Malonyl-CoA inhibition of peroxisomal carnitine octanoyltransferase

Nóirín NIC A'BHÁIRD and Rona R. RAMSAY*

Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143, and Molecular Biology Division, Veterans Administration Medical Center, 4150 Clement Street, San Francisco, CA 94121, U.S.A.

Although the malonyl-CoA sensitivity of peroxisomal carnitine octanoyltransferase (COT) is reportedly lost on solubilization, we show that malonyl-CoA does inhibit the purified enzyme. Assay conditions such as buffer composition, pH, acyl-CoA substrate and the presence or absence of BSA can affect the observed inhibition. When assayed in the absence of BSA, COT shows simple competitive inhibition by malonyl-CoA. The K_i value for inhibition of purified COT is high (106 μM) compared with physiological concentrations (1–6 μM) and other short-chain acyl-CoA esters inhibit COT to the same degree. However, when COT is assayed in intact peroxisomes, the K_i for malonyl-CoA is almost 20-fold lower than found with the purified enzyme, whereas inhibition by other short-chain acyl-CoA esters does not change significantly. Several features of the inhibition of peroxisomal COT, including the specificity of malonyl-CoA over other short-chain acyl-CoA esters, resemble those of carnitine palmitoyltransferase (CPT)-I, suggesting that the regulation of COT and CPT-I in parallel may be necessary for the control of cellular fatty acid metabolism.

INTRODUCTION

The carnitine acyltransferase enzymes catalyse the reversible transfer of acyl groups from CoA to L-carnitine. The inhibition of the overt carnitine palmitoyltransferase (EC 2.3.1.21; CPT-I) on the mitochondrial outer membrane by malonyl-CoA is important in the regulation of the β -oxidation of long-chain fatty acids and ketone-body synthesis [1,2]. Other malonyl-CoA-sensitive carnitine acyltransferase activities have been found in peroxisomes [3] and microsomes [4]. Although the proposed role of peroxisomal carnitine octanoyltransferase (COT) in shuttling chain-shortened acyl groups from peroxisomes to mitochondria remains unconfirmed, the malonyl-CoA sensitivity suggests that COT is also important in the overall pattern of fatty acid metabolism in the cell [3]. The malonyl-CoA sensitivity of peroxisomal COT, like that of mitochondrial CPT-I, is reportedly lost on solubilization of the enzyme [3,5,6].

We have now examined in detail the inhibition of COT by malonyl-CoA and related compounds and have compared the characteristics of the inhibition of the purified enzyme with the inhibition of the enzyme in intact peroxisomes.

EXPERIMENTAL

Essentially fatty-acid-free BSA, palmitoyl-CoA, decanoyl-CoA, malonyl-CoA, glutaryl-CoA, 3-hydroxy-3-methylglutaryl-CoA, methylmalonyl-CoA and L-carnitine were all obtained from Sigma. Aldrithiol (4,4'-dithiobispyridine) was obtained from Aldrich. COT was purified from bovine liver as described in [7]. Rat liver peroxisomes were prepared by centrifugation through Nycodenz [3]. COT was assayed in the forward direction by measuring the released CoA by its reaction with aldrithiol at 324 nm [3]. K_m values were determined either by fitting the initial-rate data to a rectangular hyperbola or by fitting the data from the entire progress curve of the reaction to the integrated form of the Michaelis–Menten equation. Kinetic and inhibition constants

were determined by linear or non-linear regression analysis as appropriate.

RESULTS

Assay conditions

As is well known for CPT, the activity of COT is sensitive to changes in assay conditions. The observed reaction velocity and sensitivity to inhibition are strongly influenced by the particular assay conditions employed. Since there is no universally used 'standard assay', comparison of the data obtained from different laboratories using different assay systems is difficult. BSA is commonly included in the assay of COT and CPT in order to minimize the problems associated with micellar formation and the detergent properties of fatty acyl-CoA esters [8,9]. However, the inclusion of BSA in the assay medium itself causes complications in the interpretation of results, owing to the binding of fatty acyl-CoAs to BSA and the resultant change in free substrate concentration [10,11]. Table 1 shows some of the effects of changes in the assay conditions on the observed activity of purified COT and on its sensitivity to malonyl-CoA inhibition. When assayed in 20 mM-potassium phosphate buffer at pH 7.4, in the absence of BSA, and with a substrate concentration of 10 μM acyl-CoA ($20 \times K_m$), no inhibition was observed even with 100 μM -malonyl-CoA, a concentration almost 3 orders of magnitude above the physiological range. However, in the presence of 1.3 mg of BSA/ml significant inhibition is observed at the same concentration of malonyl-CoA and a higher fatty acyl-CoA concentration. With decanoyl-CoA rather than palmitoyl-CoA as the substrate, the observed inhibition by malonyl-CoA is much less (Table 1), presumably because decanoyl-CoA binds less effectively to BSA than does palmitoyl-CoA, resulting in a smaller decrease in substrate availability. Initial-rate measurements for CPT-I activity with palmitoyl-CoA in the presence of BSA yield sigmoidal plots for the dependence of rate on palmitoyl-CoA concentration, suggesting complex kinetic behaviour [11]. Similarly, when assayed in the presence of BSA

