Target size analysis by radiation inactivation of carnitine palmitoyltransferase activity and malonyl-CoA binding in outer membranes from rat liver mitochondria

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The functional molecular sizes of the protein(s) mediating the carnitine palmitoyltransferase I (CPT I) activity and the [^{14}C]malonyl-CoA binding in purified outer-membrane preparations from rat liver mitochondria were determined by radiation-inactivation analysis. In all preparations tested the dose-dependent decay in [^{14}C]malonyl-CoA binding was less steep than that for CPT I activity, suggesting that the protein involved in malonyl-CoA binding may be smaller than that catalysing the CPT I activity. The respective sizes computed from simultaneous analysis for molecular-size standards exposed under identical conditions were 60 000 and 83 000 Da for malonyl-CoA binding and CPT I activity respectively. In irradiated membranes the sensitivity of CPT activity to malonyl-CoA inhibition was increased, as judged by malonyl-CoA inhibition curves for the activity in control and in irradiated membranes that had received 20 Mrad radiation and in which CPT activity had decayed by 60 %. Possible correlations between these data and other recent observations on the CPT system are discussed.

INTRODUCTION

Recent developments in the study of the carnitine palmitoyltransferase system of mitochondria have centred around two aspects: the distinction between the overt (CPT I) and latent (CPT II) forms of the enzymic activity, and the localization of the site of action of malonyl-CoA inhibition of CPT I with respect to the active site of the enzyme. In addressing the first of these questions, Murthy & Pande [1–3] established that CPT I resides in the outer membrane of mitochondria, thus providing an explanation for the observation that the initial digitonin-elicited release of CPT activity from rat liver mitochondria coincides with that of monoamine oxidase [4]. McGarry and co-workers [5,6] used different detergents and antibodies raised against CPT II to demonstrate that this is distinct from CPT I. In addition, they found, in near-agreement with Kiorpes *et al.* [7], that the only protein that bound [^3H]tetradecylglycidyl-CoA (TDG-CoA) in intact liver mitochondria had a molecular mass of about 90 000 Da. On the basis of the observation that malonyl-CoA binding to mitochondria was inhibited by both TDG-CoA and bromopalmitoyl-CoA plus carnitine, these authors inferred that this TDG-CoA-binding protein is both the catalytic entity (CPT I) and the malonyl-CoA-binding species, and, consequently, that malonyl-CoA binding to CPT I occurs at the active site [5]. These authors therefore assigned molecular masses of 94 000 and 80 000 to CPT I and II respectively [5]. The former value represents an indirect assignment, as the catalytic activity of the protein species concerned could not be verified directly. A more direct study of the catalytic entities represented by CPT I and II was adopted in our laboratory by performing radi-

ation-inactivation studies to determine their functional target sizes [8]. We obtained a direct determination of the size of CPT II as approx. 70 000 Da. This was in agreement with the size of the subunit of the easily solubilized mitochondrial CPT as determined by polyacrylamide-gel electrophoresis [5,9,10]. The size of CPT I, however, had to be computed from composite decay curves and two-component equations that included a number of other parameters, such as the size of CPT II (above) and the CPT I/CPT II activity ratio in frozen-thawed mitochondria [8]. Consequently, the accuracy with which the size of CPT I (approx. 96 000 Da) could be determined was necessarily limited. The subsequent demonstration that CPT I resides in the outer membrane has provided a physical means by which CPT I and CPT II activities can be separated. In this paper we report direct size determinations of CPT I in purified outer-membrane preparations.

Another question that we have addressed is whether the apparent functional size of the CPT I catalytic species is the same as, or different from, that of the high-affinity malonyl-CoA binding protein in outer-membrane preparations. The two most recent contributions on this subject have led to conflicting results. In contrast with the conclusions of McGarry and co-workers ([5,6]; see above), Murthy & Pande [1] obtained data that strongly suggest (i) that the malonyl-CoA binding site and the CPT I catalytic site reside on opposite sides of the outer membrane and (ii) that the malonyl-CoA and TDG-CoA binding sites are separate, as indicated by the differential effects of protease treatment of intact mitochondria on the efficacy of action of the two ligands [1].

