Evaluation of the Affinity and Turnover Number of Both Hepatic Mitochondrial and Microsomal Carnitine Acyltransferases: Relevance to Intracellular Partitioning of Acyl-CoAs†

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ABSTRACT: Mitochondrial carnitine palmitoyltransferase I (CPT I) and microsomal carnitine acyltransferase I (CAT I) regulate the entry of fatty acyl moieties into their respective organelles. Thus, CPT I and CAT I occupy prominent positions in the pathways responsible for energy generation in mitochondria and the assembly of VLDL in the endoplasmic reticulum, respectively. Previous attempts to determine the intrinsic kinetic properties of CPT I and CAT I have been hampered by the occurrence of sigmoidal velocity curves. This was overcome, in this study, by the inclusion of recombinant acyl-CoA binding protein in the assay medium. For the first time, we have determined the concentrations of total functional enzyme (E_t) by specific radiolabeling of the active site, the dissociation constants (K_d) and the turnover numbers of CPT I and CAT I toward the CoA esters of oleic acid (C18:1) and docosahexaenoic acid (C22:6). The data show that carnitine inhibits CAT I at physiological concentrations which are not inhibitory to CPT I. Thus, carnitine concentration is likely to be a significant factor in determining the partitioning of acyl-CoAs between mitochondria and microsomes, a role which has not been previously recognized. Moreover, the finding that CAT I elicits a lower turnover toward the CoA ester of C22:6 (25 s^{-1}) than toward that of C18:1 (111 s^{-1}), while having similar K_d values, suggests the use of this polyunsaturated fatty acid to inhibit VLDL biosynthesis.

Overt mitochondrial carnitine palmitoyltransferase (CPT I)[1] and microsomal carnitine acyltransferase (CAT I) catalyze the conversion of acyl-CoAs into the corresponding acylcarnitines (*1*). This facilitates the entry of the acyl moieties by carnitine-acylcarnitine translocase into the mitochondrial matrix and endoplasmic reticular lumen, where acyl-CoAs are regenerated from the acylcarnitines by latent acyltransferases, for subsequent mitochondrial β-oxidation (*2*) and triacylglycerol (TAG) synthesis within microsomes (*3*).

Several in vitro studies of the substrate selectivity of CPT I have been performed in this (*4*−*6*) and other laboratories (*7*, *8*). Despite their importance, it remains to be established whether these studies reflect the situation in vivo. The most serious drawbacks of these in vitro studies are that they are unable to mimic the vast combinations of substrates that exist in vivo and to take into account competition from nonmitochondrial carnitine acyltransferases. Using only K_m (substrate concentration at half-maximal velocity) and V_{max} (maximum velocity) values, it is difficult to investigate substrate selectivity and partitioning among different biochemical pathways. On a theoretical basis, a lower K_m does not always indicate a lower dissociation constant (K_d), especially in more complex kinetic models, and thus cannot be taken as an accurate index for substrate partitioning in vivo. However, if the values of K_d and turnover number (TN; also known as K_{cat}) of the binary complexes between the key enzymes of different pathways and various substrates are obtained from in vitro studies, a better indication of the extent of substrate partitioning and selectivity occurring in vivo can be obtained. To get an accurate picture, the active concentration (operational molarity) of the enzyme must also be known, because recently it has been shown that the number of CPT I sites is altered by changes in the energy state of the mitochondria (*9*).

To our knowledge, this is the first report on the measurement of the K_d and TN values for CPT I and CAT I, together with the concentrations of the active enzymes. These kinetic

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[1] Abbreviations: CPT I, overt mitochondrial carnitine palmitoyltransferase; CAT I, overt microsomal carnitine acyltransferase; VLDL, very low-density lipoprotein; C22:6, 6-CoA, docosahexaenoyl-CoA; C18:1, 1-CoA, oleoyl-CoA; TN, turnover number; Ac, acyl-CoA; Cn, carnitine; $[E]_t$, total active enzyme concentration; ACBP, acyl-CoA binding protein; K_m, Michaelis−Menten constant; V_{max}, enzyme maximum velocity; K_d, dissociation constant.

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parameters were determined for both oleoyl-CoA (C18:1-CoA) and docosahexaenoyl-CoA (C22:6-CoA), because these acyl-CoAs have been shown, when taken as dietary supplements, to have pronounced effects upon mitochondrial β-oxidation (*10*, *11*) and hepatic secretion of TAG (*12*). For CAT I, carnitine was shown to inhibit the enzyme at physiological concentrations, as illustrated in a new quadratic plot, suggesting that it might affect the partitioning of acyl-CoAs between mitochondria and microsomes.

EXPERIMENTAL PROCEDURES

Background of the Kinetic Models and Analytical Procedure. Since CPT I and CAT I have two substrates, namely acyl-CoA and carnitine, the kinetic parameters were determined by independently varying the concentration of each substrate. The true kinetic parameters for each substrate (at a saturating level of the other substrate) were then estimated using the appropriate kinetic model. The varying substrate is indicated by the superscript Ac or Cn for acyl-CoA or carnitine, respectively. Kinetic parameters are defined, for example, as follows: V_{max} is the maximum velocity obtained at infinite concentrations of the two substrates acyl-CoA ([Ac]) and carnitine ([Cn]); K_m^{Ac} and K_m^{Cn} are the concentrations of acyl-CoA or carnitine which give half-maximum velocity at a saturating concentration of the other substrate, respectively.

