Acyl-CoA chain length affects the specificity of various carnitine palmitoyltransferases with respect to carnitine analogues

Possible application in the discrimination of different carnitine palmitoyltransferase activities

Madiraju S. R. MURTHY,* Rona R. RAMSAY† and Shri V. PANDE*‡

*Laboratory of Intermediary Metabolism, Clinical Research Institute of Montreal (affiliated to the University of Montreal), Montreal, Quebec, Canada H2W 1R7, and †Molecular Biology Division, Veterans Administration Medical Center, San Francisco, CA 94121 and Department of Biochemistry/Biophysics, University of California, San Francisco, CA 94143, U.S.A.

The activities of carnitine palmitoyltransferases (CPTs) of mitochondrial outer and inner membranes and of peroxisomes have been studied with carnitine analogues, namely DL-thiolcarnitine, DL-sulphocarnitine and L-aminocarnitine, using palmitoyl-CoA or octanoyl-CoA as co-substrate. With sulphocarnitine, both of the mitochondrial CPTs and the malonyl-CoA-sensitive CPT of peroxisomes showed appreciable activity with palmitoyl-CoA, but relatively lower activity when octanoyl-CoA was the co-substrate. The soluble CPT of peroxisomes did not show any activity with sulphocarnitine in the presence of either acyl-CoA. With thiolcarnitine, all of the CPTs showed more activity with palmitoyl-CoA than with octanoyl-CoA. None of the CPTs showed any activity with aminocarnitine and palmitoyl-CoA, but when the acyl donor was octanoyl-CoA, both of the malonyl-CoA-sensitive CPT enzymes showed considerable activity, unlike the malonyl-CoA-insensitive CPT isoenzymes. Aminocarnitine inhibited palmitoylcarnitine formation by both of the mitochondrial CPTs and by the CPT of gradient-purified peroxisomes, but the purified peroxisomal soluble CPT was not inhibited. These results show that the interaction of CPT enzymes with carnitine analogues, as substrates or inhibitors, is influenced by the chain length of the acyl-CoA substrate, and that the use of the appropriate carnitine analogue and acyl-CoA is likely to be useful for the discrimination of the various CPT activities in CPT deficiency disorders.

INTRODUCTION

The existence of three major carnitine palmitoyltransferase (CPT) activities in liver and other tissues is well recognized. Two of these, CPT_o and CPT_i, reside in the outer and inner membranes respectively of mitochondria, whereas the third one, CPT_s, also called carnitine octanoyltransferase, is of peroxisomal origin (Bremer, 1983; Murthy & Pande, 1987a,b; Bieber, 1988; McGarry *et al.*, 1989). These CPT enzymes catalyse the reversible transfer of long- and medium-chain acyl groups between carnitine and CoASH and play a key role in fatty acid oxidation (Bremer, 1983; Bieber, 1988; McGarry *et al.*, 1989). The substrate specificities of the CPT enzymes with respect to acyl-CoA chain length and certain analogues of carnitine (Bremer, 1983; Bieber, 1988; Gandour *et al.*, 1988) have been studied previously. It is not known, however, which alterations in the carnitine molecule affect its ability to serve as a substrate or as an inhibitor for the CPT activities, whether these alterations are the same for the different CPT enzymes and whether they are influenced by variations in the acyl-CoA co-substrates present.

Using both palmitoyl- and octanoyl-CoA as the acyl substrates, we have in the present paper examined aminocarnitine, sulphocarnitine and thiolcarnitine as substrates/inhibitors of the three CPT activities. We knew that aminocarnitine is an effective inhibitor of mitochondrial CPT (Jenkins & Griffith, 1985, 1986; Kanamaru *et al.*, 1985; Shinagawa *et al.*, 1987), and that sulphocarnitine is a substrate for the mitochondrial but not the peroxisomal CPT (Griffith *et al.*, 1987). We are not aware of any report on thiolcarnitine and CPT enzymes, but in a preliminary note, Tubbs & Chase (1970) observed that thiolcarnitine could substitute for carnitine in supporting the mitochondrial oxidation

of palmitoyl-CoA. For carnitine acetyltransferase, thiolcarnitine has been described as a poor substrate analogue of carnitine (Duhr *et al.*, 1983). Our results described here show that the acceptability of the carnitine analogues as substrates or inhibitors is not only dependent on the nature of CPT but is also markedly influenced by the chain length of the acyl-CoA co-substrate. It seems that the appropriate choice of carnitine analogue with specific acyl-CoA substrate can provide a basis of discrimination between the different CPT activities mentioned above.