In spite of attempts in several laboratories, the purification of the separate or combined CPT I catalytic

and malonyl-CoA-binding proteins has proved difficult to accomplish unequivocally, probably because of the highly hydrophobic properties of this protein(s). Radiation-inactivation analysis would therefore appear to be a suitable technique with which to try to resolve the above questions by providing information about functional sizes *in situ* without the need for isolation of the protein(s) from the membrane.

MATERIALS AND METHODS

Animals

These were male Wistar rats (250–400 g) that were starved for 48 h before being killed.

Preparation of outer membrane from liver mitochondria

Preliminary attempts at obtaining an outer-membrane fraction from mitochondria prepared by differential centrifugation indicated that substantial contamination of outer membrane with peroxisomes and inner membrane persisted even after sucrose-density-gradient purification of crude outer membranes [11], as judged by marker enzyme determinations (uricase and catalase for peroxisomes; cytochrome *c* oxidase and succinate dehydrogenase for inner membrane). Consequently, it was necessary to alter the standard methods (i) to purify further the mitochondria from peroxisomal contamination, and (ii) to minimize the extent of inner-membrane contamination after sucrose-gradient centrifugation. Briefly, the method that was chosen for routine use was as follows. Mitochondria were isolated in a medium containing 300 mM-sucrose, 5 mM-Tris/HCl and 1 mM-EGTA (pH 7.4 at 0 °C) by differential centrifugation as described previously [8]. They were then further purified by resuspension in Percoll medium (final concn. of Percoll 31% in sucrose medium) and centrifuged at 20000 rev./min for 20 min in a Sorvall Combi ultracentrifuge, with an A841 rotor. This procedure gave mitochondria that were essentially free of endoplasmic reticulum and peroxisomes (see also ref. [12]). Density-bead studies indicated that the mitochondria were recovered in fraction of density 1.068–1.098. The mitochondria were washed free of Percoll in extraction buffer and were then resuspended in hypo-osmotic medium containing 20 mM-potassium phosphate (pH 7.0) and 0.2% fatty-acid-free albumin. (The concentration of albumin used was 10-fold higher than used previously [1,11] and was found to protect against inner-membrane contamination on resuspension.) Where indicated, proteolysis inhibitors (leupeptin, antipain, pepstatin at 1 µg/ml) were included. After 20 min on ice, 2 mM-ATP-Mg was added and the incubation was continued at 0 °C for a further 5 min. The mitochondria were sedimented at 70000 *g* for 20 min and resuspended with a loosely fitting Teflon/glass homogenizer. The 2500 *g* supernatant of the resulting suspension was used as the source of outer membranes. In order to dissociate cytochrome *c* from cytochrome *c* oxidase present in inner-membrane contaminants [13], this fraction was made 0.15 M with respect to KCl, and crude outer membranes were sedimented at 100000 *g* for 30 min. The pellet was resuspended in 0.15 M-KCl again (thus providing a second KCl wash [13]) and layered on top of a sucrose step gradient and centrifuged as described previously [11]. The membranes were collected from the interface between the 1.0 M- and 0.77 M-sucrose fractions

and diluted with water to produce a sucrose concentration of 0.25 M. Inner-membrane contamination was largely (80–95%) removed by batch incubation with freshly prepared Sepharose–cytochrome *c* [13] at 25 °C for 20 min. The Sepharose was removed by light centrifugation (100 *g* for 10 min) and the membranes were sedimented at 100000 *g* for 30 min. The membrane pellet was finally resuspended in medium containing 150 mM-KCl, 5 mM-Tris, 5 mM-EGTA and 10 mM-dithiothreitol (pH 7.4 at 0 °C) at a concentration of 2–3 mg of protein/ml. Membranes prepared by this protocol routinely had an enrichment of monoamine oxidase over cytochrome *c* oxidase and succinate dehydrogenase of approx. 200-fold when compared with whole mitochondria. They had no detectable activity of uricase, catalase or NADP–cytochrome *c* reductase. The yield was 10–20 mg of outer-membrane protein from 70 g of rat liver.

