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TOPOLOGY OF HEPATIC MITOCHONDRIAL CARNITINE PALMITOYLTRANSFERASE I

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1. INTRODUCTION

Before long-chain fatty acids can enter the mitochondria and get access to the β-oxidation pathway, they must first be activated to acyl-CoA in a reaction that requires ATP and coenzyme-A. The acyl-CoA still cannot cross the mitochondrial inner membrane and must react with carnitine to form the corresponding carnitine ester. This reaction is catalyzed by the enzyme carnitine palmitoyltransferase (CPT).[1] The acylcarnitine itself is also unable to diffuse into the mitochondrial matrix so that the transport is achieved by a specific protein, the carnitine acylcarnitine translocase.[2] Following transport across the mitochondrial inner membrane, acylcarnitines are converted back to the corresponding acyl-CoA and carnitine. This reaction is catalyzed by another carnitine palmitoyltransferase which is a different enzyme than that involved in the formation of the acylcarnitine outside the mitochondria.[1] Hence, there are two CPTs, one associated with the inner aspect of the mitochondrial inner membrane, CPT-II[3–7] and one that lies

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outside the mitochondrial inner membrane barrier, CPT-I. This overt or outer CPT is of physiological importance since it is regulated through inhibition by malonyl-CoA, an intermediate in the biosynthetic pathway of fatty acids.[8–12] Also of major importance in the hepatic regulation of the overt CPT are changes in the activity and sensitivity to inhibition by malonyl-CoA in changing physiological and pathophysiological states such as starvation and diabetes.[12–16] Quantitatively, the most significant change that occurs with the onset of diabetes[15] or the feeding-starvation transition[17] is at least a 10-fold increase in the apparent K_i for malonyl-CoA.

1.1. Location of CPT-I

The exact location of CPT-I has been the subject of much debate. Until recently its location was thought to be on the outer surface of the mitochondrial inner membrane.[18] However, data presented by Murthy and Pande[7] suggested that CPT-I is associated with the mitochondrial outer membrane. Using limited proteolysis of intact mitochondria and isolated mitochondrial outer membranes, their data indicated that the protease Nagarse had little effect on CPT activity but that the inhibitory effects of malonyl-CoA were greatly reduced. These data suggested that the outer CPT, (CPT-I) has a malonyl-CoA binding site facing the cytosol and an active acyl-CoA site facing the intermembrane space, clearly suggesting two separate binding sites. They also reported that other proteases such as trypsin and papain had essentially the same effects but we reported that the effects of trypsin were somewhat different from the actions of the other two proteases,[19] and we presented an explanation for those differences.[20]

1.2. Do all Inhibitors of CPT-I Act at a Common site?

The relationship between acyl-CoA and malonyl-CoA binding sites has also been subject to debate. It is not clearly established whether they bind at the same site or even whether they bind to the same polypeptide.[21–25] Data presented by Murthy and Pande suggests two different binding sites. The suggestion that all inhibitors of CPT-I act at a common site did not seem logical to us since many of these inhibitors have very different structures and mechanisms of inhibition. For example, malonyl-CoA is a competitive inhibitor with respect to acyl-CoA and noncompetitive with respect to carnitine,[17,26] 4-hydroxyphenylglyoxylate (HPG), the active metabolite of oxfenicine, is a competitive inhibitor with respect to carnitine but noncompetitive with respect to acyl-CoA.[27] N-Benzyladriamycin-14-valerate (AD 198), an analogue of adriamycin, is a noncompetitive inhibitor with respect to carnitine but uncompetitive with respect to acyl-CoA[28] while glyburide is noncompetitive with respect to acyl-CoA but uncompetitive with respect to carnitine.[26] DL-2-Bromopalmitoyl-CoA was synthesized as a substrate analogue,[29] and is expected to bind to the active site. One of the first studies we did was to examine the effects on the inhibitory actions of these different chemical compounds that are produced by exposure of intact mitochondria to three different proteases—Nagarse, papain and trypsin—with the hope of learning whether they bind inside or outside the mitochondrial outer membrane. As indicated in Table 1, exposure of intact mitochondria to Nagarse at 37°C for 20min had relatively slight effects on activity of the outer CPT (13% and 30% reduction with 5 and 10 μg/mL Nagarse, respectively), but the inhibitory effects of malonyl-CoA were reduced to a greater extent (60% or greater loss of inhibition in all experiments). Experiments conducted with papain gave almost identical results to those with Nagarse when higher concentrations of papain were used (Table 1). Effects of

Table 1. Effects of Protease Treatment of Intact Mitochondria on Carnitine Palmitoyltransferase Activity and its Inhibition by Malonyl-CoA. Intact mitochondria were incubated with proteases at the concentrations indicated and then assayed for outer CPT activity using 40 μM palmitoyl-CoA and 0.5 mM carnitine. Results are means ± S.E.M. of 3–10 different preparations. Where no S.E.M. is indicated, results are means of 2 separate experiments with different preparations of mitochondria. Percentage inhibition by malonyl-CoA is indicated in parentheses. Abbreviation: n.d., not determined. Data are reproduced with permission from ref. 20.

