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Hysteretic behaviour of carnitine palmitoyltransferase

The effect of preincubation with malonyl-CoA

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Continuous assays of carnitine palmitoyltransferase were used to study the hysteretic behaviour of the enzyme. When reactions were started by adding mitochondria to complete reaction mixtures, there was a lag in the assay even in the absence of malonyl-CoA. When mitochondria were preincubated with malonyl-CoA in the absence of palmitoyl-CoA, there was a greater lag period in the assay of carnitine palmitoyltransferase, but this lag was less prominent at 37 °C than at 30 °C. Preincubation of mitochondria with malonyl-CoA did not change the sensitivity of the enzyme to inhibition by malonyl-CoA.

INTRODUCTION

Robinson & Zammit (1982) have reported that high sensitivity to malonyl-CoA inhibition occurs in physiological states with the highest hepatic contents of malonyl-CoA. These authors concluded that the tissue malonyl-CoA could have induced malonyl-CoA sensitivity. An alternative explanation is that malonyl-CoA levels and malonyl-CoA sensitivity are both increased by a co-ordinated system of hormonal control. We have shown that the hepatic content of malonyl-CoA can be controlled by glucagon (Cook *et al.*, 1977, 1978) and the sensitivity of carnitine palmitoyltransferase to inhibition by malonyl-CoA can be regulated by insulin (Gamble & Cook, 1985). Furthermore, carnitine palmitoyltransferase recently has been shown to be phosphorylated and activated in response to glucagon (Harano *et al.*, 1985). Co-ordinated control by insulin and glucagon could easily explain the observations of Robinson & Zammit (1982).

Zammit (1983) has proposed that 'preincubation of liver mitochondria with micromolar concentrations of malonyl-CoA increases significantly the sensitivity of CPT I to malonyl-CoA inhibition'. That interpretation is not consistent with data demonstrating that when mitochondria from fed or fasting animals are exposed to exactly the same concentration of malonyl-CoA the carnitine palmitoyltransferases of those mitochondria show vastly different amounts of inhibition (Cook *et al.*, 1980). The data of Zammit (1983) might be explained, however, by the lack of removal of all of the added malonyl-CoA since it was reported that 'successive washes of the mitochondria tended to result in loss of CPT I sensitivity'. There is also some concern that washing of mitochondria with KCl was the actual cause of changes in the enzyme, and that the addition of malonyl-CoA only served to protect the enzyme from the effects of KCl. KCl has been shown to affect malonyl-CoA inhibition of carnitine palmitoyltransferase in exactly that manner (Saggerson, 1982).

Another report by Zammit (1984) suggested that the nonlinearity of carnitine palmitoyltransferase assays following the addition of malonyl-CoA supports the former conclusion that the sensitivity is being changed by malonyl-CoA itself. In that paper, however, assays were not continued for a period long enough to reach a steady reaction rate. Using a continuous assay, it has been shown subsequently that the changes caused by the malonyl-CoA immediately after addition to the assay medium are caused by hysteretic behaviour of the enzyme that may be associated with positive co-operative inhibition (Cook, 1984*a*, *b*). Furthermore, it can be seen, after reaching a steady state, that the carnitine palmitoyltransferases from fed and fasting rats still possess a large difference in sensitivity to malonyl-CoA (Cook, 1984*b*), i.e. the sensitivity is not changed by incubation with malonyl-CoA.

More recently Zammit (1985) suggested that the lag in the assay of carnitine palmitoyltransferase after the addition of the acyl-CoA substrate may be an artifact caused by the addition of substrate in a micellar form. The data presented in that paper show a small deviation from linearity with respect to time when acyl-CoA was added in a micellar form and a somewhat lesser, but still perceptible, deviation from linearity when the substrate was added bound to albumin (Zammit, 1985). The results of Zammit (1985) confirmed previous results (Cook, 1984*b*) showing that the lag in the assay after the addition of substrate in the absence of malonyl-CoA was indeed small when compared with the lag seen in the presence of malonyl-CoA (see Fig. 1 of Cook, 1984*b*). We present here data derived from experiments in which the problems associated with addition of acyl-CoA are avoided by starting all reactions with the addition of enzyme to reaction mixtures containing palmitoyl-CoA bound to albumin. These experiments show a clear and pronounced hysteretic effect caused by malonyl-CoA and also indicate that the hysteretic effect is more pronounced at the lower assay temperature (30 °C) used by us compared with the higher temperature (37 °C) used by Zammit and coworkers.

