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Evidence for distinct functional molecular sizes of carnitine palmitoyltransferases I and II in rat liver mitochondria

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1. Estimates of the functional sizes of the molecular species responsible for the overt (I) and latent (II) activities of carnitine palmitoyltransferase (CPT) in 48 h-starved rat liver mitochondria were obtained from radiation inactivation experiments. 2. The decay in the activity of total CPT and that of CPT II only (after inhibition of CPT I) was measured in mitochondrial samples exposed to different doses of high-energy ionizing radiation. 3. The decay curves obtained by plotting residual activity of total CPT as a logarithm function of irradiation dose suggested the contribution of more than one target towards total CPT activity. 4. By contrast, in mitochondria in which CPT I activity was ~ 95% inhibited, the activity of CPT decayed in a simple mono-exponential manner. Target-size analysis yielded an approximate M_r of 69700 for this component (CPT II). 5. This information, as well as that on the relative non-irradiated activities of CPT I and CPT II, was used in graphical and statistical methods to obtain the parameters of the decay curve for CPT I. These analyses yielded an approximate M_r of 96700 for CPT I.

INTRODUCTION

It is well established that there are two activities of carnitine palmitoyltransferase (CPT) in mammalian mitochondria, functionally located on either side of the acyl-CoA barrier presented by the inner membrane [1–3]. Conflicting results have been obtained, however, about the possible identity, or otherwise, of the proteins that catalyse the two activities. West *et al.* [4] reported the separation of two forms with distinct properties, notably different isoelectric points, susceptibility to extraction in organic solvents and to inhibition by bromo derivatives of acyl-CoA substrates. The work of Brosnan *et al.* [5] also suggested the presence of two immunologically distinct forms. However, Bergstrom & Reitz [6] could only extract one type of CPT activity from rat liver mitochondria. Similar results were obtained in other studies [7–9]. Antibody raised by Miyazawa *et al.* [8] against their preparation of total CPT from liver mitochondria also revealed only one CPT product in a translation system *in vitro* using total rat liver mRNA. Brady & Brady [10] have recently reported that antibody against a single polypeptide with CPT activity, purified from whole mitochondria, immunoneutralized all CPT activity in sonicated mitochondria (see also [11]). One common feature in all these purification studies on CPT is that the purified polypeptide has a disaggregated M_r of 65000–72000, although it has been suggested that the enzyme may exist as a much larger aggregate *in vivo* [7]. The purification of only one type of CPT activity has prompted suggestions [7,9,10] that what determines the different properties of the overt (CPT I) and latent (CPT II) forms of CPT in intact mitochondria (notably selective inhibition of CPT I by malonyl-CoA) is not so much that they are different proteins, as that the membrane environments in which the two forms exist *in situ* are different. In particular, it has been suggested that what confers malonyl-CoA sensitivity to CPT I is its putative interaction with a regulatory subunit that binds the inhibitor [12,13].

In contrast with the apparent consensus about a unique subunit M_r of CPT of about 70000, Kiorpes *et al.* [14] have reported that [^{14}C]tetradecylglycidyl-CoA covalently binds to a protein of M_r 90000 in intact mitochondria. Tetradecylglycidyl-CoA inhibits CPT I activity irreversibly and has been suggested to be an active-site-directed inhibitor of the enzyme [14,15]. Consequently, these authors have assigned an M_r of 90000 to CPT I. This is considerably larger than the value reported for the solubilized and purified CPT from whole mitochondria (see above), and raises the possibility that, during purification, there is selective loss of one form of CPT activity. Such a possibility has also been raised by recent attempts at the separation of a malonyl-CoA-sensitive form of CPT in the soluble state [16,17]. The resolution of this uncertainty is complicated by the possibility that tetradecylglycidyl-CoA may interact not with the active site of CPT I but with a separate site, possibly on the putative malonyl-CoA-binding regulatory polypeptide (see above). Indeed, Declercq *et al.* [18] have suggested that the M_r-90000 species labelled by [^{14}C]tetradecylglycidyl-CoA may be such a regulatory polypeptide. The separation of a malonyl-CoA-binding protein from a polypeptide with CPT catalytic activity has also been reported [19]. However, it is evident that the solubilization of, and distinction between, two or possibly three membrane proteins present formidable obstacles in determining whether distinct molecular species are present, owing to possible differential effects of the detergents used on both solubilization and selective inactivation.