Abbreviations used: CPT, carnitine palmitoyltransferase; COT, carnitine octanoyltransferase.

* To whom correspondence should be addressed, at the Veterans Administration Medical Center.

Table 1. Effect of assay conditions on the inhibition of purified COT by malonyl-CoA

COT was assayed spectrophotometrically with 10 μM -decanoyl-CoA (CDT) or -palmitoyl-CoA (CPT) in the absence of fatty-acid-free BSA, or with 40 μM in its presence. L-Carnitine was 1 mM. The buffers were either 20 mM-potassium phosphate (phosphate) or 220 mM-sucrose/40 mM-KCl/10 mM-Tris/HCl/1 mM-EGTA (SKTE). The values in parentheses refer to the percentage inhibition of CDT or CPT activity in the presence of malonyl-CoA compared with the activity in the absence of inhibitor.

Buffer (pH)	BSA (mg/ml)	[Malonyl-CoA] (μM)...	CDT (nmol/min per mg)			CPT (nmol/min per mg)		
			0	10	100	0	10	100
Phosphate (7.4)	—		25.6	—	26.0 (0)	30.3	—	29.5 (0)
Phosphate (7.4)	1.3		22.6	21.7 (4 %)	20.8 (8 %)	12.0	10.9 (9 %)	5.9 (51 %)
SKTE (7.4)	1.3		11.4	—	10.9 (5 %)	5.3	—	2.4 (55 %)
SKTE (6.8)	1.3		12.0	12.5 (0)	7.8 (35 %)	7.7	4.5 (41 %)	1.7 (78 %)

Fig. 1. Inhibition of purified COT by malonyl-CoA in the presence of BSA

Assays were carried out in 20 mM- KH_2PO_4 , pH 7.4, containing 125 μM -aldrithiol and 10 mM-L-carnitine. The concentrations of palmitoyl-CoA refer to the total concentration of substrate added to the assay. The concentrations of malonyl-CoA used were: ■, 0; □, 50 μM ; ○, 100 μM ; ●, 200 μM .

Table 2. pH-dependence of kinetic parameters of purified COT

Parameter	K_m or K_i (μM)	
	pH 6.8	pH 7.4
K_m for carnitine*	$227 \pm 25^{(a)}$	$102 \pm 11^{(a)}$
K_m for decanoyl-CoA	$0.24 \pm 0.07^{(b)}$	$0.34 \pm 0.07^{(b)}$
K_m for palmitoyl-CoA	$0.47 \pm 0.02^{(b)}$	$0.46 \pm 0.05^{(b)}$
K_i for malonyl-CoA*	$35 \pm 11^{(a)}$	$106 \pm 10^{(a)}$

* The substrate was palmitoyl-CoA.

^(a) Values shown are means $\pm$ S.D. of two determinations.

^(b) Values shown are means $\pm$ S.E.M. of ten determinations.

purified COT displays non-linear kinetics. Double-reciprocal plots for the inhibition of COT by malonyl-CoA (shown in Fig. 1) are also upwardly curving. In contrast, when the assays were carried out in the absence of BSA, linear kinetics and simple competitive inhibition by malonyl-CoA was observed (Fig. 2). This effect of BSA means that true kinetic constants cannot be determined in the presence of BSA unless the binding of the substrate to BSA (or indeed to other proteins and membranes) is

Fig. 2. Inhibition of COT by malonyl-CoA in the absence of BSA

Assays were carried out in 20 mM- KH_2PO_4 , pH 7.4, containing 125 μM -aldrithiol and 10 mM-L-carnitine. The concentrations of malonyl-CoA used were: ○, 0; ●, 50 μM ; □, 100 μM ; ■, 200 μM . Inset: slope replot.

taken into consideration. All the kinetic values reported in this work were determined in the absence of BSA.

