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Proteinase treatment of intact hepatic mitochondria has differential effects on inhibition of carnitine palmitoyltransferase by different inhibitors

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Proteolysis of intact mitochondria by Nagarse (subtilisin BPN′) and papain resulted in limited loss of activity of the outer-membrane carnitine palmitoyltransferase, but much greater loss of sensitivity to inhibition by malonyl-CoA. In contrast with a previous report [Murthy & Pande (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 378–382], we found that trypsin had no effect on malonyl-CoA sensitivity. Even when 80% of activity was destroyed by trypsin, there was no difference in the malonyl-CoA sensitivity of the enzyme remaining. Trypsin caused release of the intermembrane-space enzyme adenylate kinase, indicating loss of integrity of the mitochondrial outer membrane, whereas Nagarse and papain caused no release of that enzyme. Citrate synthase was not released by any of the three proteinases, indicating no damage to the mitochondrial inner membrane. When we examined the effects of proteolysis on the inhibition of carnitine palmitoyltransferase by a wide variety of inhibitors having different mechanisms of inhibition, we found differential proteolytic effects that were specific for those inhibitors (malonyl-CoA and hydroxyphenylglyoxylate) that have their inhibitory potencies diminished by changes in physiological state. Both of those inhibitors protected carnitine palmitoyltransferase from the effects of proteolysis, but did not inhibit the proteinases directly. Inhibition by two other inhibitors (DL-2-bromopalmitoyl-CoA and *N*-benzyladriamycin 14-valerate) was not altered by proteinase treatment, even when most of the enzyme activity had been destroyed. Inhibition by glyburide, which is minimally affected by physiological state, was affected only to a slight extent at the highest concentration of trypsin tested. Proteolysis by Nagarse appeared to produce loss of co-operativity in malonyl-CoA inhibition. The effects of proteolysis are discussed and compared with changes in K_i occurring with changing physiological states.

INTRODUCTION

Carnitine palmitoyltransferase (CPT; EC 2.3.1.21) is a regulatory enzyme of the hepatic fatty acid oxidation pathway which has enzyme activities expressed on either side of the mitochondrial-inner-membrane barrier to diffusion of the acyl-CoA substrates into the mitochondrial matrix preceding their β-oxidation [1]. The inner-membrane CPT activity, which is not detected when intact isolated mitochondria are assayed for CPT activity, is catalysed by a protein that resides on the inner aspect of the mitochondrial inner membrane [2,3]. However, the overt, or outer-membrane, CPT is recognized as the most physiologically important of the two mitochondrial activities, as it is regulated through inhibition by malonyl-CoA, its physiological inhibitor [4], and its activity and sensitivity to inhibition by malonyl-CoA are regulated by changes in physiological and pathophysiological states [5–10]. Quantitatively, the most significant change that occurs with the onset of diabetes [9] or the feeding–starvation transition [11] is a 10-fold increase in the apparent K_i for malonyl-CoA. The exact location of the protein catalysing the outer CPT reaction has been the subject of some debate over the past few years, but that question seems to have been resolved by evidence presented by Murthy and Pande that strongly suggests that this enzyme is located in the mitochondrial outer membrane [12,13]. Recently, it has been confirmed that the outer membrane is the location of the enzyme that changes its sensitivity to inhibition by malonyl-CoA in the diabetic state [10] and during starvation [14,15].

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 [16–20]. Data presented by Murthy & Pande [12], who used proteinase treatment of intact mitochondria and isolated outer membranes, suggested that the mitochondrial-outer-membrane CPT has its substrate-binding site facing the intermembrane space and the malonyl-CoA-binding site facing the cytosol, clearly suggesting two different binding sites. Those authors reported that Nagarse (subtilisin BPN′), papain and trypsin had essentially the same effects on the outer CPT [12], but we recently found that trypsin has somewhat different effects from the other two proteases [21], and we present here an explanation of those differences.

There are a number of different inhibitors of the outer CPT that have different mechanisms of inhibition in relation to its substrates. Whereas malonyl-CoA is a competitive inhibitor with respect to acyl-CoA and non-competitive with respect to carnitine [11,22], hydroxyphenylglyoxylate (HPG), the active metabolite of oxfenicine, is a competitive inhibitor with respect to carnitine but non-competitive with respect to acyl-CoA [23]. *N*-Benzyladriamycin 14-valerate (AD 198), an analogue of adriamycin, is a non-competitive inhibitor with respect to carnitine but uncompetitive with respect to acyl-CoA [24], whereas glyburide is non-competitive with respect to acyl-CoA but uncompetitive with respect to carnitine [22]. DL-2-Bromopalmitoyl-CoA was synthesized as a substrate analogue [25], and is expected to bind to the active site. We are not aware of any data on the location of binding sites for these inhibitors. The purpose of the present study 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 proteinases

Abbreviations used: CPT, carnitine palmitoyltransferase; PMSF, phenylmethanesulphonyl fluoride; AD 198, *N*-benzyladriamycin 14-valerate; HPG, hydroxyphenylglyoxylate.