		Carnitine Palmitoyltransferase Activity (nmol/min/mg protein)		
Protease, μg/ml		Control	+50 μM malonyl-CoA	+100 μM malonyl-CoA
None		9.4 ± 0.9	3.8 ± 0.8 (62 ± 5)	2.0 ± 0.6 (80 ± 4)
Nagarse,	5	8.2 ± 1.4	6.4 ± 1.0 (21 ± 1†)	5.5 ± 0.9 (32 ± 1†)
	10	6.6 ± 0.7*	5.2 ± 0.8 (22 ± 3†)	4.7 ± 0.6 (29 ± 2†)
Papain,	20	8.2 ± 0.4	6.0 ± 0.4 (26 ± 1†)	5.5 ± 0.5 (34 ± 1†)
	40	5.7 ± 0.5*	4.4 ± 0.3 (23 ± 1†)	4.0 ± 0.3 (31 ± 1†)
Trypsin,	5	7.0 ± 0.3*	2.6 ± 0.5 (63 ± 6)	1.0 ± 0.2 (83 ± 2)
	10	6.3 ± 0.2†	2.0 ± 0.3 (69 ± 3)	0.9 ± 0.2 (86 ± 3)
	25	5.2 ± 0.3†	1.5 ± 0.2 (73 ± 4)	0.8 ± 0.1 (84 ± 1)
	100	3.7†	1.4 (62)	n.d.
	200	2.2 ± 0.5†	0.7 ± 0.6 (66 ± 4)	0.6 ± 0.2 (79 ± 5)

*P < 0.1; †P < 0.05; ‡P < 0.001 (compared to control, Student's *t*-test)

trypsin on CPT activity and inhibition by malonyl-CoA are also given in Table 1. Exposure to increasing concentrations of trypsin decreased the activity of CPT in a concentration-dependent manner. However, in contrast to the suggestions of Murthy and Pande,[7] the inhibitory effects of malonyl-CoA were not decreased as a result of exposure to trypsin, in fact, virtually identical inhibition values were obtained at all concentrations of trypsin examined for both 50 μM and 100 μM malonyl-CoA, even though up to 80% of the activity was lost.

Table 2 shows the inhibitory effects on outer CPT activity of five different inhibitors before and after exposure to Nagarse, papain and trypsin. Inhibition by malonyl-CoA was greatly reduced by protease treatment and inhibition by HPG was affected to an equivalent extent. However, trypsin had no effect on inhibition by either malonyl-CoA or HPG. These results are especially interesting in view of the fact that HPG is the only inhibitor other than malonyl-CoA that undergoes a major change in its inhibitory potency by alterations in the physiological state of the animal.[30] Inhibition of CPT by AD 198 and DL-2-bromopalmitoyl-CoA were not affected by prior treatment of the mitochondria with any concentration of Nagarse, papain or trypsin that we evaluated. Inhibition of CPT by glyburide was altered only slightly by the highest concentration of trypsin used in Table 2 and was not affected by either Nagarse or papain. These data indicate that neither AD 198, glyburide nor 2-bromopalmitoyl-CoA bind at the same site as malonyl-CoA.

There are several other natural endobiotic compounds such as CoA, acetyl-CoA, succinyl-CoA, methylmalonyl-CoA and propionyl-CoA that are inhibitors of CPT-I and are structurally similar to malonyl-CoA.[21] Therefore, it was of interest to find out whether the inhibitory effects of these compounds were also affected by proteolysis.[31] As reported by Murthy and Pande[7] and us,[19,20] the inhibitory effects of malonyl-CoA were greatly reduced as a result of exposure of the intact hepatic mitochondria to Nagarse. The

Table 2. Inhibition of Carnitine Palmitoyltransferase by Several Known Inhibitors in Control and Protease-Treated Mitochondria. Intact mitochondria were assayed for outer CPT activity in presence and absence of different inhibitors in control and protease-treated mitochondria. All inhibitors were present throughout a 5 min preincubation with palmitoyl-CoA and a 5 min assay that was initiated by adding carnitine. Results are means ± S.E.M. of 3 different preparations, where no standard error is indicated, results are means of 2 separate experiments with different mitochondrial preparations. For specific activity of CPT see Table 1. Data are reproduced with permission from ref 20.

	Percent Inhibition					
Inhibitor	No Protease	Nagarse (5 μg/ml)	Trypsin (5 μg/ml)	Trypsin (10 μg/ml)	Trypsin (25 μg/ml)	Papain (20 μg/ml)
Malonyl-CoA (100 μM)	80 ± 4	32 ± 1†	83 ± 2	86 ± 3	85 ± 1	36 ± 1†
HPG (5 mM)	86 ± 4	6 ± 4†	85 ± 2	83 ± 1	84 ± 1	23†
AD 198 (300 μM)	92 ± 1	93 ± 1	93 ± 2	91 ± 3	96 ± 2	92 ± 1
Glyburide (200 μM)	82 ± 1	80 ± 2	81 ± 3	80 ± 3	67 ± 3	83 ± 1
2-Bromopalmitoyl-CoA (1 μM)*	84 ± 2	76 ± 5	79 ± 3	83 ± 3	86 ± 1	81

*When 2-Bromopalmitoyl-CoA was preincubated with mitochondria in the presence of 0.5 mM L-carnitine, inhibition was 100% in all cases.
†$P < 0.001$ (compared to control, Student's t-test)

inhibitory effects of succinyl-CoA and methylmalonyl-CoA were likewise reduced as a result of Nagarse treatment. However, Nagarse had no effect on the inhibitory actions of CoA or acetyl-CoA or propionyl-CoA. There were no differences between inhibition obtained with acetyl-CoA and propionyl-CoA.[31] These data suggest that inhibitors that are CoA esters of short chain dicarboxylic acids bind identically to malonyl-CoA and the others (monocarboxylic acid esters of CoA and free CoA) act at the acyl-CoA binding site. The order of potency of these inhibitors was: malonyl-CoA >> succinyl-CoA ≥ CoA > methylmalonyl-CoA > acetyl-CoA = propionyl-CoA. Therefore, malonyl-CoA cannot be considered as the only physiological inhibitor of CPT-I, though it is clearly the most potent.

