Biochem. J. (1985) **230**, 389–394
Printed in Great Britain

Altered release of carnitine palmitoyltransferase activity by digitonin from liver mitochondria of rats in different physiological states

Victor A. ZAMMIT and Clark G. CORSTORPHINE
Hannah Research Institute, Ayr, Scotland KA6 5HL, U.K.

(*Received 30 January 1985/25 April 1985; accepted 10 May 1985*)

1. The release of carnitine palmitoyltransferase (CPT) activity from rat liver mitochondria by increasing concentrations of digitonin was studied for mitochondrial preparations from fed, 48 h-starved and diabetic animals. 2. A bimodal release of activity was observed only for mitochondria isolated from starved and, to a lesser degree, from diabetic rats, and it appeared to result primarily from the enhanced release of approx. 40% and 60%, respectively, of the total CPT activity. 3. This change in the pattern of release was specific to CPT among the marker enzymes studied. 4. For all three types of mitochondria there was no substantial release of CPT concurrently with that of the marker enzyme for the soluble intermembrane space, adenylate kinase. 5. These results illustrate that the bimodal pattern of release of CPT reported previously for mitochondria from starved rats [Bergstrom & Reitz (1980) *Arch. Biochem. Biophys.* **204**, 71–79] is not an immutable consequence of the localization of CPT activity on either side of the mitochondrial inner membrane. 6. Sequential loss of CPT I (i.e. the overt form) from the mitochondrial inner membrane did not affect the concentration of malonyl-CoA required to effect fractional inhibition of the CPT I that remained associated with the mitochondria. The results are discussed in relation to the possibility that altered enzyme–membrane interactions may account for some of the altered regulatory properties of CPT I in liver mitochondria of animals in different physiological states.

The properties of overt and latent carnitine palmitoyltransferase (CPT) of mitochondria are dependent on the interaction of the enzyme(s) with the outer and inner aspects, respectively, of the mitochondrial inner membrane. Whereas some studies have indicated that the enzymes may be different protein species, with different immunological and catalytic properties (Brosnan *et al.*, 1973; West *et al.*, 1971), others have suggested that they are identical and differ primarily through the influence of the membrane environment to which they are exposed, e.g. through interaction with different membrane components and different conditions (e.g. pH) in the matrix and intermembrane space (Bergstrom & Reitz, 1980; Fiol & Bieber, 1984). The importance of the membrane environment in determining the catalytic and regulatory properties of CPT is perhaps most dramatically illustrated by the observation that inhibition of the overt activity (CPT I) by malonyl-CoA occurs only for the enzyme that is in the membrane environment. Malonyl-CoA does not inhibit the enzyme solubilized from mitochondria (e.g. by a single extraction with non-ionic detergent; McGarry *et al.*, 1978; Saggerson, 1982) or the purified enzyme (Fiol & Bieber, 1984). This observation has led to suggestions either that the effects of malonyl-CoA on the enzyme are mediated through a second component of the outer face of the inner membrane and/or that it requires the enzyme to be in a specific conformation determined by the membrane environment in which the enzyme resides (see the Results and discussion section). More recently, Bremer *et al.* (1985) have reported that, after successive extractions of rat liver mitochondria with Triton, some of the CPT activity solubilized is malonyl-CoA-sensitive. They suggested that this observation adds to the evidence for the interaction of CPT with another membrane component that is less easy to solubilize, and that confers malonyl-CoA-sensitivity to the enzyme.

A marked decrease in the sensitivity of CPT I to

Abbreviations used: CPT, carnitine palmitoyltransferase; CPT I, its overt form; CPT II, its latent form.

malonyl-CoA inhibition occurs in rat liver mitochondria under conditions characterized by a decrease in the hepatic content of malonyl-CoA (Robinson & Zammit, 1982), e.g. during starvation and diabetes (see Zammit, 1984, for review). It is possible that an altered interaction between the enzyme and other component(s) of the membrane could contribute towards the altered sensitivity to the inhibitor and/or vice versa.