It is noteworthy that neither of the two substrates can be practically added at saturating concentrations as they have been shown to have inhibitory effects at very high concentrations (*13*). Therefore, a mathematical procedure is adopted here to obtain the kinetic parameters at a saturating level of either or both substrates. Although some investigators reported no inhibition at very high concentrations of carnitine, this was likely to be due to the use of an inappropriate buffer, which probably lowered the active concentration of carnitine in the assay system. For example, Hoppel (*14*) used 15 mM carnitine (compared with the commonly used 0.5 mM) but also used 50 mM Mops, a buffer which competes with carnitine with a K_i of 65 mM (*15*). Hepes, which is a good buffer for the assay of carnitine acetyltransferase (*16*), was also avoided in the present study because it has an inhibitory effect on CPT with a K_i of 55 mM (*15*).

Whereas CPT I is known to follow a compulsory-ordered Bi−Bi mechanism in which acyl-CoA must bind prior to carnitine and acylcarnitine must leave before CoASH (*17*, *18*), no definitive mechanism has been established for microsomal CAT I. However, the most likely mechanism for CAT I can be gleaned from previously published structural studies (*1*, *19*, *20*). Two important details have come to light from these studies. First, carnitine- and acyl-CoA-binding sites reside within the large C-terminal domain. Second, CPT I and CAT I are very similar, if not identical (*1*), with apparent differences residing in the short N-terminal domain. Therefore, we assume that there is no structural difference in the carnitine-binding site between CPT I and CAT I. In all of the carnitine acyltransferases that have been studied, differences between the carnitine-binding sites were assumed to dictate the kinetic mechanism (*18*). That is, a random-order mechanism is followed when the structure of the carnitine-binding site is well formed in the free enzyme, and a compulsory-ordered Bi−Bi mechanism is followed when the carnitine-binding site is less well formed (*18*). Indeed, in the latter mechanism, carnitine cannot bind unless acyl-CoA is first bound to the enzyme, thus creating a suitable carnitine-binding site. As the carnitine-binding site appears to be identical in both CPT I and CAT I (see above), it is assumed that the kinetic mechanism assigned to CAT I is the same as that assigned to CPT I, that is a compulsory-ordered mechanism.

Preparation of Highly Purified Subcellular Fractions and Their Assay for CPT I and CAT I. Mitochondria were isolated as previously described (*21*), and microsomes were isolated as in Shepherd et al. (*22*), except that peroxisomal contamination was minimized by centrifugation through iodixanol (*23*). These highly purified organelles were the only choice available to conduct the present study. Purified enzyme cannot be used because, as has been well documented, CPT I is completely inactivated when it is removed from its natural environment (*24*−*26*). Both CPT I and CAT I assays were performed as described elsewhere (*6*), except that ATP was added according to the recommendation of McGarry et al. (*27*). Also potassium phosphate was added for its stimulatory effect, especially on microsomal CAT (*28*). The assay buffer was thus as follows: 100 mM KCl, 5 mM Tris-HCl (pH 7.4), 1 mM EGTA, 1 mM dithiothreitol, 4 mM ATP, 100 mM potassium phosphate, and the indicated concentrations of carnitine. 4,4′-Dithiobispyridine (DTBP), a commonly used CoASH trapping agent (*29*) which has no inhibitory effect on the acyltransferase activity (*30*), was included in the assay mixture at a concentration of 125 μM. This was to prevent the involvement of CoASH in the reverse reaction. The reaction was monitored by following the incorporation of [^{3}H]carnitine into acylcarnitine as previously described (*21*). It was assumed that a 1 min reaction duration was short enough to permit initial velocity studies. Acyl-CoA-binding protein (ACBP) replaced bovine serum albumin (BSA) in the assay mixture. An acyl-CoA/ACBP ratio of 0.8 was used which is similar to the physiological molar ratio (*31*). Two acyl-CoAs (oleoyl-CoA and docosahexaenoyl-CoA) were tested, and the reaction was initiated by the addition of different concentrations of carnitine as indicated in the legend to the figures.

Determination of Turnover Numbers and Operational Molarity of the Enzymes. To determine the concentration of the acyl-CoA−enzyme binary complex per milligram of organelle (mitochondrial or microsomal) protein, the reaction mixture was incubated in a manner similar to a typical CPT I and CAT I assay except that carnitine was not added and a fixed concentration of acyl-CoA was used (50 μM). The reaction mixture (5 mL) was layered in 10 mL of polycarbonate tubes on top of a mixture (2 mL) of dibutyl phthalate and diisononyl phthalate (2:1, v/v), which had been layered on 1.5 mL of a buffer consisting of 5 mM Tris-HCl (pH 7.4), 250 mM sucrose, and 1 mM EGTA at 0−2 °C. The tubes were centrifuged at 12000*g* for 1 min for mitochondria and 150000*g* for 60 min for microsomes.

