PROTEOLYTIC MICRODISSECTION OF SMOOTH-SURFACED VESICLES OF LIVER MICROSOMES

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ABSTRACT

Digestion of rabbit liver microsomal smooth vesicles with Bacillus subtilis protease released proteins and peptide fragments from the vesicles, without solubilizing phospholipids and cholesterol. The proteolysis was, however, limited when about 30% of the protein had been solubilized. The same limitation was observed when the vesicles were treated with trypsin, chymotrypsin, or their combinations with the bacterial protease. The limited proteolysis was accompanied by selective solubilization of cytochrome b₅ and microsomal NADPH-specific flavoprotein, leaving the CO-binding hemoprotein and some other enzymes still attached to the vesicular membranes. Sucrose density gradient centrifugation of protease-treated vesicles indicated that all the vesicles had been attacked by the protease to similar extents. The behavior of intact and digested vesicles in dextran density gradient centrifugation suggested that the vesicles, even after proteolytic digestion, existed in the form of closed sacs which were impermeable to macromolecules such as dextran and proteases. It was concluded that only the outside surface of the vesicles is susceptible to the proteolytic action and that cytochrome b₅ and the NADPH-specific flavoprotein are located in the susceptible area.

INTRODUCTION

The hepatic microsomal fraction consists mainly of smooth- and rough-surfaced vesicles derived from fragmentation of the endoplasmic reticulum (1), and numerous enzymes are associated with membranes of these vesicles (2, 3). To understand the functions and reaction mechanisms of these enzymes, it is desirable to elucidate how they are incorporated into the membrane architecture. As a biochemical approach to this problem, it seems worthwhile to "dissect" microsomal vesicles by using purified proteases of established substrate specificity.

Trypsin and crude pancreatic extracts (steapsin) containing protease and lipase activities have been successfully employed to solubilize cytochrome b₅ (4–8) and microsomal NADPH-specific flavoprotein (NADPH-cytochrome c reductase) (9–11) from liver microsomes. However, many microsomal enzymes including glucose-6-phosphatase (12) have been shown to resist proteolytic solubilization. Lust and Drochmans (13) have reported that treatment of rat liver microsomes with trypsin resulted in removal of ribosomes from rough vesicles, without affecting the structure of vesicular membranes within the resolution power of the electron microscope employed. The persistence of so-called unit-membrane structure in trypsin-treated liver microsomes has recently been confirmed by Omura et al. (7).

The present paper reports the action of proteases...
on smooth-surfaced vesicles of rabbit liver microsomes and the behavior of intact and protease-treated vesicles in both sucrose and dextran density gradient centrifugations. From the results obtained, it is suggested that smooth vesicles are closed, semipermeable sacs into which macromolecules such as dextran and proteases are unable to enter, though the vesicular membrane is permeable to sucrose. It is also suggested that cytochrome b₅ and the NADPH-specific flavoprotein are located at the outside surface of the vesicular membranes. A preliminary account of this work has been presented (14).

**Materials and Methods**

**Proteases**

A protease purified and crystallized from *Bacillus subtilis* N' (15) was kindly supplied from Nagase Company, Ltd., Osaka, Japan. Twice recrystallized trypsin and chymotrypsin were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Nutritional Biochemicals Corporation (Cleveland, Ohio) respectively. These proteases were used without further purification.

**Preparation of Microsomal Smooth Vesicles**

Male rabbits, weighing 2.5-3 kg, were fasted overnight and sacrificed by intravenous injection of air.

The livers were excised, perfused thoroughly with 1.15% KCl to remove hemoglobin, and homogenized in 0.25 m sucrose with the aid of a Potter glass-Teflon homogenizer to give a 10% homogenate (w/v). The homogenate was centrifuged at 10,000 g for 10 min, and a microsomal pellet was collected by centrifugation of the supernatant at 78,000 g for 90 min in a Hitachi 40P centrifuge. The pellet was resuspended in 1.15% KCl containing 0.02 m Tris-HCl buffer, pH 7.4, at a protein concentration of about 25 mg/ml.