MATERIALS AND METHODS

Male Wistar rats weighing 180–200 g were fed *ad libitum* (Purina Lab Chow; Ralston Purina Company, Richmond, IN, U.S.A.) and had free access to water. Liver mitochondria were isolated by the method of Johnson & Lardy (1967) after rats were decapitated. The final mitochondrial pellet was resuspended to a protein

concentration of 10 mg/ml in 0.25 M-sucrose/1 mM-EDTA/3 mM-Tris, pH 7.2. Protein was determined by the biuret procedure summarized by Layne (1957). Using 10 mM-glutamate and 0.5 mM-malate as substrates, respiratory control ratios (Estabrook, 1967) were 5 or greater for all mitochondrial preparations used. The outer carnitine palmitoyltransferase activity was measured as described previously (Gamble & Cook, 1985). Each 1 ml of assay mixture contained: 82 mM-sucrose, 70 mM-KCl, 35 mM-Hepes, 35 mM-imidazole, 5 mM reduced glutathione, 2 mg of bovine serum albumin, 0.5 mM-L-carnitine (0.4 μCi of L-[*methyl*-^{3}H]carnitine/μmol), 1 μg of antimycin A, 2 mM-ATP, 2 mM-$MgCl_2$, and palmitoyl-CoA and malonyl-CoA at the concentrations indicated. Assays were conducted at 30 °C or 37 °C, pH 7.0, in 25 ml Erlenmeyer flasks. Incubation medium containing palmitoyl-CoA with or without malonyl-CoA was preincubated for 5 min before starting reactions by the addition of mitochondria. The mitochondrial suspensions used to start the reactions were also preincubated at 30 °C; some mitochondrial suspensions were preincubated with malonyl-CoA at a concentration of 500 μM so that the addition of 200 μl of mitochondrial suspension to 9.8 ml of reaction mixture gave a final concentration of 10μM-malonyl-CoA. Samples (1 ml) of the total reaction mixture were removed at the times indicated and transferred to test tubes containing 2 ml of 1 M-$HClO_4$ to stop the reactions.

Malonyl-CoA, palmitoyl-CoA, L-carnitine hydrochloride, reduced glutathione, EGTA, imidazole, Hepes and essentially fatty acid-free bovine serum albumin were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-[*methyl*-^{35}H]Carnitine hydrochloride was obtained from Amersham (Arlington Heights, IL, U.S.A.). Wistar rats were purchased from Harlan Industries (Indianapolis, IN, U.S.A.).

RESULTS

Fig. 1 indicates that, except for the first 30 s, the time course of the carnitine palmitoyltransferase reaction was linear with respect to time. The small lag in the time course was present even though palmitoyl-CoA was preincubated in the presence of albumin and even though mitochondria, added to start the reaction, were also preincubated at 30 °C. The lag was identical with the lag seen when oleoyl-CoA was added to start the reaction after preincubating mitochondria in the reaction mixture (Cook, 1984*b*).

When mitochondria were preincubated with malonyl-CoA there was a lag period that lasted for about 1.5 min, but the reaction rate was constant thereafter (●, Fig. 1). These results confirm our previous results (Cook, 1984*b*) indicating a longer lag period in the presence of malonyl-CoA, a lag period that cannot be explained by the binding of the acyl-CoA substrate to albumin since palmitoyl-CoA was preincubated with albumin in all experiments. It should be noted that after about 1.5 min there was no further change in the reaction rate. When mitochondria were added to incubation mixtures containing both palmitoyl-CoA and malonyl-CoA the lag was very slight and the average of six experiments indicated no apparent lag (○, Fig. 1). A small lag, such as that seen with acyl-CoA alone, is difficult to detect when reaction rates are this slow, however. The final concentrations of all components in the two assays