In order to further discriminate the exact orientation of the malonyl-CoA and acyl-CoA binding sites with respect to the mitochondrial outer membrane, we[32] made use of (+)-hemipalmitoylcarnitinium which is a substrate analogue for CPT[33] and Ro 25-0187 which is a malonyl-CoA analogue. Using isolated mitochondrial outer membranes we confirmed that (+)-hemipalmitoylcarnitinium is a potent inhibitor of CPT-I ($I_{50} = 20\,\mu M$). Nagarse treatment of the outer membranes caused a 20% loss of CPT-I activity (data not shown), but there was no change in the sensitivity of CPT-I to inhibition by this active site-directed inhibitor. The malonyl-CoA analog Ro 25-0187 is also a potent inhibitor of CPT-I ($I_{50} = 0.2\,\mu M$) and in contrast to results with the substrate analogue, Nagarse treatment of the outer membranes resulted in complete loss of CPT-I sensitivity to inhibition. These results are very important because: (i) they confirm that the outer CPT of isolated mitochondrial outer membranes behaves exactly as the overt CPT of intact mitochondria; (ii) CPT-I located on the mitochondrial outer membrane has its malonyl-CoA binding site facing the cytosol and its active site is protected within the mitochondrial outer membrane; (iii) these data confirm that the malonyl-CoA site and the active site are entirely different; and finally (iv) since there is no remaining inhibition by Ro 25-0187 after Nagarse treatment and since there is no difference in inhibition by (+)-hemipalmitoylcarnitinium before and after Nagarse treatment, there seems to be no interaction of either of these inhibitors with an alternate site.

1.3. Protection of CPT-I from Proteolysis and its Implications

We have reported that it is possible to protect CPT against the effects of proteolysis by preincubating the intact mitochondria with malonyl-CoA or HPG before protease treatment, and that preincubation with the other inhibitors does not have any protective effects.[19,20] We have also performed experiments in order to ascertain whether it was possible to protect against proteolytic actions of Nagarse by preincubating the mitochondria with natural endobiotic compounds such as CoA, acetyl-CoA, succinyl-CoA, methylmalonyl-CoA and propionyl-CoA inhibitors or with palmitoyl-CoA and carnitine the other natural substrates of CPT-I.[31] Data presented in Fig. 1 shows that succinyl-CoA and methylmalonyl-CoA protect against the actions of Nagarse but not to the same extent as malonyl-CoA; however, propionyl-CoA offered no protection and CoA and acetyl-CoA not only did not protect but, in fact, enhanced the proteolytic actions of Nagarse. Preincubation of CPT with carnitine gave no protection against the actions of Nagarse but preincubation with palmitoyl-CoA enhanced proteolysis in a similar manner to CoA and acetyl-CoA. These data offer additional proof that malonyl-CoA, succinyl-CoA, and methylmalonyl-CoA bind to a site that is distinct from the substrate binding site to alter proteolytic effects and that acetyl-CoA and propionyl-CoA bind, as CoA does, to the active site. Substrate of the CPT reaction cause a probable conformational change in the enzyme that results in increased proteolytic effects.

1.4. Kinetic Analysis of CPT-I Inhibition Using Pairs of Inhibitors

On the basis of competitive binding alone, it has been concluded that all analogues of malonyl-CoA bind to the same site.[21] Since our proteolysis data did not support the conclusion that all inhibitors of CPT-I act at a common site, we performed additional studies in which pairs of CPT-I inhibitors were analyzed.[32] The theory behind double inhibitor studies has been developed by Yonetani and Theorell.[34] In their studies two

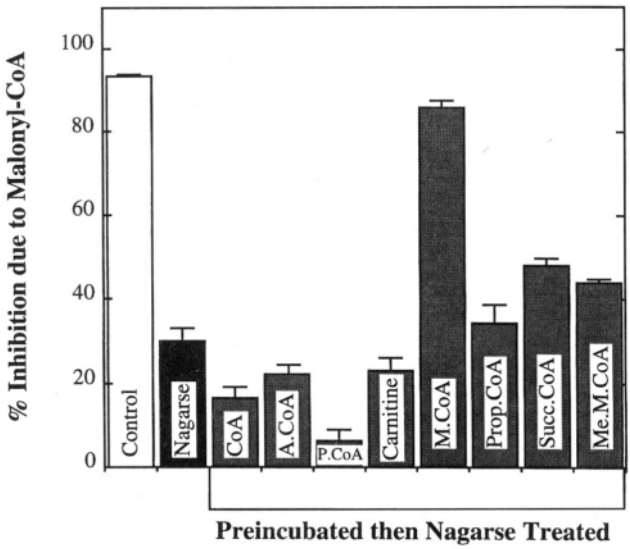

Figure 1. Protection Against Actions of Nagarse by Inhibitors and Substrates of carnitine palmitoyltransferase I.