Irradiation of membranes

Samples (0.2 ml) of membrane suspensions were dispensed into glass vials that were sealed under vacuum [8]. Molecular-size standards [glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from *Escherichia coli*, yeast alcohol dehydrogenase (EC 1.1.1.1) and pig heart malate dehydrogenase (EC 1.1.1.37)] were either included with the membranes or irradiated simultaneously in the same medium but in separate vials; preliminary trials suggested that there was no difference in their behaviour between the two conditions. Moreover, it was more convenient to expose the standards in separate adjacent vials, as this gave lower blank values for [¹⁴C]malonyl-CoA binding to the membranes. Irradiation was performed at –120 °C with the 30 MeV linear accelerator at the Kelvin Laboratory (University of Glasgow), East Kilbride, Scotland. The irradiation parameters were as described previously [8]. Amber Perspex dosimeters were used to quantify the dose rate by exposing them to 3 min of radiation delivered at the same rate as that used for the samples. A calibration curve for electron irradiation of the same batch of Perspex was constructed at the National Physical Laboratory (Teddington, Middx., U.K.).

After irradiation, the ampoules were kept at –80 °C for at least 24 h. They were subsequently opened, flushed with N₂, and their contents were rapidly thawed at 35 °C and placed on ice. Assays of CPT activity and [¹⁴C]malonyl-CoA binding were performed within 30 min.

Measurement of CPT activity

CPT activity was measured at 37 °C radiochemically by monitoring the palmitoyl-CoA-dependent incorporation of [³H]carnitine into acyl[³H]carnitine [8]. Two assay conditions were used. For the measurement of the decay in activity with increasing doses of radiation, the high palmitoyl-CoA/albumin ratio conditions described by Saggerson & Carpenter [14] were used to obtain maximal activity of CPT I. The assay medium contained 150 mM-KCl, 5 mM-Tris/HCl, 1 mM-EGTA, 1 mM-dithiothreitol, 0.5 mM-[³H]carnitine (0.36 Ci/mol), 100 µM-palmitoyl-CoA and 1.3 mg of albumin (defatted and dialysed)/ml (pH 7.4 at 37 °C). For measurement of the malonyl-CoA sensitivity of CPT activity, the conditions described by Grantham & Zammit [15] were used so as to provide a direct comparison with data obtained previously on intact mitochondria. The assay medium

was the same as above, except that $133 \mu\text{M}$ -palmitoyl-CoA and 10 mg of albumin/ml were used. Malonyl-CoA was added at the concentrations indicated. In all instances, membranes ($20\text{--}40 \mu\text{g}$ of protein) were incubated with assay medium for 2 min at 37°C and the reactions were started by addition of carnitine; incubations were terminated after a further 2 min by addition of HCl, and acyl[^3H]carnitine was quantified as described previously [16].

Measurement of [^{14}C]malonyl-CoA binding

This was performed by a filtration technique. Samples of membranes ($140 \mu\text{l}$ containing $0.2\text{--}0.3 \text{ mg}$ of protein) were incubated, at 0°C , in binding mixture (0.36 ml) containing 150 mM-KCl , 5 mM-Tris/HCl , 1 mM-EGTA , $1.4 \text{ mM-dithiothreitol}$ ($\text{pH } 7.4$ at 0°C) and the indicated concentrations of [^{14}C]malonyl-CoA (50 Ci/mol). After 20 min at 0°C , the total incubation volume was rapidly filtered under suction (Millipore, $0.2 \mu\text{m}$ pore diameter) and the filters were rapidly washed ($< 5 \text{ s}$) with 2 ml of ice-cold KCl medium. The dry filters were digested in Filtercount (Canberra Packard Ltd., Pangbourne, Berks., U.K.), and the radioactivity associated with them was measured in a liquid-scintillation counter. Values for non-specific binding were obtained (i) by inclusion of [^3H]inulin in the incubation mixture to quantify the amount of residual bulk liquid associated with the filters, and (ii) by inclusion of samples to which $200 \mu\text{M}$ unlabelled malonyl-CoA was added. The latter routinely gave negligible background values, suggesting that specific malonyl-CoA binding was being measured.

Measurement of marker and standard enzyme activities

These were performed by published methods as follows: NADP-cytochrome *c* reductase [17], monoamine oxidase [18], cytochrome *c* oxidase [19], succinate dehydrogenase [20], catalase [21], uricase [22].

