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Studies on the activation *in vitro* of carnitine palmitoyltransferase I in liver mitochondria from normal, diabetic and glucagon-treated rats

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1. The activation of overt carnitine palmitoyltransferase activity that occurs when rat liver mitochondria are incubated at near-physiological temperatures and ionic strengths was studied for mitochondria obtained from animals in different physiological states. 2. In all instances, it was found to be due exclusively to an increase in the catalytic capacity of the enzyme and not to an increase in affinity of the enzyme for palmitoyl-CoA. 3. The enzyme in mitochondria from fed animals always showed a larger degree of activation than that in mitochondria from starved animals. This was the case even for mitochondria (e.g. from fed diabetic animals) in which the kinetic characteristics of carnitine palmitoyltransferase were more similar to those for the enzyme in mitochondria from starved rats. 4. Glucagon treatment of rats before isolation of the mitochondria did not affect the characteristics either of the kinetic parameters of overt carnitine palmitoyltransferase or of its activation *in vitro*.

INTRODUCTION

When rat liver mitochondria are incubated *in vitro* in a KCl-based medium maintained at near-physiological temperatures, there is a gradual increase in the rate of formation of palmitoylcarnitine from palmitoyl-CoA and L-carnitine, owing to the activity of overt carnitine palmitoyltransferase (CPT I) (Zammit *et al.*, 1984). The rate of activation of the enzyme is accelerated if the mitochondria are preincubated in the presence of palmitoyl-CoA before initiation of the assay by addition of carnitine (Bremer *et al.*, 1985). This phenomenon has been re-described by Cook & Cox (1986) and by Lloyd *et al.* (1986), in the form of lags in time courses of acylcarnitine formation.

This time-dependent increase in the rate of product formation by CPT I is analogous to the time-dependent effects of malonyl-CoA on CPT I activity (Zammit, 1984; Zammit *et al.*, 1984). In this instance too, addition of the inhibitor of rat liver mitochondria results in a gradual increase in the potency of inhibition (for any given sub-optimal concentration of the inhibitor) until the degree of inhibition reaches a steady-state value characteristic of the physiological state of the rats from which the liver mitochondria are prepared (Zammit *et al.*, 1984). This time-dependent increase in the effect of malonyl-CoA is responsible for the 'dips' in time courses of CPT I assays on initial exposure of mitochondria to malonyl-CoA (Lloyd *et al.*, 1986). Such time courses of continuous assays (Cook & Cox, 1986; Lloyd *et al.*, 1986) have also confirmed previous observations (Zammit *et al.*, 1984) that the steady-state rate of acylcarnitine formation achieved, after the respective effects of palmitoyl-CoA and malonyl-CoA on CPT I have reached equilibrium, is independent of the order of addition of substrate and inhibitor (Fig. 1 in Zammit *et al.*, 1984).

Although these properties of CPT I have been known for some time, their mechanism remains unknown. Moreover, other observations indicate that their pattern may differ in detail for CPT I in mitochondria from different rat tissues (Lloyd *et al.*, 1986). In the case of the effects of malonyl-CoA, it is known that the time-dependent increase in potency of the inhibitor is not due to a time-dependent increase in the maximal degree of inhibition of CPT I, but rather to a gradual increase in the apparent affinity of CPT I for the inhibitor. Thus, the sensitivity of CPT I to inhibition by malonyl-CoA increases over several minutes, during incubation of mitochondria with the inhibitor, until a degree of inhibition characteristic of the physiological state of the animal from which the mitochondria are prepared is attained (Zammit *et al.*, 1984). A possible explanation of this effect may be the occurrence of a slow conformational change in CPT I induced in the presence of malonyl-CoA (Zammit, 1984).

By contrast, the mechanism(s) through which palmitoyl-CoA (at near-physiological temperatures and concentrations of KCl) affects the increase in CPT I activity is less well studied. In particular, it is not known whether the increase in the rate of formation of product is due to an increase in affinity of CPT I for acyl-CoA substrate, or to an increase in the maximal catalytic capacity of the enzyme. In the present paper we have sought to address this question. In view of the reports by Bremer *et al.* (1985) and Bergseth *et al.* (1986*b*) that there may be differences in the pattern of activation of CPT I by palmitoyl-CoA in mitochondria isolated from animals in different pathophysiological states, we have performed the experiments on mitochondria isolated from the liver of fed, starved, diabetic and glucagon-treated rats.

