Further Observations on the Effects of Trypsin on the Volume and Functions of Mitochondria Isolated from Normal Liver and AH-130 Yoshida Ascites Hepatoma

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SUMMARY

Evidence is given indicating that the outer membrane of mitochondria from AH-130 Yoshida ascites hepatoma has low resistance to trypsin. The outer membrane of freshly-isolated hepatoma mitochondria is impermeable to cytochrome c and dextran, but it seems to be easily digested by trypsin. The proteolytic enzyme partially inactivates the adenylate kinase and cytochrome c oxidase of these mitochondria. Inhibition of both enzymes by trypsin is also observed in rat liver mitochondria, but only after the outer membrane has been made permeable to large molecules. The succinate-cytochrome c reductase of both hepatoma and liver mitochondria is not inactivated by trypsin even if the outer membrane is made permeable to large molecules by digitonin. Inactivation of succinate-cytochrome c reductase occurs when trypsin is added after a strong sonic treatment of mitochondria. The adenosine triphosphatase of both types of mitochondria is resistant to trypsin also after the sonic fragmentation of the mitochondria. A marked inhibition of adenylate kinase by proteolysis takes place in phospholipase C-defatted mitochondria from hepatoma and liver when incubation with trypsin precedes the reconstitution of the enzyme by phospholipids extracted from liver. Swelling-inducing amounts of trypsin affect some inner membrane-linked functional activities of hepatoma and liver mitochondria; however, in liver mitochondria, the time of onset of swelling is much later than in hepatoma mitochondria, since the swelling in the former is preceded by a lag phase during which no functional impairment occurs.

These results are interpreted as indicative of structural alterations of the outer membrane of hepatoma mitochondria, whereas no differences with regard to sensitivity of trypsin seem to exist between the inner membrane of liver and hepatoma mitochondria.

INTRODUCTION

The effect of lytic enzymes on membranes has been used to dissect the molecular organization of the phospholipids and proteins in membranes (19, 24, 27, 33, 43). If an abnormal structure exists in membranes of tumor mitochondria (10, 39), one might also expect to find, as a possible consequence, an abnormal response to lytic enzymes. Mitochondria from Yoshida ascites hepatoma have indeed shown high sensitivity to trypsin. Small amounts of the lytic enzyme induce rapid swelling, increased permeability of the inner membrane to sucrose, and inhibition of the respiratory activity (11). These features contrast with the relative insensitivity of normal liver mitochondria to trypsin (11, 20).

In this communication we have extended the results of our previous investigations on the effect of trypsin on different enzymatic and functional activities of hepatoma mitochondria (11). In particular, we have investigated the accessibility of trypsin to the different compartments of hepatoma mitochondria by evaluating the sensitivity to proteolysis of various enzymes according to their topographic distribution in the mitochondrion. Our results indicate that the differing susceptibilities to trypsin of mitochondria from hepatoma and from rat liver can be ascribed to the higher sensitivity to proteolysis of the outer membrane of hepatoma mitochondria, which makes their inner membrane more accessible to the proteolytic enzyme.

MATERIALS AND METHODS

Preparation of Mitochondria. Long-Evans rats, weighing 150 to 200 g, were used to transplant AH-130 Yoshida ascites hepatoma or as a source of normal livers. The animals were housed no more than 4 to a cage and given tap water ad libitum. They were maintained on a semisynthetic diet (Piccioni, Brescia, Italy). The cells from 6-day-old tumors were housed no more than 4 to a cage and given tap water ad libitum. They were maintained on a semisynthetic diet (Piccioni, Brescia, Italy). The cells from 6-day-old tumors were separated from the ascitic fluid by low-speed centrifugation. Heavy mitochondria were isolated, as described in detail in a previous paper (13), except that a medium containing 220 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl (pH 7.4), 2 mM EDTA, and 0.2% (w/v) crystalline bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) was used. The albumin was defatted according to the method of Goodman (16); the albumin solutions were dialyzed overnight against water prior to use. Livers were removed after the rats were killed by decapitation; then the livers were chilled in the above-described ice-cold medium. The minced tissue was suspended in a volume of isolation medium equivalent to 5 times the weight of the tissue. The homogenization procedure used for the AH-130 hepatoma (13) was also adopted for rat liver. Heavy...
mitochondria were isolated from liver according to the method of De Duve et al. (6). The final mitochondrial pellets were suspended in the isolation medium, free of EDTA, to a final concentration of 20 mg of mitochondrial protein per ml.