The elucidation of the dual localization of overt (CPT I) and latent (CPT II) activities was made possible by the differential release of the two enzymic activities from mitochondria by treatment with different concentrations of digitonin or with phospholipase C (see Hoppel, 1982, for review). Previous studies have yielded conflicting results about the ease with which CPT activity could be solubilized from rat liver mitochondria by digitonin. Thus Bergstrom & Reitz (1980), who observed a bimodal release of CPT activity with increasing digitonin concentrations, concluded that the earlier of the two phases coincided with the release of the intermembrane-space marker enzyme, adenylate kinase. They suggested that CPT released at these concentrations of digitonin represented CPT I and that this either could be loosely associated with the outer face of the mitochondrial inner membrane or could actually reside in the soluble intermembrane space. On the contrary, in the studies of Hoppel & Tomec (1972), CPT was considerably less easily released by comparison with adenylate kinase. These differences between the two studies suggested that the association of CPT (and especially CPT I) with the inner membrane could be variable. Therefore a series of experiments was performed in which the pattern of release of CPT activity by digitonin was studied for mitochondria isolated from fed, starved and diabetic rats.

Materials and methods

Animals

These were female Wistar rats weighing 200–250g. Their source and maintenance were as described previously (Zammit, 1980). Starved rats had their food removed 48h before being killed, but had continuous access to water. Rats were made diabetic as described previously (McNeillie & Zammit, 1982) and were given a solution of 5% (w/v) glucose to drink. They were used after 48h (Topping & Tang, 1975) when their blood [glucose] was 20–30mM.

Preparation of mitochondria

Rats were killed by cervical fracture. The livers were rapidly excised, minced and homogenized with a Teflon/glass homogenizer in 10vol. of medium containing 250mM-sucrose, 5mM-Tris/HCl and 1mM-EGTA (pH7.4 at 0°C) as described by Chappell & Hansford (1972). The homogenate was centrifuged at 300*g* for 10min in a Centra 3RS refrigerated centrifuge (IEC, Dunstable, Beds., U.K.). The supernatant was diluted with the same medium to the original volume (100ml) and centrifuged for 10min at 4000*g*. The pellet was washed by resuspension in a medium containing 150mM-KCl, 5mM-Tris/HCl and 1mM-EGTA (KCl medium; pH7.4 at 0°C) and centrifuged at 2000*g* for 10min. The final pellet was diluted with KCl to the required protein concentration (66–70mg of protein/ml) and used for digitonin extraction studies. This protocol yielded preparations that were minimally contaminated with microsomal membranes (<5%, as judged by microsomal marker-enzyme activities) in a relatively short time (about 50min).

Solubilization of mitochondrial proteins with digitonin

This was performed as described by Hoppel & Tomec (1972). Equal volumes (0.25ml) of mitochondrial suspension and of freshly sonicated (2–3min at maximum power with an MSE Soniprep 150 instrument) digitonin stock solution (in KCl medium at 0°C) were mixed. After 2min the suspension was diluted with 0.75ml of cold KCl medium, and the tubes were centrifuged at 12000*g* for 90s in an Eppendorf 4312 centrifuge. In experiments in which the malonyl-CoA-sensitivity of CPT activity that remain associated with the pellet was studied, the pellets were washed twice with cold KCl medium and finally resuspended in 1.25ml of the same medium.

Marker-enzyme assays were performed at 25°C as described previously (Schnaitman *et al.*, 1967), except that cytochrome *c* oxidase was assayed spectrophotometrically (Smith, 1955). CPT activity was assayed as described previously (Zammit *et al.*, 1984). Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results and discussion

The general pattern of release of outer-membrane (monoamine oxidase), intermembrane-space (adenylate kinase), inner-membrane (3-hydroxybutyrate dehydrogenase, cytochrome *c* oxidase) and matrix (glutamate dehydrogenase) enzymes by digitonin was similar to that described by Schnaitman *et al.* (1967) and Schnaitman & Greenawalt (1968) (results not shown). Preliminary experiments established that maximal release of all the enzymes studied occurred at 0.9mg of digitonin/mg of protein. The protocol used in the present study was the same as that of Hoppel &

Tomec (1972), and the proportion of the marker enzymes released by the same absolute concentrations of digitonin (mg/mg of mitochondrial protein) was very similar to that reported by those authors. The concentration of digitonin required to effect any degree of solubilization of the marker enzymes was, however, higher than that required in the studies of Schnaitman *et al.* (1967) and Bergstrom & Reitz (1980), who used a longer incubation period of mitochondria with digitonin. However, as explained by Hoppel & Tomec (1972), the pattern of release is essentially the same in both types of study. The maximal amount of CPT released by 0.9mg of digitonin/mg of protein was approx. 2.5 times that released by sonication (3 × 15s at maximum power; see the Materials and methods section) and about 5 times that released by freezing and thawing of the mitochondria three times. For glutamate dehydrogenase the maximal amounts released by digitonin and sonication were very similar (results not shown).