inhibitors were present at the same time, but the concentration of one inhibitor was kept constant while the concentration of the second inhibitor was varied. From the shape of a plot of 1/activity *vs* the inhibitor concentration, it is possible to deduce whether the two inhibitors compete for the same site (the two lines are parallel), or bind at two independent sites (the two lines converge). We used this procedure to study the effects of CoA with malonyl-CoA and HPG with malonyl-CoA in control and Nagarse treated mitochondria.[32] Using a system in which the concentration of malonyl-CoA was varied but the concentration of CoA was kept fixed at 50 μM, for the control system it was observed that as the concentration of malonyl-CoA was varied at a fixed concentration of CoA, the plots converged consistent with two inhibitors binding at two different sites.[34] This suggests that malonyl-CoA binds at a specific malonyl-CoA binding site (or with a malonyl-CoA binding protein) that is quite different from the active (acyl-CoA binding) site, while CoA inhibits CPT activity by binding at the acyl-CoA binding site. However, after Nagarse treatment, this plot gave two parallel lines indicating that both inhibitors were now binding to the same site. The suggestion here is that Nagarse eliminates to a large extent the malonyl-CoA binding site (or the malonyl-CoA binding protein) so that both inhibitors must now be acting at one common site. Similar plots were obtained when the concentration of malonyl-CoA was fixed and the concentration of CoA was varied.[32]

With HPG a different picture emerged. The plots of 1/v as a function of HPG or malonyl-CoA showed parallel lines in agreement with two inhibitors competing for one site.[34] After Nagarse treatment, these plots were also in accord with two inhibitors binding at the same site.

Recent data of Fraser, Corstorphine, and Zammit[35] using trypsin and proteinase K treatment of intact mitochondria and outer-membrane-ruptured mitochondria suggest that both the active site and the malonyl-CoA binding site are exposed on the cytosolic side of the membrane and that CPT-I has two transmembrane domains. Thus, we have conducted additional studies of the topology of CPT-I using intact hepatic mitochondria and isolated mitochondrial outer membranes with Nagarse (subtilisin BPN'), papain, and trypsin using a variety of incubation conditions.

2. MATERIALS AND METHODS

Male Sprague-Dawley rats (180–240g), obtained from Harlan Industries, Inc. (Indianapolis, IN, U.S.A.), were fed Purina Rat Chow (Ralston Purina Co., Richmond, IN, U.S.A.) and water *ad libitum.* On the day of the experiment, rats were killed by decapitation and their livers were removed rapidly for preparation of mitochondria.

Intact mitochondria were isolated by the method of Johnson and Lardy[36] with the modifications previously published.[17] Mitochondrial outer membranes were isolated by the method of Parsons *et al.*[37] and their purity assessed as described previously.[16] Protease treatment of intact mitochondria and protection experiments were carried out as described previously.[19,20] Briefly, this method consisted of incubating the mitochondria (5mg/ml), and outer membranes (1 mg/mL) with Nagarse (5 μg/mL) at 37°C for 10min after which the proteolytic activity was stopped by addition of 200 μl of 20% (w/v) BSA/mL of incubation volume plus 40mL of ice-cold isolation medium. After centrifugation (5,600 *xg* for 10min), the mitochondria were resuspended (4mg/mL) in isolation medium and used as indicated. Protein determination was by a biuret method.[38] In some experiments intact mitochondria or the isolated outer membranes were first incubated

with malonyl-CoA at the concentrations indicated at 37°C for 5min prior to protease treatment. In those experiments the inhibitors were present during protease treatment, but were removed by the washing procedure.

Carnitine palmitoyltransferase was assayed using the method of Bremer[11] as modified and reported previously.[14] Each assay contained, in a total volume of 1 mL:82mM sucrose, 70mM KC1, 70mM imidazole, 1µg antimycin A, and 2mg bovine serum albumin. For assaying the outer CPT each assay also contained 0.5 mM L-carnitine (0.4 mCi of L-[methyl-^{3}H] carnitine) and 40µM palmitoyl-CoA. Inhibitors were added at the concentrations indicated in legends to tables and figures. Adenylate kinase was assayed using the procedure of Bergmeyer[39] as modified by Janski and Cornell.[40]

Palmitoyl-CoA, imidazole, L-carnitine hydrochloride, EDTA, acetyl-CoA, ATP, AMP, KC1, NADH, $MgCl_2$, oxaloacetate, phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, 5,5'-dithiobis-(2-nitrobenzoic acid), essentially fatty acid-free bovine serum albumin, malonyl-CoA, Nagarse (subtilisin BPN', P 4789), papain (P 4762) and trypsin (T 0134) were purchased from Sigma(St. Louis, MO, U.S.A.). Catalogue numbers are given in parentheses following each protease. L-[methyl-^{3}H]Carnitine hydrochloride was obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.). Hydroxy-phenylglyoxylate was a gift from Pfizer (Sandwich, Kent, U.K.). Ro 25-0187 was a gift from Dr. Guy Heathers (Hoffman-LaRoche, Nutley, NJ, USA) and (+)-Hemipalmitoylcarnitinium was a gift from Dr. Richard D. Gandour (Virginia Polytechnic Institute and State University, Blacksburg, VA, USA).