**Trypsin Treatment.** To study the effect of trypsin on the adenylate kinase activity, we suspended mitochondria (6 to 10 mg of protein) in 1 ml of a reaction mixture containing 290 mM sucrose and 20 mM Tris-Cl (pH 7.4) (isotonic medium). After 10 min of incubation at 23° with different amounts of trypsin (Type III; Sigma Chemical Co.), the reaction was arrested by the addition of 3 times the weight excess of soybean trypsin inhibitor (Type I-S; Sigma Chemical Co.). In some experiments, the adenylate kinase was extracted from mitochondria, according to the method of Pfaff and Schwalbach (29), and was submitted to trypsin digestion, either before or after separation from the residual particulate material by 10 min of centrifugation at 15,000 × g.

For an investigation of the effect of trypsin on the activities of the cytochrome c oxidase, succinate-cytochrome c reductase, and adenosine triphosphatase, the mitochondria (10 mg of protein per ml) were suspended in the isotonic medium and treated with trypsin, as in the adenylate kinase experiments. Some samples were submitted to digitonin or sonic treatment (see below) before incubation with trypsin. The proteolysis was arrested by suitable amounts of trypsin inhibitor. The suspensions were then submitted to an activating treatment (see below), according to the need of the particular experiment, and were used as a source of enzymes after dilution in the isotonic medium or a buffer suitable for the specific assay.

**Assays.** The spectrophotometric determinations of the adenylate kinase and succinate-cytochrome c reductase activities were performed according to the method of Sottocasa et al. (40, 41), and the assay of malate dehydrogenase was performed according to the method of Schnaitman and Greenawalt (36). The cytochrome c oxidase activity was determined polarographically according to the method of Sottocasa et al. (41). We determined the adenosine triphosphatase activity by incubating mitochondria (0.4 mg of protein) in 1 ml of a reaction medium containing 145 mM KCl, 20 mM triethanolamine-Cl (pH 7.4), 5 mM ATP, 4 mM MgCl₂, and 0.1 mM 2,4-dinitrophenol. The reaction was started by the addition of ATP and was arrested, after 15 min at 23°, by 270 mM HClO₄. The inorganic phosphorus was determined according to the method of Lowry and Lopez (22), after removal of the acid as KClO₄.

Mitochondrial respiration was measured with a vibrating platinum electrode (Oxygraph; Gilson Medical Electronics, Middleton, Wis.) coated with collodion. Mitochondria (2 mg of protein) were added to 2 ml of assay medium containing 130 mM KCl, 20 mM Tris-Cl (pH 7.4), 10 mM L-glutamate, 5 mM L-malate, and 1 mM Tris-phosphate. The acceptor control ratio and respiratory increment by Ca²⁺ were determined according to the methods of Chance and Williams (4) and Rossi and Lehninger (34), respectively. To evaluate the respiratory increment induced by K⁺ plus valinomycin, we suspended 0.2-ml aliquots of mitochondria (4 mg of protein) in 2 ml of a reaction mixture containing 10 mM KCl, 270 mM sucrose, 20 mM Tris-Cl (pH 7.4), 4 mM Tris-phosphate, 10 mM L-glutamate, and 5 mM L-malate. The respiratory release was induced by addition of 0.3 μg of valinomycin.

**Enzyme Activation.** Various activation procedures of the mitochondrial enzymes were performed. For sonic activation, mitochondrial suspensions (6 to 10 mg of protein per ml) in a solution containing 10 mM Tris-phosphate (pH 7.4) and 1 mM EDTA were submitted to five 30-sec periods of sonic oscillation with the use (at 60% of its maximal output) of a Biosonik III sonifier provided with an intermediate tip (Browning Scientific, Inc., Rochester, N. Y.). To avoid warming during sonic treatment, the suspensions were immersed in a mixture precooled at −20°. Lubrol activation was performed according to the method of Schnaitman and Greenawalt (36), with 0.3 mg of Lubrol WX (I.C.I. Organics, Inc., Providence, R. I.) per mg of protein. For disruption of the mitochondrial outer membrane by digitonin, mitochondria (10 mg of protein per ml suspended in the isotonic medium) were incubated 15 min at 0° in the presence of 0.4 mg (unless otherwise stated) of digitonin (twice recrystallized from ethanol) per mg of protein.

**Determination of Swelling and Proteolytic Activity.** We investigated the effect of trypsin on the mitochondrial volume by suspending mitochondria (0.6 to 1.0 mg of protein) in 3 ml of isotonic medium. One to 30 μl of trypsin solution were rapidly mixed with the mitochondrial suspensions, and changes in absorbance were followed at 520 nm (at 23°) in a Beckman DB spectrophotometer, with a standard of suitable opacity in the reference cell. For determination of the NRC₂, 0.2-ml aliquots were taken at different time intervals and added to 1 ml of a ninhydrin reagent (9).