Acyl-CoA bound to either CPT I or CAT I migrated through the oil layer and pelleted in the medium beneath the oil where some of the bound acyl-CoA dissociated to maintain the equilibrium. The supernatant above the oil was removed, and the side of each tube and the surface of the oil layer were washed with acyl-CoA-free assay medium.

The pellet was resuspended by vigorous shaking in the remaining buffer, after the removal of the oil layer. The total acyl-CoA (bound and free) in this suspension, which originally existed in a bound form before centrifugation took place, was determined by an end-point enzymatic assay. An aliquot (1 mL) of the suspension was transferred to another tube to which was added 0.5 mL of a CPT II-enriched solution, which was prepared as described elsewhere (*26*). The reaction volume was made up to 2 mL with buffer to give final concentrations of 100 mM KCl, 5 mM Tris-HCl, 250 mM sucrose, 0.5 mM [^{3}H]carnitine, 1 mM EGTA, 1 mM dithiothreitol, 4 mM ATP, 100 mM potassium phosphate, and 125 μM DTBP. After an incubation of 15 min at 37 °C, acylcarnitine was extracted into butanol, which was repeatedly washed with butanol-saturated water, and the radioactivity was measured. The protein content of the mitochondria and microsomes, which had migrated through the oil layer, was determined as previously described (*32*) in parallel samples where no acyl-CoA had been added.

The radioactive L-carnitine obtained commercially (85 Ci/mmol) contained small quantities of radioactive butanol-soluble contaminants and, hence, generated a high background in the CPT assay where these contaminants co-separated with the product acylcarnitine. This problem was eliminated when the carnitine solution was purified, by extraction of the contaminants into butanol, prior to use. Also, the acylcarnitine-containing butanol layer was repeatedly washed (five times) with butanol-saturated water. Following this procedure, the background radioactivity was almost eliminated. Recovery at 100% of acyl-L-carnitine into the butanol layer has been reported as a characteristic of this phase partitioning procedure (*33*). Therefore, the radioactivity measured in this experiment was taken as an accurate determination of the enzyme-acyl-CoA binary complex plus nonspecifically bound acyl-CoA.

Nonspecific binding was subtracted from the total count after being determined as follows. In parallel test tubes containing a reaction mixture identical with that described for the binding experiment above, 300 μM malonyl-CoA was added to the assay mixture before the centrifugal filtration step. Malonyl-CoA has been shown to exert its inhibitory effect on CPT I and CAT I by either causing a conformational change or by sterically hindering the approach of acyl-CoA to the active site (*29*, *34*, *35*). Moreover, nonspecific binding of acyl-CoA to the membrane, presumably through the hydrophobic moiety of acyl-CoA, is not affected by malonyl-CoA (*36*). Therefore, the amount of acyl-CoA remaining bound after centrifugation of these samples through oil represented the nonspecific binding of acyl-CoA to membranous sites other than the CPT I and CAT I catalytic sites.

The total active enzyme concentration $[E]_t$ equals the free enzyme concentration [E] plus the concentration of enzyme-acyl-CoA binary complex [E·Ac]. Let $[Ac]_t$ denote the total concentration of acyl-CoA.

Since

$$K_d^{Ac} = \frac{[E][Ac]}{[E\cdot Ac]} = \frac{([E]_t - [E\cdot Ac])([Ac]_t - [E\cdot Ac])}{[E\cdot Ac]}$$

then

$$[E]_t = \frac{[E\cdot Ac]K_d^{Ac}}{[Ac]_t - [E\cdot Ac]} + [E\cdot Ac] \quad (1)$$

The total enzyme concentration obtained by eq 1 is a measure of the active concentration of the enzyme per milligram of organelle protein (i.e., operational molarity). The number of reaction processes (turnovers) that each acyl-CoA-saturated enzyme unit catalyses per unit time is therefore obtained by applying the following equation:

$$\text{turnover number } (s^{-1}) = \frac{V_{max}(\text{nmol/mg of protein/s}) \times 1000}{[E]_t \text{ (pmol/mg of protein)}} \quad (2)$$

Note that in eqs 1 and 2, $[Ac]_t = 50\ \mu$M, [E·Ac] was obtained experimentally (as explained above), and K_d^{Ac} and V_{max} were obtained from kinetic data as described below.

Determination of K_d^{Ac}, V_{max}, K_m^{Cn}, and K_m^{Ac} for CPT I. For the kinetic model of CPT I, the initial velocity (V_0) is given (*37*) by the equation

$$V_0 = \frac{V_{max}}{1 + \frac{K_m^{Ac}}{[Ac]} + \frac{K_m^{Cn}}{[Cn]} + \frac{K_d^{Ac}K_m^{Cn}}{[Ac][Cn]}} \quad (3)$$

The kinetic parameters were determined by nonlinear least-squares regression implemented with the MATLAB computer package (The MathWorks, Inc., Natick, MA). Simple weighting was chosen in all of the regressions since the residuals were found to be independent of substrate concentrations. To obtain good starting values of the kinetic parameters, which are critical for the regression analysis, and to provide a visual representation of the data, we also produced a series of primary and secondary plots. In the secondary plots, the *y*-axis represents the slopes and ordinal intercepts obtained from primary double-reciprocal plots and the *x*-axis represents the reciprocal of the nonvaried substrate concentration, as described elsewhere (*37*) (see Figures 1−3).