We have therefore sought to determine the functional molecular sizes of CPT I and CPT II by radiation inactivation. This is a technique that provides information on the molecular mass of proteins *in situ* (e.g. in isolated membranes, intact organelles or cells) without

Abbreviations used: CPT I and CPT II, overt and latent forms of carnitine palmitoyltransferase; MDH, malate dehydrogenase.

requiring purification of the proteins of interest. We have exposed preparations of rat liver mitochondria to high-energy electron irradiation and monitored the decay in CPT activity with increasing radiation dose. By performing experiments on control mitochondria and on preparations in which CPT I activity was largely inhibited by an active-site-directed mechanism (see [20]), we were able to obtain combined decay profiles for CPT I and CPT II activities (control) and for CPT II only. Since target analysis gives the minimum molecular size required for biological function [21], we were able to obtain the size of CPT II directly and that of CPT I by mathematical derivation from the decay of the combined activities of the two enzymes. Our data indicate that CPT I and CPT II have markedly different molecular sizes, with the overt enzyme being the larger of the two.

MATERIALS AND METHODS

Animals

These were female Wistar rats (A. Tuck and Son, Rayleigh, Essex, U.K.) maintained on a standard rat chow diet. Their food was removed 48 h before being killed, but they had constant access to water.

Preparation of mitochondria for irradiation

Each preparation was made from the pooled livers of six starved rats, because starvation increases CPT I activity such that it becomes slightly higher than that of CPT II [22,23]. After rapid excision, livers were cooled and homogenized in a medium containing 300 mM-sucrose, 5 mM-Tris/HCl and 1 mM-EGTA (pH 7.4 at 0 °C). Mitochondria were prepared by differential centrifugation as described previously [24]. They were further purified by resuspension of the final washed pellet in the above medium containing, in addition, 17.5% Percoll, and re-sedimented at 9500 *g* for 15 min, as described previously [25]. They were finally washed in the above sucrose medium. The resultant pellet was resuspended in medium (6 ml) containing 150 mM-KCl, 5 mM-Tris/HCl and 1 mM-EGTA (pH 7.4 at 0 °C) and divided into two 3 ml portions. One portion ('control') was added to 22 ml of KCl medium of the same composition maintained at 25 °C (pH 7.4 at this temperature) and containing 20 μM-bromopalmitic acid and 2 mM-ATPMg. The other portion ('CPT I inhibited') was similarly treated, except that the medium contained, in addition, 250 μM-CoA and 2 mM-L-carnitine (cf. [20]). Both sets of mitochondria were incubated under their respective conditions at 25 °C for 10 min. They were then sedimented and the pellets were washed in ice-cold KCl medium (above). The mitochondrial samples were finally resuspended in medium (6 ml) containing 150 mM-KCl, 5 mM-Tris/HCl, 1 mM-EGTA and 10 mM-dithiothreitol (pH 7.4 at 0 °C). As a means of monitoring the irradiation procedure and verifying the quantitative aspects of the radiation-induced decay in activity, dialysed preparations of glucose-6-phosphate dehydrogenase (EC 1.1.1.49; M_r 106000), malate dehydrogenase (MDH; pig heart, cytosolic, EC 1.1.1.37; M_r 70000) β-galactosidase (EC 3.2.1.23; subunit M_r 116000) and yeast alcohol dehydrogenase (EC 1.1.1.1; M_r 148000) were added as molecular-size internal standards to the final mitochondrial suspensions (see [26]). The amount of pig MDH added to the samples had at least 30–40 times the activity of endogenous MDH in the mitochondria, such that it accounted for 97–98% of total MDH activity in the samples. Portions (0.2 ml) of the mitochondrial suspensions were dispensed into glass ampoules and rapidly frozen by immersion in liquid N_2. The ampoules were sealed under vacuum and the samples stored at −80 °C until irradiated. In all instances, 'control' and 'CPT I inhibited' mitochondria from any one preparation were irradiated simultaneously.

