Effects of incubation at physiological temperatures on the concentration-dependence of [2-¹⁴C]malonyl-CoA binding to rat liver mitochondria

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1. Specific binding of [2-¹⁴C] malonyl-CoA to rat liver mitochondria was measured at different temperatures and after various periods of time of exposure of the mitochondria to the ligand. 2. Incubation of mitochondria at 37 °C in the absence of malonyl-CoA resulted in a decrease in their ability to bind malonyl-CoA at all concentrations tested (up to 55 μ M). However, incubation of mitochondria in the presence of malonyl-CoA resulted in the loss of the binding only by a low-affinity component. By contrast, there was an increase in the binding that occurred at low, physiological, concentrations of malonyl-CoA. 3. These differences in the response of the two binding components to incubation conditions were used to obtain quantitative data about their respective saturation kinetics. Evidence was obtained that, whereas the high-affinity component approached saturation hyperbolically with respect to malonyl-CoA concentration, the low-affinity component had sigmoidal characteristics. The concentrations of malonyl-CoA required to half-saturate the two components were 2–3 μ M and 30 μ M for the high- and low-affinity components respectively. 4. Evidence was also obtained for the involvement of a temperature-dependent transition, that occurred at around 25 °C, in the modulation of malonyl-CoA binding to the mitochondria. 5. The possible physiological roles of the two components of malonyl-CoA binding in relation to the regulation of overt carnitine palmitoyltransferase (CPT I) activity *in vivo* are discussed.

INTRODUCTION

Evidence has emerged from previous studies to suggest that malonyl-CoA may interact with overt carnitine palmitoyltransferase (CPT I) of rat liver mitochondria with variable affinity (Zammit, 1984; Zammit *et al.*, 1984). Incubation of mitochondria at physiological temperatures in a KCl-containing medium results in the loss of sensitivity of the enzyme to malonyl-CoA inhibition when the incubations are performed in the absence of malonyl-CoA. If the effector is present during the incubation, there is a gradual increase in the sensitivity of the enzyme to malonyl-CoA. These time-dependent changes appear to have characteristics that are intrinsic to the type of mitochondria used, i.e. the physiological state of the animals from which they are isolated. Thus the increase in malonyl-CoA-induced sensitivity is several times faster for CPT I in mitochondria from fed rats compared with that in mitochondria from 48 h-starved rats (Zammit *et al.*, 1984). Similarly starvation appears to enhance the rate at which loss of sensitivity occurs on incubation of the mitochondria in the absence of the effector before assay (Zammit *et al.*, 1984).

Experiments conducted with a single concentration of malonyl-CoA (3.5 μ M) showed that the specific binding of [¹⁴C]malonyl-CoA to rat liver mitochondria underwent changes over the same time-scale reciprocal to those observed for the activity of CPT I. Thus the loss in sensitivity of CPT I to malonyl-CoA inhibition that occurred during the first 4 min of incubation of mitochondria at 37 °C was accompanied by the loss of

ability of mitochondria to bind [¹⁴C]malonyl-CoA. Moreover the differences in the time-dependence of the effects on the enzyme activity between mitochondria from fed and starved animals (see above) were reflected in those for the binding of the effector to the mitochondria, e.g. the loss in binding capacity that occurred during incubation at 37 °C was faster for mitochondria from starved than from fed animals (Zammit *et al.*, 1984). These observations suggest that time-dependent changes in malonyl-CoA sensitivity of CPT I may be due not only to possible events that occur subsequent to the binding of malonyl-CoA to the mitochondria (e.g. conformational changes; Zammit, 1984) but also to actual changes in the extent of binding of malonyl-CoA to the enzyme or an associated regulatory component (Zammit *et al.*, 1984).

The experiments described above were performed at a single physiological concentration of malonyl-CoA. However, studies in our laboratory (Zammit *et al.*, 1984) and those by Bird & Saggerson (1984) have indicated that the concentration-dependence of malonyl-CoA binding to rat liver mitochondria is complex, involving the existence of at least two components. These are a low-capacity component with high affinity for malonyl-CoA, and a high-capacity low-affinity component. We were interested, therefore, in investigating the changes in concentration-dependence of malonyl-CoA binding that accompany the previously observed changes in the sensitivity of CPT I activity to malonyl-CoA inhibition (Zammit, 1984; Zammit *et al.*, 1984) in an attempt to elucidate the function of these two components of malonyl-CoA binding.