Statistical analysis of data

The data obtained on radiation-induced decay in activity and binding (from the numbers of experiments indicated, see legends to Figures) and performed on separate mitochondrial outer-membrane preparations were pooled on the basis of their consistency of performance and individual conclusions. Maximum-likelihood estimations [23] for the radiation-induced decay in CPT I activity and [^{14}C]malonyl-CoA binding, as well as the activity of molecular-size standards, were performed with a modified Newton Algorithm implemented in the NAG Library routine E04KCF [24] on a Prime 750 computer (Scottish Agricultural Statistics Service, Edinburgh, U.K.). Confidence intervals were generated by using the likelihood profile. Data on the effect of irradiation on the sensitivity of CPT activity to malonyl-CoA inhibition were subjected to split-plot analysis of variance that took account of the nested structure of the design of the experiments.

Materials

Biochemicals and proteolysis inhibitors were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). [^3H]Carnitine, [^3H]inulin and [^{14}C]malonyl-CoA were from Amersham International (Aylesbury, Bucks., U.K.). Percoll, CNBr-activated Sepharose 4B and density beads were from Pharmacia. Perspex dosimeters were obtained from the Atomic Energy Research Establishment (Harwell, Oxfordshire, U.K.).

RESULTS AND DISCUSSION

Decay curves for CPT I activity and [^{14}C]malonyl-CoA binding in mitochondrial membranes after irradiation with high-energy electrons are shown in Fig. 1. Also shown are the slopes obtained for the decay in activity of malate dehydrogenase ($70\,000 \text{ Da}$), glucose-6-

Fig. 1. Effect of increasing dose of radiation on CPT activity and malonyl-CoA binding in outer membrane of rat liver mitochondria

Membranes and molecular-size standards were irradiated as described in the Materials and methods section. Values shown are means ($\pm \text{S.E.M.}$) for 4–13 experiments on separate membrane preparations. Six time points were used for each membrane preparation. Radiation dose was computed by multiplying the duration of irradiation by the dose rate delivered as measured by Perspex dosimeters (see the Materials and methods section). To simplify the graphical representation, the data are plotted against duration of exposure only. However, for statistical analysis of the data, the doses actually received by the samples were used and the slopes of the lines shown are those computed from those analyses. Data for binding at $1 \mu\text{M}$ - and $10 \mu\text{M}$ -malonyl-CoA have been combined. The average dose rate was $0.309 \pm 0.005 \text{ Mrad/min}$ ($\pm \text{S.E.M.}$, $n = 13$). (a) Decay of CPT activity (Δ) and of [^{14}C]malonyl-CoA binding ($\blacksquare$); the CPT activity in non-irradiated membranes was $184.7 \pm 8.4 \text{ nmol/min per mg}$ of protein, and [^{14}C]malonyl-CoA bound was 104.6 ± 20.8 and $134.4 \pm 9.8 \text{ pmol/mg}$ at $1 \mu\text{M}$ - and $10 \mu\text{M}$ -malonyl-CoA respectively. (b) Decay of activities of malate dehydrogenase ($\blacktriangle$), glucose-6-phosphate dehydrogenase ($\square$) and alcohol dehydrogenase ($\blacklozenge$).

phosphate dehydrogenase (106 000 Da) and yeast alcohol dehydrogenase (148 000 Da). These molecular-size standards have been shown previously [25] to yield reliable estimates of molecular size in radiation-inactivation studies.

Size of the CPT I catalytic moiety

Absolute radiation dose rates were measured in all experiments such that a direct comparison could be made between the apparent sizes of the standards derived from the equations of target-inactivation theory (and empirically derived constants) and the relative decay characteristics of the standards. When molecular-size estimates were performed by the empirical method using the equation $M_r = S(6.4 \times 10^5)/D_{37}$, where S is a temperature-dependent parameter (2.26 at -120°C [26]) and D_{37} is the radiation dose that decreased the activity to 37% of the original, the sizes obtained for the standards used were consistently low: 128 000 Da for alcohol dehydrogenase, 93 000 for glucose-6-phosphate dehydrogenase and 54 000 for malate dehydrogenase. These values were 15–25% lower than the known molecular masses of these enzymes. Consequently this theoretical approach would have given us unrealistically low values for CPT I and [^{14}C]malonyl-CoA binding activity. Other experimenters, too, have found that they needed to correct for the underestimation of molecular sizes by using the above equations, although usually only one standard was included (see, e.g., [27,28]). We have therefore preferred to use the internal-standard method of Lo *et al.* [29], combined with a much more rigorous statistical analysis of the data (see the Materials and methods section). We combined the results obtained on 13 different preparations of outer membranes. This latter approach has also been used recently by Kempner and co-workers [30].