MATERIALS AND METHODS

The sources of animals and chemicals were as described previously (Zammit, 1980; Zammit & Corstor-

Abbreviation used: CPT I, overt carnitine palmitoyltransferase.

phine, 1985). Animals were made diabetic by injection of streptozotocin (100 mg/kg, intraperitoneally; Easom & Zammit, 1985). They were given 5% (w/v) glucose solution to drink for the first 48 h and were used 7–8 days after treatment, when their blood glucose concentration was greater than 23 mM.

The preparation of mitochondria was as described previously (Grantham & Zammit, 1986). Rats were anaesthetized with pentobarbitone (60 mg/kg) 20 min before excision of the liver. Animals receiving glucagon were given an intraperitoneal injection of the hormone (50 μg/100 g body wt.) at the same time as the anaesthetic.

Assay of CPT I activity was performed as described previously (Grantham & Zammit, 1986). The assay medium contained 150 mM-KCl, 5 mM-Tris, 1 mM-EGTA, 1 mM-dithiothreitol, 0.5 mM-L-[^{3}H]carnitine (0.08 Ci/mol), 10 mg of defatted albumin/ml, 1 μg of rotenone/ml and 1 μg of antimycin A/ml. The final pH was 7.4 at 37 °C. Additions of palmitoyl-CoA were made as indicated. Samples of mitochondrial suspension (50 μl, containing about 1.5 mg of protein) in KCl medium (at 0 °C) were added to 1.95 ml of assay medium at 37 °C, and the amount of [^{3}H]acylcarnitine formed after 15 s was measured. In experiments in which mitochondria were preincubated for 2 min at 37 °C, assays were started by addition of a prewarmed portion (50 μl) of a solution of carnitine, and the reactions were terminated after 15 s.

RESULTS AND DISCUSSION

CPT I assays were performed over very short periods of time (15 s) to enable us to obtain rates of acylcarnitine formation before and after the occurrence of the effects of palmitoyl-CoA (and temperature) on the activity of the enzyme. In effect, this method provides the slopes of tangents drawn to time courses of continuous assays. Preliminary experiments established that neither the physiological conditions of the animals used, nor the length of incubation of the mitochondria at 37 °C, affected the K_m for L-carnitine. They also indicated that the effect on CPT I activity of incubation in palmitoyl-CoA containing assay medium was complete after 2 min. Consequently, in all further experiments, the amount of acylcarnitine formed during 15 s was measured either on initial addition of mitochondria to the complete assay medium or after addition of carnitine to assay medium in which mitochondria had been incubated with palmitoyl-CoA for 2 min. It can be estimated that the initial effect of adding a cold (0 °C) sample (50 μl) of mitochondrial suspension to the assay medium without preincubation was to lower the temperature by 0.9 °C. Consequently this would be expected to have a minimal affect on CPT I activity. In addition, this effect would be identical for the different types of mitochondria used.

(i) Effects on affinity for palmitoyl-CoA

The activity of CPT I was measured at several palmitoyl-CoA concentrations up to 540 nmol/ml in the presence of 10 mg of albumin/ml. Higher concentrations of palmitoyl-CoA exposed latent CPT (CPT II) activity (see Grantham & Zammit, 1986). When the dependence of CPT I activity on total palmitoyl-CoA concentration was studied it was found that, irrespective of the mode of starting the assays, the enzyme in mitochondria from

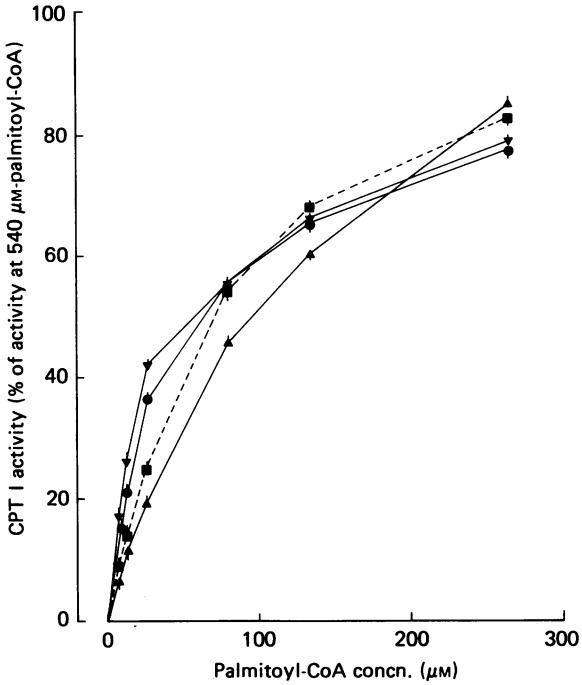

Fig. 1. Decreased affinity of CPT I for palmitoyl-CoA in mitochondria from starved and diabetic rats, but not from glucagon-treated rats