Irradiation of mitochondrial samples

The ampoules were irradiated with a 15 MeV electron beam generated by a linear accelerator at the Kelvin Laboratory, University of Glasgow, East Kilbride. Accelerator parameters were as follows: 30 rad/pulse, 2.5 μs/pulse and 100 pulses/s. Ampoules were maintained at a temperature of −120 °C by a stream of gaseous N_2 cooled by passage through a copper coil immersed in liquid N_2. Control of cooled gas flow was by means of a solenoid valve operated by a thermostatic control unit linked to a platinum resistance thermometer, which was used to monitor temperature in the irradiation chamber.

Assays

Two series of CPT assays (at 37 °C) were performed: one on the freshly prepared mitochondria before irradiation, and the other on irradiated samples. CPT assays on freshly prepared mitochondria were performed in two ways: (A) by quantifying the rate of formation of palmitoyl[^{3}H]carnitine from [^{3}H]carnitine and palmitoyl-CoA under $V_{max.}$ conditions as described previously [27], and (B) by using the isotope-exchange assay, which measures the rate of formation of palmitoyl[^{3}H]carnitine from [^{3}H]carnitine and palmitoylcarnitine [28]. Briefly, the methods were as follows. For assay A, intact or frozen–thawed mitochondria (approx. 0.5 mg of protein) were added to tubes containing (in 1.90 ml) 150 mM-KCl, 5 mM-Tris/HCl, 1 mM-EGTA, 100 μM-palmitoyl-CoA, 0.13 mg of albumin/ml, 1 μg of antimycin A/ml and 1 μg of rotenone/ml (the final pH was 7.4 at 37 °C). After a 2 min preincubation period, reactions were started by the addition of 50 μl of a solution of L-[^{3}H]carnitine (1 Ci/mol) to give a final concentration of 0.5 mM-L-carnitine. Reactions were terminated by addition of 0.3 ml of 6 M-HCl, and the palmitoyl[^{3}H]carnitine was quantified by butanol extraction as described previously [29]. For assay B the same procedure was followed, except that palmitoyl-CoA was replaced by 125 μM-palmitoylcarnitine and the albumin concentration was 10 mg/ml, as described previously [28].

After irradiation, the ampoules were opened and flushed with N_2. The samples were then thawed and assayed immediately for CPT activity by assay A. The other enzymes were assayed as described previously [26], after suitable dilution of the samples.

Statistical analysis

Data for 'control' mitochondria were subjected to non-linear regression analysis by using the Genstat V programme (Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, Herts., U.K.) to obtain optimized fits to the experimental points, as well as values for the decay parameters K_I and K_{II} (see eqn. 1) and the respective correlation coefficients (see legend to Fig. 2). Data for 'CPT I-inhibited' mitochondria were

analysed by linear regression constrained to intercept the initial (non-irradiated) value of CPT II activity [30].

Materials

Glucose-6-phosphate dehydrogenase (from baker's yeast), MDH (cytosolic, from pig heart), β-galactosidase (from *Escherichia coli*), yeast alcohol dehydrogenase and other biochemicals were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Percoll was obtained from Pharmacia (Uppsala, Sweden). L-[*Me*-^{3}H]Carnitine was obtained from Amersham International (Amersham, Bucks., U.K.). Dichromate dose meters were obtained from the National Physical Laboratory, Teddington, Surrey, U.K.

