Biochem. J. (1985) **229**, 273–275
Printed in Great Britain

Effects of the mode of addition of acyl-CoA on the initial rate of formation of acylcarnitine in the presence of carnitine by intact rat liver mitochondria *in vitro*

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

(*Received 11 March 1985/9 April 1985; accepted 23 April 1985*)

Time courses for the formation of palmitoylcarnitine from palmitoyl-CoA and carnitine, catalysed by the overt activity of carnitine palmitoyltransferase (CPT I) in rat liver mitochondria, were obtained. Significant initial non-linearity was observed only when reactions were started by addition of a concentrated solution of palmitoyl-CoA (4mM, to give a final concentration of 100μM) uncomplexed to albumin. Minimal effects were observed when the reactions were started by addition of palmitoyl-CoA–albumin mixtures, even though the final palmitoyl-CoA/albumin molar ratios in the assay medium were identical in the two sets of experiments.

The use in different laboratories of disparate conditions to assay the overt activity of carnitine palmitoyltransferase (CPT I) in rat liver mitochondria has given rise to considerable controversy as to the quantitative interpretation of the data obtained, especially with respect to the absolute requirements of the enzyme for its acyl-CoA substrate and for malonyl-CoA (see McGarry & Foster, 1981, 1982; Saggerson, 1982; Cook *et al.*, 1983). These two parameters are intimately linked because the relative amounts of total acyl-CoA and albumin play a crucial role in determining the amount of free acyl-CoA available to interact with the enzyme (McCormick & Notar-Francesco, 1983).

Another variable was introduced into the quantitative evaluation of malonyl-CoA effects, by the observation that the sensitivity of CPT I to malonyl-CoA inhibition can be reversibly increased or decreased *in vitro*, depending on the duration of incubation of rat liver mitochondria in the presence or absence of malonyl-CoA (Zammit, 1983*a*,*b*, 1984). Since this effect occurs at micromolar concentrations, a physiological role has been suggested for it. An antagonizing effect of acyl-CoA on this effect has further been demonstrated for CPT activity in Triton-extracted rat liver mitochondrial membranes (Bremer *et al.*, 1985). Cook (1984) observed that initiation of CPT I assays by addition of concentrated solutions of oleoyl-CoA to the medium was accompanied by transient lags in activity before the establishment of a steady-state rate of acylcarnitine formation. In the same paper the time-dependence of malonyl-CoA inhibition was also confirmed; the time-course for this latter effect was considerably longer (approx. 4min for completion) than for the lag effects obtained for oleoyl-CoA [< 30s at the lower concentration (50μM) used]. However, Cook (1984) suggested that the mechanistic origins of the lag effects for acyl-CoA and of the time-dependent effects of malonyl-CoA were the same. This suggestion implies that, like the effects of malonyl-CoA (Zammit, 1984; Zammit *et al.*, 1984), the lag effects with respect to acyl-CoA should be an intrinsic property of the enzyme and should not be dependent on factors other than those that alter the enzyme itself. In the present work we have sought to investigate whether the mode of addition of acyl-CoA to the assay medium to initiate the CPT I reaction *in vitro* affects the time course of acylcarnitine formation, in order to test this implication.

Abbreviation used: CPT I, overt activity of carnitine palmitoyltransferase (EC 2.3.1.21).

Materials and methods

Mitochondria were prepared from livers of fed female Wistar rats (Zammit, 1980) as described previously (Zammit *et al.*, 1984). CPT I assays were performed at 37°C as described by Zammit *et al.* (1984). Mitochondria (approx. 1.5mg of protein) were added to the medium (2ml) and preincubated for 4min in assay medium before addition (50μl) of a prewarmed solution containing

palmitoyl-CoA (4mM) and L-[^{14}C]carnitine (20mM; 2μCi/mol) either containing albumin (1 or 10mg/ml, see below) or from which albumin was omitted. When albumin was included in the starting mixture, it was present at the same final concentrations as in the assay medium. Two series of experiments were performed. In the first, the final palmitoyl-CoA/albumin molar ratio was 7.2 (100μM-palmitoyl-CoA and 0.1% albumin). In the second, the molar ratio was 0.72 (100μM-palmitoyl-CoA, 1% albumin). The reactions were terminated after the indicated time intervals by addition of 0.3ml of 6M-HCl, and the acyl[^{14}C]carnitine formed was quantified as described previously (Robinson & Zammit, 1982).

