Chymotrypsin activates cardiac mitochondrial carnitine–acylcarnitine translocase

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The carnitine–acylcarnitine translocase facilitates carnitine and acylcarnitine transport into the mitochondrial matrix during β -oxidation. Our results demonstrate that chymotrypsin can activate the maximal velocity of *N*-ethylmaleimide (NEM)-sensitive carnitine or palmitoylcarnitine exchange 7-fold, while doubling the affinity of the translocase for carnitine. Chymotrypsin activation is strictly dependent on the presence of free or short-chain acylcarnitine in the proteolysis medium, the extent of activation decreasing as the acylcarnitine chain length in the proteolysis medium increases. Chymotrypsin treatment decreases the apparent I_{50} value (inhibitor concentration required to give half-maximal inhibition) of the translocase for inhibition by NEM only under conditions which produce translocase activation. Modification of sub-mitochondrial particle membranes by chymotrypsin does not result in gross ultrastructural changes or in an increase in the passive permeability of these membranes to carnitine. The data suggest that carnitine binding produces a change in translocase conformation which allows chymotrypsin modification to occur. This modification alters the kinetic and inhibitor-binding properties of the translocase.

INTRODUCTION

The carnitine–acylcarnitine translocase is an intrinsic protein associated with the mitochondrial inner membrane. This protein is characterized as a 1:1 antiport which exchanges extramitochondrial carnitine or acylcarnitine for matrix carnitine [1,2]. It also catalyses a slow unidirectional transport of carnitine into the mitochondrial matrix [3]. The carnitine translocase activity is believed to be distinct from the carnitine–acyl-CoA transferase proteins (EC 2.3.1.21) associated with the mitochondrial inner membrane. However, recent evidence indicates that these two activities may be coupled during the transport of fatty acids into the mitochondrion [4]. While a detailed description of the transport properties of the translocase exists, less is known about the possible conformational and/or structural properties which may regulate its transport activity.

A number of physical and enzymic approaches have provided information on the conformational characteristics of proteins. Among these, limited proteolysis has proved useful in defining the regulatory and catalytic domains of proteins [5] and in examining ligand-induced conformational changes occurring during catalysis or transport [6]. It has been demonstrated that binding of substrate or effector molecules alters protein structural domains accessible to protease [7]. Proteolysis under these conditions often results in distinct alterations of the kinetic properties of the protein rather than simple enzymic inhibition. We report here the results of experiments which demonstrate that the carnitine translocase is susceptible to proteolysis by chymotrypsin. Proteolytic activation of the translocase requires the presence of carnitine and results in changes in the kinetic properties of the protein.

EXPERIMENTAL

Preparation of ox heart sub-mitochondrial particles

Ox heart mitochondria were isolated using the general procedure described by Azzone *et al.* [8], frozen in liquid N_2 and stored at -70°C . Sub-mitochondrial particles (SMPs) were prepared by sonication. Following thawing, mitochondria were suspended at approx. 30 mg/ml in a medium containing 0.1 M-KCl, 0.02 M-potassium phosphate, 0.02 M-DL-carnitine and 0.002 M-EDTA (pH 7.4) and subjected to 4 min of sonic disruption at 4°C with a Branson model 350 Sonifier at 130 W and 50% duty cycle. Following sonication, the mitochondria were sedimented from the suspension by centrifugation at 5000 *g* for 10 min ($r_{av.} = 8.95$ cm). The mitochondrial pellet was resuspended in sonication buffer and the procedure was repeated twice. The three low-speed supernatants were combined and centrifuged at 100000 *g* for 60 min ($r_{av.} = 6.98$ cm). The SMP pellet was suspended in 0.1 M-KCl/0.03 M-Mops (pH 7.2) at a protein concentration of 15 mg/ml, rapidly frozen in liquid N_2 and stored at -70°C until used.

SMP carnitine content was determined following centrifugation of 0.5 ml of a preparation through a 5 ml tuberculin syringe containing Sephadex G-50–80 in order to remove extra-luminal carnitine. The eluate was recovered, treated with perchloric acid and neutralized, and carnitine was measured using the method of McGarry & Foster [9]. The protein concentration in all experiments was determined using the biuret method [10], with bovine serum albumin as a standard.

