MALONYL CoA INHIBITION OF CARNITINE ACYLTRANSFERASE ACTIVITIES

Effects of thiol-group reagents

E. David SAGGERSON and Carol A. CARPENTER
Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, England

Received 3 December 1981

1. Introduction

The overt form of carnitine palmitoyltransferase (CPT_1) in mitochondria from rat liver [1,2] and other rat tissues, including heart [3,4] is potently inhibited by malonyl CoA. This inhibition is competitive with respect to palmitoyl CoA and results in considerable sigmoidicity in the kinetics of CPT_1 with respect to this substrate [4,5]. These findings suggest that CPT_1 might show regulation of an allosteric nature. One novel aspect of the malonyl CoA inhibitory effect is that it is lost, or greatly diminished when the association of CPT_1 with the mitochondrial membrane is disrupted [1,4]. In studies [3,5] CPT_1 activity was measured as the incorporation of [^{3}H]carnitine into palmitoylcarnitine. In parallel studies the enzyme was assayed by following the reaction of released CoASH with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). It was noted that the effect of malonyl CoA was considerably muted when the DTNB-linked assay was used. In this study therefore we have examined, using the [^{3}H]carnitine-based assay, the effects of thiol-group protection and of DTNB upon the putative allosteric effect of malonyl CoA on CPT_1.

Rat liver mitochondria also appear to contain an overt carnitine acyltransferase activity which will use medium chainlength substrates [6–8]. This is usually referred to as carnitine octanoyltransferase (COT) and, although not well characterised, appears to be distinct from CPT [4,6–8]. Hepatic COT activity is also inhibited by malonyl CoA [9], showing greater sensitivity to this effector than CPT_1. In addition, the effect of malonyl CoA is non-competitive with respect to octanoyl CoA and does not cause sigmoidicity in the kinetics with this substrate [5]. This activity is also considered in this study and shown to differ from CPT_1, in its response to DTNB and reduced glutathione (GSH).

2. Materials and methods

Sources of chemicals are described in [9]. In addition, GSH was from Boehringer and DTNB from Sigma. Fed male Sprague-Dawley rats (160–180 g) were used throughout. Liver from a single rat or hearts from 4 rats were collected, finely chopped with scissors, washed to remove blood and homogenized in ice-cold 0.25 M sucrose containing 10 mM Tris–HCl (pH 7.4), 1 mM EGTA and defatted albumin (10 mg/ml). The volume of the homogenate was adjusted in the proportion 10 ml sucrose/albumin medium per g tissue. Centrifugation conditions for isolation and washing of the mitochondria were as in [10]. Mitochondrial pellets were washed once with 0.25 M sucrose/10 mM Tris–HCl (pH 7.4)/1 mM EGTA. Finally, the mitochondria were resuspended in 0.3 M sucrose/10 mM Tris–HCl (pH 7.4)/1 mM EGTA (2 or 4 ml medium/g original tissue for heart or liver mitochondria, respectively). The entire mitochondrial isolation procedure was carried out at 0–4°C and took 20–25 min. The protein contents of these stock mitochondrial suspensions were determined [11] and were (mg/ml as means ± SEM): liver 4.0 ± 0.2 (n = 12); heart 3.0 ± 0.1 (n = 5). Enzyme assays were performed within 30 min of the isolation of mitochondria. For assay of CPT_1, 50 μl mitochondria (200 ± 8 and 150 ± 6 μg protein for liver and heart, respectively) were preincubated at 25°C for 2 min in 1.0 ml containing 220 mM sucrose, 40 mM KCl, 10 mM Tris–HCl (pH 7.4), 1 mM EGTA, defatted albumin (1.3 mg/ml), the indicated concentration of

Published by Elsevier/North-Holland Biomedical Press
00145793/82/0000–0000/$02.75