EXPERIMENTAL

L-Aminocarnitine/Cl/HCl (L-3-amino-4-trimethylaminobutyrate; emeriamine) from Takeda Chemical Industries, Osaka Japan, DL-sulphocarnitine (DL-2-hydroxy-3-trimethylaminopropanesulphonate) from Dr. Owen Griffith and DL-thiolcarnitine/HCl (DL-3-mercapto-4-trimethylaminobutyrate) from Sigma-Tau Co., Rome, Italy, were obtained as gifts. L-Carnitine/HCl (L-3-hydroxy-4-trimethylaminobutyrate) was supplied by the Sigma Chemical Co., St. Louis, MO, U.S.A., and [1-¹⁴C]octanoyl-CoA by P-L Biochemicals. [1-¹⁴C]Palmitoyl-CoA was synthesized from [1-¹⁴C]palmitic acid (Amersham) according to Hajra & Bishop (1986).

Rat liver mitochondrial outer membranes were prepared by the procedure of Parsons *et al.* (1966) with the modifications described in Murthy & Pande (1987a). These outer membranes contained CPT_o activity that was 90% inhibited by malonyl-CoA and were judged to be 95% pure based on the criteria described in Murthy & Pande (1987a). Peroxisomes from normal rat livers were prepared by the Nycodenz-density-gradient centri-

Abbreviations used: CPT, carnitine palmitoyltransferase; CPT_o, malonyl-CoA-sensitive CPT of outer membranes; CPT_i, malonyl-CoA-insensitive CPT of inner membranes; CPT_s, soluble CPT of peroxisomes; OMV, outer membrane vesicles; IMV, inner membrane vesicles.

‡ To whom correspondence should be addressed, at: Laboratory of Intermediary Metabolism, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec, Canada H2W 1R7.

fugation method (Ghosh & Hajra, 1986). Rat liver mitochondrial CPT₁ was purified by the procedure of Miyazawa *et al.* (1983) with minor modifications. Rat liver peroxisomal CPT_s was purified as described in Ramsay *et al.* (1987); in the assay system used, the enzyme preparation gave lower specific activities than those described previously (Ramsay *et al.*, 1987). Rat heart mitochondrial outer membranes were prepared as described by Comte & Gautheron (1979). CPT enzymes were assayed using 50 μ M-[1-¹⁴C]palmitoyl-CoA or -[1-¹⁴C]octanoyl-CoA. The details of the assay system with palmitoyl-CoA were as in Pande *et al.* (1986), except that 5 mM-L-carnitine or the indicated concentration of carnitine analogue was employed. Assays of CPT with 50 μ M-octanoyl-CoA were in a final volume of 100 μ l in a medium containing 50 mM-potassium phosphate, pH 7.4, and 5 mM concentrations of L-carnitine or of the carnitine analogue indicated. Incubations were at 30 °C. The reactions were initiated by adding enzyme and terminated by adding 0.7 ml of a charcoal suspension [charcoal, 7% (w/v); ethanol, 91% (v/v); phosphoric acid, 2% (v/v)]. After vortex-mixing and centrifugation, the supernatant was monitored for [1-¹⁴C]octanoylcarnitine radioactivity.

RESULTS AND DISCUSSION

During the characterization of CPT_o, CPT₁ and CPT_s from rat liver, we found not only that the activities of these CPT isoenzymes are affected differently by alterations in the carnitine molecule, but that the ability of the carnitine analogues to act as an acyl-group acceptor substrate is influenced by the chain length of the acyl-CoA co-substrate present. Thus we observed that thiocarnitine could serve as an acyl acceptor with all three CPT isoenzymes with either palmitoyl-CoA or octanoyl-CoA as acyl donor substrate (Table 1). However, under these conditions, 10 mM-DL-thiocarnitine was less effective than 5 mM-L-carnitine, and this difference was more marked with octanoyl-CoA as the acyl donor than with palmitoyl-CoA. Interestingly, under the conditions of these experiments, the activity of CPT₁ was the least affected of all three CPT isoenzymes by the substitution of carnitine with thiocarnitine when palmitoyl-CoA was the acyl-CoA substrate, but the same enzyme activity was affected most when octanoyl-CoA was the acyl-CoA substrate. Clearly, the decrease in the acyl-CoA chain length from C₁₆ to C₈ decreased the ability of CPT₁ to use thiocarnitine more than for the other CPT isoenzymes.