**Volume Determinations.** Mitochondria (5 mg of protein) were suspended in 1 ml of a medium containing 290 mM sucrose, 20 mM Tris-Cl (pH 7.4), and either 274 μg of dextran-carboxyl-¹⁴C (2 × 10⁶ dpm/mg; M.W., 50,000; The Radiochemical Center, Amersham, Bucks, England) or 5 × 10⁻⁴ mmole of uniformly labeled sucrose-¹⁴C (8.8 × 10⁸ dpm/mmmole; The Radiochemical Center). After 5 min incubation at 23°, the suspensions were sedimented at 89,000 × g for 4 min. The tubes were inverted, carefully washed to eliminate the excess of medium, and drained. The pellets were suspended in 500 mM HClO₄, and the proteins were sedimented at 5,000 × g for 3 min. The ¹⁴C content was counted, after removal of the acid as KClO₄, on 0.2-ml aliquots of supernatants in a liquid scintillation counter. The volumes of the pellet were determined as described in a previous work (13). In some experiments the mitochondria were incubated 30 min at 23° in 10 mM Tris-phosphate (pH 7.4). The inner membrane was caused to shrink by the addition of 5 mM ATP and 3 mM MgCl₂, followed by an additional 20 min of incubation at 23°. One-tenth-ml aliquots of the suspensions were then added to 1 ml of the aforementioned medium for the determination of the volumes of the pellet.

**Treatment with Phospholipase C and Rebinding of Phospholipids.** Mitochondria (4 mg of protein per ml) and 80 μg of phospholipase C (Type I; Sigma Chemical Co.) were incubated for 30 min at 23° in 1 ml of a medium containing 290 mM sucrose, 20 mM Tris-Cl (pH 7.4), and 4 mM MgCl₂. The reaction, initiated by addition of phospholipase C, was
arrested by the addition of 10 mM EDTA. The samples and controls (the latter not incubated with phospholipase C) were sedimented at 125,000 × g for 4 min, washed once in isotonic medium containing 4% (w/v) defatted crystalline bovine serum albumin and, finally, suspended in 1 ml of isotonic medium. For the rebinding experiments, phospholipids extracted from liver were added to the samples according to the method of Fleischer and Fleischer (14). Some samples were incubated 15 min at 23° with 200 µg of trypsin per mg of protein, either before or after the phospholipid rebinding. Lipids were extracted with a 2:1 (v/v) chloroform:methanol mixture, and phospholipids were purified through Unisil columns (Clarkson Co., Williamsport, Pa.). (21). Phospholipid phosphorus was determined according to the method of Skipski et al. (38).

Protein Determination. The mitochondrial protein was determined according to the method of Gornall et al. (17).

RESULTS

Effect of Trypsin on the Adenylate Kinase Activity. It follows from the data in Chart 1 that the adenylate kinase of hepatoma mitochondria, suspended in isotonic medium, is inactivated by trypsin. The degree of inactivation is a function of the amount of proteolytic enzyme; about 50% inactivation is reached with 200 µg of trypsin per mg of mitochondrial protein. This amount of trypsin induced only 16% inactivation of the adenylate kinase in liver mitochondria; with amounts of proteolytic enzyme between 10 and 100 µg/mg, no inactivation is observed in these mitochondria. The adenylate kinase of both liver and hepatoma mitochondria can be solubilized by treating the mitochondria with hypotonic phosphate in the presence of EDTA (29). The data in Chart 1 indicate that the solubilized enzyme is equally sensitive to trypsin in hepatoma and liver mitochondria, regardless of whether it is separated from the residual particulate material. The apparent lower sensitivity to trypsin of the solubilized enzyme, when not separated from the particulate material, could depend upon the presence of the bulk of mitochondrial proteins acting as substrate for the proteolytic enzyme.

The adenylate kinase is an enzyme located in the intermembrane compartment of mitochondria (5, 36, 40). A preexisting high degree of permeability to large molecules of the outer membrane could be responsible for the increased sensitivity to trypsin of hepatoma mitochondria. Normally, the intact outer membrane of mitochondria was found to be impermeable to cytochrome c (46), so that the cytochrome c oxidase activity was completely masked when the mitochondria were incubated in an isotonic medium. The rupture of the outer membrane (but with an intact inner membrane) was associated with the complete activation of the enzyme (46). Consequently, when the mitochondria are suspended in an isotonic medium, the determination of this enzymatic activity could represent a suitable marker for the integrity of the outer membrane. As shown in Table 1, a very low percentage of cytochrome c oxidase activity is detected in hepatoma mitochondria suspended in an isotonic medium.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amount (mg/mg)</th>
<th>Cytochrome c oxidase (%)</th>
<th>Malate dehydrogenase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Hypotonic</td>
<td>1.2 ± 0.3a</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Digitonin</td>
<td>70.5 ± 3.4</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Digitonin</td>
<td>7.7 ± 1.2</td>
<td>4.1 ± 1.3</td>
<td>4.1 ± 1.3</td>
</tr>
<tr>
<td>Digitonin</td>
<td>35.8 ± 7.3</td>
<td>20.8 ± 3.6</td>
<td>20.8 ± 3.6</td>
</tr>
</tbody>
</table>

— Mean ± S.D.
Various amounts of enzyme activity are released by either hypotonic or digitonin treatments, which are known to induce the rupture of the outer mitochondrial membrane (25, 45, 46). Maximal activation is obtained with amounts of digitonin that induce only a moderate release of the matrix enzyme, malate dehydrogenase (8, 35), indicating that the inner membrane is largely preserved under these conditions.

