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Topological orientation of mitochondrial GDPmannose : dolichyl-monophosphate mannosyltransferase in the outer membrane

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Previous studies have shown the existence of an autonomous mitochondrial GDPmannose : dolichylmonophosphate mannosyltransferase, located in mitochondrial outer membrane of liver cells. As nothing is known about glycosylation sites in mitochondria, we have investigated the topological orientation of this enzyme in intact mitochondria, using controlled proteolysis with trypsin. Mitochondria were purified sequentially by mild ultrasonic treatment and sucrose density gradient. Purity and homogeneity of mitochondrial fraction were assessed by electron microscopy and specific marker enzymes measures. Our data provide evidence for a mitochondrial GDPmannose : dolichylmonophosphate mannosyltransferase facing the cytoplasmic side of the outer membrane. However, the exposure of this enzyme to the water phase has been shown to be dependent on the ionic strength of the environment.

Introduction

During the past thirty years, research on mitochondrial function focused almost exclusively on mitochondrial inner membrane proteins as they are involved in fundamental processes: electron transport, ATP synthesis and ion pumping. Conversely the outer membrane was either ignored or limited to morphological observations.

In view of recent findings [1], mitochondria have been shown to interact with each other and with other cellular structures: cytoskeleton, nucleus and cytosol and all these interactions are mediated by outer membrane proteins. Therefore, the mitochondrial outer membrane appears as the key to understanding how the mitochondrion interacts with the other components of the cell.

Few data about mitochondrial outer membrane proteins have been reported. This membrane has been shown to contain a rotenone-insensitive NADH–cytochrome-*c* reductase, glycerophosphate acyltransferase, kynurenine hydroxylase, monoamine oxidase and porin [2–5]. More recently, enzymes involved in the biosynthesis of glycoconjugates have been localized in the mitochondrial outer membrane [6–10]. Particularly, a GDPmannose : dolichylmonophosphate mannosyltransferase has been solubilized from mitochondrial outer membrane, purified and reconstituted in artificial liposomal vesicles [11].

Since little is known about the molecular architecture of the outer membrane, especially concerning the topography of glycosylation reactions, we have investigated the orientation of the GDPmannose : dolichylmonophosphate mannosyltransferase 'in situ' in intact purified mitochondria. This was achieved by the use of (i) trypsin as a non-permeant probe and (ii) adenylate kinase and creatine kinase as controls for mitochondrial outer membrane integrity.

In this paper, we report that mitochondrial mannosyltransferase is located on the outer side of the outer membrane. Nevertheless, its exposure to the cytosolic side of the outer membrane is a function of the ionic strength of the environment, as has been reported for other mitochondrial outer membrane enzymes [5].

Experimental procedures

Materials

Trypsin (EC 3.4.21.4) was obtained from Difco (Detroit, Michigan). Soybean trypsin inhibitor (type II s), dolichyl monophosphate (grade III) and sphingomyelin (from bovine brain) were from Sigma.

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GDP[^{14}C]mannose (9.25 GBq/mmol) was purchased from New England Nuclear.

All other reagents were of analytical grade.

Animals

Mice, strain OF1 (IFFA Credo, Les Oncins, France) with an average weight of 20 g were used. They were killed and underwent laparatomy. The livers were rapidly removed and placed in a solution containing 250 mM sucrose and 10 mM Tris-HCl buffer (pH 7.4) at 4°C.

Preparation and purification of mitochondria

Mitochondria were isolated according to the procedure described by Weinbach [12] and modified by Bustamente et al. [13]. Mouse livers were homogenized in a Dounce homogenizer in 250 mM sucrose, 10 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at $1000 \times g$ for 15 min. Further centrifugation of the supernatant at $7500 \times g$ for 15 min led to the sedimentation of crude mitochondria. Mitochondria were washed twice [6]. Washed mitochondria were suspended in the same buffer and submitted to mild ultrasonic treatment: 2.5-ml aliquots containing 65 mg protein were sonicated with a 3 mm probe at 70 V during 10 s (Sonimass type 75 T) and washed again in order to eliminate contaminants. The mitochondrial pellet was resuspended in 250 mM buffered sucrose and laid on a discontinuous sucrose gradient (2.0/1.6/1.4/1.2/1.0 M). Gradients were centrifuged for 2 h at 20000 rpm (rotor SW 27). The fraction localized between 1.4 M and 1.6 M layers and identified as 'purified mitochondria' fraction was withdrawn, diluted to 250 mM buffered sucrose, sedimented at $7500 \times g$ and immediately used for enzymatic assay.