MATERIALS AND METHODS

Sources of animals and chemicals were as described previously (Zammit, 1984). Rat liver mitochondria were prepared from fed rats also as described previously (Zammit *et al.*, 1984). Measurement of time-dependence of specific binding of radiolabelled ligand to mitochondria was performed as described by Rial & Nicholls (1983), except that $[2\text{-}^{14}\text{C}]\text{malonyl-CoA}$ was used in place of labelled nucleotides. Briefly, mitochondria (about 1 mg of protein in 50 μl) were added to the medium (150 mM-KCl, 5 mM-Tris/HCl, 1 mM-EGTA, 10 mg of albumin/ml, 2 μg of antimycin A/ml and 1 μg of rotenone/ml), which either contained the appropriate amount of $[^{14}\text{C}]\text{malonyl-CoA}$ or lacked the latter. The pH of the medium was adjusted to 7.4 at the required temperature. After mixing, the tubes were either centrifuged for 90 s at 12000 g in an Eppendorf 4312 bench-top centrifuge or incubated for the indicated times at the required temperature before centrifugation. When the tubes were centrifuged immediately, the effective time of exposure of the mitochondria to the medium was 5–10 s (Rial & Nicholls, 1983). After sedimentation of the mitochondria, the supernatants were removed and kept. The sides of the tubes were dried and the undisturbed pellets treated with 3% (w/v) HClO_4 as described previously (Zammit *et al.*, 1984). The ^3H and ^{14}C radioactivities in the pellet and in the supernatants were measured to provide an estimate of the proportion of $[^{14}\text{C}]\text{malonyl-CoA}$ that was due to extramitochondrial water space associated with the pellet. Preliminary experiments were performed in which this space was determined in parallel by inclusion of a large excess (2 mM) of unlabelled malonyl-CoA in the medium. The $[^3\text{H}]\text{sucrose}$ and malonyl-CoA spaces of the mitochondrial pellets under these two conditions were identical (cf Zammit *et al.*, 1984). Because very high concentrations of unlabelled malonyl-CoA (2 mM) were required if interference from the low-affinity binding component was to be avoided, it was more economical to use $[^3\text{H}]\text{sucrose}$ routinely.

Protein determinations were performed by the method of Lowry *et al.* (1951).

RESULTS

The use of the technique of Rial & Nicholls (1983; see the Materials and Methods section) enabled the determination of specific binding of malonyl-CoA for shorter or longer periods of exposure of mitochondria to the ligand under different experimental conditions. Using this technique we have previously obtained detailed time-courses for changes in malonyl-CoA binding when the ligand was present at a concentration of 3.5 μM . In those studies we found that malonyl-CoA binding to rat liver mitochondria occurred in two phases: (1) rapid binding, which occurred at the earliest time-point achieved (5–10 s) and which was independent of the temperature of the incubation medium, and (2) a slower increase in binding that occurred over the following 2–3 min. This second phase was only apparent at 0 $^\circ\text{C}$ (Zammit *et al.*, 1984). In the present study we used the same methods to study the changes that occur in the concentration-dependence of malonyl-CoA binding to mitochondria during incubation at 37 $^\circ\text{C}$ and 0 $^\circ\text{C}$. As previously observed (Zammit *et al.*, 1984), when mitochondria were exposed to malonyl-CoA for 5–10 s

Fig. 1. (a) Effects of duration of incubation of rat liver mitochondria at 0 $^\circ\text{C}$ on the concentration-dependence of specific binding of $[2\text{-}^{14}\text{C}]\text{malonyl-CoA}$, and (b) Scatchard plots of mean values

Before sedimentation, mitochondria were incubated as follows: 5–10 s in the presence of malonyl-CoA (●); 4 min in the presence of malonyl-CoA (□) or in its absence followed by 5–10 s exposure to malonyl-CoA (○). Values are means ($\pm$ S.E.M.) for three determinations on separate mitochondrial preparations.

