Carnitine palmitoyltransferase in human erythrocyte membrane

Properties and malonyl-CoA sensitivity

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Carnitine palmitoyltransferase located in the erythrocyte plasma membrane is sensitive to inhibition by malonyl-CoA and 2-bromopalmitoyl-CoA plus carnitine. Although this inhibition and other properties suggest similarities to the intracellular enzymes in other tissues, no cross-reaction was observed with antisera to the peroxisomal or to the mitochondrial inner-membrane enzyme. The activity was solubilized by and was stable in Triton X-100, which destroys the enzymes found in microsomes and in the mitochondrial outer membrane. The substrate specificity is broader than for the intracellular enzymes, the activities with stearoyl-CoA (114 %) and arachidonoyl-CoA (97 %) being equal to that with palmitoyl-CoA, and the activities with linoleoyl-CoA (44 %) and erucoyl-CoA (46 %) about half that with palmitoyl-CoA. The function of this carnitine palmitoyltransferase is probably to buffer the acyl-CoA present in the erythrocyte for turnover of the fatty acyl groups of the membrane lipids.

INTRODUCTION

When the presence of carnitine palmitoyltransferase (CPT) in human erythrocyte membranes was first reported (Wittels & Hochstein, 1967), it was suggested that its role might be similar to that in mitochondria, i.e. to mediate the translocation of long-chain fatty acyl groups across the membrane. However, non-esterified fatty acids cross membranes with relative ease. The problem in mitochondria is that, although β -oxidation takes place in the matrix, long-chain fatty acids are activated on the outer membrane to acyl-CoA, which does not cross the inner membrane at a rate sufficient to support β -oxidation (Fritz, 1963). Also militating against a translocation role is the observation that neither carnitine nor acetylcarnitine exchanges freely across the erythrocyte membrane (Cooper *et al.*, 1988), in contrast with the rapid exchange observed in mitochondria (Pande, 1975; Ramsay & Tubbs, 1975).

From our observations that carnitine inhibits the acylation of membrane phospholipids, and that acylcarnitine is a substrate for phospholipid acylation (Arduini *et al.*, 1990), together with the data herein reported, we propose that the CPT in erythrocyte membranes serves the same function as does carnitine acetyltransferase in mitochondria (Bieber, 1988), namely, it buffers the free CoA concentration by catalysing the transfer of activated acyl groups to carnitine.

CPT is found in four intracellular locations. The mitochondrial inner-membrane enzyme (CPT-II) is not regulated by malonyl-CoA (Murthy & Pande, 1987) nor irreversibly inhibited by 2-bromopalmitoyl-CoA (Murthy & Pande, 1990), and antiserum to it does not cross-react with the other forms of the enzyme. The mitochondrial outer-membrane enzyme, the peroxisomal enzyme (also called carnitine octanoyltransferase) and the microsomal enzyme are all malonyl-CoA sensitive and irreversibly inhibited by 2-bromopalmitoyl-CoA (Ramsay, 1990; West *et al.*, 1971). The mitochondrial outer-membrane enzyme appears to be unique (Declercq *et al.*, 1987; Zammit *et al.*, 1989; Murthy & Pande, 1990), but the peroxisomal and microsomal enzymes do cross-react immunologically.

The sensitivities of the various CPT enzymes to solubilization and inhibition by detergents are quite different, although the distinctions are not clear-cut, with considerable discrepancies between laboratories. It is generally accepted that the mitochondrial inner-membrane enzyme is solubilized by most detergents. The outer-membrane CPT is solubilized by octyl β -D-glucopyranoside with high salt concentrations (Murphy & Pande, 1987), but not by Tween-20 (Woeltje *et al.*, 1987). The peroxisomal enzyme is easily solubilized (Derrick & Ramsay, 1989) and can be completely extracted with either Triton X-100 or octyl β -D-glucopyranoside plus KCl. The microsomal enzyme is solubilized in stable form by CHAPS and by Tween-20 (Lilly *et al.*, 1990). Triton X-100 destroys the CPT activity in microsomes (Lilly *et al.*, 1990) and in the mitochondrial outer membrane (Woeltje *et al.*, 1987), but slightly enhances the activities of the peroxisomal CPT and of the mitochondrial inner CPT (R. R. Ramsay, unpublished work).

We have examined the kinetics, inhibitor sensitivity, detergent solubilization and immunoreactivity of the erythrocyte plasma-membrane enzyme for comparison with the intracellular forms.