Mitochondrial outer membranes were prepared by swelling of ultra-sonified mitochondria in hypotonic buffer and were purified on a discontinuous sucrose gradient as previously described [6].

Preparation of microsomes

The post-mitochondrial supernatant was sedimented at $20000 \times g$ for 30 min. The pellet was discarded. Further centrifugation of the 20000 g supernatant at $145000 \times g$ for 1 h led to the sedimentation of crude microsomal membranes.

Purity of mitochondria

(a) Assays for marker enzymes. The activity of glucose-6-phosphatase (EC 3.1.3.9) was assayed according to Schachter et al. [14]. Alkaline phosphatase (EC 3.1.3.1) was measured according to Fishmann et al. [15]. Assays of the activity of monoamine oxidase (EC 1.4.3.4) were done following the procedures of Tabor et al. [16]. β-*N*-acetylglucosaminidase (EC 3.2.1.30) was measured according to Svennerholm et al. [17]. Every step of purification of mitochondria was checked according to the method of De Duve et al. [18].

(b) Electron microscopy. For electron microscopy, mitochondria pellets were fixed for 2 h in 2% glutaraldehyde, 100 mM phosphate buffer (pH 7.4). Electron micrographs were performed at the Centre de Microscopie Electronique Appliquée à la Biologie (Université Claude Bernard, Lyon) and taken with JEOL 1200 Ex.

Control of outer membrane integrity

To monitor the extent of outer membrane damage during trypsin treatment, adenylate kinase (EC 2.7.4.3) and creatine kinase (EC 2.7.3.2) were assayed.

Adenylate kinase was measured spectrophotometrically according to the procedure of Schnaitman et al. [19].

Creatine kinase was assayed using the Boehringer Mannheim CK NAC-activated UV-system.

Protein measures

Protein was assayed routinely according to the procedure of Gornall et al. [21]. Bovine serum albumin was used as a standard.

Treatment with trypsin

As trypsin is a site-specific proteinase, its effect on mannosyltransferase was first investigated on the solubilized enzyme. Mannosyltransferase was solubilized from mitochondria outer membrane by 0.1% Nonidet P-40 as previously described [11]. To 150 μl of solubilized enzyme preparation (15 μg protein) were added various amounts of trypsin (0.5 μg, 1 μg or 5 μg). The suspension was preincubated for 5 min at 30°C. The trypsin reaction was stopped by the addition of soybean trypsin inhibitor to 0.12 mg/ml. The samples were chilled and used for mannosyltransferase activity measures.

Purified mitochondria were suspended in 250 mM sucrose, 10 mM 4-morpholineethanesulfonic acid (Mes) buffer (pH 6.5), 5 mM $MgCl_2$ to a protein concentration of 2 mg/ml. To 500 μl of this suspension was added 20 μg of trypsin. The suspension was preincubated at 30°C. At given times, the trypsin reaction was completely inhibited by the addition of soybean trypsin inhibitor to 0.2 mg/ml. The samples were immediately chilled and used for enzyme activity measures. In the experiments where the ionic strength of the medium was increased, a concentrated salt solution (250 mM $MgCl_2$, $CaCl_2$, $(NH_4)_2SO_4$ or 1 M CH_3COONa, CH_3COONH_4) was prepared. Addition of the appropriate salt to experimental aliquots prior to the preincubation achieved the indicated ionic strengths. Ionic strength (I) is calculated from the formula, $I = 1/2\Sigma C_i Z_i^2$ where C_i is the molar concentration and Z_i is the charge of each ion. In some experiments, the

osmolarity of the medium was decreased from 250 mM to 50 mM by adding Mes buffer in order to abolish outer membrane integrity.