at 0 $^\circ\text{C}$ rapid binding occurred. The concentration-dependence of this binding consisted of at least two components (Fig. 1). A very similar concentration-dependence of initial binding was obtained if the experiments were performed at 37 $^\circ\text{C}$ (Fig. 2, and Zammit *et al.*, 1984). The pattern of binding can briefly be described as having a low-affinity high-capacity component and a high-affinity low-capacity component (or groups thereof). Independent confirmation of this pattern of binding has come from the work of Bird & Saggerson

Fig. 2. (a) Effects of duration of incubation of rat liver mitochondria at 37 °C on the concentration-dependence of specific binding of [2-¹⁴C]malonyl-CoA, and (b) Scatchard plots of mean values

Before sedimentation, mitochondria were incubated as follows: 5–10 s in the presence of malonyl-CoA (■); 4 min in the presence of malonyl-CoA (●) or in its absence followed by 5–10 s exposure to malonyl-CoA (○). Values are means ($\pm$ S.E.M.) for three determinations.

(1984), who incubated rat liver mitochondria for 20 min at 0 °C in the presence of malonyl-CoA. However, as expected from the results obtained by Zammit *et al.* (1984), there were major differences in the capacity and concentration-dependence of malonyl-CoA binding when mitochondria were incubated for a period of 4 min either at 37 °C or at 0 °C. Two sets of experiments were performed at the two temperatures. In the first, mitochondria were incubated for 4 min in the absence of malonyl-CoA and then exposed to the labelled ligand for 5–10 s before sedimentation (see the Materials and methods section). When the experiments were performed at 0 °C, there was no change in the concentration-dependence or capacity of binding of malonyl-CoA to the mitochondria (Fig. 1) compared with that which occurred after 5–10 s. This result indicated that dilution of a

Fig. 3. Changes in capacity of low-affinity and high-affinity malonyl-CoA-binding components during incubation of rat liver mitochondria at 37 °C

Before sedimentation, mitochondria were incubated at 37 °C as follows: 5–10 s in the presence of malonyl-CoA (●); 4 min in the absence of malonyl-CoA, followed by 5–10 s (○) or 4 min (▲) in the presence of malonyl-CoA. The experiment shown is representative of three experiments which gave similar results.

portion of mitochondrial suspension into the incubation medium did not alter the ability of mitochondria to express the initial rapid phase of the binding of malonyl-CoA if subsequent incubation was performed at low temperature. By contrast, when mitochondria were incubated at 37 °C before addition of malonyl-CoA, a substantial proportion of the rapid binding of the ligand was lost. This was expected from the results of Zammit *et al.* (1984), who used a single concentration of malonyl-CoA (3.5 μM). The present results also showed that this loss of binding occurred at all concentrations of malonyl-CoA tested. It is noteworthy that under these conditions the sensitivity of CPT I to malonyl-CoA inhibition is much diminished after 4 min incubation at 37 °C (Zammit *et al.*, 1984).

In the second series of experiments, mitochondria were incubated for 4 min either at 0 °C or at 37 °C in the presence of the different concentrations of malonyl-CoA indicated in order to study the changes in binding that occurred after the rapid initial binding described above. At 0 °C, there was an increase in the absolute amount of binding (Fig. 1; see also Zammit *et al.*, 1984). This increase appeared to occur for both components of malonyl-CoA binding (Fig. 1b). However, when experiments were performed at 37 °C the two components behaved differently. Thus there was a major loss of malonyl-CoA binding at high concentrations of the ligand, but minimal change at lower concentrations (10 μM) of malonyl-CoA (Fig. 2). This observation explains why we did not previously observe any appreciable change in binding with time of incubation of the mitochondria at 37 °C with 3.5 μM -malonyl-CoA

Fig. 4. Computation of the characteristics of the two components of malonyl-CoA binding to rat liver mitochondria

Details of the methods used to derive the plots are given in the text.

(Zammit *et al.*, 1984). We interpret these observations as suggesting that incubation of mitochondria at physiological temperatures in the presence of malonyl-CoA results in the loss of the low-affinity component of malonyl-CoA binding, with the retention, and probably increase, in the capacity of the high-affinity component (Fig. 2).