MATERIALS AND METHODS

Erythrocytes and resealed erythrocyte-membrane preparation

Human blood was obtained from healthy human volunteers by venipuncture into heparinized tubes, and processed within 30 min of collection. Erythrocytes were isolated by centrifugation at 400 g for 5 min, followed by four resuspensions and washings in PBS buffer (150 mM-NaCl/5 mM-sodium phosphate, pH 7.4). The buffy coat was carefully removed. Neither leucocytes nor platelets were detected by counting washed blood cells by standard manual techniques. Membrane ghosts were prepared from washed cells which were lysed in 30 vol. of cold lysing buffer (5 mM-NaH₂PO₄, pH 7.4, 0.1 mM-phenylmethanesulphonyl fluoride). The lysed cells were then centrifuged for 10 min at 30000 g at 4 °C, and the resulting pelleted ghosts were washed four times. In the last two washing cycles, phenylmethane-

Abbreviation used: CPT, carnitine palmitoyltransferase.

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sulphonyl fluoride was omitted from the lysing buffer. Spectrin/actin-depleted ghosts were prepared as described by Tyler *et al.* (1979). Ghosts were incubated in 30 vol. of low-ionic-strength buffer (0.3 mM- NaH_2PO_4 /0.2 mM-EDTA, pH 7.4) for 30 min at 37 °C. Spectrin and actin were removed by centrifuging at 30000 *g* for 15 min. The ghosts were collected after two washes in 5 mM- NaH_2PO_4 , pH 7.4. Spectrin/actin-depleted ghosts were further depleted of ankyrin and band 4.1 by incubating them in the above buffer containing 1 M-KCl for 30 min at 37 °C (Tyler *et al.*, 1979). Stripped ghosts were collected after two washes in phosphate buffer.

CPT assay

The activity was assayed by the radiochemical procedure (Derrick & Ramsay, 1989) in 220 mM-sucrose/40 mM-KCl/10 mM-Tris/HCl (pH 7.4 at 30 °C)/1 mM-EGTA, except that the final concentration of palmitoyl-CoA (or decanoyl-CoA) was 50 μM , with 1.3 mg of BSA/ml, L-carnitine was 1 mM, and the time of incubation was 30 min.

Others

Antisera were the same as used by Ramsay *et al.* (1987). Protein was assayed by the micro method of Bradford (1976). SDS/PAGE gels (7.5% acrylamide) were blotted and treated with antisera as in previous work (Ramsay, 1988).

RESULTS

Functional aspects

Amounts of CPT. Because the CPT assay is affected by the amounts of protein and lipid present, the linearity of the assay was checked for each preparation. Fig. 1 shows the linearity of the appearance of [^3H]palmitoyl-L-carnitine with protein and time (Fig. 1*b*) for erythrocyte ghosts. The amounts of CPT found in three preparations of ghosts were 0.11, 0.12 and 0.35 nmol/min per mg, similar to that found previously (0.147 nmol/min per mg; Wittels & Hochstein, 1967). Extraction of spectrin/actin at low ionic strength does not affect the CPT activity of the membranes, but the removal of other cytoskeleton components with 1 M-KCl (Tyler *et al.*, 1979) results to a loss of 30% CPT activity with respect to normal ghosts.

Inhibitors. Table 1 shows that the membrane-bound activity is completely inhibited by 30 μM -malonyl-CoA (92%), as also observed for mitochondrial and peroxisomal CPT. 2-Bromopalmitoyl-CoA, the irreversible inhibitor of the mitochondrial overt CPT and peroxisomal CPT (West *et al.*, 1971; Murthy & Pande, 1987; Ramsay, 1990), completely inhibited both the erythrocyte membrane activity and the extracted CPT. Dithioerythritol, reported to inhibit the mitochondrial overt CPT (Bird & Saggerson, 1984), was also a strong inhibitor (Table 1).

Malonyl-CoA regulates intracellular CPT activity and is important in the modulation of fatty acid metabolism (McGarry & Foster, 1980). The absence of β -oxidation and fatty acid synthesis in erythrocytes suggests that regulation of the enzyme would be superfluous. However, the erythrocyte enzyme is sensitive to malonyl-CoA (Table 1 and Fig. 2), with an IC_{50} (concn. giving 50% inhibition) of 2.5 μM , similar to that found for the peroxisomal and overt mitochondrial enzymes from fed-rat liver (Derrick & Ramsay, 1989).