Mannosyltransferase assay

Mannosyltransferase activity in the presence of exogenous dolichyl monophosphate and sphingomyelin was assayed by the following standard procedure. Dolichyl monophosphate (0.2 mg) and sphingomyelin (1 mg) were evaporated under a stream of nitrogen. The dry lipid film was then swollen in 0.5 ml of 10 mM Mes buffer (pH 6.5) and sonicated with a probe sonicator (Sonimass, type 75T) at 100 V for 3 × 1 min at 37°C. 25 μl of this liposome suspension were added to 0.2 ml of mitochondria (0.4 mg protein) in 250 mM sucrose, 10 mM Mes buffer (pH 6.5). The mixture was sonicated in an ultrasonic water bath (4 × 10 s) at 37°C. 5 mM $MgCl_2$ was then added. The reaction was initiated by adding 212 pmol GDP[^{14}C]mannose and was carried out for 20 min at 37°C. Incubations with mitochondrial outer membranes or microsomal membranes were performed under the same conditions in presence of 0.1 mg proteins of each sub-cellular fraction. The reaction was stopped by addition of 10 vols. of chloroform/methanol (2:1, v/v). The suspension was mixed vigorously, kept at room temperature for 10 min and centrifuged after addition of 3 vols. of water. The chloroform/methanol extract was collected as previously described [6]. Radioactivity was measured in a Minaxi Packard with a liquid scintillation counting mixture.

Results

Previous studies of our laboratory have shown that the mitochondrial outer membrane of liver cells contains a GDPmannose : dolichylmonophosphate mannosyltransferase [6]. This enzyme has been solubilized from purified mitochondrial outer membrane by the non-ionic detergent Nonidet P-40 and purified by an original method consisting of the incorporation of the enzyme in sphingomyelin liposomes loaded with dolichyl monophosphate, followed by separation of biologically active vesicles on sucrose density gradient [11].

In order to improve our knowledge about mitochondrial glycosylation processes we have investigated the transbilayer orientation of mitochondrial mannosyltransferase in the outer membrane. Because preparation of isolated mitochondrial outer membrane vesicles disturb membranous organization by disrupting the junctions between outer and inner membranes, this topological study was performed on purified intact mitochondria. The first part of our work was therefore the obtention of purified mitochondria devoid of contaminating membranes.

I. Purification of intact mitochondria

(1) Distribution of specific marker enzymes activities during purification of crude mitochondria. To assess the purity of the mitochondria the following marker enzymes were used: monoamine oxidase for mitochondria outer membrane, glucose-6-phosphatase for microsomes, alkaline phosphatase for plasma membranes and β-*N*-acetylglucosaminidase for lysosomes.

The first step of purification was obtained by mild ultrasonic treatment of washed mitochondria, resulting in significant elimination of the major contaminating membranes. However, as the specific activity of glucose-6-phosphatase still represented 5.5% of the specific activity of the enzyme in microsomes (data not shown), the purification was not sufficient for our purpose, since glycosylation processes also occur in microsomal membranes. To further purify these mitochondria, ultrasonified mitochondria were laid on a discontinuous sucrose gradient. After centrifugation, three fractions were obtained. The fraction sedimenting between 1.4 M and 1.6 M was identified as purified mitochondria (Table I):

This fraction exhibited a high specific activity of monoamine oxidase. This fraction was considered free of microsomal contaminants, as glucose-6-phosphatase activity was only 0.7% of the specific activity of this enzyme measured in microsomic membranes.

As seen in Table I, purified mitochondria exhibited significant transfer of GDPmannose onto exogenous dolichyl monophosphate (96 pmol/mg) resulting from

TABLE I

Distribution of mannosyltransferase activities in mitochondria and microsomes correlatively with marker enzymes activities

Monoamine oxidase, glucose-6-phosphatase and β-*N*-acetylglucosaminidase are expressed as nmol/min per mg protein. Alkaline phosphatase is expressed in μg phenol/min per mg protein. Mannosyltrasnferase is expressed as pmol/mg protein after 20 min of incubation. n.d., not determined.