RESULTS

A combination of two assay methods on intact or frozen–thawed, 'control' or 'CPT I-inhibited', mitochondria was used to obtain quantitative information about the relative activities of CPT I and CPT II, the degree of permeabilization of mitochondria by freeze–thawing and the extent of inhibition of CPT I achieved by the formation of the *S*-carboxypalmitoyl-CoA ester of carnitine at the active site of CPT I (see the Materials and methods section). The percentage inhibition of CPT I achieved was obtained from CPT activity measured in intact 'control' and 'CPT I-inhibited' mitochondria measured by using assay A (see the Materials and methods section). Estimates of the CPT I/CPT II activity ratio were obtained (i) by using assay A to measure CPT activity in 'control' and 'CPT I-inhibited' frozen–thawed mitochondria, (ii) by using assay A for intact and frozen–thawed mitochondria and (iii) by using assay B for intact and frozen–thawed mitochondria. They were very similar to each other and to values published elsewhere for mitochondria from starved rats [22,23]. The similarity between the values obtained by using assays A and B suggested that, in freeze–thawed mitochondria, assay A measured total (CPT I + CPT II) activity in the same way that assay B measured total activity, whether used on intact or frozen–thawed mitochondria, owing to the permeability of the inner membrane to palmitoylcarnitine. Consequently, freeze–thawing appeared to permeabilize the inner membrane to palmitoyl-CoA. Further freeze–thaw cycles did not improve this permeabilization. Only a small fraction of total CPT activity (< 1%) was solubilized by one freeze–thaw cycle in KCl medium (see the Materials and methods section). Because the preparation of samples for irradiation involved freezing the mitochondria and subsequent thawing after irradiation, use was made of assay A routinely to measure CPT activity in irradiated samples, because of the higher rates of incorporation of ^{3}H into palmitoylcarnitine obtained with this method.

Effect of irradiation on affinity for substrates

Irradiation did not alter the dependence of total CPT activity on the concentration of carnitine or of palmitoyl-CoA (Fig. 1). It was important to establish this observation, in order to exclude the possibility that the decay in activity observed after irradiation of the samples could be due to a decrease in affinity for the substrates [20]. As Fig. 1 shows, the effect of irradiation was to decrease the $V_{max.}$ of the CPT activity without altering the K_m for carnitine or the $K_{0.5}$ for palmitoyl-CoA.

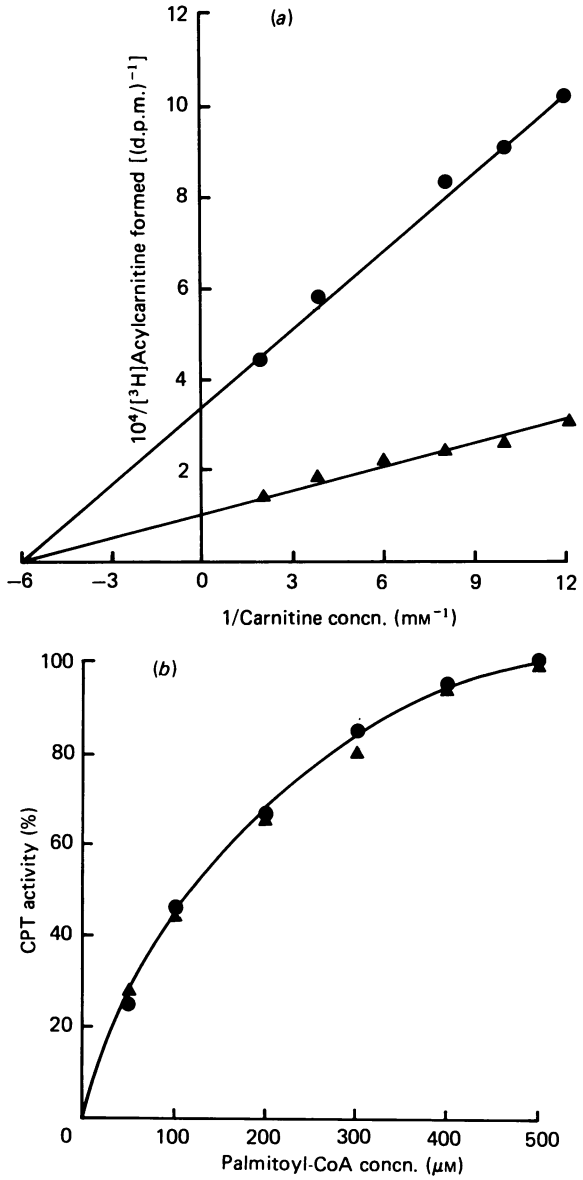

Fig. 1. Effects of high-energy radiation on (*a*) the $V_{max.}$ of total CPT and K_m for carnitine and (*b*) $K_{0.5}$ for palmitoyl-CoA in frozen–thawed rat liver mitochondria

Assays were performed by method A (see the Materials and methods section) on 'control' samples that had received either no radiation (●) or 15 Mrad of radiation (▲).