Another approach to the question of the integrity of the outer membrane of hepatoma mitochondria was the study of its permeability to dextran. This was based upon the observation that the inner membrane of mitochondria is impermeable to sucrose in low-duration experiments, whereas the outer membrane is permeable to sucrose but is not permeable to large polymers such as dextran (8, 28). If all outer membranes were ruptured, the sucrose and dextran volumes of mitochondrial pellets would be the same, if not, a larger sucrose volume would be measured (18), as shown in Table 2. The dextran volume of the mitochondrial pellet is lower than the sucrose volume in hepatoma mitochondria. After large-amplitude swelling, which involves the expansion of the inner membrane and the rupture of the outer membrane (8, 24, 44), the addition of ATP induces shrinkage of the inner but not of the outer membrane (8, 44). This would abolish or reduce the differences between the sucrose and dextran volumes of the mitochondrial pellets. As shown by data in Table 2, this is in fact our experimental observation.

**Effect of Trypsin on Inner-Membrane Enzymes of Mitochondria.** A study of the sensitivity to trypsin of the enzymes linked to the inner mitochondrial membrane could represent a suitable approach to an investigation of the accessibility of trypsin to this membrane. As can be seen in Table 3, the inner-membrane enzymes (32) show different degrees of sensitivity to trypsin. The cytochrome c oxidase of untreated liver mitochondria is insensitive to trypsin (Samples a to c).

**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume of sucrose-1^4C (μl)</th>
<th>Volume of dextran-1^4C (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated mitochondria</td>
<td>38.0 ± 2.6^a</td>
<td>27.2 ± 1.9</td>
</tr>
<tr>
<td>Mitochondria submitted to swelling and shrinkage</td>
<td>43.3 ± 3.9</td>
<td>45.0 ± 2.7</td>
</tr>
</tbody>
</table>

^a Mean ± S.D.

The cytochrome c oxidase is largely affected by proteolysis in hepatoma mitochondria. In the presence of 200 μg of trypsin per mg of protein, 42% inhibition of the enzymatic activity occurs in these particles (Samples a and c). When incubation with trypsin is preceded by treatment with digitonin or sonic fragmentation of mitochondria, only a slight increase of sensitivity of the enzyme to trypsin is observed in hepatoma mitochondria (Samples d to g). In these conditions, a strong inhibition of the cytochrome c oxidase also occurs in liver mitochondria. Table 3 also shows that the succinate-cytochrome c reductase of hepatoma and liver mitochondria is insensitive to relatively high amounts of trypsin (Samples a to e). Inactivation of the succinate-cytochrome c reductase is only effected by trypsin in sonically fragmented mitochondria from hepatoma and liver (Samples f and g). The adenosine triphosphatase activity of both types of mitochondria appears to be resistant to trypsin also after sonic fragmentation of the particles.

The sensitivity to trypsin of adenosine triphosphatase was studied in phospholipase C-treated mitochondria. The phospholipase C affects an extensive delipidation associated with structural changes very similar to those induced by sonic treatment (32, 33). The incubation of hepatoma and liver mitochondria with phospholipase C induces a 74 to 79% release of phosphatidyicholine and a 50 to 57% release of the phosphatidyethanolamine. This release represents about 62% of the total phospholipid phosphorus in both types of mitochondria (data not given). As shown in Table 4, the phospholipase C-treated mitochondria lose about 60% of their original adenosine triphosphatase activity in both hepatoma and liver mitochondria (Samples a and d). The addition of phospholipids reconstitutes the enzyme activity at about 85 and 75% of the original activity in hepatoma and liver mitochondria, respectively (Samples a and e). When the treatment with trypsin precedes the addition of phospholipids (Sample f), the latter fails to reactivate the enzyme in both types of mitochondria; moreover, a further decrease of the activity takes place (compare Samples d and f). The addition of trypsin to the reconstituted enzyme (Sample g) causes about 35% decrease of the enzymatic activity in the 2 types of mitochondria (Samples e and g). This could indicate that the reconstituted enzyme is more susceptible to proteolysis, in comparison with the untreated adenosine triphosphatase (Sample b). However phospholipids seem to exert some protection on the enzyme. In fact, trypsin induces a more marked inhibition of the adenosine triphosphatase when added to the defatted enzyme than when added to the reconstituted enzyme. No difference was found between hepatoma and liver mitochondria as a result of these treatments.

