μM MCoA	8.30 ± 0.11	40.67 ± 2.33	52.53 ± 11.01	82.14 ± 8.93	≈ 1	≈ 0

where C16 denotes the units of ex-pc. We have assumed that all carbon entering the system leaves it as either ketone bodies or carbon dioxide.

Determination of group flux control coefficients

The terms ‘control’ and ‘regulation’ are often used loosely and without strict adherence to any agreed definition [15,27]. In this paper we are considering only control and we use the term as a synonym for flux control, i.e. we consider an enzyme, step or block of reactions with a group flux-control coefficient close to unity as rate-controlling, whereas a value close to zero indicates to us almost no control over flux through the metabolic system under consideration.

Group flux-control coefficients were determined by a method described in [28]. According to the definition of the group elasticity coefficient of CPT I to changes in intermediate (see Figure 1, and [9,10]):

$$*e_{\text{ex-pc}}^{\text{CPT I}} = \frac{\partial J_{\text{carbon}}/J_{\text{carbon}}}{\partial [\text{ex-pc}]/[\text{ex-pc}]} \quad (4)$$

the elasticity of CPT I can be determined from a plot of $\Delta[\text{ex-pc}]$ (expressed as a percentage change relative to the condition with highest flux) against J_{carbon} (expressed as a percentage of the highest flux). By using the summation and connectivity theorems of MCA [9,12,14], the group control coefficient for CPT I over pathway flux can be estimated graphically from parameters A and B (see Figure 1b and the Appendix for a full derivation) as:

$$*C_{\text{CPT I}}^{\text{carbon}} = A/(A+B) \quad (5)$$

Similarly, it can be derived that the control exerted by the consumers block on pathway flux is given by:

$$*C_{\text{cons}}^{\text{carbon}} = B/(A+B) \quad (6)$$

The latter is a consequence of the summation theorem. For conditions described here, the group control coefficients of the

two blocks, linked by a common intermediate, sum to unity [9,10,28].

Note that the group elasticities as exemplified in eqn. (4) are given as their normal, scaled forms, whereas the scaling factors have been cancelled out from eqns. (5) and (6) and in the description of the slopes x and y in Figure 1(b) (see Appendix for method).

Statistics

Values obtained for J_{carbon} and $\Delta[\text{ex-pc}]$ were averaged for each of the 24 conditions defined by MCoA concentration, respiratory state and stage of development. S.D. and S.E.M. from the means were calculated (see example state 4 values in Table 1). The means $\pm$ S.D. were used to assess whether changes in flux or intermediate levels were significant for single-step changes in respiratory state or changes in MCoA concentrations. Using Student's t test, values where $P < 0.05$ were considered to indicate significantly different fluxes or levels of intermediate.

Owing to the number of incubations and the incubation times required, it was not possible to titrate complete sets of all different MCoA concentrations and transitions from state 3 to state 4 from a daily yield of isolated mitochondria. As our calculation of group flux-control coefficients is based on the evaluation of a complete graphical plot representing the three MCoA titrations and four transitions from state 3 to state 4 (which was compiled as a composite of partial grids, each arising from one day's experiments on one preparation) it is not valid to present the sets of group flux-control coefficients with error bars. For this to be a valid procedure, it would be necessary to be able to construct an entire grid from a single day's preparation and experiments. This was not feasible for the reasons given above. However, we have determined parameters A and B (see Figure 1b) and estimated the errors on these parameters from the error bars of the lines that connect the points of the MCoA and respiratory state titrations. We then calculated the respective flux control coefficients with errors. These errors are relatively high: for example, $C_{\text{CPT I}}^{\text{carbon}}$ (state 3.5, 0 μM MCoA) = 0.98 ± 0.15 or 0.81 ± 0.40 in the adult and suckling experiments respectively,

and $*C_{\text{cons}}^{\text{carbon}}$ (state 3.5, $0 \mu\text{M}$ MCoA) = 0.02 ± 0.14 in the adult and 0.19 ± 0.25 in suckling experiments. However, when assessing the data, for the reasons described above we should give the actual variability of control coefficients in replicated experiments more weight than the estimated errors (see also below).