palmitoyl CoA and other additions stated in individual figure legends. The reaction was initiated by addition of 20 μl containing 0.4 μmol L-carnitine and 1 μCi D,L-[*methyl*-^{3}H]carnitine (Radiochemical Centre, Amersham). After 4 min the reaction was terminated with 1 ml ice-cold 1.2 M HCl followed by 2 ml water-saturated butanol. After mixing and brief centrifugation, the butanol layer was washed with 5 ml butanol-saturated water and 1.0 ml of the butanol layer, containing acyl-[^{3}H]carnitine taken for liquid scintillation counting. The assay of covert COT was identical except that octanoyl CoA was used in place of palmitoyl CoA. Zero-time blanks were subtracted. Assays were linear with amount of mitochondria and with time over the range of acyl CoA concentrations used, with and without malonyl CoA, with and without DTNB. Respiratory inhibitors (rotenone or antimycin A at 1 μg/ml) were not routinely included in the assay mixtures since these were found to have no effect upon the measured rates of acyl carnitine formation (with or without GSH, DTNB or malonyl CoA). Since the mitochondria were coupled [4] and were incubated without phosphate, ADP or Mg^{2+} in the presence of EGTA, respiration was almost undetectable. Loss of the assay product by β-oxidation is therefore discounted. Under the assay conditions rates of palmitoyl CoA deacylation (measured as CoASH release by a sensitive recycling assay [12]) were 0.8 and 0.6 nmol . min^{-1} . mg protein^{-1} for liver and heart mitochondria respectively and therefore did not significantly deplete the acyl CoA substrate under the assay conditions.

3. Results and discussion

Inclusion of 0.1 mM DTNB in CPT_1 assays was found to appreciably decrease the sensitivity to malonyl CoA in both liver and heart mitochondria (fig.1). With liver mitochondria, inclusion of 1 mM GSH considerably increased the sensitivity permitting 50% inhibition with ~5 μM malonyl CoA. Similar sensitivity of CPT_1 from livers of fed rats was observed when 0.5 mM dithiothreitol (DTT) was used as the thiol-group protective agent [9]. As shown in [3] heart CPT_1 showed considerably greater sensitivity to malonyl CoA than the liver enzyme (cf. fig.1a,1b). By contrast with liver CPT_1, the sensitivity observed here for heart CPT_1 in the absence of thiol-group protecting agent was similar to that observed for the heart enzyme in the presence of 0.5 mM DDT [3]. With the heart enzyme the effect of DTNB can be examined without recourse to absurdly high malonyl CoA concentrations. It may be seen (fig.1b) that the effect of

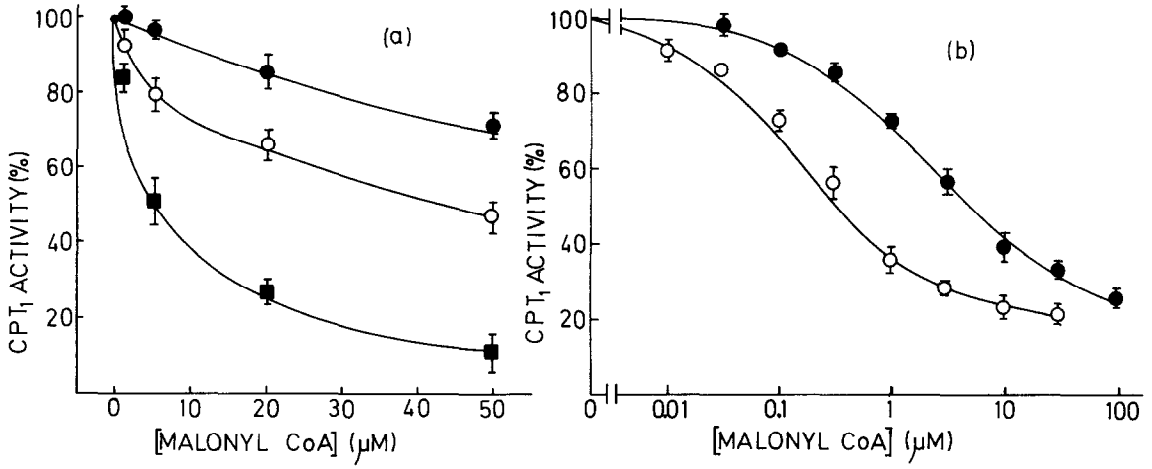

Fig.1. Effect of DTNB and GSH on inhibition of CPT_1 by malonyl CoA. Palmitoyl CoA was present throughout at 40 μM and L-carnitine at 400 μM; (○) with no additions; (●) with DTNB (0.1 mM); (■) with GSH (1 mM). The bars indicate SEM; where not shown these lie within the symbol. (a) Liver mitochondria (4 expt). Absolute activities in the absence of malonyl CoA were: 5.5 ± 0.3, 7.3 ± 0.2 and 5.0 ± 0.2 nmol . min^{-1} . mg protein^{-1}, respectively, with no additions, DTNB or GSH. (b) Heart mitochondria (3 expt). Absolute activities in the absence of malonyl CoA were: 8.4 ± 0.7 and 9.2 ± 0.3 nmol . min^{-1} . mg protein^{-1} with no additions and DTNB, respectively.