DL-Sulphocarnitine has recently been found to act as a substrate for the mitochondrial CPT but not for the soluble CPT of mouse peroxisomes (Griffith *et al.*, 1987). Consistent with this, we observed that DL-sulphocarnitine is not an active substrate for the rat liver peroxisomal (purified) CPT. This was found to be true with either palmitoyl-CoA or octanoyl-CoA as the acyl-donor substrates. However, gradient-purified peroxisomes themselves showed significant CPT activity with sulphocarnitine plus palmitoyl-CoA (nearly one-third of that seen with 5 mM-carnitine) but much less (about 8%) with sulphocarnitine plus octanoyl-CoA. This ability of the CPT in whole peroxisomes to show activity with sulphocarnitine, unlike the purified CPT obtained from the readily solubilizable CPT activity of peroxisomes, indicates either that, as in mitochondria, two different forms of CPT activity exist in peroxisomes, or, more likely, that the properties change dramatically after solubilization. It was previously observed that the solubilized CPT of peroxisomes is malonyl-CoA-insensitive, but the CPT in intact peroxisomes is malonyl-CoA-sensitive (Derrick & Ramsay, 1989). Alternatively, association with a malonyl-CoA-inhibition-conferring polypeptide could be responsible for these differences in properties cannot be entirely discounted. For the mitochondrial CPT, and

Table 1. Activities of CPT_o, CPT₁ of inner membrane vesicles (IMV), purified CPT₁, CPT of peroxisomes and purified peroxisomal CPT_s with carnitine or its analogues using either [1-¹⁴C]palmitoyl-CoA or [1-¹⁴C]octanoyl-CoA as the acyl donor.

Rat liver mitochondrial OMV, IMV, peroxisomes, purified CPT₁ and purified CPT_s were obtained as described in the Experimental section. Assays of CPT were with 50 μ M-[1-¹⁴C]palmitoyl-CoA or 50 μ M-[1-¹⁴C]octanoyl-CoA and the indicated concentrations of carnitine or carnitine analogues. For other details, see the Experimental section. Values represent averages of at least three separate experiments; N.D., not detectable.

Substrate	Enzyme...	CPT activity (% of activity with carnitine)									
		[1- ¹⁴ C]Palmitoyl-CoA				[1- ¹⁴ C]Octanoyl-CoA					
		OMV CPT _o	IMV CPT ₁	Purified CPT ₁	Purified CPT _s	Peroxisomal CPT	OMV CPT _o	IMV CPT ₁	Purified CPT ₁	Purified CPT _s	Peroxisomal CPT
L-Carnitine (5 mM)*	100 (0.043)	100 (0.018)	100 (15.5)	100 (3.0)	100 (0.005)	100 (0.075)	100 (0.11)	100 (74.0)	100 (17.3)	100 (0.027)	
DL-Sulphocarnitine (20 mM)		104	105	106	N.D.	37	7	9	9	N.D.	8
DL-Thiocarnitine (10 mM)		62	90	81	46	145	29	12	9	23	37
L-Aminocarnitine (20 mM)		N.D.	N.D.	N.D.	N.D.	N.D.	34	2	2	N.D.	13

* Activities with carnitine (μ mol/min per mg of protein) are shown in parentheses.

Rat liver mitochondrial OMV, IMV, peroxisomes, purified CPT₁ and purified CPT_s were obtained as described in the Experimental section. Assays of CPT were with 50 μ M-[1-¹⁴C]palmitoyl-CoA or 50 μ M-[1-¹⁴C]octanoyl-CoA and the indicated concentrations of carnitine or carnitine analogues. For other details, see the Experimental section. Values represent averages of at least three separate experiments; N.D., not detectable.