Smooth-surfaced vesicles in the microsomal fraction were separated from the rough elements and free ribosomes by a modification of the method of Rothchild (16, 17). A 3 ml portion of the microsomal suspension was layered over 7 ml of 1.23 m sucrose containing 0.1 m MgCl₂ and 0.02 m Tris-HCl buffer, pH 7.4, and centrifuged at 105,000 g for 60 min. The amounts of various components of the vesicles thus released into the supernatant were determined. The extent of solubilization of a component was expressed as the percentage of that component not sedimentable under this centrifugal condition relative to that present in the original incubation mixture. In estimating the solubilization of protein, correction was made for the amount of protease used. Calculation of percent solubilization of RNA, phospholipids, and cholesterol was based on the amounts remaining in the sedimented residue rather than on those liberated in the supernatant. In experiments in which solubilization of enzyme activities was studied, measurements were also made on the whole incubated mixture to evaluate the inactivation (or activation) of the enzymes caused by the protease treatment.

**Sephadex G-100 Gel Filtration of Solubilized Components**

A suspension of smooth vesicles (7.46 mg protein/ml) in 0.01 m phosphate buffer, pH 7.4, was incubated with trypsin (19 µg/mg vesicular protein) at 30°C for 2 hr as described above. The mixture was then centrifuged at 105,000 g for 60 min. The resultant supernatant (derived from 269 mg of vesicular protein) was concentrated by lyophilization and subjected to gel filtration through a Sephadex G-100 column (2.5 X 75 cm) equilibrated with 0.01 m phosphate buffer, pH 7.4; elution was conducted with the same buffer. Fractions of 2 ml were collected and analyzed for protein, cytochrome b₅, and NADPH-specific flavoprotein.

**Density Gradient Centrifugations**

For sucrose density gradient centrifugation, 0.3 ml of suspension of untreated or protease-treated...
vessicles (in 0.02 M Tris-HCl buffer, pH 7.4; about 0.6 mg of protein) was layered over 4.4 ml of a linear sucrose gradient ranging in density value from 1.04 to 1.17. The tube was centrifuged for 4 hr at 155,000 g (40,000 rpm) in an SPR-40 rotor of the Hitachi centrifuge. After centrifugation, fractions of three drops were collected by puncturing the bottom of the tube and were analyzed for protein. In some experiments, centrifugation was conducted by the method of Omura and Sato (18). The CO-binding hemoprotein, P-450, and its modification product, P-420, were determined from the dithionite-reduced minus oxidized difference spectrum. A molar extinction coefficient increment of 185 cm\(^{-1}\) mm\(^{-1}\) between 424 and 409 nm (18) was assumed in these difference spectra. The CO-binding hemoprotein, P-450, and its modification product, P-420, were determined from the CO difference spectrum of the dithionite-treated sample as described previously (18, 27). Microsomal NADPH-specific flavoprotein was determined either from its vitamin K\(_{1}\)-dependent NADPH oxidase activity as measured according to Phillips and Langdon (9). Mg\(^{2+}\)-ATPase activity was measured by a modification of the method of Ernster and Jones (29) in a reaction mixture containing 0.1 M Tris-HCl buffer, pH 7.4, 5 mM disodium ATP, and 5 mM MgCl\(_{2}\). Glucose-6-phosphatase activity was assayed by a modification of the method of Swanson (30) in a reaction mixture containing 0.1 M Tris-maleate buffer, pH 6.5, and 40 mM glucose-6-phosphate.