Chymotrypsin treatment of SMPs

SMPs (6 mg/ml) were incubated in the presence of chymotrypsin (1 unit/mg of SMP) for 20 min at 20°C .

Abbreviations used: SMP, sub-mitochondrial particle; NEM, *N*-ethylmaleimide; I_{50} , inhibitor concentration giving half-maximal inhibition; CPT, carnitine–palmitoyl CoA transferase.

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The incubation medium contained 0.1 M-KCl, 0.03 M-Mops and 0.02 M-DL-carnitine (pH 7.2). Following incubation, the SMPs were isolated by centrifugation at 100 000 *g* for 30 min ($r_{av.} = 8.1$ cm), resuspended in 0.1 M-KCl/0.03 M-Mops (pH 7.2) and stored on ice until assayed. Control experiments were carried out in an identical manner, with the exception that no chymotrypsin was present during incubation.

In experiments measuring the effect of increasing medium carnitine concentration on chymotrypsin activation of the translocase, the DL-carnitine concentration in the solution was increased by additions from either a 0.4 or a 1.0 M stock solution. In experiments measuring the dependence of translocase activation on chymotrypsin concentration, an incubation medium containing 20 mM-DL-carnitine was used and the concentration of chymotrypsin was varied by addition from a stock solution containing 6 units of chymotrypsin/ml.

Assay of carnitine translocase activity in SMPs

N-Ethylmaleimide (NEM)-sensitive carnitine translocase activity was determined in SMPs as described by Schulz & Racker [11]. SMPs at 4–5 mg/ml were incubated in 0.1 M-KCl/0.03 M-Mops in the presence or absence of NEM (30 nmol/mg of SMP) for 5 min at 20 °C. Following this preincubation, SMP carnitine exchange activity was initiated by addition of [³H]carnitine (1 mM final concentration; 9800 d.p.m./nmol) to the solutions. Following a 2-min incubation, 100 μ l aliquots were pipetted on to 1 ml tuberculin syringes containing Sephadex G-50-80, and the reaction was terminated by centrifugation as described by Penefsky [12]. Carnitine translocase activities represent NEM-sensitive transport determined as the difference between the activity measured in the absence and presence of NEM. Transport was linear up to 5 min for both control and chymotrypsin-treated SMPs under these conditions.

Measurement of carnitine permeability of control or chymotrypsin-treated SMPs

Control or chymotrypsin-treated SMPs were prepared as described. [³H]Carnitine content in the SMPs was measured under the following four conditions. (a) Following a 10-min period of [³H]carnitine uptake by control or chymotrypsin-treated SMPs, the reaction was terminated and [³H]carnitine accumulation was determined. (b) Following 10 min of [³H]carnitine accumulation, NEM was added to the control or chymotrypsin-treated SMPs. The incubation was continued for an additional 15 min and the [³H]carnitine content of the SMPs was measured. (c) As in (b), except that subsequent to NEM addition at 10 min, 40 mM-DL-carnitine was added to the control or chymotrypsin-treated SMPs at 15 min and the assay was terminated at 25 min. (d) Following a 15 min incubation with [³H]carnitine, 40 mM-DL-carnitine was added to the control or chymotrypsin-treated SMPs. After an additional 10 min incubation, the reaction was terminated and the [³H]carnitine content was measured.

NEM I_{50} for control and chymotrypsin-modified carnitine translocase

The I_{50} (inhibitor concentration giving half-maximal inhibition) for translocase inhibition by NEM for control SMPs and those exposed to chymotrypsin in the presence or absence of 20 mM-DL-carnitine was determined as

follows. SMPs were preincubated for 5 min at 20 °C with increasing concentrations of NEM from a 30 mM stock solution. Transport velocities were then measured at 2 min as described above. Values represent the percentage of translocase activity remaining following subtraction of NEM-insensitive transport. The insensitive component of carnitine transport was determined at an NEM concentration of 30 nmol/mg and represents 5–15 % of the total carnitine transport activity for control SMPs and those treated with chymotrypsin in the presence or absence of carnitine.