Fig. 1. Inhibition of the activities of OMV CPT_o (○), purified CPT_i (●), peroxisomal CPT (▲) and purified CPT_s (△) by L-aminocarnitine

Activities of the various CPTs were measured using 50 μ M-[1-¹⁴C]palmitoyl-CoA and 5 mM-L-carnitine. L-Aminocarnitine was present at 0–2 mM in the assay system. Other details were as described in the Experimental section. CPT activity measured in the presence of aminocarnitine is expressed as a percentage of the activity seen without the addition of aminocarnitine.

with palmitoyl-CoA as a substrate, 20 mM-DL-sulphocarnitine was about as effective an acyl acceptor as near-saturating (5 mM)-L-carnitine (Griffith *et al.*, 1987; O. W. Griffith, personal communication); we have found that this applied for both the CPT_o and the CPT_i isoenzymes. However, under the same conditions but with octanoyl-CoA as the acyl-donor substrate, both of the mitochondrial isoenzymes showed with sulphocarnitine less than 10% of the activity seen with carnitine (Table 1).

DL-Aminocarnitine and its naturally occurring L-isomer have been identified as potent inhibitors of mitochondrial CPT and of fatty acid oxidation (Kanamaru *et al.*, 1985; Shinagawa *et al.*, 1987; Jenkins & Griffith, 1985, 1986). Jenkins & Griffith (1985) have suggested that of the two forms of CPT in mitochondria, one form is more potently inhibited than the other. Using separate preparations of the two mitochondrial isoenzymes, we have confirmed that when palmitoyl-CoA and carnitine are present as substrates, L-aminocarnitine inhibits the activity of both of the mitochondrial CPT enzymes, and have identified the CPT_i as the isoenzyme activity which is more sensitive to aminocarnitine inhibition. The concentration of aminocarnitine needed for 50% inhibition of the CPT_i in the inner-membrane-bound or the purified form, was about 25 μ M, compared with the value of nearly 250 μ M for the outer-membrane vesicle (OMV) CPT_o (Fig. 1). Under the same conditions, however, up to 2 mM-aminocarnitine had no effect on the activity of the purified CPT_s (Fig. 1). However, CPT activity of isolated peroxisomes was nearly as susceptible to aminocarnitine inhibition as was the OMV CPT_o.

We noted also that L-aminocarnitine (20 mM) was unable to act as acyl-group acceptor with all three CPT enzymes of rat liver when palmitoyl-CoA was the co-substrate. This is in agreement with the observations of Jenkins & Griffith (1985), where significant acylation of aminocarnitine by palmitoylcarnitine was not found; however, under the experimental conditions of Kanamaru & Okazaki (1989) with liver mitochondria, such a reaction appeared to proceed. Unlike liver OMV CPT_o, the OMV CPT_o of heart seemed able to catalyse the transfer of a palmitoyl group from palmitoyl-CoA to L-aminocarnitine (20 mM) but only at rates about 2% of those seen with 5 mM-carnitine.

When octanoyl-CoA was the acyl donor, however, the OMV CPT_o proved capable of using aminocarnitine as acyl-group

acceptor with rates nearly one-third of those seen with carnitine. This was found to be true with the OMV CPT_o of both liver and heart mitochondria. Under these conditions the CPT_i, in the membrane-bound as well as in the purified form, showed only about 2% of the activity seen with carnitine, whereas purified CPT_s showed no detectable activity (Table 1). The CPT_o activity seen with L-aminocarnitine and octanoyl-CoA was found to be sensitive to malonyl-CoA inhibition as, expectedly, was the activity seen with carnitine and octanoyl-CoA (more than 90% inhibition by 100 μ M-malonyl-CoA in both cases). The CPT of gradient-purified peroxisomes showed appreciable activity with aminocarnitine and octanoyl-CoA (about 13% of that with carnitine and octanoyl-CoA), and this activity with aminocarnitine was also found to be sensitive to malonyl-CoA inhibition. Thus only the malonyl-CoA-sensitive CPTs of OMV and of peroxisomes are able to use L-aminocarnitine as a substrate, and only with octanoyl-CoA as the acyl donor co-substrate. Examinations with CPT_o of OMV showed that in terms of both the K_m and the V_{max} values, aminocarnitine was a poor substitute for carnitine (Table 2); the K_m/V_{max} value with aminocarnitine (0.136) was about 20-fold higher than the corresponding value (0.007) seen with carnitine. Because of this, despite being able to function as an acyl-group acceptor substrate, the presence of aminocarnitine proved inhibitory for CPT_o and for the other CPT isoenzymes, when 5 mM-carnitine and octanoyl-CoA were present as substrates (40 $\pm$ 10% inhibition of all CPT activities by 2 mM-L-aminocarnitine).