(Nagarse, papain and trypsin), with the hope of learning whether they bind inside or outside the mitochondrial outer membrane. Although our data support some of the original interpretations presented by Murthy & Pande [12], there are important differences in our results that may lead to a better understanding of the orientation of CPT in the mitochondrial outer membrane and to changes in the sensitivity of CPT to inhibition by malonyl-CoA.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (180–240 g) obtained from Harlan Industries (Indianapolis, IN, U.S.A.) were fed on 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.

Isolation and proteinase treatment of mitochondria

Intact mitochondria were isolated by the method of Johnson & Lardy [26], with the modifications previously published [11], except that the isolation medium contained 210 mM-mannitol, 70 mM-sucrose, 0.1 mM-EDTA and 10 mM-Tris/HCl (pH 7.4). Mitochondria (5 mg/ml) were incubated at 37 °C in isolation medium with proteinases at 5 μg/ml or as indicated. After 10 min of incubation, the proteolytic activity was stopped by addition of 200 μl of 20 % (w/v) BSA/ml of incubation volume, followed by addition of 40 ml of ice-cold isolation medium, and mitochondria were subsequently sedimented by centrifugation at 5600 ***g*** for 10 min and then resuspended in the isolation medium. Initial experiments indicated that the same data were obtained regardless of washing the mitochondria once or three times. After proteinase treatment and washing, the mitochondria were resuspended to a concentration of 4 mg of protein/ml in incubation medium. Protein was determined by a biuret method [27]. In some experiments intact mitochondria were first incubated with malonyl-CoA, HPG or 2-bromopalmitoyl-CoA at the concentrations indicated at 37 °C for 5 min before proteinase treatment. In those experiments the inhibitors were present during proteinase treatment, but were removed by the washing procedure. Preincubation experiments with 2-bromopalmitoyl-CoA were carried out in the absence of carnitine so that CPT would not be irreversibly inhibited (see the Results section). In some experiments the proteolytic actions of Nagarse and papain were stopped by addition of phenylmethanesulphonyl fluoride (PMSF) (final concn. 0.2 mM), and trypsin action was stopped by addition of soybean trypsin inhibitor. Although the trypsin inhibitor immediately blocked trypsin action in a chromogenic assay system using N^{α}-benzoyl-L-arginine ethyl ester, we found the trypsin inhibitor to act too slowly for immediate inhibition of proteolytic effects on CPT, so we depended on washing for removal of trypsin. The effects of Nagarse were shown to be caused by its proteolytic action, since heating at 100 °C for 10 min and preincubation with 0.2 mM-PMSF prevented the effects of Nagarse on CPT. PMSF, trypsin inhibitor and the inactivated proteinases had no effect on the CPT assay.

Enzyme assays

CPT was assayed by the method of Bremer [6] as modified and reported previously [9]. Each assay contained, in a total volume of 1 ml: 82 mM-sucrose, 70 mM-KCl, 70 mM-imidazole, 1 μg of antimycin A and 2 mg of BSA. For assaying the outer CPT, each assay also contained 0.5 mM-L-carnitine (0.4 mCi of L-[*methyl*-^{3}H]carnitine) and 40 μM-palmitoyl-CoA (except that 0.1 mM-carnitine and 100 μM-palmitoyl-CoA were used when assaying for glyburide inhibition). Inhibitors were added at the concentrations indicated in legends to Tables and Figures. Adenylate kinase was assayed by the procedure of Bergmeyer [28] as modified by Janski & Cornell [29]. Citrate synthase was assayed as described by Shepherd & Garland [30].