**Volume Changes Induced by Trypsin.** As observed in a previous paper (11), the time of onset of the swelling induced by trypsin is rapid in hepatoma mitochondria, while in liver mitochondria it is slower and is preceded by a lag phase. In those experiments, the NRC production was also determined during the proteolysis, but this was not done at the same time or in the presence of the same amounts of trypsin used in the induction of the swelling. Consequently, it was not possible, at the time, to directly correlate the kinetics of NRC production to that of the swelling. Chart 2 shows the time courses of the swelling and NRC production induced by trypsin. The 2...
Mitochondria (10 mg of protein per ml) were suspended in isotonic medium and then were submitted to trypsin, as in the experiments described in Chart 1. Some samples were treated with digitonin or sonic oscillation (see "Materials and Methods") before incubation in the presence of trypsin. For determination of the cytochrome c oxidase, the suspensions were submitted to activation with Lubrol; for determination of the succinate-cytochrome c reductase, the suspensions were sonically activated. The specific activities are expressed as nanomols of oxygen taken up per min per mg of protein for cytochrome c oxidase and as nmoles of inorganic phosphorus released per min per mg of protein, respectively, for succinate-cytochrome c reductase and adenosine triphosphatase. The data are the mean values of 6 experiments for the determination of the oxidase and reductase activities and of 4 experiments for the determination of adenosine triphosphatase activity.

Table 3
The effect of trypsin on the activity of inner membrane enzymes of AH-130 hepatoma and rat liver mitochondria

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>Amount (µg/mg)</th>
<th>Pretreatment of mitochondria</th>
<th>Cytochrome c oxidase</th>
<th>Succinate-cytochrome c reductase</th>
<th>Adenosine triphosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hepatoma</td>
<td>Liver</td>
<td>Hepatoma</td>
</tr>
<tr>
<td>a</td>
<td>Control</td>
<td>None</td>
<td>None</td>
<td>360 ± 30</td>
<td>109 ± 26</td>
<td>141 ± 29</td>
</tr>
<tr>
<td>b</td>
<td>Trypsin</td>
<td>100</td>
<td>None</td>
<td>300 ± 24</td>
<td>107 ± 14</td>
<td>149 ± 23</td>
</tr>
<tr>
<td>c</td>
<td>Trypsin</td>
<td>200</td>
<td>None</td>
<td>210 ± 18</td>
<td>99 ± 13</td>
<td>148 ± 22</td>
</tr>
<tr>
<td>d</td>
<td>Control</td>
<td>None</td>
<td>Digitonin</td>
<td>321 ± 44</td>
<td>119 ± 28</td>
<td>148 ± 22</td>
</tr>
<tr>
<td>e</td>
<td>Trypsin</td>
<td>200</td>
<td>Digitonin</td>
<td>146 ± 32</td>
<td>119 ± 19</td>
<td>148 ± 22</td>
</tr>
<tr>
<td>f</td>
<td>Control</td>
<td>Sonic oscillation</td>
<td>Sonic oscillation</td>
<td>400 ± 56</td>
<td>142 ± 8</td>
<td>148 ± 22</td>
</tr>
<tr>
<td>g</td>
<td>Trypsin</td>
<td>200</td>
<td>Sonic oscillation</td>
<td>210 ± 24</td>
<td>71 ± 5</td>
<td>137 ± 12</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

Table 4
The effect of trypsin on the activity of adenosine triphosphatase of AH-130 hepatoma and rat liver mitochondria following delipidation by phospholipase C

Mitochondria (4 mg of protein) were suspended in 1 ml of a medium containing 290 mM sucrose, 20 mM Tris-Cl (pH 7.4), 4 mM MgCl₂, and, where indicated, 80 µg of phospholipase C. After 30 min at 23°, 10 mM EDTA was added and the particles were sedimented, washed, and resuspended in the above mentioned medium as described in "Materials and Methods." Samples b and f were then incubated 10 min at 23° with 200 µg/trypsin/mg protein. Finally, where indicated (Samples c, e, f, g), L-PL (15 µg of phospholipid phosphorus per mg of protein) was added according to the method of Fleischer and Fleischer (14). In Sample g, the trypsin treatment was performed after the rebinding with L-PL. The adenosine triphosphatase activity is expressed as nmoles of inorganic phosphorus split per min per mg of protein. The data are mean values of 4 experiments for hepatoma mitochondria and of 2 experiments for rat liver mitochondria.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>Specific activity of adenosine triphosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hepatoma</td>
</tr>
<tr>
<td>a</td>
<td>Untreated mitochondria</td>
<td>146 ± 13</td>
</tr>
<tr>
<td>b</td>
<td>Mitochondria + trypsin</td>
<td>153 ± 21</td>
</tr>
<tr>
<td>c</td>
<td>Mitochondria + L-PL</td>
<td>175 ± 27</td>
</tr>
<tr>
<td>d</td>
<td>Phospholipase C-treated mitochondria</td>
<td>61 ± 7</td>
</tr>
<tr>
<td>e</td>
<td>Phospholipase C-treated mitochondria + L-PL</td>
<td>124 ± 9</td>
</tr>
<tr>
<td>f</td>
<td>Phospholipase C-treated mitochondria + trypsin + L-PL</td>
<td>29 ± 8</td>
</tr>
<tr>
<td>g</td>
<td>Phospholipase C-treated mitochondria + L-PL + trypsin</td>
<td>81 ± 13</td>
</tr>
</tbody>
</table>