From previous studies with a mitochondrial CPT, it is known that the substrate function of carnitine is influenced by the concentration of the co-substrate, palmitoyl-CoA (Bremer, 1983). The fact that the ability of a carnitine analogue to act as a substrate or an inhibitor for the various CPT isoenzymes is influenced also by the chain length of the acyl-CoA co-substrate, as our present data show, has not been recognized before. Among the CPT preparations and within the limits examined, the purified peroxisomal CPT_s seems to have more stringent requirements for both the hydroxyl and the carboxyl groups on the carnitine molecule, as the activity of this enzyme is affected most by changes in these groups. Inasmuch as all three CPT isoenzymes were able to use thiocarnitine as a substrate with both palmitoyl-CoA and octanoyl-CoA as the acyl donors, the most tolerable substitution in carnitine for the resulting analogue to act as an acyl-group acceptor, for CPT enzymes as a group, is the replacement of hydroxyl by thiol. For CPT_o, replacement of palmitoyl-CoA by octanoyl-CoA led to a decrease in activity when sulphocarnitine or thiocarnitine was the carnitine analogue present. In marked contrast, with aminocarnitine as the carnitine analogue, replacement of palmitoyl-CoA by octanoyl-CoA resulted in appreciable activity, compared with little detectable activity otherwise. This selective ability of the malonyl-CoA-

Table 2. Comparison of kinetic constants of OMV CPT_o obtained using aminocarnitine and carnitine, with octanoyl-CoA as the acyl donor

Liver mitochondrial OMV were used as the source of CPT_o. CPT_o activity was assayed using 50 μ M-[1-¹⁴C]octanoyl-CoA and various (25 μ M–20 mM) concentrations of L-aminocarnitine or L-carnitine. Other details were as described in the Experimental section. Values are means $\pm$ S.E.M. of four observations.

Kinetic constant	Aminocarnitine	Carnitine
K_m (mM)	4.0 $\pm$ 0.5	0.9 $\pm$ 0.1
V_{max} (nmol/min per mg of protein)	29.4 $\pm$ 1.4	133.7 $\pm$ 7.5

sensitive CPT activities of OMV and peroxisomes to show activity with aminocarnitine and octanoyl-CoA as substrates is of interest.

Recently, Derrick & Ramsay (1989) showed the presence of a membrane-bound malonyl-CoA-sensitive CPT in normal rat liver peroxisomes. However, the relatively readily solubilizable activity is insensitive to malonyl-CoA inhibition. The present findings indicate that, in both mitochondria and peroxisomes, the malonyl-CoA-sensitive CPT was able to selectively use aminocarnitine with octanoyl-CoA as the co-substrate, whereas under these conditions the malonyl-CoA-insensitive CPT enzymes in these two organelles did not show any significant activity. Even though there are some similarities between the malonyl-CoA-sensitive CPT enzymes of mitochondria and peroxisomes with respect to their inhibition with carnitine analogues, the malonyl-CoA-insensitive CPT of mitochondria (purified CPT_m) behaved much differently from the comparable peroxisomal enzyme (purified CPT_p). Thus the present findings suggest that in gradient-purified peroxisomes which have a malonyl-CoA-sensitive CPT and a malonyl-CoA-insensitive CPT, the former can be selectively assayed with either sulphocarnitine plus palmitoyl-CoA or aminocarnitine plus octanoyl-CoA as the substrates. In mitochondria, the malonyl-CoA-sensitive CPT can be assayed selectively by employing aminocarnitine plus octanoyl-CoA. Based on the above, it should be possible to set up specific procedures for the detection of deficiencies of CPT enzymes in mitochondria or in peroxisomes. Thus, like malonyl-CoA sensitivity, use of aminocarnitine with octanoyl-CoA provides another means of selectively assaying the membrane-bound CPT activities of both mitochondria and peroxisomes.