Materials

Palmitoyl-CoA, imidazole, L-carnitine hydrochloride, EDTA, acetyl-CoA, ATP, AMP, KCl, NADH, $MgCl_2$, oxaloacetate, phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, 5,5′-dithiobis-(2-nitrobenzoic acid), essentially fatty-acid-free BSA, malonyl-CoA, N^{α}-benzoyl-L-arginine ethyl ester, Nagarse (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 after each proteinase. AD 198 was kindly given by Dr. M. Israel and Dr. R. Seshadri of the Department of Pharmacology, University of Tennessee (Memphis, TN, U.S.A.). DL-2-Bromopalmitoyl-CoA was synthesized from 2-bromopalmitic acid via 2-bromopalmitoyl chloride [31] as described previously [32]. L-[*methyl*-^{3}H]Carnitine hydrochloride was obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.). HPG was a gift from Pfizer (Sandwich, Kent, U.K.). Potassium glyburide was a gift from the Upjohn Co. (Kalamazoo, MI, U.S.A.).

RESULTS

Initial experiments were conducted with Nagarse under the exact conditions detailed by Murthy & Pande [12] in order to reproduce their experiments as closely as possible. Although Nagarse had little effect on the activity of the outer CPT (Fig. 1), there was clearly a specific effect of Nagarse to decrease the ability of malonyl-CoA to inhibit the enzyme, essentially as reported previously [12]. Experiments with papain gave similar results to those in which Nagarse was used, but, when we repeated these experiments with an equivalent concentration of trypsin, we found that almost all activity was lost. In addition to the problem that the trypsin concentration used was obviously too high, our initial experiments had been carried out on ice for 1 h, and we found it difficult to control proteinase digestion reproducibly, so not only did we attempt to determine the best concentration of the proteinases to use, but we also examined carefully all the incubation and assay conditions used. We were

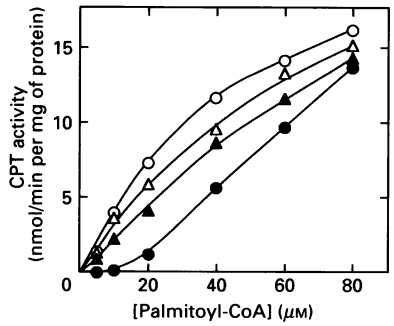

Fig. 1. Effects of Nagarse on CPT activity and its inhibition by malonyl-CoA

Intact mitochondria were exposed to 400 μg of Nagarse/ml of incubation medium for 60 min on ice. After washing to remove the proteinase, mitochondria were assayed for CPT activity in the absence or presence of 50 μM-malonyl-CoA. The symbols represent assays before Nagarse treatment without (○) and with (●) malonyl-CoA and after Nagarse treatment without (△) and with (▲) malonyl-CoA.

Table 1. Effects of proteinase treatment of intact mitochondria on CPT activity and its inhibition by malonyl-CoA

Intact mitochondria were incubated with proteinases at the concentrations indicated and then assayed for outer CPT activity as described in the Materials and methods section by 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.

		CPT activity (nmol/min per mg of protein)		
Proteinase (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±40
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)

Table 2. Effect of proteinase pretreatment of intact mitochondria on adenylate kinase and citrate synthase content

Intact mitochondria were incubated with Nagarse or trypsin at the concentrations indicated for 10 min at 37 °C. Proteinase-treated mitochondria were then washed and assayed for the marker enzymes as described in the Materials and methods section. Results are means ± S.E.M. of 4–7 different preparations of mitochondria: *$P < 0.1$, †$P < 0.05$, ††$P < 0.001$ compared with control.

		Activity (μmol/min per mg of protein)	
Proteinase (μg/ml)		Adenylate kinase	Citrate synthase
None		0.60±0.03	0.21±0.02
Nagarse	5	0.56±0.03	0.20±0.01
	40	0.61±0.05	0.21±0.01
	100	0.55±0.08	0.19±0.02
Trypsin	10	0.52±0.03*	0.21±0.02
	25	0.47±0.05†	0.21±0.02
	100	0.19±0.02††	0.20±0.02
	200	0.16±0.03††	0.20±0.01

also concerned that the mitochondrial outer membrane would be in a non-fluid and therefore non-physiological state at 0 °C. We found that incubating the intact mitochondria with trypsin or Nagarse at a concentration of 5 μg/ml at 37 °C for 10 min gave the best results and that somewhat more papain was required for equivalent effects.

As indicated in Table 1, exposure of intact mitochondria to the 40- and 80-fold lower concentrations of Nagarse at 37 °C had relatively slight effects on activity of the outer CPT (13% and 30% decreases with 5 and 10 μg of Nagarse/ml respectively), but the inhibitory effects of malonyl-CoA were decreased to a greater extent (60% or greater loss of inhibition in all experiments). Experiments conducted with papain gave results almost identical with those with Nagarse when higher concentrations of papain were used (Table 1).