Subcellular fraction	Monoamine oxidase	Glucose-6-phosphatase	Alkaline phosphatase	β-*N*-Acetyl glucosaminidase	Mannosyl-transferase
Purified mitochondria	16	3.69	0.50	5.37	96
Purified outer membranes	67	0.31	0.16	0.88	647
Microsomes	0	502	3.84	n.d.	804

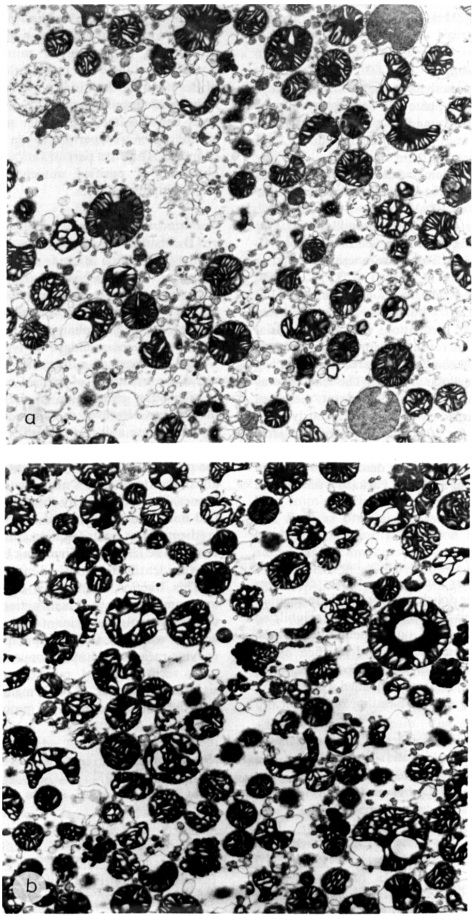

Plate I. Control of the purity of mitochondrial fractions by electron microscopy. (a) Washed mitochondria; (b) mitochondria purified on sucrose gradient. Magnification: ×9000 (original magnification: ×10000).

the activity of the mitochondrial outer membrane mannosyltransferase (as shown by the increase of mannosyltransferase specific activity in purified outer membranes commensurate with that of monoamine oxidase).

(2) Electron microscopy. The purification of intact mitochondria was confirmed by electron microscopy. As shown on Plate I, the purification of ultrasonified mitochondria on sucrose gradient resulted in a loss of contaminating membranes. Moreover, all mitochondria showed a continuous outer membrane and a condensed, densely staining matrix, therefore demonstrating the integrity of these organelles.

II. Trypsin treatment of purified mitochondria

In order to determine the transbilayer orientation of the GDPmannose : dolichylmonophosphate mannosyltransferase in the mitochondrial outer membrane, we used limited proteolysis of intact mitochondria with trypsin. As trypsin is a site-specific proteinase, its effect on mitochondrial mannosyltransferase was first investigated on the solubilized enzyme. The addition of trypsin to solubilized enzyme preparation led to a marked decrease in the activity of mannosyltransferase, which demonstrated the existence of sites of trypsinization in the enzyme (data not shown).

The results of limited tryptic digestion of mannosyltransferase 'in situ' in the mitochondria are presented in Fig. 1. Treatment of mitochondria for 15 min with increasing amounts of trypsin progressively inhibited the activity of mannosyltransferase. A proteinase-to-membrane protein ratio of 1 : 50 resulted in the loss of 70% of mannosyltransferase activity and a ratio of 1 : 12.5 completely inhibited the activity of the mitochondrial enzyme. The integrity of mitochondrial outer membrane during the course of the experiments was assessed by measuring the activities of two enzymes: adenylate kinase and creatine kinase. Adenylate kinase is a soluble enzyme present in the intermembrane space, sensitive to trypsin attack [5]. As this enzyme has been reported to be quite labile [19], creatine kinase, an extrinsic protein bound to the outside of the inner mitochondrial membrane was also used as a probe for outer membrane intactness. The sensitivity of this enzyme to trypsin had been previously demonstrated using disrupted mitochondria (data not shown). As shown in Fig. 1, trypsin at a proteinase-to-membrane protein ratio of 1 : 50 did not affect the intactness of the outer membrane since adenylate kinase and creatine kinase were not attacked by the proteinase. But the outer membrane integrity was abolished by a ratio of 1 : 12.5 as judged by the inhibition of adenylate kinase and creatine kinase activities (−16% and −42%, respectively).