In order to test this inference, we performed a third series of experiments in which, after incubation of mitochondria for 4 min at 37 °C in the absence of the ligand, malonyl-CoA was added and incubations were allowed to proceed for a further 4 min. As expected (from Fig. 2), after the first 4 min binding capacity was lost at all concentrations, but, after a further 4 min in the presence of malonyl-CoA, binding at concentrations lower than 10 μM increased, whereas that at higher concentrations continued to decrease (Fig. 3). Therefore these experiments confirmed that at 37 °C there occurred an increase in high-affinity binding but a continual decrease in the capacity of the low-affinity component. Under the same conditions, the sensitivity of CPT I to malonyl-CoA inhibition is enhanced (Zammit, 1984; Zammit *et al.*, 1984; see also Bremer *et al.*, 1985). It is to be noted that these changes in concentration-dependence of malonyl-CoA binding could not have resulted from artefacts arising from the metabolism of malonyl-CoA. Preliminary experiments (see also Zammit, 1984) confirmed the findings of Stephens *et al.* (1983) that metabolism of malonyl-CoA by rat liver mitochondria under similar conditions was not extensive. Moreover, the destruction of malonyl-CoA could not possibly account for an increase in binding at the lowest concentrations of the ligand used. The cyclical variation in binding of malonyl-CoA at low concentrations in these experiments confirms the observations made previously (Zammit *et al.*, 1984).

The data from these experiments enabled the quantitative and separate assessment of the saturation kinetics of the high- and low-affinity components. The characteristics of the high-affinity component were derived by subtracting the mean values obtained for the binding at different malonyl-CoA concentrations when mitochondria were incubated at 37 °C for 4 min in the absence of malonyl-CoA from those obtained after incubation

Fig. 5. Effect of temperature on [^{14}C]malonyl-CoA binding to rat liver mitochondria at 60 μM -malonyl-CoA

Binding was measured at the temperature shown after exposure of the mitochondria to [^{14}C]malonyl-CoA for 5–10 s (●), or for 1 min in the presence of [^{14}C]malonyl-CoA (□), or for 5–10 s after incubation for 1 min in the absence (○) of malonyl-CoA. The pH of the incubation medium was adjusted to 7.4 for all the temperatures tested. Values are means ($\pm$ s.e.m.) for three separate mitochondrial preparations and are expressed as a percentage of the degree of binding obtained initially after 5–10 s at 37 °C by the same mitochondrial preparation.

(4 min) in the presence of malonyl-CoA (Fig. 4). It will be recalled that only the high-affinity component appears to increase in capacity at 37 °C during 4 min incubation in the presence of malonyl-CoA (Fig. 2). The characteristics of the low-affinity component were derived by subtracting the values obtained after 4 min incubation at 37 °C in the presence of malonyl-CoA from those observed initially (after 5–10 s). Under the former conditions only the low-affinity component appeared to be lost (Fig. 2). As shown in Fig. 4, these analyses suggested that the two components differed not only with respect to their avidity for malonyl-CoA and their capacity, but also with respect to their saturation kinetics. Thus the high-affinity component appeared to approach saturation hyperbolically and was half-saturated at concentrations of malonyl-CoA in the region of 2–3 μM . By contrast, the low-affinity component appeared to approach saturation sigmoidally, with half-maximal saturation requiring 30 μM -malonyl-CoA. Such sigmoidicity may arise from allosteric interactions between subunits of a protein species responsible for low-affinity binding of malonyl-CoA.

The different results obtained for changes in capacity of the low-affinity component at 0 °C and 37 °C suggested that expression of this component depended on the temperature at which mitochondrial incubations were performed. Thus at 0 °C there was an increase in binding