Results and discussion

The experiments were designed such that for the two values of palmitoyl-CoA/albumin molar ratios chosen [7.2, cf. Cook (1984); and 0.72] the palmitoyl-CoA that was added to start the assays either was complexed to albumin or was added as an albumin-free solution (i.e. in true solution plus that present as micelles). However, the final total concentrations of palmitoyl-CoA and albumin in the assay medium were identical irrespective of the mode of addition of palmitoyl-CoA. As shown in Fig. 1, when the time course of the reaction was monitored until attainment of a steady rate of acylcarnitine formation (for the same preparations of mitochondria), the pattern of increase in acylcarnitine formation was different for reactions started with palmitoyl-CoA–carnitine or palmitoyl-CoA–albumin–carnitine mixtures. When the reactions were started with the latter, the rate was linear after the earliest time point (15s). On the other hand, when reactions were started by addition of an albumin-free solution of palmitoyl-CoA (plus carnitine), the time course of the reaction was non-linear for approx. 60s. This was due to a more pronounced and prolonged lag period, followed, after 45s, by a decrease in activity to achieve the steady-state rate. This complex but distinctive time course of acylcarnitine formation under these conditions was very reproducible (it was observed in each of four experiments performed on separate mitochondrial preparations). The combined data from these experiments are given in Fig. 1, expressed as a percentage of the amount of acylcarnitine formed after 3min, in order to minimize variations in absolute rates owing to slight differences in protein concentration and variation between different rats used for each experiment. In an attempt to quantify the linearity of the rates of acylcarnitine formation obtained under the two conditions, linear regression analyses were performed for the

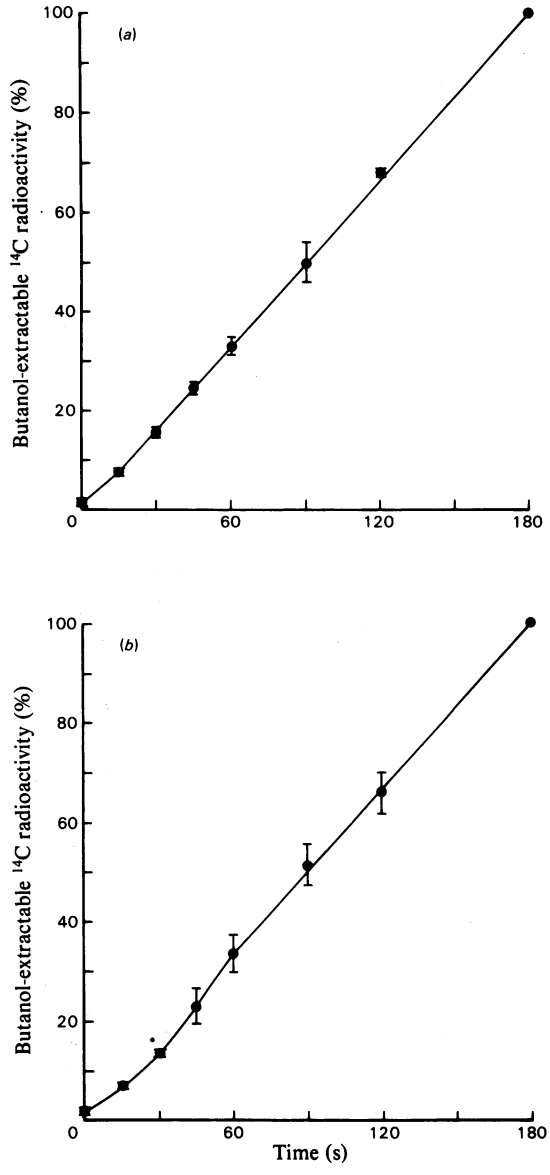

Fig. 1. *Initial rates of incorporation of ^{14}C label into palmitoylcarnitine from [^{14}C]carnitine and palmitoyl-CoA by isolated rat liver mitochondria*