Using the ratio of 1 : 50, the kinetic of inhibition of mitochondrial mannosyltransferase activity by trypsin was investigated. The results presented in Fig. 2 show that the activity of mannosyltransferase could be completely inhibited by trypsin after 40 min of preincubation. However, measurements of adenylate kinase and creatine kinase activities under the same conditions, indicated that the mitochondrial outer membrane did not remain intact after more than 15 min of preincubation: 20 min of preincubation with trypsin caused 5% inhibition of adenylate kinase activity and 30% of creatine kinase activity and 40 min of preincubation with trypsin resulted in the loss of 15% and 55% of adenylate

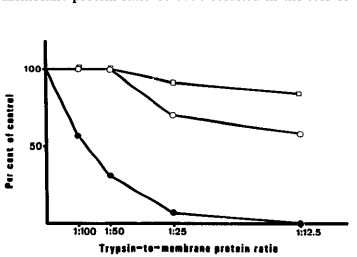

Fig. 1. Inactivation of mitochondrial mannosyltransferase by trypsin. Mitochondria (2 mg/ml) suspended in 250 mM buffered sucrose, 5 mM $MgCl_2$ were preincubated with varying amounts of trypsin (0–80 μg per mg mitochondrial protein) for 15 min at 30 °C and assayed for mannosyltransferase, adenylate kinase and creatine kinase activities (expressed as percentages of controls obtained with samples preincubated in the absence of trypsin). Control specific activities for mannosyltransferase, adenylate kinase and creatine kinase were 93 pmol/mg per 20 min, 152 nmol/mg per min and 220 nmol/mg per min, respectively. Each value represents an average of duplicate assays. ●, mannosyltransferase activity; ○, creatine kinase activity; □, adenylate kinase activity.

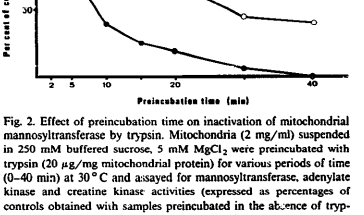

Fig. 2. Effect of preincubation time on inactivation of mitochondrial mannosyltransferase by trypsin. Mitochondria (2 mg/ml) suspended in 250 mM buffered sucrose, 5 mM $MgCl_2$ were preincubated with trypsin (20 μg/mg mitochondrial protein) for various periods of time (0–40 min) at 30 °C and assayed for mannosyltransferase, adenylate kinase and creatine kinase activities (expressed as percentages of controls obtained with samples preincubated in the absence of trypsin). Each value represents an average of duplicate assays. ●, mannosyltransferase activity; ○, creatine kinase activity; □, adenylate kinase activity.

kinase and creatine kinase activities, respectively. Nevertheless, the data indicated that a trypsin-to-membrane protein ratio of 1 : 50 and a pretreatment of 15 min did not affect the integrity of the outer membrane. Moreover, this ratio was not limitant for the attack of mitochondrial mannosyltransferase.

In view of these results, two hypotheses arise:

-- either a crucial domain of mitochondrial mannosyltransferase is located on the outer side of the outer membrane

-- or the mitochondrial mannosyltransferase is located on the inner side of the outer membrane and trypsin inhibits a sugar nucleotide carrier protein as it has been described in the case of microsomal glycosylation processes [22].

To decide between both hypotheses, intact mitochondria in 250 mM buffered sucrose were pretreated with trypsin at a proteinase-to-membrane protein ratio of 1 : 50 for 15 min. The reaction was stopped by adding soybean trypsin inhibitor. The outer membrane was then ruptured by decreasing the tonicity of the incubation to 50 mM osmolarity and the samples were used for mannosyltransferase activity analyses (Table II). Parallel to this, the extent of outer membrane damage in hypotonic media was monitored by measuring the activities of adenylate kinase and creatine kinase after trypsin treatment of mitochondria in media consisting of buffered sucrose at 175, 100 and 50 mM osmolarity. As shown in Fig. 3, outer membrane integrity was abolished at 50 mM osmolarity as judged by the maximal inhibitions of adenylate kinase and creatine kinase by trypsin. However, disruption of mitochondrial outer membrane by decreasing osmolarity after trypsin treatment had no effect on GDPmannose utilization for dolichylmonophosphate-mannose synthesis (Table II).