RESULTS AND DISCUSSION

Western blotting of CPT I immunoreactive protein

To show that comparison of results from experiments performed in mitochondria from suckling and adult rats as two distinct groups is justified with regard to CPT I expression levels, it was necessary to demonstrate similar expression levels of CPT I immunoreactive protein within the range of ages of the suckling rats. Our results (Figure 2) are in qualitative agreement with those described in [6]. In suckling rats the amount of CPT I immunoreactive protein expressed is higher per mg of mitochondrial protein than in adult rats and within the age range 11–15 days the apparent expression level of CPT I does not change.

Changes in intermediate levels and in fluxes

Single-step changes in the sets of $\Delta[\text{ex-pc}]$ values were significantly different when either respiratory state or MCoA concentration was changed, except in state 4 where $\Delta[\text{ex-pc}]$ values were generally not significantly different for single-step changes in MCoA (Table 1). In suckling and adult rats, single-step changes in J_{carbon} values were generally not significantly different when either respiratory states or MCoA concentrations were changed. For small changes (1 or $2 \mu\text{M}$) in [MCoA], one would not predict a significant change in J_{carbon} . Even where there are significant changes in O_2 consumption, J_{carbon} would not be expected to change markedly, as can be seen from eqns. (2) and (3). Indeed, where oxygen consumption rates were significantly decreased in state 4, relative to state 3, J_{carbon} decreased disproportionately and much less (Table 1).

Flux control exerted by CPT I over total carbon flux

Group flux control coefficients calculated for CPT I and the consumers block are shown in Figure 3. They quantify the control exerted by the system parameter CPT I (or consumers) activity over the system variable J_{carbon} .

The value of $*C_{\text{CPT I}}^{\text{carbon}}$ is generally very high and for a few conditions almost unity, indicating that CPT I exerts very significant control under all conditions chosen. However, in states 3.5 and 4, control is lower in suckling rats (with values ranging from 0.73 to 0.96) than in adult rats (where values are

between 0.87 and 1.0). Indeed, in state 3.5, which might be considered to be closest to the physiological metabolic state, control of CPT I over total carbon flux is decreased for all conditions by a range of values (10–20%).

Statistical analyses of certain results (where we calculated flux-control coefficients as means $\pm$ S.E.M. for a series of experiments performed under a single set of conditions, as described in the Statistics subsection above) indicate that we can be confident in our observation of apparent differences between the numerical values of flux-control coefficients for the two distinct developmental groups. In suckling rats, a general trend of decrease in control is observed when the system ATP turnover rate is changed (i.e. with a state 3 to state 4 transition). Increasing MCoA concentrations shift the control towards CPT I in both suckling and adult rats, although this effect is less pronounced in adults.

An intuitive conclusion that CPT I might not be the only 'rate-limiting' enzyme of fatty acid oxidation and ketogenesis during development in rats has been drawn from the fact that hepatocytes from full-term rat fetuses, incubated with octanoate, show ketogenesis rates as low as those determined from oleate [29]. Furthermore, octanoate or palmitoylcarnitine oxidation was found to be lower in liver mitochondria isolated from rat fetuses than in those from 1-day-old newborns [30]. Our work supports this conclusion with quantitative results.

As the control exerted by CPT I over pathway flux is decreased in our experiments under all the sets of conditions studied in suckling relative to adult rats, control is redistributed between the other enzymes of the pathway beyond the intermediate, *ex-pc*. The consumers block takes up these differences (see Figure 3); however, it cannot be said which particular steps within the consumers block increase their flux control coefficient without repeating the analyses around different intermediates.