Determination of K_d^{Ac}, V_{max}, K_m^{Cn}, K_m^{Ac}, and K_i of Cn for CAT I. In the case of CAT I, carnitine displayed an inhibitory effect when used at high concentrations. As previously shown (*37*), this inhibition can be taken into account by the use of the following equation:

$$V_0 = \frac{V_{max}}{1 + \frac{K_m^{Ac}}{[Ac]}\left(1 + \frac{[Cn]}{K_i}\right) + \frac{K_m^{Cn}}{[Cn]} + \frac{K_d^{Ac}K_m^{Cn}}{[Ac][Cn]}\left(1 + \frac{[Cn]}{K_i}\right)} \quad (4)$$

The kinetic parameters were determined by fitting the initial velocity data to this equation by nonlinear regression analysis. It should be recognized that there is a potential nonuniqueness in the estimation of K_m^{Ac} and K_i, which is intrinsic to the model. By using different starting values in the optimization algorithm for the regression, each of two possible estimates of K_m^{Ac} and K_i could be obtained (with the same residuals). The rationale for choosing the most appropriate estimates is explained below.

Starting values of V_{max} and K_m^{Cn}, for the regression analysis, were determined from classical double-reciprocal

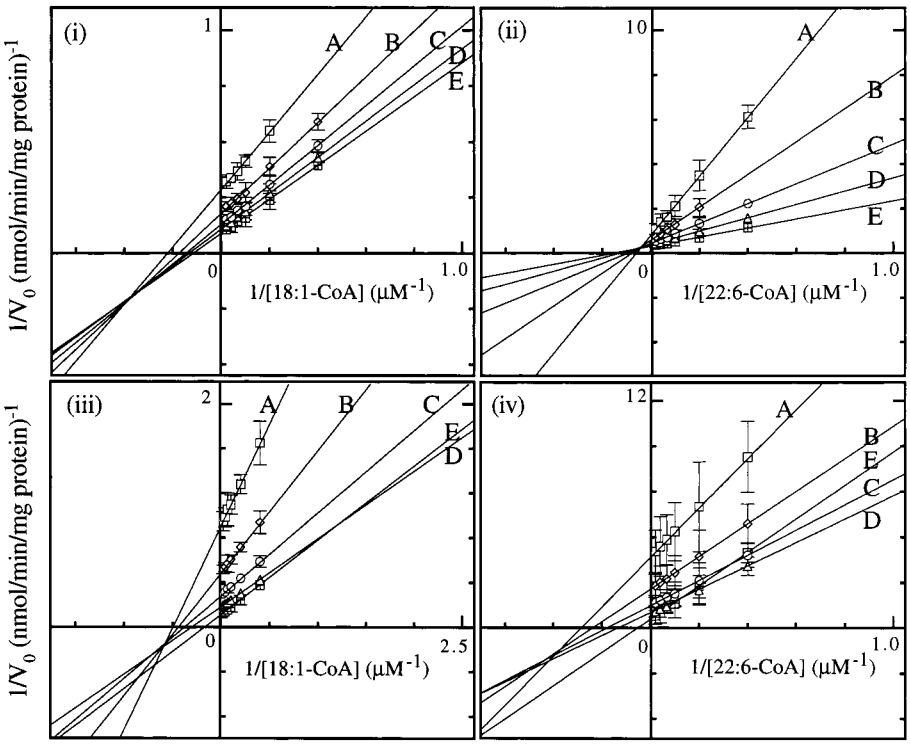

FIGURE 1: Primary double reciprocal plots of $1/V_0$ vs 1/[acyl-CoA]. The data were derived from the initial velocity values (V_0) for mitochondrial CPT I (panels i and ii) and microsomal CAT I (panels iii and iv) against 1/[acyl-CoA] (oleoyl-CoA, C18:1-CoA, and docosahexaenoyl-CoA, C22:6-CoA) (2.5−50 μM) at different fixed concentrations of carnitine. The fixed carnitine concentrations were as follows: A, 25 μM; B, 50 μM; C, 100 μM; D, 200 μM; and E, 600 μM. The data represent the mean ± SEM of three separate experiments.