Palmitoylcarnitine–carnitine exchange activities in SMPs

Palmitoylcarnitine–carnitine exchange was measured in control or chymotrypsin-treated SMPs as follows. Control or chymotrypsin-treated SMPs were suspended at 15 mg/ml in 0.1 M-KCl/0.03 M-Mops and loaded with [³H]carnitine by incubation with 1 mM-[³H]carnitine (9800 d.p.m./nmol) for 20 min at 20 °C. The loaded SMPs were then isolated by centrifugation at 100 000 *g* for 30 min ($r_{av.} = 8.1$ cm). The SMP pellets were resuspended in 0.1 M-KCl/0.03 M-Mops and incubated for 5 min at 20 °C in the presence or absence of 30 nmol of NEM/mg. Palmitoylcarnitine–carnitine exchange was initiated by addition of increasing concentrations of L-palmitoylcarnitine (0.5 mM stock), and the reaction was terminated after 2 min. Velocities of exchange were calculated from the measured percentage exchange values [13] and the vesicle L-carnitine concentration was determined for either untreated SMPs loaded with [³H]carnitine or those treated with chymotrypsin and subsequently loaded with [³H]carnitine.

Activation by chymotrypsin: dependence on acylcarnitine chain length

Control and chymotrypsin-treated SMPs were prepared as described with the following substitutions for DL-carnitine in the proteolysis medium: 0.02 M-DL-carnitine, 0.03 M-DL-acetylcarnitine, 0.025 M-DL-butyrylcarnitine, 0.006 M-DL-octanoylcarnitine and 0.02 or 0.04 mM-DL-palmitoylcarnitine. Following the standard 20 min incubation at 20 °C in the presence or absence of chymotrypsin, the particles were isolated by centrifugation and NEM-sensitive carnitine exchange activity was determined following a 20 min incubation at 20 °C.

Electron microscopy

Aliquots of untreated and chymotrypsin-treated SMPs were diluted to a final protein concentration of 0.25 mg/ml. For negative staining, a drop of sample was placed on the surface of a Formvar-coated, carbon-stabilized 400 mesh copper grid and allowed to stand for 1 min, after which most of the fluid was withdrawn by touching the edge of the grid with filter paper. A droplet of 1 % (w/v) sodium phosphotungstate (pH 7.2) was placed on the grid and after 1 min withdrawn with filter paper. The grid was allowed to air dry before being examined in a Philips 300 electron microscope at an accelerating voltage of 60 kV.

Materials

Ox hearts were obtained from a local slaughterhouse. Chymotrypsin (EC 3.4.21.1, Type VII from bovine pancreas, lot 75F-8025; 60 units/mg of protein), NEM, Mops, EDTA, DL-acetyl-, -butyryl- and -octanoyl-

carnitine chloride, L-palmitoylcarnitine chloride and Sephadex G-50-80 were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. DL-Carnitine and L-carnitine chloride were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. L-[methyl- ^3H]Carnitine chloride (71 Ci/mmol) was obtained from Amersham/Searle, Arlington Heights, IL, U.S.A. D-Carnitine was a gift from Dr. R. Bressler. All other chemicals were of laboratory grade.

RESULTS

The sensitivity of carnitine translocase activity to chymotrypsin modification was tested in ox heart SMPs. SMPs treated with 30 μg of chymotrypsin/mg of SMP as described in the Experimental section show little if any alteration in translocase activity if carnitine is not present in the chymotrypsin incubation medium (Fig. 1a). However, SMPs treated with chymotrypsin in the presence of increasing external carnitine concentrations demonstrate marked increases in their NEM-sensitive carnitine translocase activity (Fig. 1a). Incubation of SMPs with increasing concentrations of carnitine in the absence of chymotrypsin results in no change in the transport activity of these particles (Fig. 1a). Half-maximal stimulation of transport by chymotrypsin occurs with approx. 5 mM-L-carnitine in the proteolysis medium. Maximal stimulation of translocase activity requires approx. 30 μg of chymotrypsin/mg of SMP (Fig. 1b). The activation is

essentially complete in 5 min and the resulting translocase activity is stable to further chymotrypsin incubation for up to 1 h (results not shown).

To determine if chymotrypsin treatment alters the permeability of SMPs to carnitine, we measured [^3H]carnitine transport in control and chymotrypsin-treated SMPs after a 10 min incubation (Fig. 2, bar A). Translocase inhibition with NEM at 30 nmol/mg following 10 min of transport prevents further [^3H]carnitine uptake in either control or treated SMPs (Fig. 2, bar B). Adding 40 mM unlabelled DL-carnitine 5 min after NEM inhibition produces no change in the [^3H]carnitine content in either preparation of SMPs (Fig. 2, bar C). However, if control or chymotrypsin-treated SMP translocase activities are not inhibited with NEM prior to addition of unlabelled carnitine, then release of all previously accumulated [^3H]carnitine occurs (Fig. 2, bar D).