Detailed studies were performed in the range of digitonin concentrations up to 0.5mg/mg of protein on mitochondria isolated from livers of fed, 48h-starved or diabetic rats. The data for CPT, adenylate kinase, monoamine oxidase and cytochrome *c* oxidase are shown in Figs. 1–3. The release of none of the marker enzymes was changed significantly after starvation or diabetes, but there were marked differences in the pattern of release of CPT activity.

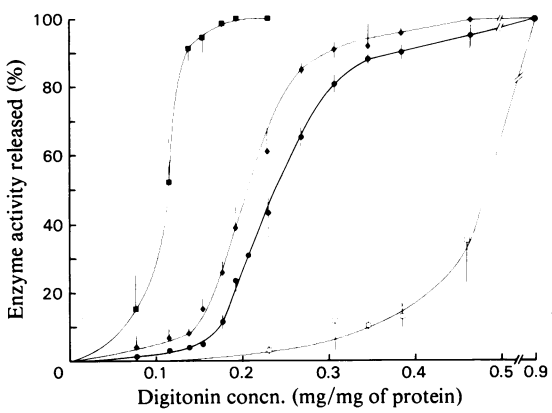


Fig. 1. *Release of carnitine palmitoyltransferase activity from mitochondria isolated from livers of fed rats*
The maximal activities of the enzymes released were (expressed as nmol/min per mg of mitochondrial protein at 25°C): ●, carnitine palmitoyltransferase, 1.15±0.04; ◆, monoamine oxidase, 64±11; □, cytochrome *c* oxidase, 290±80; ■, adenylate kinase, 680±150. The data are averages (±S.E.M.) of three to six determinations on separate mitochondrial preparations, except for points for which error bars are not shown, in which case the number of separate mitochondrial preparations used was two.

The unimodal curve obtained for the release of CPT from mitochondria of fed rats (Fig. 1) was unexpected, since the work of Bergstrom & Reitz (1980) had suggested that release of CPT I and CPT II occurs in two discrete phases separated by a plateau. This distinctive plateau, however, was observed for the release of CPT activity from mitochondria isolated from starved animals (Fig. 2). Since Bergstrom & Reitz (1980) also used starved animals in their experiment, it would

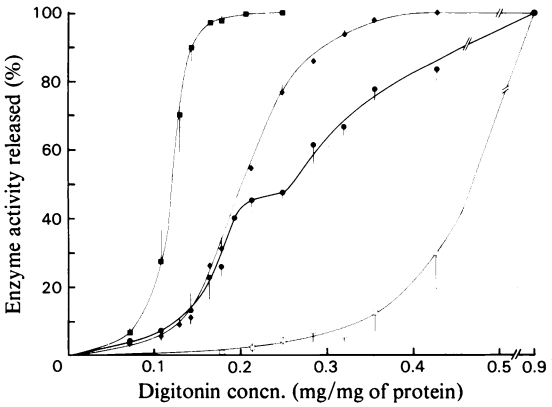


Fig. 2. *Release of carnitine palmitoyltransferase activity from mitochondria isolated from livers of 48h-starved rats*
The symbols and other details are the same as for Fig. 1. The activities of the enzymes (nmol/min per mg of mitochondrial protein) were: carnitine palmitoyltransferase, 1.36±0.06; monoamine oxidase, 40±18; cytochrome oxidase, 360±20; adenylate kinase, 560±190.