Our recent bottom-up control analyses in hepatocytes from fed adult rats [31] give similar numerical values (1.06 ± 0.29 , $n = 5$ hepatocyte preparations; 0.99 ± 0.01 for state 3.5, $4 \leq n \leq 6$ mitochondrial preparations) for the flux-control coefficients of CPT I over total palmitate oxidation to ketone bodies and carbon dioxide, suggesting that our mitochondrial model is a valid reflection of the system behaviour of palmitate flux *in situ* in cells.

Although under some of the experimental conditions CPT I seemingly approaches the status of the 'rate-limiting step' in the model pathway of total carbon flux from PCoA to ketone bodies and carbon dioxide, we should point out that CPT I does not necessarily control ketogenic flux to the same extent. This conclusion is a direct consequence of the requirement to define a pathway [9]. If the pathway were to be reorganized conceptually as a multibranch system, the control exerted by CPT I over the respective sub-branches might vary greatly. Indeed, we are finding that in isolated hepatocytes from adult fed rats, by using the bottom-up approach of MCA, the flux-control coefficients for CPT I over Krebs cycle flux (0.23 ± 0.06 , $n = 5$ preparations) and over ketogenic flux (0.85 ± 0.20 , $n = 5$ preparations) are markedly different [31].

It has repeatedly been pointed out [1,6] that an increase in the expression of CPT I protein at birth to a distinctly high level lasting the whole suckling period (as described in [6] and confirmed in this work) is accompanied by an increased activity of the enzyme. However, the level of gene expression in respective developmental stages and different nutritional status does not itself necessarily support the 'key regulatory role' of CPT I in the control of hepatic LCFA oxidation. Furthermore, the activity of CPT I is determined not only by its level of expression but also by its sensitivity to MCoA inhibition, which itself is a function of

Figure 2 Developmental changes in the expression of rat liver immunoreactive CPT I protein

Solubilized mitochondrial protein (100 μg or 50 μg respectively) from suckling [11, 13 or 15 days (d)] and adult (A) rats were blotted with anti-CPT I antiserum as described in the Materials and methods section. The bands give a semiquantitative measure of expression of CPT I protein.

Figure 3 Group flux control coefficients for CPT I and the consumers block over total pathway carbon flux

Group flux control coefficients of CPT I and the consumers block over total pathway carbon flux (see Scheme 1) were determined for a set of 12 conditions (defined by respiratory state and MCoA concentration) for suckling (a) and adult (b) rats. Note that only the intercepts of the surfaces represent data points (mean values, $4 \leq n \leq 6$ preparations); the lines are drawn for clarity. Errors are not shown (see the Statistics subsection).

membrane composition and prevailing MCoA concentration [3]. A prediction of flux control of CPT I for a given physiological state is not possible. Our results show clearly that any assumption of a straightforward, positively linked relationship between CPT I gene expression and control by CPT I over LCFA oxidation is not valid. This is because flux control coefficients of CPT I over pathway fluxes are properties of the defined system under specified conditions and not of the individual enzyme.

MCA predicts that allosteric enzymes promote regulation, not control, in that they can be seen as homeostatic mechanisms providing rapid stabilization of pathways and regulation of metabolite levels, and are typically of limited significance for flux control [11]. In this sense, an increased expression of CPT I in rats after birth can be interpreted as a way of rapidly adjusting metabolism to an abruptly different hormonal and nutritional situation [1]. This does not establish an important role of CPT I in determining pathway flux, but suggests that it might be important in controlling metabolite concentrations. According to Small and Kacser's theory of finite changes [19,20], the effect on pathway flux of changing the amount of a single enzyme can be restricted and limited to small changes in flux. To our knowledge, it has not been investigated how the expression levels of enzyme proteins other than CPT I, CPT II or mHMG-CoA synthase [6,7,32–34] change in rat immediately after birth owing to developmental, hormonal or nutritional factors. The assumption that CPT I is rate-limiting for fatty acid oxidation and ketogenesis in ketogenic states should not preclude an investi-

gation of other enzyme activities and expression profiles within the pathway of interest.