Mitochondria were incubated in assay medium at 37°C for 4min, followed by initiation of the reactions by addition of carnitine plus palmitoyl-CoA (*a*) or of carnitine plus palmitoyl-CoA–albumin (*b*). When albumin was present in the starting mixture, the concentration was 0.1% and the concentration of palmitoyl-CoA was 4mM. The final concentrations of palmitoyl-CoA and albumin in the assay mixture were identical for (*a*) and (*b*), namely 100μM and 0.1% respectively. The average activity of CPT I after attainment of a steady rate was 3.0$\pm$0.4nmol/min per mg of protein (n = 4) for both sets of conditions.

data from 0 to 60s. The correlation coefficients were 0.988 when palmitoyl-CoA was added unbound to albumin, and 0.999 when added bound to albumin.

In experiments in which the palmitoyl-CoA/albumin molar ratio was 10-fold lower (0.72), very similar results (not shown) were obtained to those shown in Fig. 1, except that, as expected, the absolute rates of acylcarnitine formation were lower (about 40%), owing to the lower equilibrium concentrations of free, unbound, palmitoyl-CoA available to the enzyme in the presence of a 10-fold higher concentration of albumin (McCormick & Notar-Francesco, 1983).

The present results indicate that the lag effects of CPT I with respect to palmitoyl-CoA may depend on factors other than those that may be intrinsic to the enzyme itself. One such factor appears to be the form in which concentrated solutions of acyl-CoA substrate are added to initiate the reaction. The dependence of this effect on the prior binding of acyl-CoA to albumin suggests that lag effects with respect to acyl-CoA may represent the time necessary to obtain an equilibrium between free, micellar and albumin-bound forms of the substrate. It is possible that transient high concentrations of free and micellar acyl-CoA could result in initially lower CPT I activities. Irrespective of the precise mechanism, it is evident that, when palmitoyl-CoA was added in a form that most approximated to the situation *in vivo* (i.e. when most of the acyl-CoA is protein-bound), any lag effect must have been exceedingly short-lived. In this respect it displayed time-dependent properties different from those shown by malonyl-CoA on CPT I. These require up to 4–8min to attain their maximal effect (Zammit, 1984; Zammit *et al.*, 1984). Another difference between the acyl-CoA and the malonyl-CoA effects is that the lag effects of acyl-CoA are very similar for CPT I in mitochondria from fed and starved rats (Cook, 1984), whereas the time course of the increase in malonyl-CoA sensitivity on addition of mitochondria to a malonyl-CoA-containing medium is dependent on the source of the mitochondria, being markedly longer for CPT I in mitochondria from starved than in those from fed rats (Zammit *et al.*, 1984). This is suggestive of intrinsic differences in the two types of mitochondria with respect to time required, at physiological temperatures, for malonyl-CoA to induce the appropriate changes in the properties of CPT I and/or those of the mitochondrial inner membrane (Zammit *et al.*, 1984).

References

Bremer, J., Woldergiorgis, G., Schalinske, K. & Shrago, E. (1985) *Biochim. Biophys. Acta* **833**, 9–16

Cook, G. A. (1984) *Biochem. J.* **224**, 1015–1018

Cook, G. A., Otto, D. A. & Cornell, N. W. (1983) *Biochem. J.* **212**, 525–527

McCormick, K. & Notar-Francesco, V. J. (1983) *Biochem. J.* **216**, 495–498

McGarry, J. D. & Foster, D. W. (1981) *Biochem. J.* **200**, 217–223

McGarry, J. D. & Foster, D. W. (1982) *Biochem. J.* **208**, 527–528

Robinson, I. N. & Zammit, V. A. (1982) *Biochem. J.* **206**, 177–179

Saggerson, E. D. (1982) *Biochem. J.* **208**, 525–526

Zammit, V. A. (1980) *Biochem. J.* **190**, 293–300

Zammit, V. A. (1983*a*) *Biochem. J.* **210**, 953–956

Zammit, V. A. (1983*b*) *Biochem. J.* **214**, 1027–1030

Zammit, V. A. (1984) *Biochem. J.* **218**, 379–386

Zammit, V. A., Corstorphine, C. G. & Gray, S. R. (1984) *Biochem. J.* **222**, 335–342