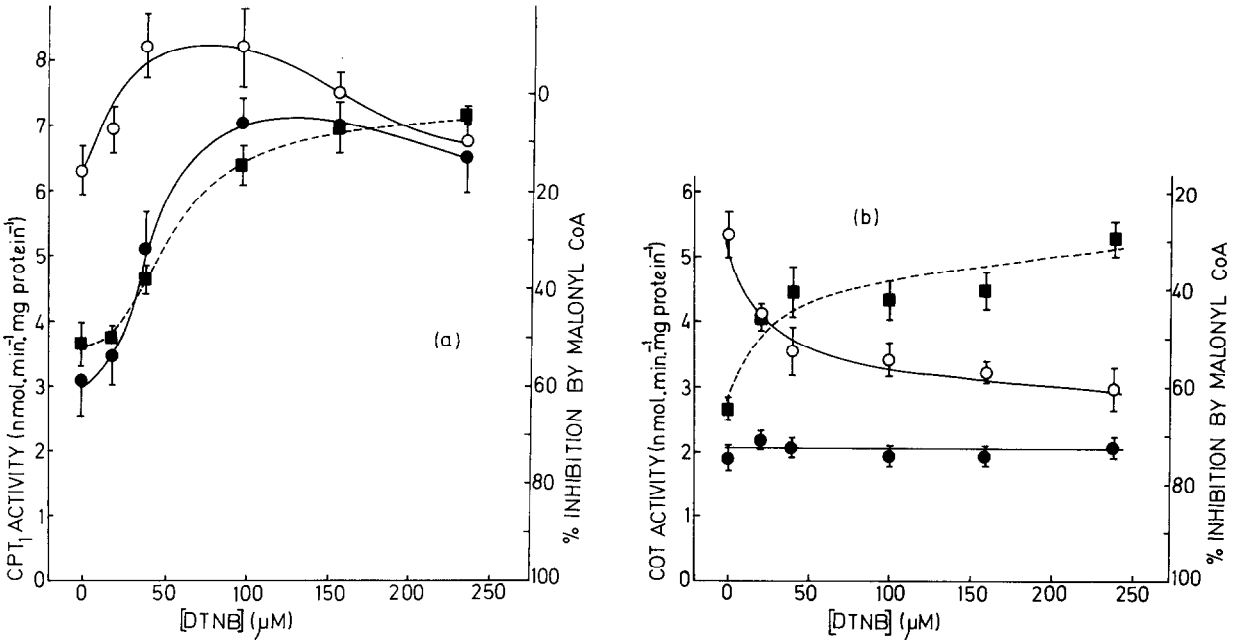

Fig.2. Effect of DTNB concentration on liver CPT_1 and overt COT activities. Palmitoyl CoA and octanoyl CoA were present throughout at 40 μM and L-carnitine at 400 μM: (○) activity without malonyl CoA; (●) activity with malonyl CoA (50 μM for CPT_1 and 10 μM for COT assays); (■) percentage inhibition by malonyl CoA. The bars indicate SEM: (a) CPT_1 (3 expt); (b) overt COT (4 expt).

DTNB is apparently competitive with malonyl CoA, simply shifting the inhibition curve by ~1 order of magnitude.

Fig.2 summarises an experiment in which the responses of CPT_1 and overt COT activities to DTNB are compared. These were found to differ considerably. DTNB increased both basal CPT_1 and CPT_1 activity in the presence of 50 μM malonyl CoA. This effect was maximal at ~0.1 mM DTNB. DTNB at 0.24 mM was sufficient to virtually abolish the effect of malonyl CoA under the chosen conditions (fig.2a). The corresponding experiment with overt COT (fig.2b) was performed using 10 μM malonyl CoA since liver COT is more sensitive to malonyl CoA than CPT_1 and this concentration gives near-maximal inhibition [9]. By contrast with its effect on CPT_1, DTNB did not stimulate, but inhibited basal COT, had no effect on the malonyl CoA-inhibited activity and, even with 0.24 mM DTNB, did not achieve full abolition of the malonyl CoA effect.

Fig.3 shows another set of differences in the properties of CPT_1 and overt COT. The effect of GSH to increase the percentage effect of malonyl CoA on CPT_1 (fig.1a) is achieved by a GSH dose-dependent decrease in CPT_1 activity when malonyl CoA is present but with negligible change in the basal activity (fig.3a). By contrast, GSH decreased basal COT but had little effect on the malonyl CoA-inhibited activity (fig.3b). At present no explanation is advanced for the effects of DTNB and GSH on COT, but the disparity in responses of CPT_1 and COT to these agents further exemplify the differences between these activities [4,5,9] and add support to the idea that these may be separate enzymes.