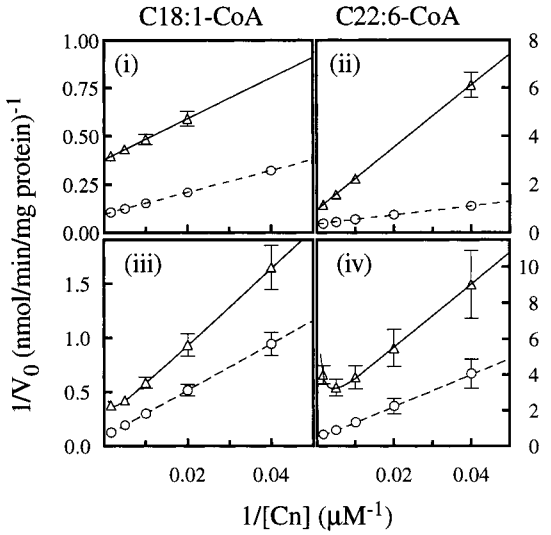

FIGURE 2: Primary double reciprocal plots of $1/V_0$ vs 1/[Cn]. The data were derived from the initial velocity values (V_0) at [Cn] = 25−600 μM for mitochondrial CPT I (panels i and ii) and microsomal CAT I (panels iii and iv) at different fixed concentrations of acyl-CoA (oleoyl-CoA, C18:1-CoA, and docosahexaenoyl-CoA, C22:6-CoA). Although six different concentrations of the acyl-CoAs were used, for the sake of clarity only the minimum and maximum concentrations [2.5 μM (△) and 50 μM (○)] are shown in the figure. The data represent the mean ± SEM of three separate experiments. The curves for CAT I in panels iii and iv are the graphs defined by eq 4 with the parameter estimates in Table 2.

plots (*37*), whereas the starting values of the other kinetic parameters (namely, K_d^{Ac}, K_m^{Ac}, and K_i) were obtained using the following equation:

$$\text{slope}_{\text{plot } 1/V_0 \text{ vs } 1/[Ac]}[Cn]V_{max} = (K_m^{Ac}[Cn] + K_d^{Ac}K_m^{Cn})\left(1 + \frac{[Cn]}{K_i}\right) = \frac{K_m^{Ac}}{K_i}[Cn]^2 + \left(K_m^{Ac} + \frac{K_d^{Ac}K_m^{Cn}}{K_i}\right)[Cn] + K_d^{Ac}K_m^{Cn} \quad (5)$$

The application of this quadratic formula appears to be less complicated than previously described procedures (*38*, *39*). Note that in the plot of eq 5, the degree of curvature is inversely related to the magnitude of K_i and that at very high K_i values the plot approaches linearity.

Using the method of least squares, a quadratic function ($y = ax^2 + bx + c$) was fitted through the data plotted according to eq 5, where $x = [Cn]$, $a \cong K_m^{Ac}/K_i$, $b \cong (K_m^{Ac} + K_d^{Ac}K_m^{Cn}/K_i)$, and $c \cong K_d^{Ac}K_m^{Cn}$. Then K_d^{Ac} was obtained by dividing the last term c in the quadratic expression by K_m^{Cn}, which was previously obtained. As is consistent with the intrinsic nonuniqueness discussed above, eq 5 leads to two feasible estimates of K_i, namely $[b \pm \sqrt{(b^2-4ac)}]/(2a)$. The larger value of K_i and the corresponding value of K_m^{Ac} were chosen since, in this case, eq 4, without the inhibition factor $(1 + [Cn]/K_i)$, gives a better fit to the data for smaller values of [Cn]. The corresponding value of K_m^{Ac} is then obtained by multiplying the coefficient a by the estimated K_i.

RESULTS AND DISCUSSION

Kinetic Behavior of CPT I and CAT I. CPT I activity displayed simple linear Michaelis−Menten kinetics, and there was no evidence of the sigmoidal kinetics reported by others

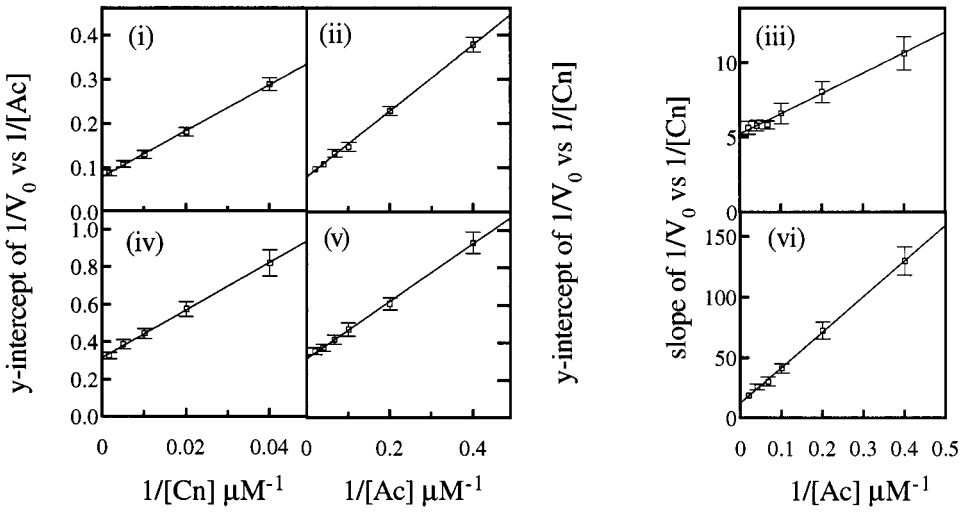

FIGURE 3: CPT I secondary plots of the ordinal intercepts and line slopes in the primary plots against the reciprocal of substrate concentration. CPT I activity was measured as described in Experimental Procedures using two acyl-CoAs ([Ac]): oleoyl-CoA, C18:1-CoA (panels i, ii, and iii) and docosahexaenoyl-CoA, C22:6-CoA (panels iv, v, and vi). Three types of secondary plots were constructed using these data. Panels i and iv are secondary plots of the *y*-intercepts in the primary plots of $1/V_0$ vs 1/[Ac] (Figure 1, panels i and ii). Panels ii and v are secondary plots of the *y*-intercept in the primary plots of $1/V_0$ vs 1/[Cn] (Figure 2, panels i and ii). Panels iii and vi are secondary plots of the slope of the line in the primary plot of $1/V_0$ vs 1/[Cn] (Figure 2, panels i and ii). The data represent the mean ± SEM of three separate experiments.