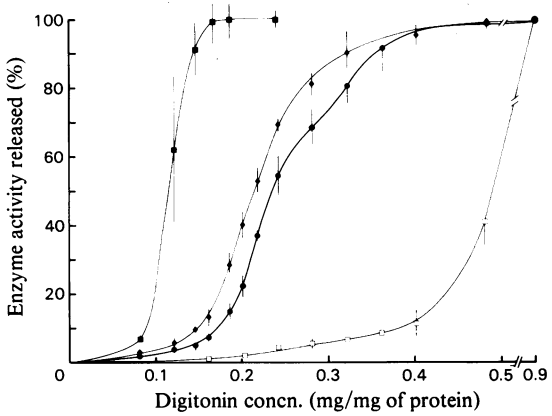
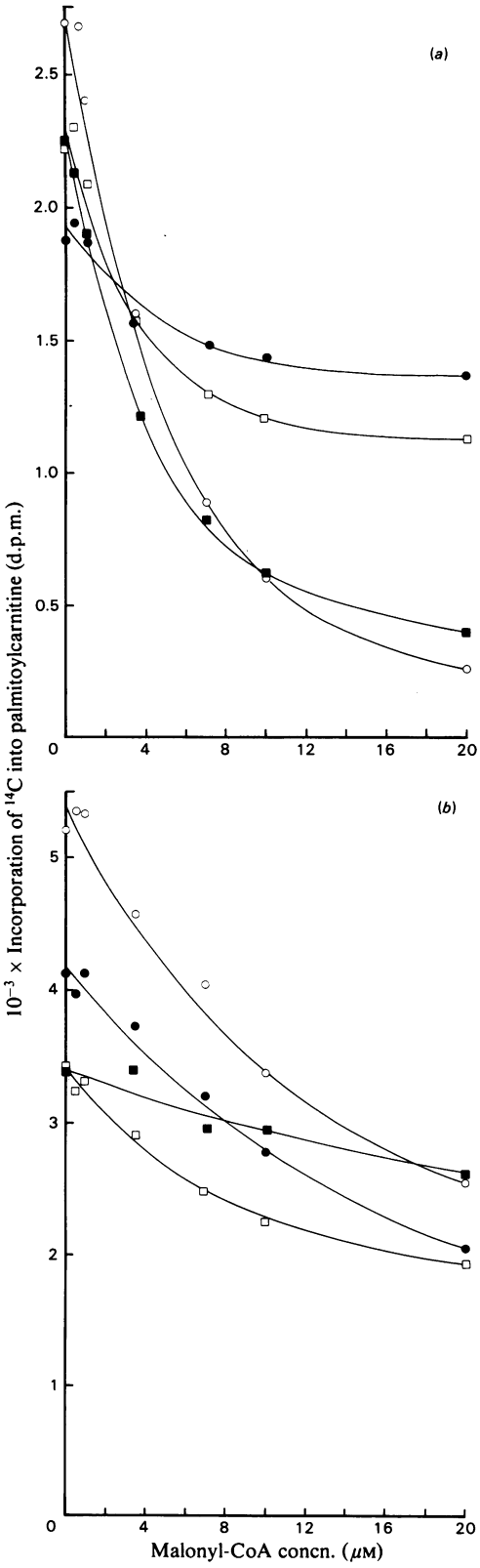


Fig. 3. *Release of carnitine palmitoyltransferase activity from mitochondria isolated from livers of diabetic rats*
The symbols and other details are the same as for Fig. 1. The activities of the enzymes (nmol/min per mg of mitochondrial protein) were: carnitine palmitoyltransferase, 1.21±0.56; monoamine oxidase, 63±5; cytochrome oxidase, 202±15; adenylate kinase, 340±50.



appear that a bimodal release of CPT does not necessarily result from the dual localization of CPT in the mitochondria, but is a result of changes that occur in liver mitochondria on starvation of the rats. Furthermore, since none of the other enzymes studied showed any significant change in their mode of release, it would appear that the change was specific to CPT. Because some properties of CPT I change in a similar manner in starvation and diabetes (see Zammit, 1984, for review) it was decided to study the pattern of release of CPT activity from mitochondria isolated from diabetic animals (Fig. 3). In these too a bimodal pattern was apparent, although it was less well defined, owing to an overall enhancement of CPT release even at higher digitonin concentrations (e.g. at 0.45mg of digitonin/mg of protein 98% of CPT activity was released from diabetic-rat mitochondria, whereas only 85% was released from those isolated from fed and starved rats).

It was apparent that under none of the conditions studied (fed, starved or diabetic) did CPT release resemble that of the intermembrane-space marker adenylate kinase, which was always almost completely released before significant release of CPT started. Therefore the possibility raised by the work of Bergstrom & Reitz (1980) that CPT I may be an enzyme residing in the intermembrane space appeared not to be confirmed. In this respect our conclusions are more similar to those of Hoppel & Tomec (1972).

The major change that accounted for the establishment of the bimodal release characteristic of the starved state appeared to be the enhanced release of approx. 40% of the total CPT activity of the mitochondria. By analogy with the results of Solberg (1974), this early release of CPT activity is likely to represent primarily that of CPT I. To test this inference, experiments were performed in which mitochondria from fed or starved rats were exposed to different digitonin concentrations as described above, but were subsequently washed twice with KCl medium. The CPT activity that remained associated with the pellet was then assayed in the absence or in the presence of several different concentrations of malonyl-CoA (Fig. 4).