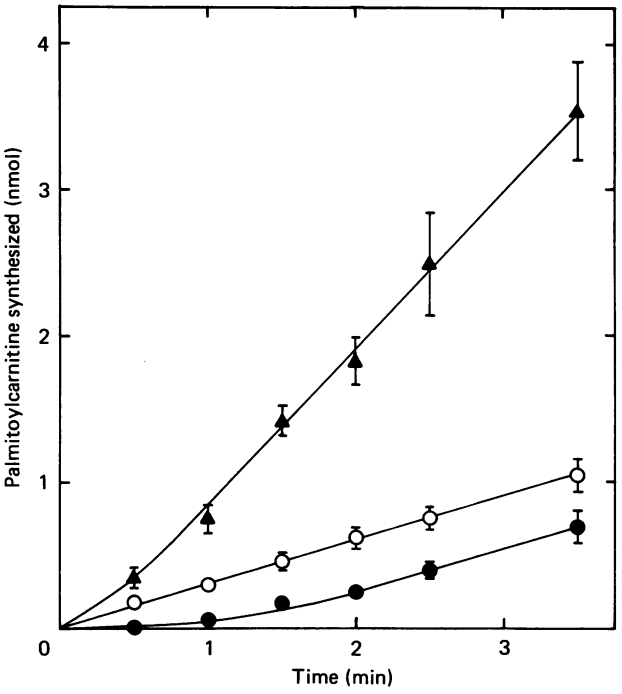

Fig. 1. Time course of the carnitine palmitoyltransferase reaction using intact rat liver mitochondria

Reaction mixtures were incubated at 30 °C for 5 min before mitochondria, also incubated separately at 30 °C, were added to start the reactions. ▲, Reaction mixtures containing 50 μM-palmitoyl-CoA but no malonyl-CoA; ○, ●, reaction mixtures containing 50 μM-palmitoyl-CoA plus 10 μM-malonyl-CoA. Experiments illustrated by ○ and ● contained exactly the same final concentrations of all components after mitochondria were added, but in experiments illustrated by ○ preincubated mitochondria were added to reaction mixtures already containing malonyl-CoA, whereas in experiments illustrated by ● mitochondria were preincubated in the presence of 500 μM-malonyl-CoA before addition to reaction mixtures not previously containing any malonyl-CoA (see the text for details). Mitochondrial protein concentration was 0.2 mg/ml in all assays.

containing malonyl-CoA (○ and ●, Fig. 1) were identical; the difference in preincubation conditions was the only difference in the two assays. The slopes of the linear portions on the latter two curves were identical, indicating that preincubation of the mitochondria with a very high concentration of malonyl-CoA (500 μM) did not change the sensitivity of the enzyme. The final, linear rate of reaction illustrated by these two curves showed 75% inhibition compared with the rate in the absence of malonyl-CoA, almost identical with previous results (Cook, 1984*b*). A more important aspect, shown previously (Cook, 1984*b*), was that preincubation of mitochondria from starved rats with malonyl-CoA did not increase the sensitivity of these mitochondria to inhibition by malonyl-CoA.

Mitochondria preincubated at 37 °C and subsequently assayed at 37 °C (Fig. 2) showed a less pronounced lag in the assay compared with experiments conducted at 30 °C, suggesting that differences in assay temperature may be responsible for part of the difference between the results reported by Zammit and coworkers and the results reported by us.