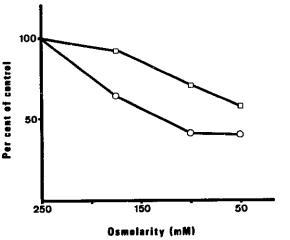

Fig. 3. Effect of decreasing medium osmolarity on the susceptibility of creatine kinase and adenylate kinase to trypsin. Mitochondria were suspended to a final concentration of 5 mg/ml in media consisting of buffered sucrose at the indicated concentration and 5 mM $MgCl_2$. They were treated with trypsin (20 μg/mg mitochondrial protein) for 15 min at 30 °C and assayed for adenylate kinase and creatine kinase activities. Results are expressed as a percentage of the control obtained with mitochondria preincubated in the absence of trypsin. ○, creatine kinase activity; □, adenylate kinase activity.

TABLE II

Effect of outer membrane disruption on the accessibility of mannosyltransferase to trypsin

10 mg of mitochondria in 1 ml of 250 mM buffered sucrose were pretreated with trypsin (20 μg/mg mitochondrial protein) for 15 min at 30 °C. The reaction was stopped with soybean trypsin inhibitor. A portion of the suspension was assayed for mannosyltransferase activity (0.4 mg mitochondrial protein/assay). The osmolarity of the other portion was decreased to 50 mM which caused disruption of mitochondrial outer membrane. The disrupted mitochondria were then assayed for mannosyltransferase activity (0.4 mg mitochondrial protein/assay). Controls in the absence of trypsin were also performed under the same conditions of osmolarity.

Treatment	Mannosyltransferase activity (pmol/mg per 20 min)
Intact mitochondria (in 250 mM sucrose)	93
Mitochondria outer membrane disruption (50 mM sucrose)	89
Pretreatment of intact mitochondria with trypsin (250 mM sucrose)	25
Pretreatment of intact mitochondria with trypsin, then disruption of outer membrane	22

As the inhibition of mannosyltransferase activity was not relieved by rupture of mitochondrial outer membrane, it appeared that it could not be attributed to interaction with specific transport proteins but rather to direct interaction of the proteinase with the mitochondrial enzyme.

The above results therefore demonstrate that a crucial domain of mitochondrial mannosyltransferase is located on the outer side of the outer membrane. But they do not rule out the possibility of an enzyme spanning the bilayer with another crucial domain facing the intermembrane space. To probe the existence of such a domain in the inner side of the outer membrane, mitochondrial samples were incubated at 30 °C for 15 min in the presence of trypsin (ratio 1 : 50) after gradually disrupting the outer membrane. Adenylate kinase and creatine kinase were assayed to monitor the extent of outer membrane damage during the course of the experiments as previously described (Fig. 3). Fig. 4 documents the results of such an experiment where the outer membrane was ruptured by gradually decreasing the tonicity of the incubation medium. Although outer membrane integrity was abolished at 50 mM sucrose, no additional inhibition of mannosyltransferase activity was observed. These results are therefore consistent with a

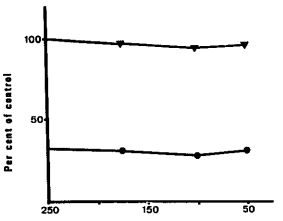

Fig. 4. Effect of mitochondrial outer membrane disruption on the susceptibility of mannosyltransferase to trypsin. Mitochondria were suspended to a final concentration of 2 mg/ml in media consisting of buffered sucrose at the indicated concentration and 5 mM $MgCl_2$. They were preincubated with trypsin (20 μg/mg mitochondrial protein) for 15 min at 30°C and assayed for mannosyltransferase activity. Controls in the absence of trypsin were also performed. Control specific activities for mannosyltransferase ranged from 84 to 93 pmol/mg per 20 min. Each value represents an average of duplicate assays. ▼, mannosyltransferase activity in the absence of trypsin; ●, mannosyltransferase activity in the presence of 20 μg trypsin per mg mitochondrial protein.