* L-PL, phospholipids extracted from liver.
* Mean ± S.D.

Chart 2. Time courses of swelling and proteolysis in trypsin-treated mitochondria from AH-130 hepatoma and rat liver. Mitochondria (0.6 to 1.0 mg of protein) were suspended in 3 ml of isotonic medium. The reaction was started by the addition of trypsin. We measured the swelling by observing the absorbance changes at 520 nm at 23°; 0.1-ml aliquots of the suspensions were taken at the indicated times and used to determine the NRC. Numbers near the tracings indicate the µg of trypsin per mg of protein. The swelling is expressed as decrease in absorbance per mg of protein; the proteolysis is expressed as NRC produced per mg of protein. The NRC were determined colorimetrically, according to the method of Fasold and Gundlach (9), and were expressed in arbitrary units (absorbance at 570 nm).

Phenomena were determined at the same time, during the trypsin digestion of mitochondria. It appears that low amounts of trypsin are able to induce proteolysis (NRC production), coupled with rapid swelling, in hepatoma mitochondria. In liver mitochondria, the swelling is induced by high trypsin concentrations, after a lag phase. On the other hand, a lag

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Effects of Trypsin on Hepatoma Mitochondria

The extent of swelling induced by trypsin in AH-130 hepatoma and rat liver mitochondria

The same conditions existed as in Chart 2. The changes in absorbance induced by trypsin were observed until no further appreciable decrease occurred (30 to 40 min). The data (mean values of 5 experiments) are expressed as absorbance differences (ΔA) per mg of protein, at 520 nm.

<table>
<thead>
<tr>
<th>Trypsin (µg/mg)</th>
<th>Swelling (ΔA/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hepatoma</td>
</tr>
<tr>
<td>1</td>
<td>0.150 ± 0.029a</td>
</tr>
<tr>
<td>3</td>
<td>0.159 ± 0.026</td>
</tr>
<tr>
<td>9</td>
<td>0.175 ± 0.038</td>
</tr>
<tr>
<td>30</td>
<td>0.212 ± 0.036</td>
</tr>
<tr>
<td>60</td>
<td>0.305 ± 0.039</td>
</tr>
<tr>
<td>100</td>
<td>0.365 ± 0.047</td>
</tr>
</tbody>
</table>

a Mean ± S.D.

The effect of trypsin on functional activities of AH-130 hepatoma and rat liver mitochondria

Mitochondria (20 µg of protein per ml) were submitted to 200 µg of trypsin per mg of protein, as in the experiments illustrated in Chart 1. At the indicated times, 0.2-ml aliquots were taken and used to determine the swelling and functional activities. For measurement of the swelling, the mitochondria were appropriately diluted with isotonic medium, and the absorbance changes were read at 520 nm. To measure the acceptor control ratios and respiratory increments by Ca**, we suspended mitochondria (2 µg of protein) in 2 ml of a medium containing 130 mM KCl, 20 mM Tris-Cl (pH 7.4), 10 mM L-glutamate, 5 mM L-malate, and 1 mM phosphate. The respiratory release was induced by either 0.1 mM ADP or 0.3 mM CaCl₂. For measurement of the respiratory increments by K* plus valinomycin, the test system contained the following in 2 ml: 10 mM KCl, 270 mM sucrose, 20 mM Tris-Cl (pH 7.4), 10 mM L-glutamate, 5 mM L-malate, 4 mM phosphate, and mitochondria (4 mg of protein). The respiratory release was induced by 0.3 µg of valinomycin. The respiratory increments by either Ca** or K* plus valinomycin are calculated as "extra" oxygen uptakes after the addition of Ca** or valinomycin, respectively. The data are the mean values of 4 experiments.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Swelling (ΔA/mg)</th>
<th>Acceptor control ratio</th>
<th>Respiratory increment by Ca**</th>
<th>Respiratory increment by K*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hepatoma</td>
<td>Liver</td>
<td>Hepatoma</td>
<td>Liver</td>
</tr>
<tr>
<td>0</td>
<td>0.180 ± 0.062</td>
<td>0.000</td>
<td>5.1 ± 0.4a</td>
<td>4.4 ± 1.0</td>
</tr>
<tr>
<td>10</td>
<td>0.400 ± 0.054</td>
<td>0.430 ± 0.038</td>
<td>3.6 ± 0.2</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td>1.0b ± 0.0</td>
<td>2.3 ± 0.2</td>
</tr>
</tbody>
</table>

a Mean ± S.D.