Mitochondria were isolated from normal fed (▼), 48 h-starved (▲), diabetic (■) or fed glucagon-treated (●) rats. All assays were started by addition of mitochondria (see the text). Values are means (±S.E.M.) for four separate determinations and are normalized with respect to the activity obtained at 540 μM-palmitoyl-CoA (100%) for each type of mitochondria preparation.

starved or diabetic rats always had a lower affinity for acyl-CoA (Fig. 1). This observation indicated that the difference in $K_{0.5}$ for palmitoyl-CoA reported previously for the enzyme from starved and diabetic rats (Brady *et al.*, 1985; Grantham & Zammit, 1986) does not depend on the length of time of preincubation of the mitochondria in the assay medium. In contrast, glucagon treatment of rats did not result in any change in the affinity of CPT I for palmitoyl-CoA.

The concentration-dependence of CPT I activity with respect to palmitoyl-CoA was not affected by the period of incubation of the mitochondria (Fig. 2). Thus the curves of relative velocity versus [palmitoyl-CoA] for CPT I in mitochondria from rats in any one physiological condition were almost identical irrespective of which protocol was followed before the assay period. This observation indicates that the changes in CPT I activity observed on incubation of mitochondria in KCl-based media at near-physiological temperatures (see the Introduction) are not due to an increase in the affinity of the enzyme for palmitoyl-CoA.

(ii) Effects on activity of CPT I

When the absolute activities of CPT I were plotted against palmitoyl-CoA concentration (Fig. 3), it was apparent that after 2 min incubation of the mitochondria there was an increase in the activity of CPT I at high palmitoyl-CoA concentrations. However, there were large differences between the degree of activation

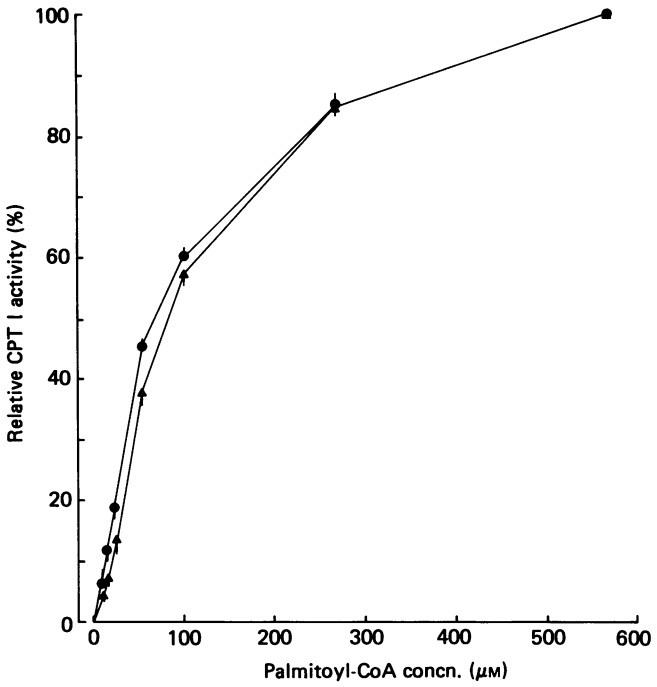

Fig. 2. Effect of preincubation of mitochondria at 37 °C on the relative dependence of CPT I velocity on palmitoyl-CoA concentration as illustrated by mitochondria from normal fed rats

Plots for mitochondria from rats in other physiological conditions were similarly almost identical. Values are means (±S.E.M.) for four separate determinations and are expressed relative (%) to the activity obtained at 540 μM-palmitoyl-CoA under the respective conditions used, i.e reaction started with mitochondria (●) or with carnitine (▲). See the legend to Fig. 3 for details.

achieved in mitochondria isolated from animals in different physiological states. Thus the activation in mitochondria from normal fed animals was approx. 80%, whereas for starved animals it was less than 10%. Interestingly, although CPT I in mitochondria from diabetic animals showed $K_{0.5}$ characteristics similar to those from starved animals (above), the activation of the enzyme was more similar to that observed for the enzyme in mitochondria from fed animals. CPT I in mitochondria from glucagon-treated rats was also activated considerably (approx. 60%) after 2 min preincubation. The net effect of this activation (in the absence of changes in affinity for palmitoyl-CoA) was to diminish the difference between CPT I activity in mitochondria from fed and starved animals (from 4-fold to only 2-fold; see legend to Fig. 3). Therefore, it is unlikely that the well-established increase in CPT I activity that occurs in mitochondria isolated from animals in ketogenic states (see Zammit, 1984) can be accounted for by differential activation *in vitro*. Differences in activity of CPT I observed in the steady state subsequent to the changes induced by preincubation (above) appear to be an intrinsic property of the mitochondria (cf. malonyl-CoA sensitivity), although whether they reflect changes in the amount of enzyme present remains to be ascertained.