Chymotrypsin treatment of SMPs changes the sensitivity of the translocase to inhibition by NEM (Fig. 3). The concentration of NEM required to produce half-maximal inhibition of the initial rates of carnitine transport in control SMPs is approx. 7 nmol/mg of SMP. Treatment of the SMPs with chymotrypsin in the presence of carnitine lowers this value to approx. 3 nmol/mg. If chymotrypsin treatment occurs in the absence of carnitine in the proteolysis medium then the I_{50} for NEM inhibition of the translocase is not significantly different from the control value. NEM-insensitive carnitine transport

Fig. 1. (a) Chymotrypsin-induced activation of carnitine translocase depends on the medium carnitine concentration; (b) the concentration-dependence of chymotrypsin for activation of carnitine translocase

(a) Ox heart SMPs were incubated in the presence (○) or absence (△) of 30 μg of chymotrypsin per mg of SMP and increasing levels of carnitine for 20 min at 20 °C. SMPs were re-isolated and assayed for NEM-sensitive carnitine translocase activity as described in the Experimental section. Values represent means $\pm$ S.E.M. of three separate measurements. S.E.M. values in the absence of chymotrypsin fall within the symbol $\triangle$. (b) Ox heart SMPs were incubated for 20 min at 20 °C in the presence of 20 mM-DL-carnitine and various concentrations of chymotrypsin. SMPs were recovered and assayed for NEM-sensitive carnitine translocase activity as described in the Experimental section. Values represent means $\pm$ S.E.M. of three separate experiments.

Fig. 2. Chymotrypsin proteolysis of SMPs does not increase their permeability to carnitine

Control and chymotrypsin-activated SMPs were loaded with [^3H]carnitine using the scheme depicted in the inset and described in the Experimental section. [^3H]Carnitine content of the particles was determined as described in the inset and in the Experimental section. Values represent means $\pm$ S.E.M. of between three and seven separate experiments.

measured at 30 nmol of NEM per mg of SMP is essentially unaffected by chymotrypsin treatment (0.012 ± 0.001 and 0.015 ± 0.001 nmol/min per mg for control and chymotrypsin-treated SMPs respectively).

Kinetic analysis of control and chymotrypsin-treated SMP carnitine uptake shows that the K_m is halved by chymotrypsin treatment (2.24 ± 0.07 to 1.0 ± 0.12 mM, control and chymotrypsin-treated SMPs respectively) while the $V_{\max}$ for carnitine uptake increases approx. 7.5-fold from 0.160 ± 0.03 to 1.19 ± 0.10 nmol of carnitine/min per mg (see also Fig. 4). Modification of the translocase activity by chymotrypsin also alters the kinetics of palmitoylcarnitine-for-carnitine exchange (Fig. 5). The K_m value for palmitoylcarnitine increases slightly from 45 to 56 μM following chymotrypsin treatment. The $V_{\max}$ for this exchange increases from 0.17 ± 0.05 to 1.84 ± 0.89 nmol of L-carnitine/min per mg with chymotrypsin treatment.

Since chymotrypsin treatment markedly increases the velocity of exchange, the possibility that the treated SMPs contain more carnitine was investigated. Untreated SMPs contain 1.43 ± 0.3 nmol of L-carnitine/mg. Upon

Fig. 3. Chymotrypsin-induced activation of the translocase increases its sensitivity to NEM inhibition

Control SMPs ($\circ$) or SMPs treated with chymotrypsin ($30 \mu\text{g}/\text{mg}$ of SMP) in the absence ($\triangle$) or presence ($\square$) of 20 mM-DL-carnitine as described in the Experimental section were assayed for translocase activity following a 5-min preincubation with the indicated concentrations of NEM. NEM-insensitive carnitine transport determined as described in the Experimental section was subtracted from the velocities at sub-saturating NEM concentrations to obtain the reported values. These results represent means $\pm$ S.E.M. of four separate experiments.