at 55 μM -malonyl-CoA after 4 min (Fig. 1, and Zammit *et al.*, 1984), whereas at 37 °C there was a decrease of binding (Fig. 2). We were therefore interested to investigate the temperature-dependence of these effects and especially to find out at which temperature the transition from loss of binding to an increase in binding occurred as the temperature of incubation was lowered. The results of the relevant experiments are given in Fig. 5. Binding was measured at 60 μM -malonyl-CoA to minimize the contribution of the high-affinity component (see also Bird & Saggerson, 1984). Binding was measured for three sets of conditions: (i) exposure of mitochondria to malonyl-CoA for 5–10 s; (ii) 5–10 s exposure after 1 min preincubation of the mitochondria; and (iii) incubation of the mitochondria with malonyl-CoA for 1 min before sedimentation. These three conditions enabled an estimate of the rate of increase or decrease in the binding by the low-affinity component that occurred after the initial binding. The initial binding showed a modest but distinct optimum around 25–30 °C (Fig. 5). This temperature range also coincided with the transition between loss (above 25 °C) and gain (below 25 °C) of binding that occurred in the presence of 60 μM -malonyl-CoA (Fig. 5). The rate of increase in binding was enhanced at lower temperatures, but was similar at 10 °C and 0 °C. These observations suggested that at temperatures around 25 °C the component in rat liver mitochondria responsible for low-affinity binding underwent a transition: binding was enhanced below 25 °C, but was lost above this temperature. A similar observation was made for the retention of binding capacity after a period of mitochondrial incubation in the absence of malonyl-CoA (Fig. 5). By contrast, as indicated in Figs. 1 and 2, the time-dependent increase in binding by the high-affinity component appeared to occur independently of temperature. These observations suggest that the two components of malonyl-CoA binding in rat liver mitochondria are affected differently by changes in the temperature of the incubation medium. Whether such temperature-dependent transitions are indicative of the involvement of membrane fluidity in determining the pattern of malonyl-CoA binding to rat liver mitochondria will need to be confirmed by studies involving other means of altering membrane fluidity.

DISCUSSION

We have confirmed that binding of malonyl-CoA to rat liver mitochondria occurs at two classes of sites with very different avidity for the ligand. The high-affinity component became saturated at malonyl-CoA concentrations similar to those that occur *in vivo* (0.4–8 μM ; Zammit, 1981). Evidence was also obtained that the two components approached saturation differently: the high-affinity component obeyed Michaelis–Menten kinetics, whereas the low-affinity component showed sigmoid saturation kinetics. In this respect, although we have used Scatchard plots for illustrative purposes (Figs. 1 and 2), we appreciate that, if the above analysis is valid, the use of such plots to derive quantitative estimates of binding parameters for the two components can present problems if the data for total binding are analysed by a two-hyperbola model.

The two components also differed in their response to different temperatures of the mitochondrial incubation medium. Binding of malonyl-CoA by the high-affinity component occurred at all temperatures between 0 and 37 °C, whereas that by the low-affinity component was progressively lost with time above 25 °C. It is suggested that the micro-environment of the molecular species responsible for low-affinity binding of malonyl-CoA undergoes temperature-related transitions that may derive from changes in membrane fluidity that occur around 25 °C. Parallel experiments on the temperature-dependence of the catalytic activity of CPT I and its sensitivity to malonyl-CoA inhibition could indicate the degree of involvement of the enzyme protein in the time- and temperature-dependence of malonyl-CoA binding to rat liver mitochondria observed in the present study.

A comparison of the changes in CPT I sensitivity to malonyl-CoA inhibition (Zammit, 1984; Zammit *et al.*, 1984) that occur during incubation at 37 °C with the changes in binding observed in the present study suggests that increased inhibition of the enzyme is accompanied not only by an increase in binding by the high-affinity component of malonyl-CoA binding but also by a decrease in binding by the low-affinity component. Such comparisons may start to elucidate the function and identity of the sites responsible for the two components. Evidently, only binding at the high-affinity site is required to elicit inhibition of CPT I. It is noteworthy that the concentration required to elicit half-maximal saturation of this component (about 3 μM -malonyl-CoA) is similar to the concentration of malonyl-CoA required to produce 50% inhibition of CPT I activity measured under very similar conditions (Zammit & Corstorphine, 1985). It would be expected that under conditions *in vivo* such concentration-dependence characteristics of the high-affinity component would enable CPT I to respond optimally to the changes in concentration of malonyl-CoA that occur in rat liver (Zammit, 1981). The very high concentrations of malonyl-CoA required to half-saturate the low-affinity component suggest that *in vivo* this site may not be occupied by malonyl-CoA to any significant extent. We have obtained evidence that under conditions *in vivo* this site may be occupied preferentially by long-chain acyl-CoA (B. D. Grantham & V. A. Zammit, unpublished work).

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