The effect of trypsin on functional activities of mitochondria and submitochondrial particles has been the subject of several investigations (7, 19, 20, 43). In general, results have shown a high resistance to the proteolysis of intact rat liver mitochondria. This was proved by the fact that large amounts of trypsin were needed to obtain functional and structural damage of mitochondria (7, 43). If small amounts of trypsin were used, a low degree of damage occurred which was essentially limited to the outer membrane. Contrary to these findings, however, is the observation (19) that some functional activities of sonically disrupted mitochondria were very susceptible to low amounts of trypsin.

Our results with liver mitochondria are in line with these observations, confirming the high resistance of these particles to proteolysis. We found somewhat different behavior with hepatoma mitochondria. Apparently, the outer membrane of these mitochondria offers relatively low resistance to trypsin.

DISCUSSION

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Our results with liver mitochondria are in line with these observations, confirming the high resistance of these particles to proteolysis. We found somewhat different behavior with hepatoma mitochondria. Apparently, the outer membrane of these mitochondria offers relatively low resistance to trypsin.

Changes of Functional Activities during the Trypsin-induced Swelling. We studied the acceptor control ratios and the respiratory increments by Ca** or K* plus valinomycin during tryptic digestion, in order to correlate these phenomena with the volume changes induced by trypsin. As shown in Table 6, no changes in the acceptor control ratios are observed in liver mitochondria during the lag phase of the swelling. A marked decrease of acceptor control ratios and respiratory increments by Ca** occurs in swollen liver mitochondria. In hepatoma mitochondria, the fast swelling is accompanied by a faster decrease of these functional activities, proportional to the amplitude of swelling. Among the reactions investigated, the K* translocation appears resistant to trypsin. In fact, no appreciable inhibition of the respiratory increment by K* plus valinomycin is induced by trypsin in hepatoma and liver mitochondria. These last results are analogous to those reported by Junitti et al. (19) on submitochondrial particles submitted to trypsin. The oligomycin-induced respiratory control and some energy-utilizing reactions were highly sensitive to trypsin, while the translocation of K* was not affected by the proteolytic enzyme.

Changes of Functional Activities during the Trypsin-induced Swelling. We studied the acceptor control ratios and the respiratory increments by Ca** or K* plus valinomycin during tryptic digestion, in order to correlate the behavior of these inner-membrane-linked functions to the volume changes induced by trypsin. As shown in Table 6, no changes in the functional activities are observed in liver mitochondria during the lag phase of the swelling. A marked decrease of acceptor control ratios and respiratory increments by Ca** occurs in swollen liver mitochondria. In hepatoma mitochondria, the fast swelling is accompanied by a faster decrease of these functional activities, proportional to the amplitude of swelling. Among the reactions investigated, the K* translocation appears resistant to trypsin. In fact, no appreciable inhibition of the respiratory increment by K* plus valinomycin is induced by trypsin in hepatoma and liver mitochondria. These last results are analogous to those reported by Junitti et al. (19) on submitochondrial particles submitted to trypsin. The oligomycin-induced respiratory control and some energy-utilizing reactions were highly sensitive to trypsin, while the translocation of K* was not affected by the proteolytic enzyme.

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This conclusion is based upon the observation that adenylate kinase, an enzyme located in the intermembrane space of mitochondria (5, 8, 41), is highly sensitive to trypsin in hepatoma mitochondria but not in liver mitochondria, when suspended in an isotonic medium. The cytochrome c oxidase follows the same pattern in its response to trypsin in the 2 types of particles. The cytochrome c oxidase is located between the 2 faces of the inner mitochondrial membrane (32, 33), probably occupying the total thickness of the membrane. Hence, the enzyme should be accessible to trypsin in its more external portion [i.e., on the “C face” of the inner membrane, according to Racker’s terminology (32)] if the outer membrane of mitochondria is ruptured [i.e., in digitonintreated mitochondria (8, 46)] or digested by trypsin. The cytochrome c oxidase of liver mitochondria becomes sensitive to proteolysis only after the outer membrane has been rendered permeable to large molecules by digitonin. On the other hand, the enzyme in hepatoma mitochondria is sensitive even in conditions of complete latency, that is, when masked by an intact outer membrane (46). The permeabilization of this membrane in hepatoma mitochondria by either digitonin or high-amplitude swelling is additional proof that large molecules do not cross the outer membrane of freshly isolated particles.