Therefore the decay in activity observed with increasing radiation dose could be analysed according to classic target analysis [31], which predicts that decay of enzyme activity with increasing radiation dose occurs exponentially and that, for two or more structures that contribute independently to the total catalytic activity in a sample, the decay is described by an additive series of exponentials. The M_r value(s) of the target(s) are obtained from the slope of a plot of ln A/A_0 versus radiation dose, where A is the surviving activity and A_0 is the original (non-irradiated) activity.

Inactivation of CPT by irradiation

Preliminary experiments established that, as expected (see [26]), the activities of glucose-6-phosphate dehydro-

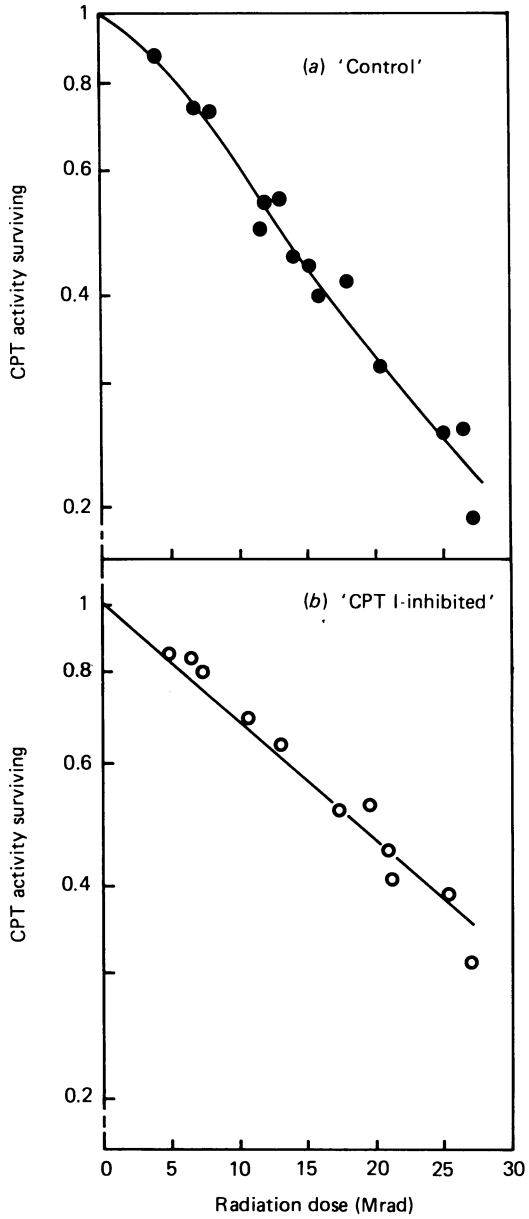

Fig. 2. Representative decay curves for CPT activity in an experiment in which (*a*) 'control' mitochondria and (*b*) 'CPT I-inhibited' mitochondria isolated from the same rat liver were exposed to increasing doses of radiation

The values for K_I and K_{II} derived from the data of 'control' mitochondria (see eqn. 1, in the text) were 0.06990 (S.E.M. 0.008448) and 0.04246 (S.E.M. 0.00306) respectively. The corresponding correlation coefficients were 0.7026 and 0.7712. In (*b*) a line corresponding to the value of K_{II} (above) is drawn through the actual experimental data obtained for 'CPT I-inhibited' mitochondria (constrained to intercept the initial, non-irradiated, activity of CPT II, i.e. at 1.0).

genase, MDH, β-galactosidase and alcohol dehydrogenase, included with the mitochondria as M_r standards, all decayed in a simple exponential manner with increasing radiation dose. By contrast, the loss of activity of total CPT activity (CPT I and CPT II) in 'control' mitochondria was complex and did not decay in a monoexponential manner (Fig. 2*a*). At low radiation