(*36*, *40*). It has been shown that sigmoidal kinetics can arise from the use of BSA in the assay medium (*36*), which was replaced by ACBP in the present study. The difference observed using these two binding proteins might be related to the fact that BSA has multiple binding sites for acyl-CoAs whereas the stoichiometry of ACBP binding is 1 (*41*). The kinetic behavior of carnitine acyltransferases is notoriously sensitive to the assay conditions used, as has been illustrated by Zierz and Engel in their studies of the human skeletal muscle CPT (*35*, *42*). Therefore, the choice of assay conditions is critical in the interpretation of experimental results. The use of the sensitive radiochemical assay, the inclusion of the CoASH-trapping agent DTBP and the short reaction duration made the initial velocity studies feasible and prevented a possible inhibitory effect of CoASH on the activity of the enzyme in the forward direction (*43*). The initial velocity data, in which the concentration of either substrate (acyl-CoA or carnitine) was varied in the presence of a series of fixed concentrations of the other substrate, resulted in a series of primary double reciprocal plots, as shown in Figures 1 and 2.

Acyl-CoA Substrate Delivery System. Since the acyl-CoA to ACBP molar ratio was maintained at 0.8 and the K_d values of ACBP for C18:1-CoA and C22:6-CoA, under the experimental conditions employed in this study, were 14.1 and 16.2 nM, respectively (*3*), most of the acyl-CoA (97.7−99.9%) was bound to ACBP (Table 1). This raises the question as to whether CPT I and CAT I utilize free acyl-CoA and/or ACBP-bound acyl-CoA. The data presented in Table 1 provide an answer to this question. For a 20-fold increase in the total acyl-CoA concentration, the free concentration of C18:1-CoA and C22:6-CoA only increased 9.8 and 11.2%, respectively. However, the CPT I activity increased 270% for C18:1-CoA and 200% for C22:6-CoA. Likewise the CAT I activity increased 200% for C18:1-CoA and 485% for C22:6-CoA. Therefore, the increase in the free concentration of acyl-CoA is insufficient to account for the large increases in enzyme activity. It is thus apparent that the enzymes must be able to recognize acylCoA−ACBP binary complexes rather than only the free acyl-CoAs as their substrates. This highlights the role of ACBP in the intracellular delivery of acyl-CoA to CPT I and CAT I. Similarly, in vitro studies have suggested that acylCoA−BSA complexes may act as substrates for CPT (*36*). Although CPT I is also known to be capable of utilizing free acyl-CoA (*44*), under our assay conditions where ACBP was used as the delivery system for acyl-CoA, the concentration of free acyl-CoA was exceptionally low (51−64 nM, see Table 1). Using the K_m value for free palmitoyl-CoA of 2.55 μM for CPT I (*45*), these low concentrations of free acyl-CoA could only account for less than 2% of the activity.

Table 1: Enzyme Activity with Respect to Free and Bound Acyl-CoA

parameters	C18:1-CoA		C22:6-CoA	
total Acyl-CoA (μM)	2.5	50.0	2.5	50.0
acyl-CoA bound to ACBP (%)[a]	98.0	99.9	97.7	99.9
free acyl-CoA[a] (nM)	51.1	56.1	57.9	64.4
CPT I activity[b]	2.5	9.5	0.9	2.6
CAT I activity[b]	2.6	7.9	0.3	1.5

[a] The concentrations of free and ACBP-bound acyl-CoAs were calculated using the values mentioned in the text for the dissociation constants of ACBP bound to the two acyl-CoAs. [b]The enzymatic activity (nmol/min/mg of protein) toward acyl-CoA was measured at a carnitine concentration of 600 μM as described in Experimental Procedures. Values are the means of three experiments.