Effects of trypsin on CPT activity and inhibition by malonyl-CoA are also shown in Table 1. Exposure to increasing concentrations of trypsin decreased the activity of CPT in a concentration-dependent manner. However, in contrast with the suggestions by Murthy & Pande [12], 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.

Proteinase treatment of intact mitochondria with Nagarse did not alter the integrity of the mitochondrial membranes, since there was no release of adenylate kinase, an intermembrane-space enzyme, or of citrate synthase, a matrix enzyme (Table 2). However, treatment of mitochondria with trypsin apparently damaged the mitochondrial outer membrane, but not the inner membrane, since adenylate kinase was released in concert with increasing concentrations of trypsin, but citrate synthase was not released even at the highest concentration of trypsin tested.

In order to examine further the effects of proteinases on inhibitors of the outer CPT, we synthesized DL-2-bromopalmitoyl-CoA and examined its effects on intact mitochondria. 2-Bromoacetyl-CoA is an active-site-directed inhibitor for carnitine acetyltransferase [25], and has been shown to react with the enzyme–carnitine intermediate to form a stable, irreversible, complex with that enzyme [33]. It has also been suggested that the same type of complex is formed by 2-bromopalmitoyl-CoA with the outer CPT [34], but other workers have suggested that 2-bromopalmitoyl-CoA inhibits in a strictly reversible, competitive, manner [35]. Preincubation of intact mitochondria with DL-2-bromopalmitoyl-CoA in the presence of carnitine (1 mM) afforded better inhibition than did preincubation in the presence of 40 μM-palmitoyl-CoA. When mitochondria were either diluted 70-fold or washed by centrifugation and resuspension in fresh isolation medium after exposure to 1 μM-DL-2-bromopalmitoyl-CoA, we found that inhibition was reversible if the mitochondria were preincubated with palmitoyl-CoA or the inhibitor alone (95% inhibition before dilution or washing; 4% inhibition remaining after dilution and no inhibition remaining after washing), but inhibition was essentially irreversible when mitochondria were preincubated with the inhibitor in the presence of carnitine (95–97% inhibition remaining in all experiments after dilution and/or washing). We chose to use conditions that produced reversible inhibition for all further experiments.

Table 3 shows the inhibitory effects on outer CPT activity of five different inhibitors before and after exposure to Nagarse,

Table 3. Inhibition of CPT by several known inhibitors in control and proteinase-treated mitochondria

Intact mitochondria were assayed for outer CPT activity in the presence and absence of different inhibitors in control and proteinase-treated mitochondria as described in the Materials and methods section. 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 S.E.M. is indicated, results are means of 2 separate experiments with different mitochondrial preparations. For specific activity of CPT see Table 1.

Inhibitor	No proteinase	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.

papain and trypsin. Inhibition by malonyl-CoA was much decreased by proteinase treatment (also shown in Fig. 1), 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 [36]. 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 3 and was not affected by either Nagarse or papain. These data indicate that AD 198, glyburide and 2-bromopalmitoyl-CoA do not bind at the same site as malonyl-CoA.

When mitochondria were preincubated in the presence of malonyl-CoA or HPG and then exposed to Nagarse in the presence of the inhibitors, both inhibitors protected CPT from the actions of the proteinase in a concentration-dependent manner (Fig. 2). In these experiments, significant protection against loss of sensitivity to inhibition by subsequently added malonyl-CoA was achieved by preincubation in the presence of physiological concentrations of malonyl-CoA (5–10 μM), and, as shown previously [21], higher concentrations of malonyl-CoA seemed to be able to block the action of Nagarse completely. We have also shown previously [21] that malonyl-CoA protects against loss of CPT activity by the actions of both Nagarse and trypsin, but does not inhibit proteinase action directly. HPG was also able to protect CPT from loss of malonyl-CoA sensitivity and from loss of HPG sensitivity, but sensitivity to inhibition by 2-bromopalmitoyl-CoA was not lost during Nagarse treatment and was not affected by HPG (Fig. 2*b*). HPG also protected CPT from loss of activity during Nagarse treatment (0.5 mM-, 1 mM- and 2 mM-HPG afforded 11, 56 and 100% protection respectively), but preincubation of mitochondria with 2-bromopalmitoyl-CoA (1 μM, in the absence of carnitine) did not protect against loss of sensitivity to inhibition by malonyl-CoA, HPG or 2-bromopalmitoyl-CoA, and it did not protect against loss of activity (results not shown), again indicating that malonyl-CoA and 2-bromopalmitoyl-CoA do not bind at the same site. Although malonyl-CoA protected CPT from the actions of proteinases, the release of adenylate kinase caused by trypsin action was not prevented. Proteinase treatment in the presence of malonyl-CoA resulted in losses of adenylate kinase identical with those in Table 2.