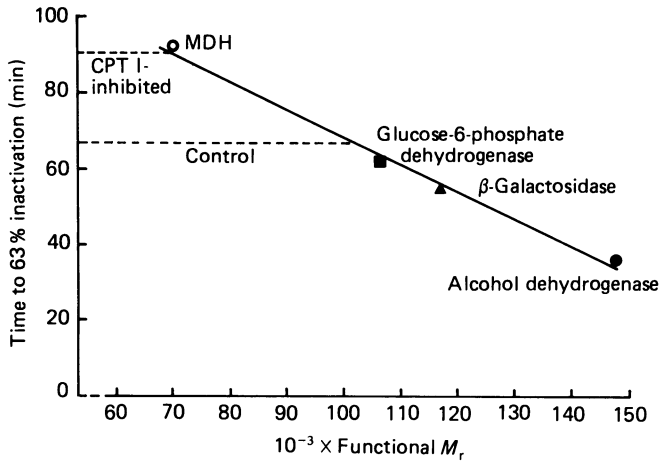

Fig. 3. Relationship between the M_r of standards included with the mitochondrial samples and the duration of irradiation (at a constant dose rate) required to diminish the activity of the enzymes to 37% of their respective initial (non-irradiated) activities

The relevant values for the initial part (5–20 Mrad) of the decay of total CPT activity in 'control' samples and for the linear decay plots for CPT II in 'CPT I-inhibited' mitochondrial samples are also shown.

doses, total CPT activity decayed inordinately little. This was probably due to the behaviour of CPT I, as it was not observed when CPT I was inhibited. This behaviour is known to occur for other enzymes (see [32],[33]). Statistical analysis (see the Materials and methods section) of inactivation data obtained at doses above 5 Mrad, however, indicated that the decay in CPT activity was not due to inactivation of a single target with a unique molecular size. A model which assumed two exponential components (see eqn. 1) invariably described the data better than one assuming a single exponential decay (see legend to Fig. 2):

$$CPT_{total} = CPT_I e^{-K_I D} + CPT_{II} e^{-K_{II} D} \quad (1)$$

When the relative non-irradiated activities of CPT I and CPT II (obtained as described above; CPT I represented 58±4% of total activity, cf. [22], [23]) were inserted into the two-component model, decay constants (K_I and K_{II}) were obtained for the two components that were in the ratio 1:1.5 (±0.2; $n = 4$). This observation suggested that the two components that contributed towards total CPT activity had markedly different functional molecular sizes, in the same ratio as that of their decay constants (the decay constants K_I and K_{II} are directly related to the molecular sizes of the targets; see [30]).

Irradiation of 'CPT I-inhibited' mitochondrial samples (in which approx. 95% of CPT I activity had been inhibited; see the Materials and methods section) gave a simple exponential decay (Fig. 2*b*) of CPT activity (presumably due almost solely to CPT II). The correlation coefficients obtained by regression analysis were all > 0.98 for four independent experiments. From the decay constants obtained from these linear plots, M_r of 69700±2300 ($n = 4$) was obtained for CPT II. In Fig. 3 are shown the relative dose-dependences of the decay of activity of the M_r standards and of CPT. The dose required for inactivation to 37% of initial activity (i.e.

$\ln A/A_0 = 1$) was used, as this amount of inactivation is normally used to calculate decay coefficients (see, e.g., [30]). The computation of the M_r of CPT I was performed by obtaining the decay constants for the initial part (5–20 Mrad) of the total CPT activity decay curves (see above). The M_r derived from this analysis was 96700 ± 2500 ($n = 4$). It is appreciated, however, that the accuracy of this computation may have been diminished by the fact that, at low radiation doses, the decay of total CPT activity was inordinately low (see above).