**RESULTS**

**Chemical Composition of Smooth Vesicles**

Table I shows the contents of several chemical components in the total microsomal fraction from rabbit liver as well as in the rough and smooth subfractions obtained therefrom. The data for phospholipids and RNA were in fairly good agreement with those reported previously for rat liver microsomes (31, 32). The content of sialic acids in the smooth subfraction was also similar to that reported previously (33). The high level of phospholipids and low content of RNA in the smooth subfraction was consistent with the presence of high levels of phospholipids and low content of RNA in the smooth subfraction.
TABLE I
Contents of Several Components in Rabbit Liver Microsomes and Smooth and Rough Subfractions Prepared Therefrom

The microsomal fractions were prepared as described in Materials and Methods. The data are expressed as µg/mg protein (± standard deviations). The figures in parentheses represent the number of determinations.

<table>
<thead>
<tr>
<th></th>
<th>Phospholipids</th>
<th>Cholesterol</th>
<th>RNA</th>
<th>Sialic acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total microsomes</td>
<td>538 ± 56 (5)</td>
<td>N. D.*</td>
<td>147 ± 9.1 (4)</td>
<td>N. D.</td>
</tr>
<tr>
<td>Smooth subfraction</td>
<td>713 ± 55 (11)</td>
<td>49.8 ± 4.0 (4)</td>
<td>31.8 ± 8.5 (11)</td>
<td>3.65 ± 0.45 (5)</td>
</tr>
<tr>
<td>Rough subfraction</td>
<td>466 ± 44 (5)</td>
<td>N. D.</td>
<td>275 ± 36 (5)</td>
<td>N. D.</td>
</tr>
</tbody>
</table>

* N. D. = not determined.

Figure 1. Time courses of solubilization of protein from liver microsomal smooth vesicles by the action of crystalline B. subtilis protease. Smooth vesicles (0.19 mg protein/ml) were incubated with various concentrations of the protease under the conditions described in Materials and Methods. After incubation for indicated periods of time, the mixture was chilled and centrifuged at 105,000 g for 60 min, and the amount of protein solubilized in the supernatant was determined. It should be noted that the protease continued to act on the vesicles during centrifugation. This could account for the apparent solubilization observed at 0 time. A, 57 µg protease/mg vesicular protein; B, 18 µg; C, 5 µg; D, no protease was added.

suggested that this subfraction consisted predominantly of smooth-surfaced vesicles. Electron microscopic observations, kindly performed by Drs. K. Hama and T. Kanaseki, actually demonstrated that this fraction was only slightly contaminated by rough vesicles and free ribosomes.

Digestion of Smooth Vesicles with Proteases

As shown in Fig. 1, anaerobic digestion of smooth vesicles with crystalline B. subtilis protease (15) resulted in progressive solubilization of protein from the vesicles. The proteolytic action, however, became limited when about 30% of the total protein had been solubilized, regardless of the protease concentration used. The time required to reach this limit was dependent on the protease concentration; at a concentration of about 20 µg protease/mg vesicular protein, this limit could be reached within 3 hr. The apparent solubilization of more than 10% of the protein without incubation (time 0) was due to the action of the added protease during centrifugal recovery of the vesicles from the suspension (0°C, 60 min). Fig. 1 also shows that 5-8% of the protein was recovered in the supernatant even when the vesicles were incubated in the absence of added protease. This result appeared to represent the detachment of adsorbed cytoplasmic proteins rather than the action of contaminating proteases, because this “solubilization” was not significantly time-dependent.

The observed limitation of protein solubilization was not due to inactivation of the protease, since reinforcement of the reaction mixture with the fresh protease, 90 min after the start of incubation, did not cause any appreciable increase in the extent of protein solubilization. Therefore, it was likely that the limitation was a reflection of the presence in the vesicles of a certain barrier against the proteolytic activity.