Fig. 4. Velocity versus carnitine concentration plot of control and chymotrypsin-activated SMP carnitine translocase activities

Control ($\triangle$) or chymotrypsin-activated ($\circ$) SMPs were assayed for NEM-sensitive carnitine transport as described in the Experimental section. Values represent means $\pm$ S.E.M. of four experiments. S.E.M. values in control SMPs fall within the symbol $\triangle$.

Fig. 5. Velocity versus palmitoylcarnitine concentration plot of control and chymotrypsin-activated SMP palmitoylcarnitine-for-carnitine exchange activity

Control (Δ) or chymotrypsin-activated ($\circ$) SMPs were loaded with [^3H]carnitine and assayed for NEM-sensitive palmitoylcarnitine-for-carnitine exchange as described in the Experimental section. Derivation of velocities from % exchange values was according to Halperin & Pande [13]. Values represent means $\pm$ S.E.M. of six separate experiments.

incubation in the presence of 20 mM-DL-carnitine, this value rises to 3.82 ± 0.17 nmol of L-carnitine/mg, with no significant effect on the maximal velocity of exchange (Table 1). Incubation of untreated SMPs in the presence of 20 mM-DL-carnitine and chymotrypsin results in

Fig. 6. Chymotrypsin-induced activation of carnitine-for-carnitine exchange in SMPs depends on the chain length of the acylcarnitine in the activation medium

SMPs were incubated with acylcarnitines of increasing chain length in the absence (Δ) or presence ($\circ$) of chymotrypsin as described in the Experimental section. Following re-isolation, NEM-sensitive carnitine-for-carnitine exchange was determined. Values represent means $\pm$ S.E.M. of between four and eight separate experiments.

5.30 ± 0.09 nmol of L-carnitine/mg in the lumen, and the $V_{\max}$ is increased 8-fold. The [^3H]carnitine-loaded SMPs used to determine the velocity of palmitoylcarnitine-for-carnitine exchange have a carnitine content of 0.99 ± 0.07 and 3.89 ± 0.36 nmol of L-carnitine/mg for control and chymotrypsin-treated particles respectively (Table 1).

Since carnitine is required to produce activation of the

Table 1. Comparison of SMP carnitine content with maximum carnitine exchange velocities

SMPs were treated, isolated from contaminating carnitine and their carnitine content determined as described in the Experimental section. The carnitine content ($[\text{Carn}]_i$) is reported as nmol of L-carnitine/mg of SMP and represents the average $\pm$ S.E.M. of three or four measurements. $V_{\max}$ values represent either carnitine-for-carnitine exchange (*) or palmitoylcarnitine-for-carnitine exchange (†), reported as nmol of carnitine/min per mg of SMP. These results also represent the average $\pm$ S.E.M. of three to four measurements.

	Untreated	20 mM-Carnitine; 20 min	20 mM-Carnitine; 1 mM-[^3H]carnitine; 20 min each
Without chymotrypsin:			
$[\text{Carn}]_i$ (nmol/mg)	1.43 ± 0.3	3.82 ± 0.17	0.99 ± 0.07
$V_{\max}$ (nmol/min per mg)	$0.14 \pm 0.019^*$	$0.16 \pm 0.033^*$	$0.17 \pm 0.046^\dagger$
With chymotrypsin:			
$[\text{Carn}]_i$ (nmol/mg)	—	5.30 ± 0.09	3.89 ± 0.36
$V_{\max}$ (nmol/min per mg)	—	$1.19 \pm 0.10^*$	$1.84 \pm 0.89^\dagger$

Fig. 7. Negatively stained electron micrographs of control and chymotrypsin-treated SMPs

SMPs were treated with chymotrypsin in the presence or absence of carnitine as described in the Experimental section. (a) Control SMPs, (b) chymotrypsin-treated in the absence of carnitine, (c) chymotrypsin-treated in the presence of carnitine (20 mM). Magnification $\times 47000$.

translocase, we tested the possibility that other translocase and carnitine-palmitoyl-CoA transferase substrates may permit activation of exchange activity by chymotrypsin. Preincubation of SMPs with the transferase substrates or inhibitors CoA, palmitoyl-CoA, acetyl-CoA or malonyl-CoA produces no alteration in the transport activity of the translocase (results not shown). D-Carnitine and DL-acetylcarnitine approximately equal DL-carnitine in their ability to mediate chymotrypsin activation of translocase activity (Fig. 6). However, as the chain length of the acylcarnitine in the chymotrypsin treatment medium increases, protease enhancement of translocase activity decreases, such that inclusion of palmitoylcarnitine as activator results in barely a 1.5-fold increases in translocase velocity. Incubation of SMPs with increasing chain length acylcarnitines in the absence of chymotrypsin produces a slight diminution in the translocase activity of these particles (Fig. 6).