The other noteworthy characteristic of this activation is that it only occurred when mitochondria were incubated at near-physiological temperatures. The preparation used in the present work involving washing the mitochondria in cold KCl medium as well as final resuspension in this medium. Therefore, mitochondria were exposed to KCl for up to 50 min before addition to the assay medium. Notwithstanding, activation occurred only on addition of the mitochondria to the assay medium. This observation may be relevant to the elucidation of the mechanism(s) responsible for the palmitoyl-CoA- and malonyl-CoA-induced effects (see below). The time-dependence of the effects of palmitoyl-CoA and malonyl-CoA on the activity of CPT I has now been confirmed in other laboratories (Cook & Cox, 1986; Lloyd *et al.*, 1986). These authors have also introduced the term 'hysteresis' to describe these slow responses, although, for the malonyl-CoA effects, they failed to distinguish between experiments in which these effects are retained by CPT I in mitochondria maintained at 0 °C after removal of the ligand (Zammit, 1983*a*,*b*) and others conducted exclusively at near-physiological temperatures (Zammit *et al.*, 1984). In the latter instance, the sequence of addition of substrates, inhibitor and mitochondria to the assay medium had already been shown to have no effect on the steady-state rate of acylcarnitine formation attained after the slow responses have occurred (Zammit *et al.*, 1984).

The present results indicate that there is a major difference in the mechanisms through which the respective slow effects of palmitoyl-CoA and malonyl-CoA are achieved. Thus the increase in CPT I activity is due exclusively to an increase in $V_{max.}$ of the enzyme and not to an increased affinity for palmitoyl-CoA. In contrast, the slow increase in the malonyl-CoA effect on CPT I on addition of the inhibitor to mitochondria is due to an apparent decrease in the $I_{0.5}$ (concentration required to inhibit activity by 50%) for the inhibitor (Zammit, 1984). Thus, although the two effects may appear to be related (especially since activation of the enzyme is accompanied by an increase in $I_{0.5}$ for malonyl-CoA), they may not be causally so. Bergseth *et al.* (1986*a*) have reported that freshly prepared mitochondria have malonyl-CoA associated with them, and that this may be metabolized on incubation of the mitochondria in assay medium. It is unlikely, however, that such removal of malonyl-CoA could account for the observed increase in CPT I activity, because malonyl-CoA inhibits CPT I activity in a competitive manner, whereas the increase in activity is due solely to an increase in $V_{max.}$ and not to a decrease in $K_{0.5}$ for palmitoyl-CoA. Similarly, it is unlikely that, as suggested by Cook (1984) and Cook & Cox (1986), retention of malonyl-CoA by mitochondria could account for the increased sensitivity of CPT I to malonyl-CoA inhibition after preincubation of mitochondria with micromolar concentrations of malonyl-CoA (Zammit, 1983*a*,*b*). If this were responsible, CPT I in these mitochondria would appear to be less, and not more, sensitive to malonyl-CoA inhibition in subsequent assays. It is therefore suggested that conformational changes occur in CPT I in the presence of malonyl-CoA (or on removal of palmitoyl-CoA) that are thermodynamically difficult to reverse at low temperatures and can only be reversed relatively slowly on incubation of the mitochondria at near-physiological temperatures and ionic strengths, giving rise to the hysteretic effects.

The physiological relevance of these putative conformational changes is unknown, but it is noteworthy that