The limitation phenomenon was not restricted to B. subtilis protease. As can be seen in Table II, practically the same limit of protein solubilization was also observed with trypsin and chymotrypsin. Furthermore, the combined action of the bacterial protease with trypsin or chymotrypsin did not cause any increased solubilization over that attainable by one of these proteases. It was thus
TABLE II
Solubilization of Protein from Smooth Vesicles by *B. subtilis* Protease, Trypsin, and Chymotrypsin

Smooth vesicles (3.87 and 6.08 mg protein/ml for Experiments 1 and 2, respectively) were incubated with indicated amounts of *B. subtilis* protease, trypsin, chymotrypsin, or their combinations for 120 min under the conditions described in Materials and Methods.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Experiment 1</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein solubilization</td>
<td>Protein solubilization</td>
<td></td>
</tr>
<tr>
<td></td>
<td>µg/mg*</td>
<td>%</td>
<td>µg/mg*</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>5.0</td>
<td>0</td>
</tr>
<tr>
<td><em>B. subtilis</em> protease</td>
<td>58</td>
<td>32.1</td>
<td>31</td>
</tr>
<tr>
<td>Trypsin</td>
<td>74</td>
<td>29.8</td>
<td>42</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>60</td>
<td>31.0</td>
<td>32</td>
</tr>
<tr>
<td><em>B. subtilis</em> protease + trypsin</td>
<td>58 + 74</td>
<td>29.3</td>
<td>31 + 42</td>
</tr>
<tr>
<td><em>B. subtilis</em> protease + chymotrypsin</td>
<td>58 + 60</td>
<td>29.1</td>
<td>31 + 32</td>
</tr>
</tbody>
</table>

* µg protease protein/mg vesicular protein.

TABLE III
Solubilization of Protein and Sialic Acids, and Loss of Phospholipids, Cholesterol, and RNA from Smooth Vesicles by the Action of *B. subtilis* Protease

Smooth vesicles (5-13 mg protein/ml) were digested with or without *B. subtilis* protease (2-8 µg/mg vesicular protein) for 120 min, and solubilization or loss of each component was determined as described in Materials and Methods. The data are expressed as per cent solubilization or loss (± standard deviations). The figures in parentheses represent the number of determinations.

<table>
<thead>
<tr>
<th>Component</th>
<th>% Solubilization or loss</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without protease</td>
</tr>
<tr>
<td>Protein</td>
<td>7.4 ± 1.9 (6)</td>
</tr>
<tr>
<td>Sialic acids</td>
<td>2.9 (1)</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>2.5 ± 2.2 (4)</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>5.0 (2)</td>
</tr>
<tr>
<td>RNA</td>
<td>18.9 ± 8.1 (5)</td>
</tr>
</tbody>
</table>

As shown in Table IV, cytochrome *b*4 was solubilized almost quantitatively, whereas P-450 was mostly recovered in the vesicular residue. The latter hemoprotein had been converted to a considerable extent (about 50%) into the spectrally modified form called P-420 (6, 18), during the digestion. Similar behavior of the two hemoproteins has also been observed on treatment of liver microsomes with steapsin (5, 6). Like cytochrome *b*4, microsomal NADPH-specific flavoprotein, as-

A. ITO AND R. SATO Proteolytic Microdissection of Smooth-Surfaced Vesicles 183
TABLE IV
Behavior of Hemoproteins, NADPH-specific Flavoprotein, Mg++-ATPase, and Glucose-6-Phosphatase on Incubation of Smooth Vesicles with B. subtilis Protease

The conditions of protease digestion were the same as in Table III. Control samples were incubated without the protease. Assays were made as described in Materials and Methods. The figures in parentheses represent the number of determinations. NADPH-specific flavoprotein was assayed from its vitamin K₃-dependent NADPH oxidase activity.