Kinetic Parameters of CPT I and CAT I. In the primary plots of CPT I activity for each acyl-CoA, all of the lines (corresponding to the different fixed concentrations of carnitine) intersect at a common point (Figure 1, panels i and ii). This is consistent with the model (eq 3) since it can be shown (*39*) that the coordinates are

$$x = \frac{-1}{K_d^{Ac}}, y = \frac{1}{V_{max}}\left(1 - \frac{K_m^{Ac}}{K_d^{Ac}}\right)$$

and thus the location of the intersection is independent of

Table 2: Summary of the Kinetic Parameters for CPT I and CAT I[a]

parameters	oleoyl-CoA		docosahexaenoyl-CoA	
	CPT I	CAT I	CPT I	CAT I
V_{max} (nmol/min/mg of protein)	12.28 ± 0.67	12.77 ± 0.27	3.13 ± 0.16	2.85 ± 0.12
K_m^{Ac} (μM)	8.80 ± 0.53	3.48 ± 0.12	4.30 ± 0.15	6.96 ± 0.86
K_d^{Ac} (μM)	2.37 ± 0.23	1.79 ± 0.10	22.71 ± 4.29	2.71 ± 0.24
K_m^{Cn} (μM)	64.2 ± 5.7	265.8 ± 29.3	41.7 ± 2.9	243.8 ± 55.1
K_i (μM) for carnitine	N/A	637.5 ± 94.9	N/A	352.8 ± 89.5
E_t (pmol of enzyme/mg of protein)	3.85 ± 0.39	1.97 ± 0.23	4.00 ± 0.66	1.97 ± 0.17
turnover number (s^{-1})	54.34 ± 5.72	111.13 ± 14.42	13.92 ± 2.72	24.50 ± 2.38

[a] The numerical values for the first five parameters were derived from nonlinear regression analysis of the data graphically illustrated in Figures 1−4. The values for E_t and turnover number were derived from the application of eqs 1 and 2, as defined in Experimental Procedures. N/A denotes not applicable. The values are the mean ± SEM of three separate experiments.

[Cn]. The intersection points in Figure 1 (panels i and ii) are below and above the *x*-axis for oleoyl-CoA and docosahexaenoyl-CoA, respectively. This indicates that, for CPT I, K_m^{Ac} is larger than K_d^{Ac} in the case of oleoyl-CoA and lower than K_d^{Ac} in the case of docosahexaenoyl-CoA, as confirmed by the values presented in Table 2. This is a good example of how a comparison between different substrates on the basis of K_m values can sometimes lead to an incorrect interpretation as far as enzyme affinity and, therefore, substrate selectivity is concerned.

In contrast to the situation for CPT I, the lines for CAT I representing $1/V_0$ vs 1/[Ac] at a series of fixed concentrations of carnitine did not have a common intersection point (Figure 1, panels iii and iv), indicating that [Cn] has an effect on the binding of acyl-CoA to this enzyme. For CAT I, it can be shown that the abscissa coordinate (1/[Ac]) of the point of intersection is

$$1/\left(\frac{K_m^{Ac}[Cn_1][Cn_2]}{K_i K_m^{Cn}} - K_d^{Ac}\right)$$

where $[Cn_1]$ and $[Cn_2]$ represent two different fixed concentrations of carnitine. It is clear that a positive value for the abscissa coordinate of the intersection point is indicative of a relatively low K_d^{Ac} value. Figure 1 (panels iii and iv) shows that carnitine has an inhibitory effect which appears only at high concentrations and that this effect on the binding of acyl-CoA to CAT I is more pronounced with docosahexaenoyl-CoA (more points of intersection) than with oleoyl-CoA. Figure 2 shows that, for CAT I (panels iii and iv), but not for CPT I (panels i and ii), there is an upward curvature of the plot of $1/V_0$ vs 1/[Cn] at a high concentration of carnitine and low concentration of acyl-CoA (2.5 μM), especially C22:6-CoA. This also demonstrates that carnitine, at high concentration, acts as an inhibitory substrate for CAT I (see next section).

A series of secondary plots for CPT I (Figure 3) and CAT I (Figure 4) were constructed from the primary plots, and good initial estimates for the kinetic parameters were readily obtained as described in Experimental Procedures. Table 2 summarizes the kinetic parameters derived by nonlinear regression using these initial estimates. The curves generated using these parameters and eq 3 for CPT I and eq 4 for CAT I accurately fitted the experimental data (Figure 2), suggesting that the corresponding models are applicable.

Substrate Inhibition. In contrast to the observation of Bremer and Norum (*38*), there was no evidence of inhibition by acyl-CoA for either CPT I or CAT I in the current study, presumably because of the inclusion of ACBP in the assay medium. Carnitine was not inhibitory to CPT I within the concentration range employed in this study. This is consistent with a previous study which showed that CPT I is inhibited by carnitine only at concentrations higher than 4 mM (*38*). In contrast to the situation with CPT I, carnitine not only behaves as the second substrate for CAT I but also, when at high concentrations, inhibits the binding of acyl-CoA in a competitive-like manner. Differences in the microenvironment of the enzymes within the mitochondrial and microsomal membranes (*29*, *46*) might be responsible for the difference in the carnitine inhibition characteristics displayed by CPT I and CAT I. For CAT I, the K_i for carnitine was determined to be 0.35 and 0.64 mM for docosahexaenoyl-CoA and oleoyl-CoA, respectively. These K_i values indicate that the inhibition of CAT I is physiologically significant because it has been shown that the concentration of carnitine within the cytosol is 2 mM (*47*). This may explain the previously published clinical observation that the administration of high levels of carnitine caused only a modest increase in the rate of VLDL secretion but a marked enhancement of β-oxidation (*48*, *49*).