Substrate-specificity. The substrate specificity of the human erythrocyte CPT is compared with that of the rat malonyl-CoA-sensitive enzymes in Fig. 3. The ratio of decanoyltransferase to

Fig. 1. Linearity of the CPT assay on erythrocyte ghosts

Erythrocyte ghosts were assayed as described in the Materials and methods section in a final volume of 260 μl of 0.15 M-sucrose/60 mM-KCl/10 mM-Tris/HCl/1 mM-EDTA, containing 50 μM -palmitoyl-CoA, 1 mg of BSA/ml and 1 mM- $[\text{^3H}]$ carnitine. In (a), the incubation time was 30 min; in (b), the amount of protein present was 50 μg .

Table 1. Inhibition of CPT in ghosts

The erythrocyte ghosts and cytoskeleton-depleted membranes were prepared as described in the Materials and methods section. The proteins extracted by the KCl treatment were concentrated and dialysed before assay. Assays contained 1 mg of BSA/ml, 50 μM -palmitoyl-CoA and 0.9 mM-L- $[\text{^3H}]$ carnitine. Malonyl-CoA (100 μM final concn.) was added to the enzyme suspension (150 μl , 110 mg) 1 min before addition of the substrate mixture, as was dithioerythritol (1 mM). 2-Bromopalmitoyl-CoA (15 μM) and L-carnitine (1 mM) were preincubated with the ghosts (110 mg) in a total volume of 25 μl for 30 min at 30 °C, then diluted to 150 μl with phosphate buffer (K_2HPO_4 , pH 7.4) immediately before the assay. Abbreviation: n.d., not determined.

Sample	Inhibition (%)		
	Malonyl-CoA	2-Bromopalmitoyl-CoA	Dithioerythritol
Ghosts	96	94	100
KCl-treated membrane	92	n.d.	n.d.
KCl extract	78	93	n.d.

Fig. 2. Malonyl-CoA inhibition of CPT in erythrocyte ghosts

Malonyl-CoA was added to 150 μl of buffer as in Fig. 1 containing 50 μg of protein at 1 min before addition of 50 μl of substrate mixture containing palmitoyl-CoA, BSA and carnitine. The incubation at 30 °C was terminated at 30 min by addition of 100 μl of 2 M-HCl. Details are given in the Materials and methods section.

Fig. 3. Substrate specificity of CPT in erythrocyte ghosts

The assay method is as described in the Materials and methods section. The value obtained for each acyl-CoA substrate was divided by that for decanoyl-CoA in order to compare the relative activities in each sample. The specific activities with 50 μ M-palmitoyl-CoA were 0.35 nmol/min per mg in ghosts, 3.58 nmol/min per mg in mitochondria, 3.19 nmol/min per mg in peroxisomes, and 1.01 nmol/min per mg in microsomes. Symbols: ▨, erythrocytes; ▩, peroxisomes; ■, microsomes; ■, mitochondria.

Table 2. Effect of detergents on the carnitine decanoyltransferase (CDT) activity in erythrocyte ghosts

Ghosts (1.44 mg in a final volume of 1 ml) were incubated for 45 min on ice in (final volume 1 ml) 20 mM-potassium phosphate, pH 7.4, containing 0.1 mg of phenylmethanesulphonyl fluoride/ml, alone or with 1.5 mg of Triton X-100 (1 mg/mg of protein), or 0.01 ml of Tween 20 [1% (v/v) or 0.007 ml/mg of protein; low], or 0.14 ml of Tween 20 (0.1 ml/mg of protein; high), or 5 mM-cholate (3.4 μ mol/mg of protein), or 40 mM-octyl glucopyranoside plus 100 mM-KCl. The samples were centrifuged at 100 000 *g* for 30 min in a Beckman type 40 rotor. The supernatants were carefully removed and the pellets resuspended in the same detergent mixture as used for the incubation.

Detergent	CDT activity (pmol/min)		Recovery	Percentage solubilized
	Supernatant	Pellet		
None	10.0	222	100	4
Triton X-100	139	54	83	60
Tween 20 (high)	29	42	30	12.5
(low)	32	234	115	13.8
Cholate	5.7	250	110	2.5
Octyl glucoside/KCl	31	< 13 > *	19	13.4

* Pellet could not be solubilized.

palmitoyltransferase activity (C_{10}/C_{16}) was 2.3 for the erythrocyte CPT, very similar to that of the organelle enzymes ($C_{10}/C_{16} = 2$). The erythrocyte enzyme has higher activity with stearoyl-CoA ($C_{18:0}$) and arachidonoyl-CoA ($C_{20:4}$) than does the organelle enzyme. Very low activity was observed with linoleoyl-CoA ($C_{18:2}$) and with erucyl-CoA ($C_{22:1}$) for the organelle enzymes (4–6% and 9–13% of the activity with palmitoyl-CoA, res-

pectively), whereas for the erythrocyte enzyme the activities were almost half that for palmitoyl-CoA (44% and 46% respectively). The higher activity with these long-chain substrates is consistent with an acyl-buffering role for CPT in the erythrocyte membrane.