Liver CPT_1 shows sigmoidal kinetics with respect to palmitoyl CoA under the assay conditions employed here and malonyl CoA increases the sigmoidicity [5]. DTNB reduces the effect of the putative allosteric inhibitor malonyl CoA (fig.1a) whilst increasing the basal activity (fig.2a) at 40 μM palmitoyl CoA, which is a sub-saturating concentration of this substrate under the assay conditions. It seemed possible that DTNB might be increasing this basal activity by decreasing the sigmoidicity of the kinetics with respect to palmitoyl CoA in the absence of malonyl CoA. This was tested for the liver (fig.4a) and the heart

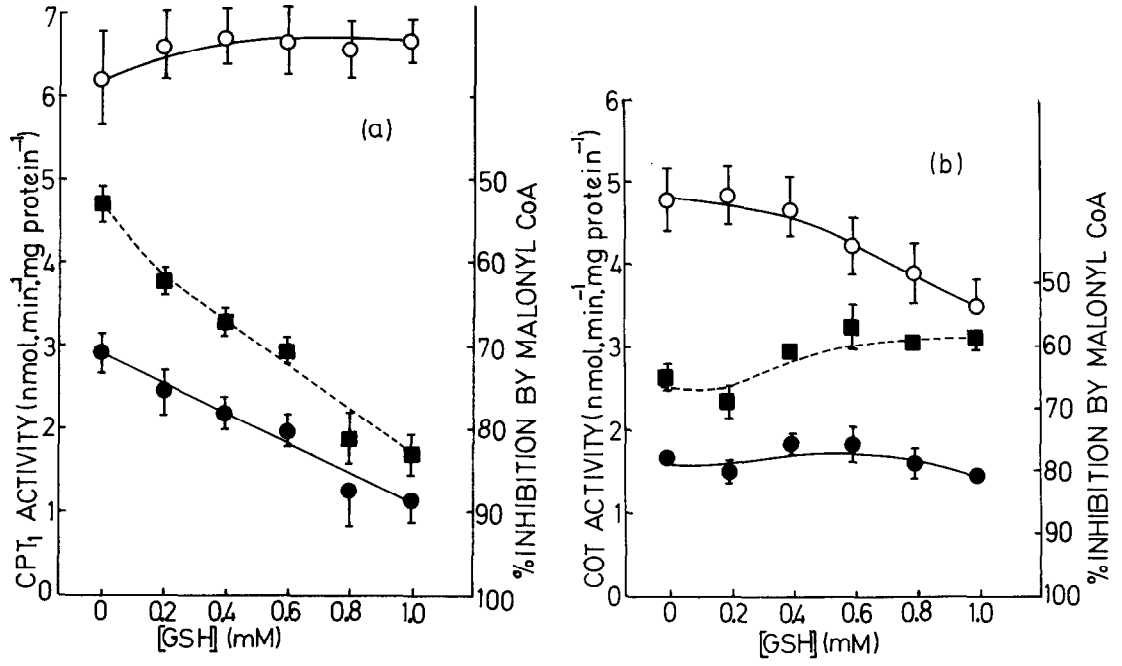

Fig.3. Effect of GSH concentration on liver CPT_1 and overt COT activities. Legend as for fig.2: (a) CPT_1 (3 expt); (b) overt COT (4 expt).