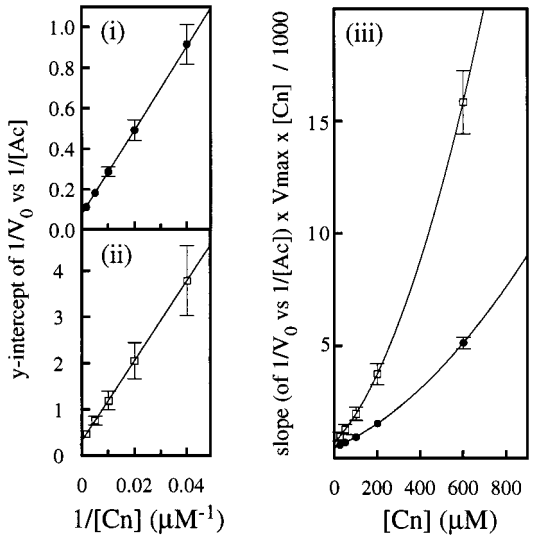

FIGURE 4: CAT I secondary plots. The kinetic behavior of CAT I is presented as the secondary plots of the *y*-intercepts, of the primary plots of $1/V_0$ vs 1/[Ac], against 1/[Cn] for the two acyl-CoAs (i) oleoyl-CoA (●) and (ii) docosahexaenoyl-CoA (□). Panel iii shows the quadratic relationship between (slope of the lines in the primary plots) × [corresponding Cn] × (V_{max}) on the *y*-axis vs [Cn] on the *x*-axis for both acyl-CoAs. The results represent the mean ± SEM of three separate experiments.

Relevance to Intracellular Partitioning of Acyl-CoA. To reach a valid conclusion on substrate partitioning between competing enzymes, it is important not only to know the dissociation constants of the enzymes under consideration for different substrates but also to determine the enzyme active "operational" concentration. For example, a low-affinity enzyme (low $1/K_d$) can still compete successfully with a high-affinity enzyme providing that the concentration of the former is relatively much higher. The total amount of active enzyme (CPT I and CAT I) per milligram organelle protein was calculated in this study from the known values of acyl-CoA concentration ([Ac]), K_d^{Ac}, and the total amount of enzyme-acyl-CoA binary complex (measured as explained in Experimental Procedures). The number of reaction processes (turnovers) that each acyl-CoA-saturated enzyme unit catalyses per unit time was then obtained from eq 2. The results of these calculations for turnover numbers (s^{-1}) and amounts of active enzymes (E_t) (pmole per mg of organelle protein) are also summarized in Table 2.

From the results, it can be concluded that although the K_ds of CAT I and CPT I for C18:1-CoA are similar, i.e., 1.8 and 2.4 μM, respectively, the turnover numbers, i.e., 111 and 54 s^{-1}, respectively, indicate that the microsomal enzyme is about twice as active as the mitochondrial enzyme in converting C18:1-CoA into its carnitine derivative. However, since the E_t value for CPT I is approximately twice that of CAT I, i.e., ~4 and ~2 pmol/mg of mitochondrial and microsomal protein, respectively, the data suggest that, in the absence of other factors, C18:1-CoA would be likely to be partitioned equally between CAT I and CPT I. In contrast to the situation for C18:1-CoA, the K_ds of CAT I and CPT I for C22:6-CoA differ markedly, i.e., 2.7 and 22.7 μM, respectively, while the turnover value for CAT I is still about twice that of CPT I, i.e., 25 and 14 s^{-1}, respectively. These data, when taken in conjunction with the E_t values, suggest that C22:6-CoA would be taken up predominantly by the microsomes.

Although CAT I has a similar affinity for C18:1-CoA and C22:6-CoA, the latter forms a less functional binary complex with the enzyme such that it is converted to its carnitine derivative at a 4.5-fold slower rate, as indicated by the turnover numbers. At least part of the synthesis of TAG required for the assembly of VLDL is believed to be via acylation of DAG by acyl-CoA generated in the lumen of the endoplasmic reticulum and therefore under the control of CAT I (*3*). Therefore, when present at a similar or higher concentration, C22:6-CoA would be expected to impair the formation of microsomal VLDL from C18:1-CoA. This provides a kinetic explanation of the recently documented observation that dietary fish oils containing eicosapentaenoic and docosahexaenoic acids reduce the plasma triglyceride concentration in humans and experimental animals (*10*, *11*). For CPT I, both the affinity and the turnover number are much higher for C18:1-CoA than C22:6-CoA (~10- and ~4-fold, respectively). This implies that, unlike the situation with microsomes, the presence of C22:6-CoA will have little effect on the rate of utilization of C18:1-CoA by mitochondria.

Although, as described above, ACBP has comparable affinity for C18:1-CoA and C22:6-CoA and is involved in the delivery of these acyl-CoAs to both CPT I and CAT I, the former enzyme has a 10-fold higher affinity for C18:1-CoA whereas the latter enzyme has comparable affinity for both substrates. This implies that it is the intrinsic characteristics of these enzymes that determine the extent to which mitochondria and the endoplasmic reticulum compete for the carnitine-dependent uptake of acyl-CoAs and thus the partitioning of these substrates between β-oxidation and TAG synthesis in the two organelles, respectively.

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