Malonyl-CoA inhibition of COT is enhanced by a decrease in pH (Table 1), as has been observed for membrane-bound CPT-I [12,13]. Although the control rate for the COT reaction is not changed between pH 7.4 and 6.8, a 20–30 % increase in inhibition by malonyl-CoA is observed when the pH is decreased from 7.4 to 6.8 (see also below).

Kinetics of inhibition of purified COT

In a simple assay system (without BSA), namely 20 mM-potassium phosphate buffer, pH 7.4, 125 μM -aldrithiol and saturating L-carnitine, the concentrations of palmitoyl-CoA and malonyl-CoA were varied (Fig. 2). The K_i for malonyl-CoA was 106 ± 10 μM , which is much higher than the K_i values reported for CPT-I [14,15]. However, a decrease in pH, which is reported to decrease the IC_{50} for malonyl-CoA inhibition of CPT-

Table 3. Kinetic parameters for COT assayed in peroxisomes or in purified enzyme preparations

COT was assayed as described in the Experimental section. For K_m and K_i determinations for the purified enzyme, COT was assayed in potassium phosphate, pH 7.4. All other assays were carried out in 220 mM-sucrose/40 mM-KCl/1 mM-EGTA/10 mM-Tris/HCl, pH 7.4 (SKTE). IC_{50} values refer to the concentration of inhibitor required to give 50% inhibition of activity at a palmitoyl-CoA concentration of 1 μ M; accompanying values in parentheses show percentage inhibition by 100 μ M acyl-CoA analogue. The values shown are means $\pm$ S.D. of two separate determinations carried out in triplicate, except the K_m for palmitoyl-CoA for purified COT, which is the mean $\pm$ S.E.M. of ten determinations.

Parameter	K_m or K_i (μ M)	
	Purified COT	Peroxisomal COT
K_m for palmitoyl-CoA	0.46 $\pm$ 0.05	0.60 $\pm$ 0.07
K_i for malonyl-CoA	106 $\pm$ 10	6.4 $\pm$ 1.5

Analogue	IC_{50} (μ M)	
	Purified COT	Peroxisomal COT
Malonyl-CoA	285 $\pm$ 26 (25 $\pm$ 1)	10 $\pm$ 2 (100)
Glutaryl-CoA	305 $\pm$ 23 (25 $\pm$ 4)	> 100 (26 $\pm$ 2)
Hydroxymethylglutaryl-CoA	293 $\pm$ 29 (24 $\pm$ 3)	> 100 (25 $\pm$ 6)
Methylmalonyl-CoA	333 $\pm$ 22 (14 $\pm$ 1)	> 100 (20 $\pm$ 0.5)

Fig. 3. Inhibition of COT, assayed in peroxisomes, by malonyl-CoA in the absence of BSA

Assays were carried out in SKTE buffer, pH 7.4 (220 mM-sucrose, 40 mM-KCl, 10 mM-Tris/HCl, 1 mM-EGTA) containing 125 μ M-aldrithiol and 10 mM-L-carnitine. The concentrations of malonyl-CoA used were: $\square$, 0; $\blacksquare$, 10 μ M; $\circ$, 20 μ M; $\bullet$, 30 μ M. Inset: slope replot.

I several-fold [12,13], has the same effect on the inhibition of purified COT. Table 2 shows that, although V_{max} and K_m values for palmitoyl-CoA and decanoyl-CoA are unchanged when the pH is decreased from 7.4 to 6.8, the K_i for malonyl-CoA falls

from 106 to 35 μ M over that range. Thus, since the change in the ionization of malonyl-CoA itself is small in that pH range, the group(s) responsible for this change in sensitivity is present in the isolated enzyme.

Kinetics of COT assayed in peroxisomes

Because the K_i value for malonyl-CoA inhibition is so much higher than the physiological range of malonyl-CoA concentrations (1–6 μ M) [16] and than the IC_{50} observed for COT in isolated peroxisomes [3], we also examined the kinetics of the enzyme in peroxisomes.

Table 3 compares some of the kinetic parameters observed for COT assayed in peroxisomes and for the solubilized enzyme. Although the K_m values for the fatty acyl-CoA substrate in the organelle are the same as for the purified enzyme, the K_i for the inhibition of COT by malonyl-CoA is dramatically lower for the enzyme assayed in peroxisomes. Fig. 3 shows a double-reciprocal plot for the inhibition of COT in peroxisomes by malonyl-CoA (in the absence of BSA). The inhibition is competitive, with a K_i of 6.4 μ M. In order to determine whether this change in sensitivity to inhibition was specific for malonyl-CoA, we examined the inhibition of COT by several other short-chain acyl-CoA esters.