Because two of the proteinases that we tested seemed to have a specific effect to decrease the inhibitory actions of malonyl-CoA, the possibility was raised that this could relate to a physiological mechanism in which malonyl-CoA sensitivity was decreased by the actions of a hepatic proteinase. Experiments presented in Fig. 3 show inhibition by several concentrations of malonyl-CoA on CPT in control and Nagarse-treated mitochondria. The data in Fig. 3(*a*) suggest partial inhibition, possibly

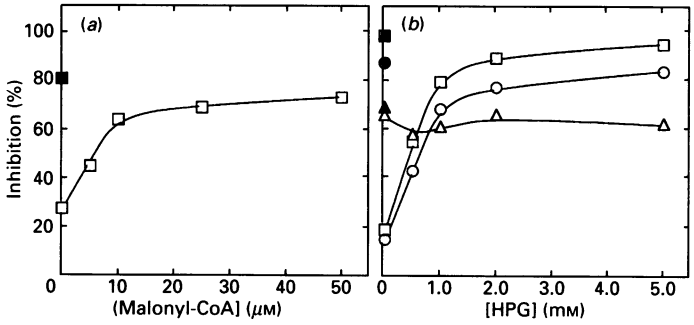

Fig. 2. Protection from proteolysis by inhibitors of CPT

Intact mitochondria were preincubated with malonyl-CoA or with HPG at the concentrations indicated at 37 °C for 5 min before addition of Nagarse (5 μg/ml) for an additional 10 min incubation at 37 °C. After washing, the mitochondria were assayed in the presence of 50 μM-malonyl-CoA (□, ■), 2 mM-HPG (○, ●), or 1 μM-2-bromopalmitoyl-CoA (△, ▲), with 0.5 mM-carnitine in (*a*) and 0.2 mM-carnitine in (*b*). The palmitoyl-CoA concentration was 40 μM in all assays. The black symbols represent percentage inhibition with mitochondria that were not treated with Nagarse.

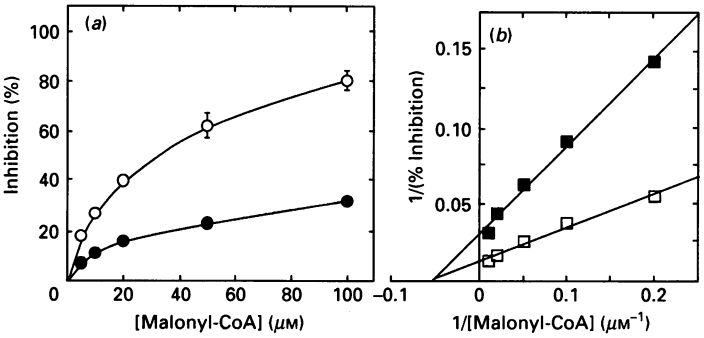

Fig. 3. (*a*) Inhibition of CPT by malonyl-CoA in control (○) and Nagarse-treated (●) mitochondria and (*b*) reciprocal plot of data from (*a*) for determination of maximum possible inhibition and I_{50}

In (*a*), points are means ± S.E.M. for 4 experiments.

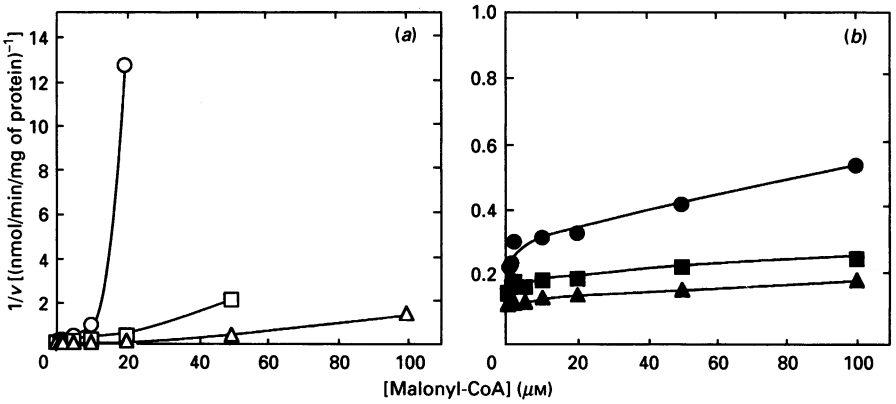

Fig. 4. Determination of the K_i value of CPT for malonyl-CoA by Dixon plots

(*a*) Representative plot from control experiments using mitochondria from fed rats before Nagarse treatment; (*b*) representative plot from experiments using Nagarse-treated mitochondria. Concentrations of palmitoyl-CoA used were 20 μM (○, ●), 40 μM (□, ■) and 60 μM (△, ▲).