3. RESULTS AND DISCUSSION

Figure 2 shows the effects of Nagarse on CPT-I activity and inhibition by malonyl-CoA as a function of incubation time. As shown, incubation of intact mitochondria up to 30 minutes in absence of Nagarse has no significant effect on CPT-I activity or malonyl-CoA inhibition. However, when the mitochondria is treated with 5µg/mL of Naragse, both CPT-I activity and malonyl-CoA inhibition decrease as a function of incubation time. As reported earlier,[19,20] treatment of intact mitochondria with 5µg/mL of Nagarse up to 10min at 37°C has little effect on CPT-I activity but the magnitude of malonyl-CoA inhibition is significantly reduced. These data suggest that the malonyl-CoA binding domain is exposed to Nagarse on the cytosolic side of the outer membrane, while the acyl-CoA binding domain is not, that is, it is facing the inter membrane space. However, when the incubation time is increased, CPT-I activity decreases even further and by 30min it has decreased by about 60%. Malonyl-CoA has no inhibitory effects after 30min of incubating the intact mitochondria with Nagrase at this concentration.

The fact that CPT-I activity is now decreased by such a large amount suggests that either the acyl-CoA binding domain is facing the cytosol and it needed more time for the protease to act upon it, or that the outer membrane is ruptured allowing exposure of the acyl-CoA binding domain to the protease. These observations also suggest that proteolysis of the intact mitochondria with Nagarse is affecting the apparent K_i for malonyl-CoA inhibition. To address this question, Dixon plots[41] were constructed as reported previously.[42] The data presented in Fig. 3 shows that exposure of intact mitochondria to Nagarse increases the apparent K_i values for malonyl-CoA. This is an important observation since changes in the apparent K_i values for malonyl-CoA inhibition have also been reported in such states as starvation[13] and diabetes.[14,15]

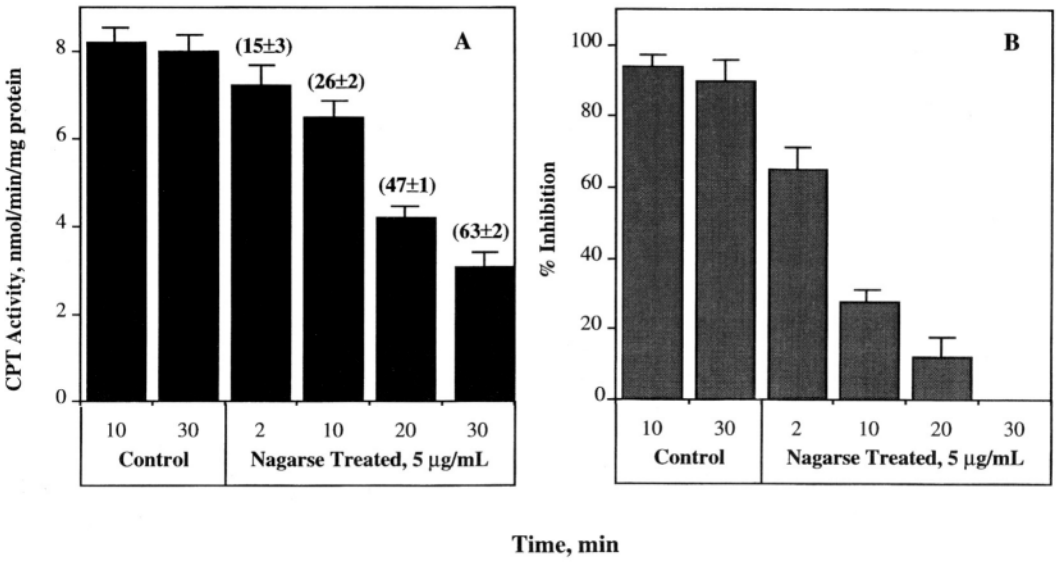

Figure 2. Effect of Nagarse on CPT Activity (A) and Malonyl-CoA inhibition (B) as a Function of Time.

Effects of trypsin (10µg/mL) on CPT-I activity time course were similar to those of Nagarse, but there was no corresponding loss of malonyl-CoA inhibition even when the enzyme activity had been destroyed by about 60%, Figure 4. This data is in contrast to a recent report by Fraser *et al.*[35] suggesting that trypsin at a concentration of 10µg/mL has no effect on CPT-I activity. Their data is presented as percent activity as a function of exposure time to trypsin with no indication of the specific activity of the enzyme before

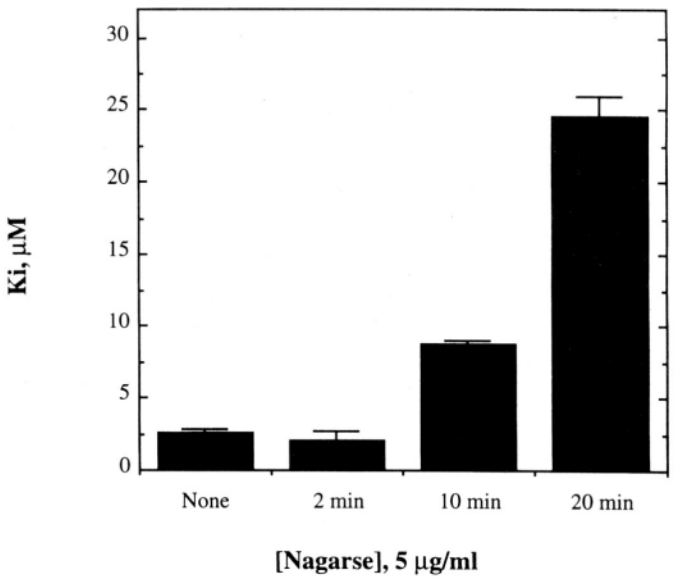

Figure 3. Effects of Nagarse on the apparent K_i of CPT-I for malonyl-CoA.