The differences in sensitivity to inhibition by malonyl-CoA and oxirane-substituted acyl-CoA derivatives have been used to distinguish CPT_p from the other CPT activities (Bremer 1983; Bieber, 1988; McGarry *et al.*, 1989). The present findings indicate that the variable ability of different CPT enzymes to use carnitine analogues as substrates and/or inhibitors provides another means of differentiation between the CPT isoenzymes, and this could be useful for the discrimination of the various CPT deficiency disorders. That each of the CPT isoenzymes has (a) unique active centre(s) is indicated by the present findings.

We thank Mr. Tak-Sing Lee for expert technical assistance, Dr. Owen Griffith of Cornell University, New York, U.S.A. for the gift of sulphocarnitine, the Sigma-Tau Co., Rome, Italy for thiocarnitine and Takeda Chemical Industries Ltd., Osaka, Japan for emeramine. Grants from the Medical Research Council of Canada (MT-4264), the Quebec Heart Foundation and the National Institutes of Health (HL-16251 and DK-41572) provided support for this work.

REFERENCES

- Bieber, L. L. (1988) *Annu. Rev. Biochem.* **57**, 261–283
- Bremer, J. (1983) *Physiol. Rev.* **63**, 1420–1480
- Comte, J. & Gautheron, D. C. (1979) *Methods Enzymol.* **55**, 98–104
- Derrick, J. P. & Ramsay, R. R. (1989) *Biochem. J.* **262**, 801–806
- Duhr, E. F., Mauro, J. M., Clennam, E. L. & Barden, R. E. (1983) *Lipids* **18**, 382–386
- Gandour, R. D., Colucci, W. J., Stelly, T. C., Brady, P. S. & Brady, L. J. (1988) *Arch. Biochem. Biophys.* **267**, 515–520
- Ghosh, M. K. & Hajra, A. K. (1986) *Anal. Biochem.* **159**, 169–174
- Griffith, O. W., Jenkins, D. L., Lilly, K. & Bieber, L. L. (1987) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **46**, 1180 (abstr. 5029)
- Hajra, A. K. & Bishop, J. E. (1986) *Methods Enzymol.* **122**, 50–53
- Jenkins, D. L. & Griffith, O. W. (1985) *J. Biol. Chem.* **260**, 14748–14755
- Jenkins, D. L. & Griffith, O. W. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 290–294
- Kanamaru, T. & Okazaki, H. (1989) in *Novel Microbiological Products for Medicine and Agriculture* (Demain, A. L., Somkuti, G. A., Hunter-Cevera, J. C. & Rossmore, H. W., eds.), pp. 135–144, Elsevier, Amsterdam
- Kanamaru, T., Shinagawa, S., Asai, M., Okajaki, H., Sugiyama, Y., Fujita, T., Iwatsuka, H. & Yoneda, M. (1985) *Life Sci.* **37**, 217–223
- McGarry, J. D., Woeltje, K. F., Kuwajima, M. & Foster, D. W. (1989) *Diabetes/Metabolism Rev.* **5**, 271–284
- Miyazawa, S., Ozasa, H., Osumi, T. & Hashimoto, T. (1983) *J. Biochem. (Tokyo)* **94**, 529–542
- Murthy, M. S. R. & Pande, S. V. (1987a) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 378–382
- Murthy, M. S. R. & Pande, S. V. (1987b) *Biochem. J.* **248**, 727–733
- Pande, S. V., Murthy, M. S. R. & Noël, H. (1986) *Biochim. Biophys. Acta* **877**, 223–230
- Parsons, D. F., Williams, G. R. & Chance, B. (1966) *Ann. N.Y. Acad. Sci.* **137**, 643–666
- Ramsay, R. R., Derrick, J. P., Friend, A. S. & Tubbs, P. K. (1987) *Biochem. J.* **244**, 271–278
- Shinagawa, S., Kanamaru, T., Harada, S., Asai, M. & Okazaki, H. (1987) *J. Med. Chem.* **30**, 1458–1463
- Tubbs, P. K. & Chase, J. F. A. (1970) *Biochem. J.* **116**, 34P

Received 21 September 1989/18 January 1990; accepted 31 January 1990