owing to a complete loss of inhibition in a fraction of the outer CPT. This is seen more clearly in reciprocal plots (Fig. 3*b*), which indicated that, although CPT in control mitochondria could be completely inhibited (112 ± 10 %) by malonyl-CoA, only 40 ± 4 % of the CPT activity in the Nagarse-treated mitochondria could be inhibited by malonyl-CoA. Nagarse treatment had no effect on the I_{50} (conc. giving half-maximum inhibition) for malonyl-CoA (I_{50} values from Fig. 3*b* were 22 ± 3 μM and 19 ± 4 μM for control and Nagarse-treated mitochondria respectively). In order to see what effects Nagarse treatment would have on the apparent K_i for malonyl-CoA, Dixon plots were constructed by using data from fed animals. Fig. 4 indicates that there is a change in the shape of the Dixon plots caused by Nagarse treatment. Positive co-operativity seen here (Fig. 4*a*) and reported previously [11] for overt mitochondrial CPT was apparently lost in response to Nagarse treatment (Fig. 4*b*). It should be pointed out that plots identical with those in Fig. 4 have been seen in the very early stages of recovery from diabetes after insulin injection [8].

DISCUSSION

In contrast with results with Nagarse and papain, we found the effects of trypsin to be substantially different from those of the other two proteinases and from previously reported effects of trypsin, since it had only the effect of decreasing activity without changing the sensitivity to inhibitors. The release of adenylate kinase indicated that trypsin produced considerable damage to 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 appears more likely, since malonyl-CoA was able to protect against loss of activity and sensitivity to inhibition, but did not protect against loss of adenylate kinase from treated mitochondria, suggesting that rupture of the outer membrane is not necessarily related to loss of CPT activity. These results represent the first indication that some protein domain that is necessary for CPT activity is exposed on the outer surface of the outer membranes. If this interpretation is correct, trypsin would have to be able to hydrolyse protein domains of CPT that are inaccessible to Nagarse and papain. We believe that our results differ from those previously reported [12] because we were using a much more highly purified preparation of trypsin (M. S. R. Murthy, personal communication). Contamination of trypsin with non-specific proteinase would be expected to yield results similar to those with Nagarse and papain.

The two inhibitors of CPT that had their inhibitory potencies altered by proteinase treatment, malonyl-CoA and HPG, were also the only two inhibitors of CPT that have their potencies substantially changed during fasting or diabetes [8–11,36]. This raises the question whether they may both bind, at different sites, to a regulatory malonyl-CoA-binding protein. Although HPG and malonyl-CoA prevent the effects of Nagarse and trypsin on CPT, it seems unlikely that such small molecules could directly block proteolytic effects without a major change in the CPT protein such as binding of a regulatory protein or a major conformational change. Because of the unique relationship between malonyl-CoA and HPG compared with all other inhibitors of CPT, and because proteolysis is most prevalent during starvation and diabetes, these results raise the question of whether endogenous proteinases may play a role in regulation of CPT. There are several reports indicating that proteolysis mimics effects of metabolic regulation and suggesting that proteolysis may have a regulatory biological role for other enzymes; for example, it has been reported that 3-hydroxyl-3-methylglutaryl-CoA reductase [37] and glycogen synthase D [38] are both substrates of the endogenous Ca^{2+}-activated proteinase, calpain II, which can alter the regulatory properties of these enzymes *in vitro*. Also, reports by Pontremoli *et al.* [39,40] indicate that isovalerylcarnitine, a product of L-leucine catabolism, decreases the activation requirements of calpain II for Ca^{2+} and reverses the effect of the endogenous inhibitor calpstatin, suggesting a possible mechanism for initiation of proteolysis in physiological states such as starvation. A recent review discusses several similar physiological functions of endogenous proteinases [41]. Much more extensive work on proteinase effects on CPT will be needed before any conclusion can be drawn about its physiological relevance.

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