The value for the size of CPT I obtained from the above studies was 83 000 ($n = 13$). The lower and upper confidence limits (95%) were given by 73 000 and 91 000 respectively. This value was considerably smaller than our earlier estimate. We consider it to be, however, a more reliable one, as it was obtained by direct determinations on pure outer membranes. Our previous estimate was obtained by mathematical analysis of two-component curves and depended on the accurate determination of the CPT I/CPT II activity ratio in frozen-thawed mitochondria. Because of the difference between the present and previous estimates, we explored the possibility that, during the preparation of outer membranes, CPT I was being subjected to proteolytic degradation, thus resulting in a lower apparent molecular size than that observed in whole mitochondria. The most likely step in the preparation of outer membranes during which proteolysis could be expected to occur would be the hypo-osmotic swelling in phosphate buffer. We considered two possible proteolytic sources that could be unmasked at this step: (i) lysosomal contamination and (ii) mitochondrial-matrix protease(s) involved in the processing of non-outer-membrane proteins to their mature forms [31]. Therefore we conducted studies in which outer membranes were prepared from the same batch of mitochondria swollen in the presence or absence of proteolysis inhibitors known to be effective either against lysosomal proteases (see the Materials and methods section) or the metal-ion-dependent matrix protease (which is inhibited by *o*-phenanthroline). The

data obtained from paired analyses indicated that there was no difference in the apparent size of CPT I in the two types of membrane preparation (results not shown). Therefore we conclude that the size that we observed for CPT I activity was not due to proteolytic degradation of the protein.

In the course of these experiments, we observed that inclusion of proteolysis inhibitors (but not *o*-phenanthroline) during the hypo-osmotic swelling stage resulted in a marked decrease (about 80%) in the subsequent yield of outer-membrane protein. This observation suggested that a certain degree of proteolysis may be required to detach outer membrane from the inner-membrane/matrix fraction, possibly through proteolytic action on some component(s) of the contact sites [32] between the two membranes. These sites are known to be enriched in specific proteins [33]. It is possible that the small amount of residual lysosomal contamination that remained after Percoll-gradient purification of the mitochondria was sufficient to provide the necessary proteolytic activity.

Size of [^{14}C]malonyl-CoA-binding protein

The size of the malonyl-CoA-binding protein in outer membranes of rat liver mitochondria was determined in the same preparations used for CPT I size determination, such that paired observations were obtained. In all of the 13 preparations used, the malonyl-CoA-binding capacity decayed at a lower rate with increasing radiation dose than did CPT I activity. The estimate for the size of the protein responsible for [^{14}C]malonyl-CoA binding was 60 000. The upper and lower confidence limits (95%) were given by 52 000 and 67 000 respectively. This value

Fig. 2. Representative Scatchard plot of [^{14}C]malonyl-CoA binding to liver mitochondrial outer membranes obtained from 48 h-starved male rats

is statistically significantly different from that for CPT I (above) ($P < 0.01$). There was no significant difference in the rate of decay of [^{14}C]malonyl-CoA binding whether it was measured at $1\ \mu\text{M}$ - or $10\ \mu\text{M}$ -malonyl-CoA. It was important to test both concentrations, as Scatchard plots for malonyl-CoA binding to outer-membrane preparations indicated that, although the low-affinity component observed in intact mitochondria [34,35] was much diminished relative to high-affinity binding, it nevertheless persisted to some extent (Fig. 2). As can be ascertained from Fig. 2, the high-affinity component of the binding accounts for 90–95% of total binding at $1\ \mu\text{M}$ -malonyl-CoA and about 75% at $10\ \mu\text{M}$ -malonyl-CoA.