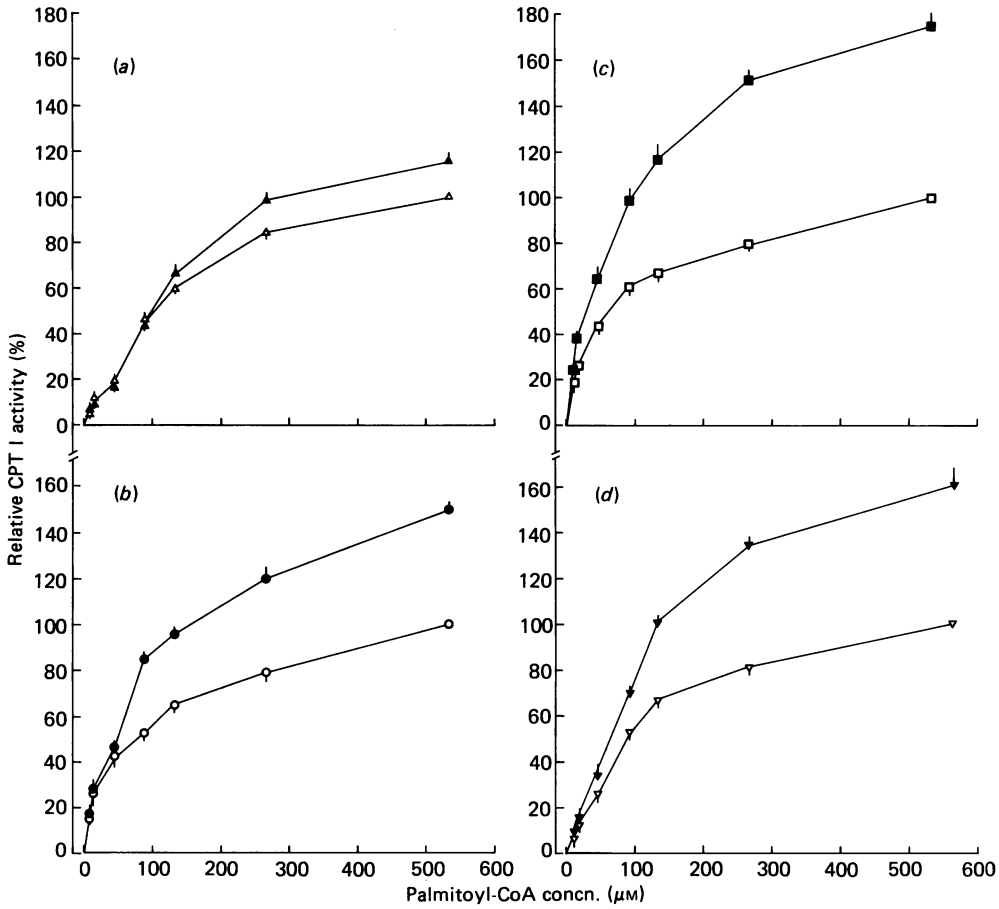

Fig. 3. Effects of preincubation of mitochondria at 37 °C for 2 min on the velocity of the CPT I reaction at different concentrations of palmitoyl-CoA

Mitochondria from (*a*) starved, (*b*) fed, (*c*) fed glucagon-treated and (*d*) diabetic fed rats were used. Assay of CPT I activity was initiated either by addition of mitochondria (white symbols) to the complete assay medium, or by addition of carnitine after incubation of mitochondria in assay medium from which carnitine had been omitted (black symbols). Values are means (±S.E.M.) for four separate determinations on different mitochondrial preparations and are expressed relative (%) to the activity obtained at 540 μM-palmitoyl-CoA on initiation of the assay with mitochondria. The absolute activities for the latter parameter were (*a*) 39.9±6.3, (*b*) 10.5±0.6, (*c*) 12.1±1.6, (*d*) 25.2±2.1 nmol/min per mg of protein at 37 °C.

they may differ for CPT I in different tissues (Lloyd *et al.*, 1986). It has previously been suggested (Robinson & Zammit, 1982) that the kinetic properties of CPT I *in vivo* could be modified by the same or closely related mechanism(s) that produce concerted changes in hepatic metabolite concentrations and enzyme activities under different physiological conditions (e.g. concerted covalent modification of two or more enzymes). Alternatively, they could be modified by direct action of effectors. More recent studies (see, e.g., Grantham & Zammit, 1986) have indicated that the properties of the enzyme in isolated mitochondria have components that appear to be intrinsic to the preparations obtained from animals in different conditions and survive mitochondrial isolation procedures (i.e. in the absence of precautions against possible dephosphorylation). In addition, however, the affinity of CPT I for malonyl-CoA, as well as the $V_{max.}$ of the enzyme, undergo slow changes *in vitro* (see the Introduction). It has proved difficult to extrapolate from results obtained *in vitro* to the situation *in vivo*. However, this should not alter the significance of the basic relationship between the kinetic characteristics of CPT I and the hepatic concentration of malonyl-CoA (Robinson & Zammit, 1982). This relationship is likely to amplify the responses of the activity of the enzyme to changes in the concentration of the effector under different physiological conditions.

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