The apparent K_m for L-carnitine was 0.18 mM at 50 μ M-palmitoyl-CoA, very similar to that for the intracellular malonyl-CoA-sensitive enzymes (Derrick & Ramsay, 1989).

Molecular aspects

Immunoreactivity of erythrocyte CPT. Antisera to bovine liver peroxisomal CPT and to CPT-II can be used to identify the equivalent human enzymes (Singh *et al.*, 1988). The peroxisomal and microsomal enzymes cross-react with each other, but not with antiserum to CPT-II. Neither antibody will cross-react with the mitochondrial outer-membrane enzyme (Murthy & Pande, 1990). When Western blots from erythrocyte membranes were incubated with antiserum either to peroxisomal CPT or to CPT-II, no reaction of any erythrocyte protein with either antibody could be detected (results not shown).

Detergent effects. Table 2 summarizes the effect of detergents on carnitine decanoyltransferase in erythrocyte ghosts. Only Triton X-100 solubilizes a large part of the activity. The activity of the extract stored on ice remained constant for over 1 week, indicating that the enzyme is stable in the presence of Triton X-100. The results obtained in Table 2 do not follow the pattern expected for any of the organelle enzymes. The stability in Triton X-100 suggests that it is neither the microsomal nor the mitochondrial-outer-membrane enzyme. The fact that only Triton X-100 extracted activity tends to rule out also the inner-membrane CPT, which can be solubilized by many detergents, and the peroxisomal enzyme likewise. It is possible that the different effects of the detergents are related to the lipid composition of the membrane and the other associated proteins present, as well as to the differences in the CPT proteins from the different locations.

DISCUSSION

CPT in the erythrocyte is a membrane-bound protein which only partially dissociates in high-salt buffer, treatment which removes the majority of the cytoskeletal and loosely associated proteins. The CPT activity is present at a level higher than the rate of acyl-CoA synthesis (Wittels & Hochstein, 1967), and at a level which is consistent with the observed rate of phospholipid acylation from palmitoylcarnitine (Arduini *et al.*, 1990). The sensitivity of the enzyme to inhibitors (Table 1) is similar to those of the intracellular enzymes which use cytoplasmic CoA substrates (Derrick & Ramsay, 1989). The inhibition by malonyl-CoA is curious, in view of the absence of both fatty acid synthesis and β -oxidation for the erythrocyte, but suggests that the enzyme is closely related to the cytoplasmic membrane enzymes. However, we do not know of any reaction generating or using malonyl-CoA in the erythrocyte, so this regulatory mechanism is likely to be vestigial rather than functional. The substrate specificity is different from the rat enzymes, giving better activity with the longer-chain and unsaturated fatty acids that are common in the erythrocyte membrane (Shohet, 1977). This enhanced activity is consistent with a role for the enzyme in buffering the acyl-CoA content of the erythrocyte, and hence facilitating the turnover of the fatty acid moieties of the lipids in the membranes.

Immunologically, the CPT in the erythrocyte membrane is distinct from the peroxisomal enzyme and from the malonyl-CoA-insensitive mitochondrial inner enzyme, CPT-II. Since the microsomal enzyme cross-reacts with antiserum to the

peroxisomal enzyme, it must also differ from that enzyme. The stability of the erythrocyte CPT in Triton X-100 suggests differences from the labile microsomal and mitochondrial overt enzymes. The erythrocyte enzyme is solubilized by Triton X-100 and can be partially extracted from the membrane in high-salt solution, whereas the overt CPT is very difficult to solubilize, requiring both high salt and detergent such as Tween 20 or octyl glucopyranoside for efficient extraction of an active enzyme. If the erythrocyte enzyme is indeed different, it is yet another variant in this family of malonyl-CoA-sensitive CPT enzymes which catalyse the equilibration of acyl-CoA and acyl-L-carnitine in the cytoplasm.

This work was supported by the National Institutes of Health (DK-41572), the Department of Veterans Affairs, and by Sigma Tau s.r.l., Pomezia, Italy.

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Received 25 September 1990/31 December 1990; accepted 22 January 1991