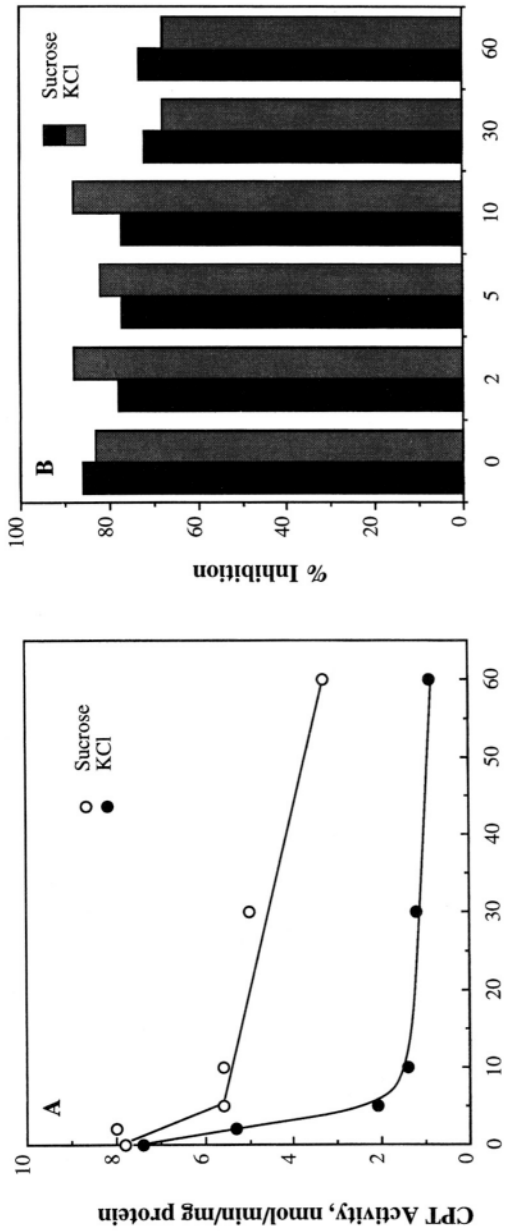

Figure 4. Effect of Trypsin on CPT Activity (**A**) and Malonyl-CoA Inhibition (**B**) as a Function of Time.

trypsin treatment. In our studies presented in Fig. 4, it is interesting to note that if the isolation medium was changed from 250mM sucrose to 150mM KC1, then loss of CPT-I activity as a function of exposure time to trypsin was much faster and also greater. This suggests that changes in salt concentration may some how expose the acyl-CoA binding domain to trypsin even further. However, the KC1 medium had no effect on malonyl-CoA inhibition as was observed when sucrose was the isolating/incubation medium.

Nagarse treatment of the intact mitochondria did not alter the integrity of the mitochondrial outer membrane, since adenylate kinase which is an intermembrane space enzyme was not released, Figure 5. However, exposure of the intact mitochondria to trypsin caused release of adenylate kinase in a concentration dependent manner. These data suggest that trypsin has damaged the mitochondrial outer membrane, possibly allowing exposure of CPT active domain to trypsin within the outer membrane. Preincubation of the intact mitochondria with malonyl-CoA prior to trypsin treatment did not protect against loss of adenylate kinase (data not shown) but did protect against loss of CPT-I activity as reported before [19,20].

Effects of Nagarse and trypsin on CPT activity and malonyl-CoA inhibition using swollen mitochondria are presented in Figure 6. Swelling alone without any protease treatment slightly increased CPT activity suggesting possible rupture of the mitochondrial inner membrane and exposure of CPT-II. Malonyl-CoA inhibition is reduced from 92 ± 1% in unswollen state to 60 ± 2% when swollen, again suggesting exposure of CPT-II or a conformational change in the malonyl-CoA binding domain such that the ligand does not have full access for optimum effects. Nagarse treatment of the swollen mitochondria did not increase CPT-I activity any further, however, malonyl-CoA inhibition was reduced even more. Exposure of the swollen mitochondria to trypsin had no additional effects on CPT-I activity or malonyl-CoA inhibition. These data suggest that whilst the mitochondria is in the swollen state, the malonyl-CoA binding domain is further exposed to Nagarse on the cytosolic side of the membrane and that the protease has limited access to the acyl-CoA binding domain that is either within the membrane or facing the inter membrane. These data further suggest that it is unlikely for CPT-II to be exposed as the result of swelling since the increase in CPT activity is very small even though malonyl-CoA inhibition is much less in this state. Figure 6 also shows the results

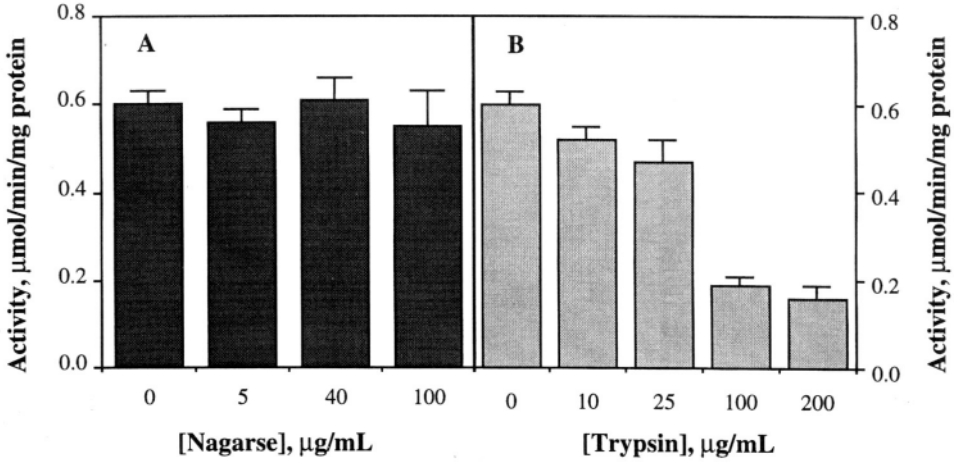

Figure 5. Effect of Protease Treatment of Intact Mitochondria on Adenylate Kinase content.