Others have suggested that a functional coupling of the translocase to the carnitine-palmitoyl-CoA transferase (CPT) occurs, implying a close proximity of these two proteins in the mitochondrial membrane [4]. To determine the effect of chymotrypsin treatment on CPT, we assayed this activity in control SMPs and following treatment with chymotrypsin. In the absence of carnitine, chymotrypsin produces a small decrease in the total CPT activity from 20.6 ± 0.6 nmol/min per mg to 17.5 ± 1.3 nmol/min per mg. In the presence of carnitine, chymotrypsin treatment further decreases CPT activity to 14.6 ± 0.66 nmol/min per mg. No apparent morphological differences can be observed between control and chymotrypsin-treated SMPs. All vesicles are of similar size and appearance and contain characteristic F_1 -ATPase knobs (Fig. 7).

DISCUSSION

The data in this paper demonstrate that the properties of the carnitine-acylcarnitine translocase are subject to modification by chymotrypsin proteolysis. Incubation of SMPs with chymotrypsin in the absence of external carnitine produces no measurable alterations in the transport activity or NEM-sensitivity of the carrier. However, in the presence of increasing concentrations of external carnitine, chymotrypsin increases the transport activity of the carrier as well as its sensitivity to inhibition by NEM. These results are similar to those reported for phenylalanine hydroxylase, which demonstrates substrate-dependent activation upon proteolysis [14]. One possible explanation for these changes in the case of the translocase is the following. Chymotrypsin may proteolyse the translocase in the presence or absence of carnitine, but the presence of carnitine is required to change the protein conformational state such that additional chymotrypsin cleavage sites are exposed. Subsequent proteolysis of these sites produces the observed effects on carrier activity and inhibitor sensitivity. Alternatively, the translocase may not be proteolysed by chymotrypsin in the absence of carnitine, while addition of carnitine induces a conformational change that exposes one or more chymotrypsin-sensitive sites. When these are proteolysed, protein function is altered. Resolution of these hypotheses requires further purification of the translocase [15] to identify the extent of proteolytic digestion in the presence and absence of carnitine.

Our results demonstrate that chymotrypsin activates the palmitoylcarnitine-for-carnitine exchange process to a similar extent as the carnitine-for-carnitine exchange. The dissimilarity in apparent substrate affinity for the two exchanges has been observed by others [2,16] and is

probably due to differences in substrate availability at the membrane-bound carrier, i.e. water-soluble carnitine versus membrane-bound acylcarnitine. The similarity of both the control $V_{\max}$ values for free and long-chain acylcarnitine exchange and the extent of its activation by chymotrypsin suggests that free and long-chain acylcarnitine exchange may be occurring through a similar process on the translocase.

Carnitine and palmitoylcarnitine exchange are activated by chymotrypsin only in the presence of free or short-chain carnitines in the incubation medium. Increasing the chain length of the acylcarnitine in the chymotrypsin incubation medium results in a marked diminution of activation. These results are again similar to those reported for the phenylalanine hydroxylase and for the uncoupling protein of brown fat mitochondria as well [14,17]. In the former case tetrahydrobiopterin, a cofactor for the hydroxylase, protects against chymotrypsin-induced activation. In the latter case, the regulatory nucleotide of the uncoupling protein, GDP, protects against extensive chymotrypsin proteolysis. Two possible explanations for the observed inhibitory effects of increasing chain length on translocase activation can be proposed. The first is that the conformation assumed by the translocase during the binding and transport of free or short-chain carnitines is different from that for long-chain acylcarnitine transport. Therefore, while the former are capable of exposing the correct chymotrypsin cleavage site for activation, binding of the latter cannot induce an appropriate conformation to produce proteolysis and activation. The observation that both carnitine and palmitoylcarnitine exhibit similar $V_{\max}$ values in the control and chymotrypsin-activated states argues against this. The second possibility is that the chymotrypsin cleavage site responsible for transport activation is found in or near an acyl-chain-binding region of the translocase. Increasing the chain length of the acylcarnitine in the activation medium could interfere with the ability of chymotrypsin to proteolyse this site, thereby lowering the extent of translocase activation.