The IC_{50} values for the inhibition of purified COT at a fixed non-saturating concentration of palmitoyl-CoA (1 μ M) were determined for malonyl-CoA (285 $\pm$ 26 μ M), glutaryl-CoA (305 $\pm$ 23 μ M), 3-hydroxy-3-methylglutaryl-CoA (293 $\pm$ 29 μ M) and methylmalonyl-CoA (333 $\pm$ 22 μ M) (Table 3). The similar IC_{50} values of approx. 300 μ M suggest that the binding is due to the common CoA moiety. In contrast, when assayed in intact peroxisomes COT shows a much greater sensitivity to inhibition by malonyl-CoA (IC_{50} = 10 μ M), whereas the IC_{50} value for each of the other compounds examined was greater than 100 μ M under the same assay conditions (Table 3).

DISCUSSION

Several factors can influence the reaction kinetics of CPT and COT. Although these complicating factors have been acknowledged, their influence on the interpretation of results has not always been appreciated. We have shown here that the assay buffer, assay pH, acyl-CoA substrate and the presence or absence of BSA in the reaction medium can significantly alter the interpretation of experimental results, especially when fixed substrate concentrations are used (Tables 1 and 2). Changes in assay conditions are particularly important in the kinetics of the inhibition of acyltransferases by malonyl-CoA, since IC_{50} values are critically dependent on the substrate concentration used.

The data presented demonstrate not only that purified COT is inhibited competitively by malonyl-CoA, but also that, when the assay conditions are changed, the observed properties of the sensitivity of COT to malonyl-CoA mimic those observed for CPT-I. A direct plot of the inhibition of COT by malonyl-CoA in the presence of BSA (Fig. 1) is sigmoidal, and malonyl-CoA increases this sigmoidicity, an effect that has been observed for CPT-I in intact mitochondria [17]. Comparison of Figs. 1 and 2 shows that this sigmoidicity is an artefact of the presence of BSA, which binds palmitoyl-CoA, in agreement with recent studies showing that plotting free rather than total substrate concentrations linearizes otherwise sigmoidal kinetic plots [10,11]. Any assay component which binds palmitoyl-CoA, such as membranes or other proteins, could give such an effect, and these parameters must be rigorously controlled if meaningful kinetic data are to be obtained.

A decrease in pH has been shown to increase the inhibition of CPT-I by malonyl-CoA and may provide a mechanism for attenuating fatty acid oxidation during periods of acidosis [12].

A pH-dependent increase in sensitivity is also observed for purified COT (Table 2) and may be due to the presence of a histidine residue in the active site. An active-site histidine which has been identified in carnitine acetyltransferase [18], and has been proposed for CPT-I [13,15], would titrate over the pH range 6.8–7.4. The effect of BSA and pH on the inhibition kinetics of COT suggests that all of these properties of malonyl-CoA inhibition of COT can be explained by features of the 62500-Da peptide of the purified preparation.

Malonyl-CoA inhibition of purified COT is competitive with respect to palmitoyl-CoA, and the degree of inhibition with malonyl-CoA is similar to that seen with other short-chain acyl-CoA esters (Table 3). Thus the inhibition is likely to be due to the common acyl-CoA moiety of those compounds, interacting with COT at the substrate-binding site. The K_i value for the inhibition of purified COT by malonyl-CoA is rather high in comparison with cellular concentrations of 1–6 μM [16]. Thus this interaction is not likely to be physiologically significant.

Although COT has been called a 'soluble' enzyme [5], incomplete liberation of the enzyme activity is obtained by sonication of peroxisomes [3]. We therefore thought that the enzyme might be membrane-bound *in vivo*, which might affect its kinetic behaviour, so we compared the properties of the enzyme in isolated peroxisomes with those of the purified enzyme. Both forms of the enzyme show similar K_m values for fatty acyl-CoA substrates and show little inhibition by several short-chain acyl-CoA esters (Table 3). However, the K_i value for inhibition by malonyl-CoA in peroxisomes was dramatically lower, at 6.4 μM (almost 20-fold lower than for the isolated enzyme) (Table 3).