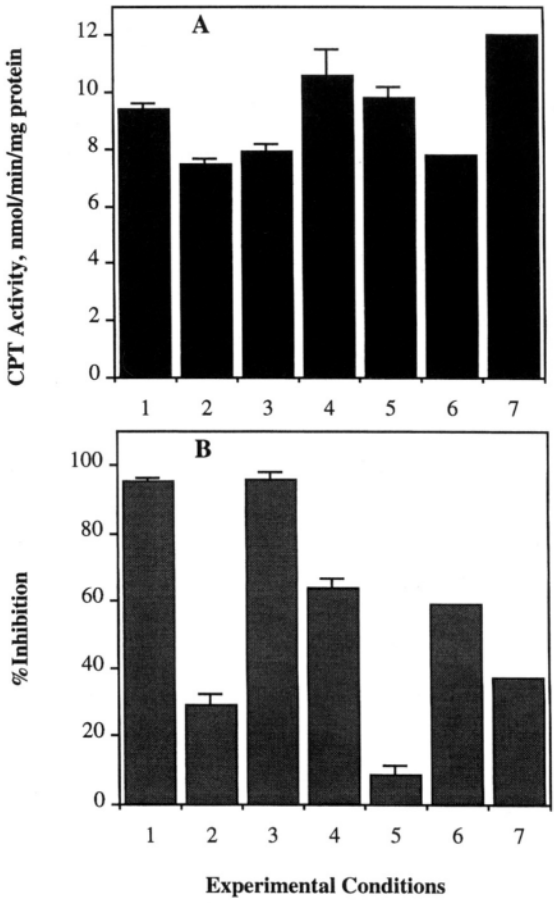

Figure 6. Effect of Proteases on CPT Activity (Panel A) and Malonyl-CoA Inhibition (Panel B) in Intact and Swollen Mitochondria.

of preincubating the swollen mitochondria with 50μM malonyl-CoA prior to Nagarse treatment. Our previous data had shown that preincubation of the intact mitochondria with malonyl-CoA protects against proteolytic actions of Nagarse.[19,20] Here we see that the protection is very limited, malonyl-CoA inhibition is now about 40% instead of being about 10% (compare conditions 5 and 7 in Figure 6). This means that under these conditions some domain of the CPT protein that is involved in malonyl-CoA inhibition is still exposed to Nagarse.

Using isolated mitochondrial outer membranes, Nagarse at increasing concentrations up to 40μg/mL had very little effect on CPT-I activity, Fig. 7 panel A, but the inhibitory effects of malonyl-CoA were reduced in a dose dependent manner still suggesting that while the malonyl-CoA domain is accesible to Nagarse, the acyl-CoA domain is not, Fig. 7 panel B. However, when the concentration of Nagarse was increased to 200 μg/mL, CPT-I activity was reduced significantly with malonyl-CoA inhibition being almost zero, Fig. 7 panels A and B. These data suggest that the malonyl-CoA binding domain is completely destroyed by this concentration of Nagarse and that the acyl-CoA binding domain is facing outwards and it needs a higher concentration of Nagarse to

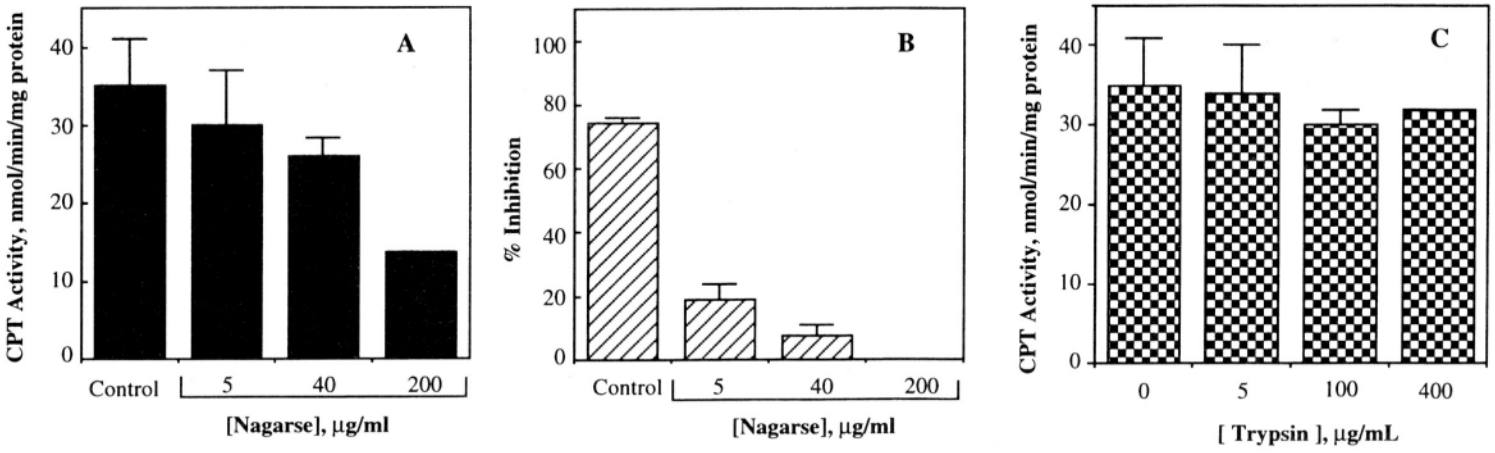

Figure 7. Effects of Proteases on CPT Activity and Inhibition by Malonyl-CoA in Isolated Mitochondrial Outer Membranes.