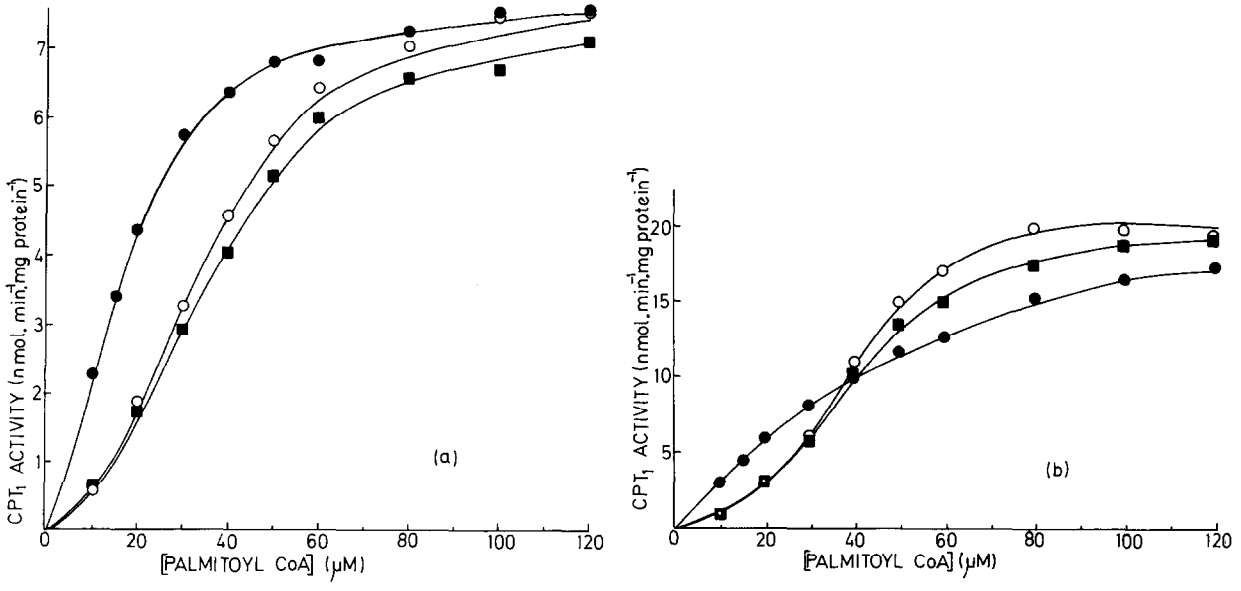

Fig.4. Effect of DTNB on dependence of CPT_1 activity on palmitoyl CoA concentration. L-Carnitine was present throughout at 400 μM: (○) with no additions; (●) with DTNB (0.1 mM); (■) with dithiothreitol (0.5 mM); (a) liver mitochondria (2 expt); (b) heart mitochondria (2 expt).

enzymes (fig.4b) and found to be so. The thiol-group protecting agent DTT had little effect whereas DTNB virtually abolished the sigmoidicity of the kinetics. With the liver enzyme DTNB decreased the Hill coefficient from 2.5–1.8. With the heart enzyme a decrease of 3.0–1.4 was observed. Detergent treatment has also been found to abolish sigmoidal kinetics of heart CPT_1 [4].

It is concluded that thiol-group protecting agents do not appreciably alter the basal kinetics of CPT_1 but appear to facilitate the effect of malonyl CoA (at least for the liver enzyme). On the other hand, DTNB both diminishes the effect of malonyl CoA and alters the basal kinetics essentially abolishing apparent cooperativity with respect to palmitoyl CoA. This effect of DTNB is strikingly similar to that seen with skeletal muscle phosphofructokinase in which titration of a single highly reactive thiol-group in the enzyme with DTNB abolishes sigmoidal kinetics with respect to fructose 6-phosphate [13]. Further studies should establish whether this parallel between the 2 enzymes is appropriate and whether membrane-associated CPT_1 can truly be regarded as an allosteric enzyme in the accepted sense.

Acknowledgement

We wish to thank Dr M. R. Hollaway for helpful discussions and suggestions.

References

[1] McGarry, J. D., Leatherman, G. F. and Foster, D. W. (1978) J. Biol. Chem. 253, 4128–4136.
[2] McGarry, J. D. and Foster, D. W. (1980) Ann. Rev. Biochem. 49, 395–420.
[3] Saggerson, E. D. and Carpenter, C. A. (1981) FEBS Lett. 129, 229–232.
[4] Saggerson, E. D. (1982) Biochem. J. in press.
[5] Saggerson, E. D. and Carpenter, C. A. (1981) FEBS Lett. 132, 166–168.
[6] Kopec, B. and Fritz, I. B. (1971) Can. J. Biochem. 49, 941–948.
[7] Solberg, H. E. (1971) FEBS Lett. 12, 134–136.
[8] Solberg, H. E. (1972) Biochim. Biophys. Acta 280, 422–433.
[9] Saggerson, E. D. and Carpenter, C. A. (1981) FEBS Lett. 129, 225–228.
[10] Harper, R. D. and Saggerson, E. D. (1975) Biochem. J. 152, 485–494.
[11] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[12] Allred, J. B. and Guy, D. G. (1969) Anal. Biochem. 29, 293–299.
[13] Forest, P. B. and Kemp, R. G. (1968) Biochem. Biophys. Res. Commun. 33, 763–768.