The specificity of the inhibition by malonyl-CoA displays a great discrimination by the membrane-bound enzyme between malonyl-CoA and other short-chain acyl-CoA esters. Mitochondrial CPT-I is also specifically inhibited by malonyl-CoA, with other short-chain acyl-CoA esters inhibiting to a much lesser extent [19]. There is some evidence that CPT-I malonyl-CoA sensitivity is conferred by a regulatory subunit bearing a specific malonyl-CoA-binding site [20–22]. A similar model could account for the malonyl-CoA inhibition of peroxisomal COT. A regulatory subunit with a malonyl-CoA-binding site may be lost on solubilization of the enzyme, with a consequent decrease in sensitivity to inhibition. Alternatively, the catalytic polypeptide could contain a separate binding site for malonyl-CoA, but on solubilization of the enzyme a conformation change occurs that results in a lowered affinity for malonyl-CoA at this regulatory site. A separate binding site for malonyl-CoA (either on the catalytic polypeptide or on a regulatory subunit) could explain why K_m values for both substrates do not change from peroxisomes to the purified enzyme (Table 3); the only kinetic parameter

to change in the K_i for malonyl-CoA. Such a site could also explain the efficacy of malonyl-CoA over other short-chain acyl-CoA esters in inhibiting COT in peroxisomes. It should be noted that the pattern of competitive inhibition observed with malonyl-CoA does not preclude the existence of a separate binding site for malonyl-CoA, even in the purified enzyme. Competitive inhibition merely indicates that the binding of malonyl-CoA interferes with substrate binding.

Lastly, since the features of the inhibition of COT by malonyl-CoA resemble those of CPT-I, peroxisomal COT could be regulated in tandem with CPT-I for effective control of cellular fatty acid metabolism. Thus the role of COT in peroxisomes and regulation of its activity must be significant in the overall pattern of lipid metabolism in the cell.

REFERENCES

1. Bieber, L. L. (1988) *Annu. Rev. Biochem.* **57**, 261–283
2. McGarry, J. D. & Foster, D. W. (1980) *Annu. Rev. Biochem.* **49**, 395–420
3. Derrick, J. P. & Ramsay, R. R. (1989) *Biochem. J.* **262**, 801–806
4. Lilly, K., Bugaisky, G. E., Umeda, P. K. & Bieber, L. L. (1990) *Arch. Biochem. Biophys.* **280**, 167–174
5. Miyazawa, S., Ozasa, H., Furuta, S., Osumi, T. & Hashimoto, T. (1983) *J. Biochem. (Tokyo)* **94**, 529–542
6. Farrell, S. O. & Bieber, L. L. (1983) *Arch. Biochem. Biophys.* **222**, 123–132
7. Ramsay, R. R., Derrick, J. P., Friend, A. S. & Tubbs, P. K. (1987) *Biochem. J.* **244**, 271–278
8. Smith, R. H. & Powell, G. L. (1986) *Arch. Biochem. Biophys.* **244**, 357–360
9. Wolkowicz, P. E. & McMillin-Wood, J. (1980) *Biochem. J.* **186**, 257–266
10. Pauly, D. F. & McMillin, J. B. (1988) *J. Biol. Chem.* **263**, 18160–18167
11. Richards, E. W., Hamm, M. W., Fletcher, J. E. & Otto, D. A. (1991) *Biochim. Biophys. Acta* **1076**, 23–28
12. Stephens, T. W., Cook, G. A. & Harris, R. A. (1983) *Biochem. J.* **212**, 521–524
13. Mills, S. E., Foster, D. W. & McGarry, J. D. (1984) *Biochem. J.* **219**, 601–608
14. Cook, G. A. & Gamble, S. M. (1987) *J. Biol. Chem.* **262**, 2050–2055
15. Declercq, P. E., Falck, J. R., Kuwajima, M., Tyminski, H., Foster, D. W. & McGarry, J. D. (1987) *J. Biol. Chem.* **262**, 9812–9821
16. Ontko, J. A. & Johns, M. L. (1980) *Biochem. J.* **192**, 959–962
17. Saggerson, E. D. & Carpenter, C. A. (1981) *FEBS Lett.* **132**, 166–168
18. Chase, J. F. A. & Tubbs, P. K. (1970) *Biochem. J.* **116**, 713–720
19. Mills, S. E., Foster, D. W. & McGarry, J. D. (1983) *Biochem. J.* **214**, 83–91
20. Lund, H. & Woldegiorgis, G. (1986) *Biochim. Biophys. Acta* **878**, 243–249
21. Kerner, J. & Bieber, L. (1990) *Biochemistry* **29**, 4326–4334
22. Murthy, M. S. R. & Pande, S. V. (1990) *Biochem. J.* **268**, 599–604