The simplest explanation of these results is that the malonyl-CoA-binding protein is smaller than that which catalyses the CPT I reaction, thus implying that these functions reside in different molecules. It is possible, of course, that this difference may arise through some artefact of the method used, although we made both determinations on the same outer-membrane preparations and we took the precautions described above. Such artefacts do arise with certain proteins during radiation inactivation (see, e.g., [36]), although, if this were the case, it would have been expected to apply equally to both CPT I activity and malonyl-CoA binding if these reside in the same molecule.

Effect of irradiation on sensitivity of CPT I to malonyl-CoA

In an attempt to verify functionally the difference between the sizes of the CPT I catalytic entity and that for malonyl-CoA binding, we studied the sensitivity of CPT I activity to malonyl-CoA inhibition before and after irradiation in four separate outer-membrane preparations. The rationale behind these experiments was that, if these two functions reside in two separate proteins that are destroyed at different rates, and if inhibition depends on interaction of these two putative proteins, then their differential decay should be reflected in a change in the malonyl-CoA sensitivity of CPT I. In Fig. 3 the sensitivity of CPT I activity to malonyl-CoA inhibition in membranes that were not irradiated is compared with that in membranes that received approx. 20 Mrad of radiation. The activity of CPT I in the latter determined in the absence of malonyl-CoA was diminished to about 40% of that in unexposed membranes. In all four experiments the CPT I activity appeared to be more sensitive to malonyl-CoA after irradiation. Split-plot analysis of variance indicated that the malonyl-CoA inhibition characteristics displayed by the two curves were highly significantly different ($P < 0.001$). This effect of radiation on CPT I sensitivity to malonyl-CoA inhibition occurred in the direction (an increase) that would be expected if a putative separate malonyl-CoA-binding protein was destroyed less extensively than the catalytic protein for a given dose of radiation (i.e. represents a smaller target).

Conclusions

The need for caution in the interpretation of data obtained from target-size analysis by radiation inactivation is widely acknowledged (see, e.g., [36]), particularly as it relates to membrane proteins. It is with this qualification that the present results may be interpreted to indicate that CPT I catalytic and malonyl-CoA-

Fig. 3. Effect of exposure to high-energy radiation of mitochondrial outer membranes on the sensitivity of CPT I to malonyl-CoA inhibition

Mitochondrial outer membranes isolated from 48 h-starved male rats were assayed for CPT I activity at increasing malonyl-CoA concentrations either before (○) or after (●) irradiation (20 Mrad). The activity of CPT in the absence of malonyl-CoA (i.e. 100%) was decreased by approx. 60% in the irradiated membranes. Under the same conditions malonyl-CoA binding would have been decreased by only approx. 45%. Values are means ($\pm$ S.E.M.) for four experiments performed on separate membrane preparations. The data were subjected to split-plot analysis of variance, which resulted in an F statistic, for the effect of irradiation, of 15.2 on 1 (12 degrees of freedom). This was significant at the $P < 0.001$ level.

binding activities reside in differently sized protein entities within the outer membrane of rat liver mitochondria. The size for CPT I obtained by direct measurement (the present study) is considerably smaller than that obtained previously by computation from data relating to whole mitochondria [8]. Nevertheless, the radiation-induced decay characteristics of malonyl-CoA binding were those of an even smaller molecule (approx. 60 000 Da). The effects of a putative differential decay between the two parameters appeared to be accompanied by increased sensitivity of CPT I to malonyl-CoA inhibition in irradiated membranes. These results will again raise the question as to whether malonyl-CoA acts on CPT I via a regulatory subunit. DeClercq *et al.* [5] presented evidence that both TDG-CoA and the *S*-carboxypalmitoyl-CoA ester of carnitine prevent malonyl-CoA binding. Consequently, they concluded that all these ligands bind at the same (catalytic) site on CPT I. The possibility exists, however, that the effects of the inhibitors are mediated allosterically between separate malonyl-CoA-binding and palmitoyl-CoA sites. Murthy & Pande [1] presented data which suggest (i) that the active site of CPT I and the malonyl-CoA-binding site