chew it up or that at this concentration Nagarse is able to compromise the outer membrane vesicle, get inside and have access to the acyl-CoA site. Trypsin up to concentrations of 400μg/mL had no effect on CPT-I activity of the outer membranes, Fig. 7 panel C, or the malonyl-CoA sensitivity (data not shown).

Effects of papain on CPT-I activity of the outer membranes were very similar to those of Nagarse (data not shown). Papain also caused a concentration dependant loss of malonyl-CoA inhibition with the outer membranes similar to that seen with Nagarse, Figure 8. We had previously shown that it was possible to protect against the proteolytic actions of these proteases by preincubating the mitochondria with malonyl-CoA prior to protease treatment. The same general observation is true when isolated outer membranes are used. Data shown in Fig. 8 shows protection against proteolytic actions of papain by preincubation of the outer membranes with malonyl-CoA. When the outer membranes were incubated with relatively low (5μg/mL) concentrations of any of the proteases and then co-sonicated, CPT-I activity was significantly reduced (data not shown). These observations suggest that co-sonication breaks open the outer membrane vesicles so that the protease can have access to the acyl-CoA binding domain.

4. SUMMARY

Our earlier work using intact mitochondria and isolated mitochondrial outer membranes confirms the observations of Murthy and Pande[7] that CPT-I is located on the mitochondrial outer membranes and supports the notion that this enzyme has a malonyl-CoA binding domain facing the cytosol and an acyl-CoA binding domain facing the inter

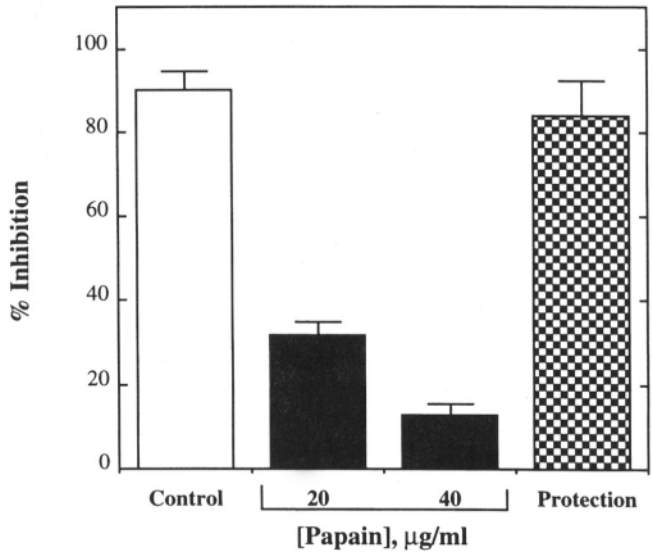

Figure 8. Effects of Papain on Malonyl-CoA Inhibition and Protection from Proteolysis by Malonyl-CoA in Isolated Mitochondrial Outer Membranes.

membrane space. Our data also suggests that coenzyme A binds at the active site of CPT-I, as does acyl-CoA, 2-bromopalmitoyl-CoA, and (+)-hemipalmitoylcarnitinium, but malonyl-CoA does not bind at that site. Inhibition of CPT-I at the malonyl-CoA binding site by HPG and Ro 25-0187, which have no CoA moiety, contributes to a resolution of this question in that the CoA itself is not essential for the binding of malonyl-CoA to its regulatory site, but the dicarbonyl function which is present in malonyl-CoA, HPG, and Ro 25-0187 is absolutely essential.

Our re-evaluation of the topology of hepatic mitochondrial CPT-I confirms the original observations that this enzyme has at least two different binding domains, one domain binding malonyl-CoA, HPG, and Ro-25-187 and the other domain binding acyl-CoA and other inhibitors of CPT-I. Furthermore, the malonyl-CoA binding domain is exposed to the cytosolic face of the membrane. Our data showing that treatment of the intact mitochondria with trypsin causes release of adenylate kinase which indicates that trypsin has damaged the mitochondrial outer membrane, possibly allowing trypsin to enter the intermembrane space and act on CPT from within the outer membrane. Since trypsin's action is limited to arginine and lysine residues, an alternative explanation could be that the portion of the protein domain responsible for malonyl-CoA inhibition may not contain these residues. The latter explanation is plausible, since malonyl-CoA was able to protect against loss of activity and sensitivity to inhibition, but did not protect against loss of adenylate kinase, suggesting that rupture of the outer membrane is not necessarily related to loss of CPT activity. These results suggest that some protein domain that is necessary for CPT activity is exposed on the outer surface of the outer membranes. Therefore, it seems likely that trypsin would have to be able to hydrolyse protein domains of CPT that are inaccessible to Nagarse and papain.

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