Our data indicate that, once trypsin reaches the inner membrane structures, no differences can be observed between hepatoma and liver mitochondria in regard to the effect of the proteolysis on the enzymatic and functional activities studied. The succinate-cytochrome c reductase of both types of mitochondria is affected by proteolysis when the incubation with trypsin follows a strong sonic treatment of the particles. This treatment is logically assumed to be accompanied by fragmentation of the inner membrane, allowing trypsin to reach the interior and/or the “M face” (32) of this membrane. This could indicate that substrate for trypsin is the succinate-cytochrome c reductase but not the binding site for cytochrome c, superficially located on the C face of the inner membrane (32). In fact, this site could be accessible to trypsin in mitochondria with an intact inner membrane, provided that the outer membrane is permeable to the proteolytic enzyme. One could suggest that the binding site for cytochrome c, as well as for the cytochrome c molecule, is resistant to proteolysis. Consequently, the inactivating effect of trypsin on the cytochrome c oxidase of hepatoma and liver mitochondria could also depend on digestion of the mitochondrial enzyme molecule.

The insensitivity to trypsin of adenosine triphosphatase has already been reported (32) and attributed to the location of the enzymatic activity in the hydrophobic interior of the protein constituting F1. The inactivation of the enzyme induced by phospholipase C is related to its well-known phospholipid dependence (14). The sensitivity to trypsin of the defatted enzyme could be caused by conformational rearrangements of the enzyme molecule that might occur after the loss of interactions with phospholipids. In agreement with this possibility, the reconstituted enzyme is partially protected against proteolysis. These results indicate that phospholipids may be involved, at least under actual conditions, in the resistance to trypsin of the mitochondrial membranes. The differences in sensitivity to trypsin of the outer membrane between the 2 types of mitochondria could explain the slow onset of swelling during proteolysis in liver mitochondria. Mitochondria from normal liver behave as perfect osmometers (42). This was true also for hepatoma mitochondria (13). Consequently, changes of structures involved in the preservation of the osmotic properties of the particles could lead to swelling. We may assume that trypsin, in liver but not in hepatoma mitochondria, digests initially some structures not responsible for the preservation of the permeability properties of the particles, so that the proteolysis is not coupled to swelling. Although our data do not permit localization of these structures, it seems reasonable to suggest that the proteolysis of liver mitochondria preceding the swelling results at least in part from digestion of the outer membrane. Analogous considerations could apply to the behavior of the inner membrane-linked functional activities in trypsin-treated mitochondria. In hepatoma mitochondria, the inner membrane of which is easily reached by trypsin, the uncoupling effect of proteolysis and the inhibition of the respiratory increment by Ca2+ could depend on the susceptibility to proteolysis of the proteins involved in the specific functions. If the outer membrane were resistant to trypsin, as in liver mitochondria, one would expect that the inner membrane functions would be protected during treatment with trypsin.

At this time we know neither the conditions leading to the low degree of resistance to trypsin of the outer membrane of hepatoma mitochondria nor whether mechanical damage during tissue fractionation is involved in the phenomenon. Perhaps an enhanced fragility of the outer membrane makes tumor mitochondria more susceptible to mechanical stress which is easily endured by normal mitochondria. In this respect, recent evidence indicates the existence of some functional and perhaps structural (26, 30, 44) alterations of the outer membrane of mitochondria from different tumors. Some changes in enzymatic activities linked to the outer membrane of liver mitochondria during hepatocarcinogenesis were also shown by Arcos et al. (1, 2). These authors also found a decrease of the acceptor control ratio and of the ability to swell, probably due to some alterations of the inner mitochondrial membrane. All these mitochondrial changes, with the exception of the decreased ability to swell, were reversed in coincidence with the onset of irreversibility in tumorigenesis. A reduced ability to swell (but with high acceptor control ratios) was indeed found in mitochondria from AH-130 hepatoma (10, 12). It should be noted, however, that our results concern only the effect of trypsin on mitochondria. The fact that the inner membranes of liver and hepatoma mitochondria are equally sensitive to trypsin does not exclude the possibility that differences exist between other properties of the inner membranes of tumor and liver mitochondria.

In conclusion, the outer membrane of mitochondria, in addition to the endoplasmic reticulum (30, 31), seems to be functionally and structurally altered in tumor cells. This is interesting in view of the proposal that functional, structural, and, perhaps, biogenetical correlations exist between the outer...
membrane of mitochondria and the endoplasmic reticulum (3, 15, 23, 25).

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Further Observations on the Effects of Trypsin on the Volume and Functions of Mitochondria Isolated from Normal Liver and AH-130 Yoshida Ascites Hepatoma

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