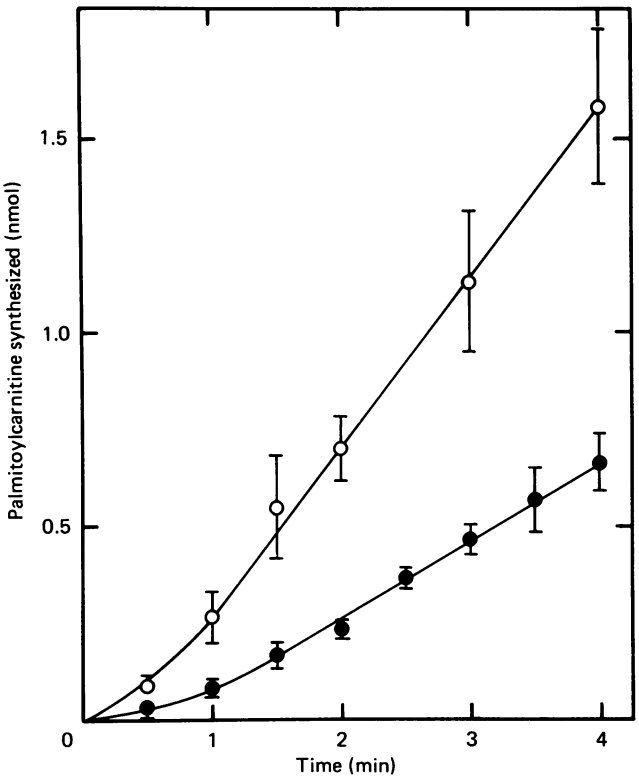

Fig. 2. Effect of incubation temperature on hysteretic behaviour of carnitine palmitoyltransferase

Mitochondria were preincubated in the presence of 500 μM-malonyl-CoA before they were added to reaction mixtures containing palmitoyl-CoA (final concentrations of 50 μM-palmitoyl-CoA and 10 μM-malonyl-CoA). ○, Experiments conducted at 37 °C; ●, experiments conducted at 30 °C. Mitochondrial protein concentration was 0.2 mg/ml.

DISCUSSION

Experiments reported here indicate that malonyl-CoA is responsible for the major part of the lag in reaction rate and that the method of addition of the acyl-CoA substrate to the reaction mixture cannot explain all of the lag even in the absence of malonyl-CoA. They also demonstrate that preincubation of mitochondria with malonyl-CoA cannot change the sensitivity of carnitine palmitoyltransferase to inhibition by malonyl-CoA.

It is of some concern that the hysteretic behaviour induced by the binding of malonyl-CoA has been described as a change in the sensitivity of carnitine palmitoyltransferase to inhibition by malonyl-CoA (Zammit, 1984). Since the discovery of differential inhibition by malonyl-CoA of fatty acid oxidation in mitochondria isolated from fed and fasting rats (Cook *et al.*, 1980; Ontko & Johns, 1980), the term *sensitivity*, as it relates to malonyl-CoA inhibition of fatty acid oxidation, has been used to describe changes caused by the physiological cycle of feeding and fasting. That the mechanism for the change in sensitivity is actually a change in the K_i of carnitine palmitoyltransferase for malonyl-CoA has recently been demonstrated for fasting rats (Cook, 1984*a*) and for diabetic and insulin-treated diabetic rats (Gamble & Cook, 1985). The best method of distinguishing between these differences in malonyl-CoA sensitivity in different physiological states is the measurement of K_i values. Such measurements have shown that there is a 10-fold difference between fed and fasting rats (Cook, 1984*a*) and between diabetic and insulin-treated diabetic rats (Gamble & Cook, 1985). Thus changes in the sensitivity of carnitine palmitoyltransferase to inhibition by malonyl-CoA are clearly defined as changes in K_i.

It should be emphasized that it is not hysteretic behaviour that will be important in the further studies of the physiological importance of changes in the malonyl-CoA sensitivity of carnitine palmitoyltransferase, since hysteretic behaviour deals only with a very short period transition. More important will be an examination of co-operative inhibition by malonyl-CoA in a steady state. Hysteretic behaviour may be simply the result of constraints on the enzyme by its membrane environment which slow down the conformational changes or other changes in the enzyme caused by the binding or dissociation of malonyl-CoA. Co-operative inhibition, on the other hand has the effect of magnifying the already large differences in sensitivity between fed and fasting animals or diabetic and insulin-treated diabetic animals so that the final effect on fatty acid oxidation *in vivo* becomes many times greater than the 10-fold difference in K_i values, resulting in a multiple amplification process that is a much more effective control mechanism than simple, competitive inhibition.

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