DISCUSSION

The simplest explanation for the multi-component exponential decay of total CPT activity after irradiation of 'control' mitochondria, in which both CPT I and CPT II were active, is that total CPT activity in these samples was due to two polypeptides of different functional molecular sizes. A non-linear semi-logarithmic decay plot would be expected if it resulted from the summation of the linear decays of the two independent activities (see above). This conclusion was corroborated by the observation that inhibition of CPT I activity by the formation of the *S*-carboxypalmitoyl-CoA ester of carnitine at the active site of CPT I (see the Materials and methods section) transformed the decay of the residual activity [mostly ($\geq 95\%$) CPT II] to a simple exponential function with a lower decay coefficient. This observation provides strong evidence that the molecular size of CPT I is larger than that of CPT II.

Consequently, it is suggested that the two CPT activities that occur in intact mitochondria are due to proteins of distinct molecular sizes, rather than to a single protein with a heterogeneous distribution within mitochondria. The latter possibility had been considered by several workers, who could only detect one species of polypeptide on gel electrophoretograms after purification of CPT from liver or heart mitochondria (see the Introduction). It was also considered that the membrane environment, rather than differences in the molecular structures of CPT I and CPT II, could account for differences in their respective properties, most notably sensitivity to malonyl-CoA inhibition. The present results suggest that CPT I and CPT II are likely to be different proteins. It is possible, as discussed elsewhere [17], that purification procedures may have resulted in the selective loss of the activity of one of the CPT enzymes.

In this context it is noteworthy that the M_r, deduced from the present studies, for CPT II was about 70000. This is similar to the subunit M_r determined from disaggregating gel electrophoresis of CPT purified in several laboratories [7–11]. Thus it appears that the size of the CPT purified in those studies is more similar to the functional molecular size of CPT II *in situ*. The present results also suggest that, if either CPT I or CPT II occurs as much larger aggregates (see [7]), these are not made up of covalently linked protomers. If that were the case, it would have been expected that CPT activity would have decayed with characteristics of a much larger molecular size than either of those calculated above. However, this does not rule out the possibility that either CPT species could exist as non-covalently associated oligomers. Examples of such enzymes exist, e.g. glutamate dehydrogenase is a hexamer whose activity decays, upon irradiation, with the molecular-size characteristics of its component protomers [34].

It may be relevant that Kiorpes *et al.* [14] found that [^{14}C]tetradecylglycidyl-CoA labels covalently a protein of M_r 90000 in isolated liver mitochondria. Because this compound was suggested to interact with the active site of CPT I, those authors ascribed this M_r to CPT I, even though it was considerably larger than that of 65000–70000 reported by several laboratories for purified CPT (see above). The present studies suggest that an M_r of 90000 may, in fact, be closer to the functional molecular size of CPT I, as deduced from radiation inactivation analysis. However, such similarity may be fortuitous, because the situation is complicated by the possibility [12,13,18,19,35] that tetradecylglycidyl-CoA may bind covalently not directly to CPT I but to a regulatory protein that interacts with the enzyme (see above). Unfortunately, the accurate determination of the molecular size of CPT I was made difficult by the behaviour of the total enzyme activity (CPT I plus CPT II) at low doses of radiation. It may be relevant that possibilities considered by other workers to be responsible for such behaviour [32,33] include the inactivation (at low doses) of a larger inhibitory polypeptide associated with the protein of interest, and transitions of a protein between different molecular-size forms. Interestingly, both these phenomena have been postulated to account for some of the characteristics of CPT I in isolated mitochondria (see [36]).

While the present paper was being reviewed, Declercq *et al.* [37] published data which indicated that the tetradecylglycidyl-CoA-binding protein and CPT purified from rat liver have different M_r values. These authors concluded that the tetradecylglycidyl-CoA-binding protein is identical with the malonyl-CoA-binding species and the protein that expresses CPT I catalytic activity; they assigned an M_r of 94000 to it (cf. [14]). This value is very similar to that of 96700 which we have obtained for CPT I from radiation inactivation analysis. They also assigned an M_r of 80000 to CPT, purified by well-established protocols [7–12], on the basis of the anomalous behaviour of bovine serum albumin standards in their SDS/polyacrylamide-gel-electrophoresis experiments. This value appears to be too high to account for the value of 69700 obtained for CPT II by radiation activation. Whether the errors involved in the two methods of molecular-size estimation are sufficiently large to reconcile the two values will need to be ascertained by further work.

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