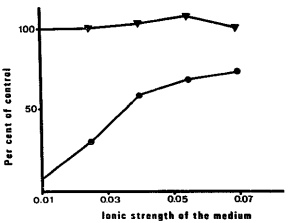

Fig. 5. Effect of different ionic environments on the susceptibility of mannosyltransferase to trypsin. The mitochondrial fraction (2 mg/ml) was suspended in 250 mM buffered sucrose with $MgCl_2$ added to the desired ionic strengths as indicated. After 5 min of preincubation at 0°C, trypsin was added (20 μg/mg mitochondrial protein). The samples were further incubated for 15 min at 30°C and assayed for mannosyltransferase activity. Controls in the absence of trypsin were also performed. Control specific activities for mannosyltransferase ranged from 93 to 103 pmol/mg per 20 min. Each value represents an average of duplicate assays. ▼, mannosyltransferase activity in the absence of trypsin; ●, mannosyltransferase activity in the presence of 20 μg trypsin per mg mitochondrial protein.

mitochondrial mannosyltransferase facing the cytoplasmic side of the outer membrane.

III. Effect of ionic strength on the inactivation of mitochondrial mannosyltransferase by trypsin

As it has been reported that the exposure of some mitochondrial outer membrane enzymes to the cytosolic side of the membrane may change according to the ionic strength of the environment [5], the susceptibility of mitochondrial mannosyltransferase to trypsin was investigated using media of increasing ionic strengths. This was achieved by adding concentrated salt solutions (magnesium chloride, calcium chloride, ammonium sulfate, ammonium acetate, sodium acetate) to a mitochondrial suspension in 250 mM buffered sucrose prior to the preincubation with trypsin (ratio 1:50). Fig. 5 illustrates the results of such an experiment where the ionic strength was increased with concentrated magnesium chloride. As shown in this figure, a high ionic environment protected mannosyltransferase against inactivation by trypsin. This protection was not salt-specific since the same effect was obtained with calcium chloride and ammonium sulfate (Table III). It must be emphasized, however, that divalent ions were more efficient than monovalent ions in preventing mannosyltransferase from proteolysis by trypsin. This effect was not due to a change in the osmolarity of the medium as the addition of 250 mM sucrose had no influence on mannosyltransferase sensitivity toward trypsin (data not shown). In order to rule out a possible inhibitory action of high ionic strengths on trypsin, solubilized mannosyltransferase was preincubated with trypsin at two different ionic strengths: 0.01 and 0.07, the latter being obtained with concentrated magnesium chloride. The proteolytic activity of trypsin on solubilized mannosyltransferase was not dependent on the ionic strength of the medium since the same inhibition of mannosyltransferase activity was obtained at both ionic strengths: −38% at $I = 0.01$ and −33% at $I = 0.07$. It appeared therefore that the different inhibitory action of trypsin on the mitochondrial mannosyltransferase at different ionic strengths was not due to

TABLE III

Effect of various salts on the inactivation of mitochondrial mannosyltransferase activity by trypsin

Mitochondria (2 mg of protein/ml) were preincubated for 15 min at 30°C in the presence of trypsin (20 μg/mg mitochondrial protein) with the addition of various salts at 0.025 and 0.07 ionic strength. Controls in the absence of trypsin were also performed. Results are expressed as percent of the controls without trypsin.

Salt	Mannosyltransferase activity after pretreatment with trypsin	
	$I = 0.025$	$I = 0.07$
Sodium acetate	5%	13%
Ammonium acetate	9%	42%
Magnesium chloride	33%	74%
Calcium chloride	61%	82%
Ammonium sulfate	41%	79%

any change in the activity of this proteinase but to a substantial change in mannosyltransferase topography.

Discussion

The present work is about the topological organization of mitochondrial outer membrane GDPmannose: dolichylmonophosphate mannosyltransferase, 'in situ' in intact purified mitochondria.

The aim of this study was two-fold: first, to improve our knowledge about the molecular architecture of the outer membrane particularly concerning glycosylation sites, and second, to make possible a comparison with the organization of the enzyme reconstituted in liposomal vesicles.

Since a GDPmannose: dolichylmonophosphate mannosyltransferase in endoplasmic reticulum is well-known [23], the first part of our work was the obtention of purified mitochondria devoid of microsomal contaminants. This was achieved by a two-step purification involving mild ultrasonic treatment of mitochondria followed by a discontinuous sucrose density gradient centrifugation.