reside on opposite sides of the mitochondrial outer membrane, and (ii) that TDG-CoA and malonyl-CoA effects on CPT I are differentially affected by treatment of isolated mitochondria with Nagarse. Other, less direct, evidence also points to the possible existence of a regulatory subunit, e.g. the number of high-affinity binding sites for malonyl-CoA does not vary in parallel with the catalytic activity of CPT I in mitochondria from livers of rats in different physiological states [35]. Moreover, Lund [37] claimed to have separated the catalytic activity of CPT I from malonyl-CoA-binding activity, although a different interpretation was put on these data by DeClercq *et al.* [5]. However, it is to be appreciated that in all studies in which malonyl-CoA binding is studied, it is possible that any malonyl-CoA-binding protein unrelated to the CPT system could interfere with the data and their interpretation.

It is instructive to relate the above size estimates (and that obtained for CPT II [8]) with the three different current lines of evidence about the CPT system. It has been suggested that CPT I and CPT II have the same molecular mass and may be identical proteins [9,38]. Our previous data [8] tend to suggest that, whereas the size of CPT II is approx. 70 000 Da when measured directly by radiation inactivation *in situ*, that of CPT I is larger. That conclusion remains valid even though accurate estimation of CPT I size by indirect computation was not possible in those experiments. The present results have confirmed that CPT I is larger than CPT II. The second possibility is that CPT I is identical with the protein that covalently binds ^{14}C - or ^3H -labelled TDG-CoA. Kiorpes *et al.* [7] first indicated that this molecule has a subunit molecular mass of 90 000 Da in rat liver mitochondria. Subsequently the data of DeClercq and colleagues [5,6] suggested that it could be as large as 94 000 Da in liver mitochondria, but that it could be smaller (approx. 86 000 Da) in mitochondria from other tissues. Whether this variability is determined by abnormal behaviour of the protein in polyacrylamide gels, or whether there are genuine differences, remains to be determined by further comparative studies. It is important to appreciate, however, that in all these studies the labelled protein was covalently modified with a relatively bulky ligand before electrophoresis and that this may have affected its behaviour in polyacrylamide gels. Most recently, Murthy & Pande [3] purified CPT I from rat liver mitochondrial outer membranes. After several stages of purification the protein was still malonyl-CoA-sensitive. On SDS/polyacrylamide-gel electrophoresis, this malonyl-CoA-sensitive preparation gave two protein bands, one at a position corresponding to M_r 77 000 and the other to M_r 61 000. It is intriguing to speculate whether these may correspond to the sizes of CPT I and malonyl-CoA-binding entities as determined in the present study. For this to be the case, the two proteins would need to have been intimately associated throughout solubilization and partial purification performed by Murthy & Pande [3]. Such an association between proteins is not implausible (see, e.g., [39]). Furthermore, it is noteworthy that polyclonal antibody raised against an easily solubilized CPT (69 000 Da) precipitated three other proteins from hepatocyte preparations (83 000, 77 000, 67 000 Da) in the studies of Harano *et al.* [40]. These authors suggested that this co-precipitation could arise through close protein association in the original membranes. If the 69 000 Da protein against which the antibody raised by Harano

et al. [40] was CPT II (see [5,6,8]), co-precipitation of CPT I could occur if the two proteins share at least some antigenic determinants, as would arise from some degree of homology between their amino acid sequences.

Note added in proof (received 25 July 1989)

Since this paper was accepted, we have explored the possibility that a putative regulatory subunit might interact sufficiently strongly with CPT I in the presence of malonyl-CoA such that the resulting complex would present an effectively larger target. We irradiated membranes that had been incubated for 2 min at 37 °C in either the presence or the absence of 30 μM -malonyl-CoA, immediately before being frozen. The size of CPT I computed from the respective decay curves was the same for the two sets of membranes. These results do not provide evidence in favour of a separate regulatory subunit; the most that can be stated is that, if a separate subunit does exist, its malonyl-CoA-induced interaction with CPT I is not of the type that allows sufficient energy transfer to occur between the two proteins for them to behave as a single target.

This work was supported, in part, by the British Diabetic Association. We thank Mr. M. Kelliher for operation of the linear accelerator at the Kelvin Laboratory and Dr. I. Bradbury for the statistical analyses.

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Received 5 April 1989/6 June 1989; accepted 13 June 1989