Conclusions

The importance of CPT I in controlling LCFA oxidation flux cannot be derived intuitively from its expression levels in different developmental stages in rat. In our defined PCoA-oxidizing system the flux control coefficient of CPT I, which is high and significant for the range of conditions studied, changes with MCoA concentration and ATP turnover rate, and increased expression levels of CPT I during the suckling period are not obviously correlated with the potential of CPT I to exert control over carbon flux. With control being shifted down the pathway in the suckling period, it is clear that other enzymes participate in the control over pathway flux. Therefore we suggest that the role of the allosteric regulation of CPT I and of increased expression levels at this stage of development is to control other pathway variables as well as to exert significant control over pathway flux.

This work was supported by grants from the BBSRC to P.A.Q. and V.A.Z. and the Sir Halley Stewart Trust and the Sir Scott of Yews Trust to P.A.Q. S.K. thanks the Konrad-Adenauer-Stiftung e.V., St. Augustin bei Bonn, for a scholarship. C.V.L. gratefully acknowledges the Krebs Memorial Scholarship.

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APPENDIX

Derivation of eqns. (5) and (6) of the main paper

Equations (5) and (6) in the text derive from the explicit solution of the summation and the connectivity theorems (references [9,10,12,14] of the main paper) for the control coefficients in terms of the elasticities.

I. Connectivity theorem for the pathway depicted in Scheme 1:

$$*C_{\text{cons}}^{\text{carbon}} \cdot \epsilon_{\text{ex-pc}}^{\text{cons}} + *C_{\text{CPT I}}^{\text{carbon}} \cdot \epsilon_{\text{ex-pc}}^{\text{CPT I}} = 0 \quad (\text{A1})$$

Summation theorem:

$$*C_{\text{CPT I}}^{\text{carbon}} + *C_{\text{cons}}^{\text{carbon}} = 1 \quad (\text{A2})$$

II. From Figure 1(b) follows:

$$x = 1/*\epsilon_{\text{ex-pc}}^{\text{CPT I}} \quad (\text{A3})$$

$$y = 1/*\epsilon_{\text{ex-pc}}^{\text{cons}} \quad (\text{A4})$$

III. Using the elasticities, a relationship between group control coefficients and parameters A and B can be developed. From the connectivity theorem and eqns. (A3) and (A4) above, we get

$$\frac{*C_{\text{cons}}^{\text{carbon}}}{*C_{\text{CPT I}}^{\text{carbon}}} = -\frac{\epsilon_{\text{ex-pc}}^{\text{CPT I}}}{\epsilon_{\text{ex-pc}}^{\text{cons}}} = -\frac{1/x}{1/y} = \frac{y}{|x|} = \frac{B/C}{A/C} = \frac{B}{A} \quad (\text{A5})$$

[Note that A and B are counted as absolute (positive) values.]

From eqn. (A2), it follows that:

$$*C_{\text{cons}}^{\text{carbon}} = 1 - *C_{\text{CPT I}}^{\text{carbon}} \quad (\text{A6})$$

This combines with eqn. (A5) to give:

$$\frac{*C_{\text{cons}}^{\text{carbon}}}{*C_{\text{CPT I}}^{\text{carbon}}} = \frac{1 - *C_{\text{CPT I}}^{\text{carbon}}}{*C_{\text{CPT I}}^{\text{carbon}}} = \frac{B}{A} \quad (\text{7})$$

and hence:

$$\frac{1}{*C_{\text{CPT I}}^{\text{carbon}}} - 1 = \frac{B}{A} \quad (\text{A8})$$

and therefore:

$$*C_{\text{CPT I}}^{\text{carbon}} = \frac{1}{B/A + 1} = \frac{A}{A + B} \quad (\text{A9})$$

Similarly, the following relation can be obtained:

$$*C_{\text{cons}}^{\text{carbon}} = \frac{B}{A + B} \quad (\text{A10})$$