The mitochondrial outer membrane has been reported to be permeable to low molecular weight compounds [24], in contrast to endoplasmic membranes. Therefore, the use of anion-specific inhibitors usually employed as non-permeant probes in topological studies of glycosylation reactions [25–28] was precluded. In the investigation reported here, we based on the sensitivity of solubilized mannosyltransferase toward trypsin to examine the topological orientation of the enzyme 'in situ' in purified isolated mitochondria. Since trypsin has a molecular weight of 24000, the outer membrane should be impermeable to this enzyme. However, trypsin, as a proteinase, can penetrate the membrane by cleaving the proteins, hence the necessity to control the intactness of outer membrane during trypsin treatment. As the existence of an outer membrane enzyme entirely located on the inner surface of the outer membrane has not been described yet, outer membrane integrity during trypsin treatment was therefore assessed by measuring the activities of two enzymes exposed to the intermembrane space: adenylate kinase, a soluble enzyme and creatine kinase, an extrinsic protein bound to the outer side of the mitochondrial inner membrane by electrostatic interactions with cardiolipin [29]. These two enzymes become easily accessible to external compounds (such as proteinases) in the case of outer membrane disruption. Both enzymes were efficient probes for outer membrane intactness as judged by the similarity of the results obtained. However, it is noteworthy that when the outer membrane integrity was abolished, the inhibition of creatine kinase by trypsin was always greater than the inhibition of adenylate kinase. This fact is probably to be related to the amounts of these enzymes in liver mitochondria, adenylate kinase being present in larger quantities than creatine kinase.

We previously reported that the activity of purified mannosyltransferase was most important in the acidic range, with a maximum at pH 6.1 [11]. Since Hanover and Lennarz [30] reported that at pH values below 6.5, the membrane permeability barrier is significantly disrupted, all the experiments were carried out at pH 6.5.

Trypsin treatment, under conditions where trypsin did not penetrate the outer membrane, resulted in the reduction of dolichylmonophosphate-mannose synthesis by at least 70%. Moreover, disruption of mitochondrial outer membrane by decreasing the osmolarity of the medium did not cause an increase in the proteinase sensitivity of mannosyltransferase. These results demonstrate that the active site of this enzyme resides on the cytoplasmic face of the mitochondrial outer membrane. However, as the existence of sugar nucleotide carrier proteins has been described in the rough endoplasmic reticulum membrane for the translocation of UDP-*N*-acetylglucosamine and UDPglucose [31] and in Golgi-derived vesicles for the transport of CMP-neuraminic acid, GDPfucose and UDPgalactose [21], and as it is not known whether sugar nucleotides can permeate the mitochondrial outer membrane, an alternative explanation was that the active site of mannosyltransferase is located on the inner side of the outer membrane and that the loss of activity is due to the proteolysis of a GDPmannose carrier protein. The inhibition of mannosyltransferase activity by trypsin was not relieved by disruption of mitochondrial outer membrane after trypsin treatment. Furthermore, no effect of outer membrane disruption (without trypsin treatment) on the utilization of GDPmannose for dolichylmonophosphate-mannose synthesis was observed. These results are therefore consistent with the absence of GDPmannose carrier protein and with a direct interaction of trypsin with mannosyltransferase.

According to Hesler et al. [5], there appear to be two groups of outer membrane proteins: the first group is composed of intrinsic proteins such as monoamine oxidase and porin, completely protected from proteinase action whatever ionic or osmotic conditions may be. The other group consists of proteins such as glycerophosphate acyltransferase and rotenone-insensitive NADH–cytochrome-*c* reductase, whose exposure to the water phase is a function of the ionic strength of the environment. In view of our results concerning the susceptibility of mitochondrial mannosyltransferase to trypsin in different ionic environments, the GDPmannose: dolichylmonophosphate mannosyltransferase belongs to the latter group. The variation in the exposure of these enzymes in media of different ionic strengths can be explained either by a change in the conformation of the proteins themselves or by an architectural change in the phospholipid environment of these proteins. Con-

cerning mannosyltransferase, the fact that divalent ions were more efficient than monovalent ions in protecting the enzyme against trypsinolysis might substantiate the second hypothesis: by making bonds between polar headgroups of phospholipids, the divalent ions might change the architecture of the membrane in a way that mannosyltransferase would become cryptic.

So, whether the variation in the cytosolic exposure of mannosyltransferase is due to a change in the conformation of the enzyme or to a change in the molecular architecture of the outer membrane cannot be deduced from the present experiments and will have to await the data from the organization of purified mannosyltransferase reconstituted in well-defined artificial membranes.

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