Fig. 4. *Effect of malonyl-CoA on CPT activity in membrane pellets after treatment of mitochondria from fed (a) and starved (b) rats with various concentrations of digitonin*

Mitochondria were treated with the following concentrations of digitonin (mg/mg of protein): ■, 0.14; □, 0.17; ●, 0.21. They were then sedimented and washed twice before CPT activity was measured in the presence of the indicated concentrations of malonyl-CoA. This experiment is representative of two similar experiments. ○, Control mitochondria.

These experiments showed that a progressively smaller proportion of CPT associated with the pellet was susceptible to inhibition by high concentrations of malonyl-CoA, as would be expected if only CPT I is sensitive to the inhibitor (McGarry *et al.*, 1978; Saggerson, 1982) and if CPT I is released preferentially by the lower concentrations of digitonin. At 0.4mg of digitonin/mg of protein less than 25% of total CPT remained associated with the pellet, and only 5–10% of this activity was inhibited by 20μM-malonyl-CoA (results not shown). It is not known whether this residual inhibition was due to CPT I that remained associated with the pellet or whether it represented some sensitivity of CPT II to high malonyl-CoA concentrations. Previous studies tend not to support the latter possibility (McGarry *et al.*, 1978; Saggerson, 1982). Solubilized CPT activity was never sensitive to malonyl-CoA inhibition for any of the digitonin concentrations tested (up to 0.9mg/mg of protein; results not shown).

Another observation from Fig. 4 was that, in spite of the decrease in CPT I associated with the pellet with increasing digitonin concentration, the apparent sensitivity of the enzyme to malonyl-CoA inhibition (i.e. the concentration required to elicit a given percentage inhibition as a fraction of that observed at 20μM-malonyl-CoA) did not vary significantly. This result suggested that partial loss of CPT I from the outer face of the mitochondrial inner membrane did not enhance the sensitivity of the remaining enzyme to malonyl-CoA, as would be expected if a higher stoichiometry or tighter association was formed between any putative regulatory subunit (Bieber & Farrell, 1983; Mills *et al.*, 1984 and CPT I after partial loss of the enzyme.

It is possible that, in the light of observations on the starvation-induced changes in the properties of CPT I (see Zammit, 1984, for review), a change in association of CPT with the mitochondrial inner membrane (as reflected by its altered pattern of release) may derive from a phenomenon that is physiologically significant. Thus several groups have suggested that some form of altered state of CPT within the inner membrane of mitochondria from starved rats could account for the diminished sensitivity of CPT I towards malonyl-CoA inhibition. These suggestions have included decreased latency of CPT (Bremer, 1981), an altered conformational state of CPT I within the membrane (Zammit, 1983), different 'positioning' of the enzyme within the membrane (McGarry *et al.*, 1983) and the 'looser' association of CPT I with a putative malonyl-CoA-binding regulatory component of the inner membrane (Bieber & Farrell, 1983; Bird & Saggerson, 1984).

Digitonin interacts with cholesterol molecules in membranes (Glauret *et al.*, 1962; Shany *et al.*, 1974). At low digitonin/cholesterol ratios the main effect may be a decreased molecular ordering of cholesterol resulting from a loose, non-stoichiometric, association between the saponin and cholesterol (Akiyama *et al.*, 1980). (This may precede the formation of stoichiometric precipitation of cholesterol and the creation of cholesterol-free domains within the membrane with consequent permeabilization; Nishikawa *et al.*, 1984). The present results suggest that the association of CPT I with the mitochondrial inner membrane may be altered (concomitantly with changes in its regulatory properties) in different physiological states in such a way as to alter its sensitivity to perturbation of the membrane by digitonin. In this respect two other observations may be relevant: (i) that the association of CPT I with the mitochondrial inner membrane is central to the expression of its regulatory properties (specifically its inhibition by malonyl-CoA; McGarry *et al.*, 1978; Saggerson, 1982; see also above); and (ii) that the major effect of the presence of cholesterol in membranes is to decrease the antisotropic motion of phospholipids, with a consequent increase in membrane rigidity (Akiyama *et al.*, 1980). Therefore the association of CPT I with the mitochondrial inner membrane (i.e. its microenvironment), the expression of its kinetic properties and the relative rigidity (or fluidity) of the membrane may be interrelated.

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