Although alterations in the velocity of the translocase may result from changes in SMP carnitine content, we found that incubation of untreated SMPs with 20 mM-DL-carnitine increases their carnitine content 2.5-fold, but does not affect their translocase activity. Therefore, in untreated SMPs, the carnitine present in the vesicular lumen (1.4 nmol/mg) is apparently sufficient to saturate the internal face of the carrier. Chymotrypsin treatment in the presence of carnitine increases the SMP carnitine content to a comparable extent, while the translocase activity is increased 8-fold (Table 1). Although chymotrypsin treatment decreases the K_m for external carnitine, it is not known to what extent the K_m for internal carnitine is affected. However, under these experimental conditions, sufficient internal carnitine should be present to saturate both the untreated and the chymotrypsin-treated particles.

Experimental conditions for [^3H]carnitine loading of SMPs require a high-specific-radioactivity label and, therefore, a low loading concentration of carnitine is used in the incubation medium. Thus, the lumen carnitine content of untreated and treated vesicles is lower for measurement of palmitoylcarnitine-[^3H]carnitine exchange than for carnitine uptake measurements. Incubation of the chymotrypsin-treated vesicles in 1 mM-[^3H]carnitine produces a 30 % decrease in lumen carnitine

content (5.30 to 3.89 nmol/mg) while an almost four-fold decrease is observed in the untreated vesicles (3.82 to 0.99 nmol/mg). Therefore, it is possible that the higher carnitine content in the treated vesicles may be responsible for the increased velocity of palmitoylcarnitine-carnitine exchange. This type of situation would require that the K_m for carnitine on the internal face is high compared with the K_m for internal carnitine during carnitine-carnitine exchange. If this were the case, however, the velocity should increase at most by 4-fold instead of the 11-fold increase observed. Therefore, it is reasonable to expect that the increase in velocity of the palmitoylcarnitine-carnitine exchange results from the same proteolytic event which affects the carnitine exchange velocity.

The observed difference in lumen carnitine content between chymotrypsin-treated and untreated SMPs with 20 mM-carnitine loading (5.30 versus 3.82 nmol/mg) and with 1 mM-[^3H]carnitine loading (3.89 versus 0.99 nmol/mg) may be ascribed to two causes. First, the elevated velocity and affinity of the carnitine translocase produced by chymotrypsin may increase the ability of these particles to sequester carnitine by a unidirectional carrier mechanism [3] when compared with untreated SMPs. Secondly, we have observed that chymotrypsin treatment of SMPs produces a 50 % decrease in their rate of carnitine loss when placed in carnitine-free medium, as compared with untreated particles (results not shown). This would reduce the net loss of lumen carnitine and result in a net increase in the level of carnitine retained.

In summary, our data suggest that the carnitine-acylcarnitine translocase undergoes conformational changes following carnitine binding to a site on the carrier. These conformational changes expose one or more chymotrypsin cleavage sites on the carrier. Since these experiments were conducted in the presence of inverted mitochondrial membranes, we assume that the operative binding site for carnitine as well as the chymotrypsin cleavage site(s) are located on the surface of the translocase which originally faced the matrix. Proteolysis produces a 7–10-fold increase in translocase velocity and an increase in the accessibility of the carrier to its inhibitor, NEM. Furthermore, this chymotrypsin-induced increase in activity depends on the presence of free or short-chain carnitines in the proteolysis medium. Increasing the chain length of the acylcarnitine in the proteolysis medium reduces the extent of translocase activation. These results demonstrate that the carnitine-acylcarnitine translocase behaves similarly to several other proteins in that its activities are markedly altered by proteolysis with specific enzymes such as chymotrypsin [6,14,17]. These alterations in the kinetic properties imply that significant conformational rearrangement of the protein occurs during the transport 'cycle'. Such changes may expose new domains of the protein structure which are sensitive to chymotrypsin proteolysis [7]. These observations provide the first evidence suggesting that substrate-induced alterations in translocase conformation may be important in controlling the transport activity of the carrier.

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