<table>
<thead>
<tr>
<th></th>
<th>Without protease</th>
<th>With protease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovery</td>
<td>Solubilization</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Cytochrome b₁</td>
<td>96.2±8.2 (6)</td>
<td>2.7 (2)</td>
</tr>
<tr>
<td>P-430 (+ P-420)</td>
<td>102.5±5.7 (3)</td>
<td>6.1 (2)</td>
</tr>
<tr>
<td>NADPH-specific flavoprotein</td>
<td>85.3±14.5 (6)</td>
<td>7.0±3.4 (6)</td>
</tr>
<tr>
<td>Mg²⁺-ATPase</td>
<td>96.8 (2)</td>
<td>0.4 (2)</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
<td>76.2 (2)</td>
<td>3.6 (2)</td>
</tr>
</tbody>
</table>

* Recovered mostly in the form of P-430.
† About 50% of the hemoprotein was in the form of P-420.

sayed by its vitamin K₃-dependent NADPH oxidase activity (11, 28), was mostly solubilized, though accompanied by severe inactivation. This inactivation could be greatly prevented when incubation was conducted at 0°C, as reported previously (36). Preliminary experiments showed that about 20% of the NADH-cytochrome b₁ reductase activity was also solubilized under the present digestion conditions. No solubilization of Mg²⁺-ATPase and glucose-6-phosphatase was observed, and the latter activity was extensively inactivated during incubation. It was concluded that the proteolytic attack, limited by a structural barrier, was accompanied by selective release of certain enzyme components from the vesicles.

Size Distribution in Solubilized Protein Components

Since all the protein of the vesicles could be recovered after proteolysis when assayed by the colorimetric method of Lowry et al. (19), it was certain that the protein components solubilized could react with the Lowry reagents to the same extent as ordinary proteins. However, it was conceivable that some of the solubilized proteins had been degraded by the protease. This possibility was examined by subjecting the supernatant, obtained after treatment of the vesicles with trypsin, to gel filtration through a Sephadex G-100 column. The elution pattern thus obtained, shown in Fig. 2, was similar to that reported for the components solubilized from total rat liver microsomes (7). It will be seen that a large amount of material, reactive with the Lowry reagents, was eluted after cytochrome b₁. Since cytochrome b₁ solubilized under similar conditions has been shown to possess a molecular weight of about 12,000 (37, 38), the material in question must have been peptide fragments having molecular weights less than 12,000. It should be noted that considerable quantities of larger proteins including NADPH-specific flavoprotein were also present in the solubilized fraction. These proteins seemed to be resistant to the proteolytic attack. The fraction solubilized by B. subtilis protease digestion also displayed a similar gel filtration pattern, but the proportion of peptide fragments was considerably higher.

Analysis of Vesicles by Sucrose Density Gradient Centrifugation

As shown in Fig. 3, intact smooth vesicles were equilibrated as a symmetrical peak at a density value of about 1.14 when subjected to sucrose density gradient centrifugation, suggesting that the vesicles were homogeneous with respect to buoyant density. A much smaller peak seen at the top of the tube seemed to represent a small amount of cytoplasmic proteins which had been adsorbed on the vesicles. On the other hand, density gradient centrifugation of the preparation which had been extensively digested with B. subtilis protease gave two large peaks, one equilibrated at a density of
FIGURE 2 Sephadex G-100 gel filtration of the 105,000 g supernatant obtained after tryptic digestion of smooth vesicles. Smooth vesicles (269 mg protein) were digested with trypsin and then centrifuged as described in Materials and Methods. 5.0 ml of concentrated supernatant (74.2 mg protein, 0.01 M phosphate buffer, pH 7.4) were subjected to gel filtration through a Sephadex G-100 column (2.5 X 75 cm) as described in Materials and Methods. Fractions of 2 ml were collected. NADPH-specific flavoprotein was assayed from its NADPH-cytochrome c reductase activity.

A. ITO AND R. SATO
Proteolytic Microdissection of Smooth-Surfaced Vesicles

1.12 and the other floating at the top. It was clear that the peak at the top represented the solubilized protein components and the protease protein. The peak, having a density of 1.12 and representing the digested vesicles, was also symmetric and showed no shoulder at the position corresponding to the untreated vesicles. These observations indicated that all the vesicles in the incubation mixture had been attacked by the protease to similar extents. The reduction in median density from 1.14 to 1.12 by the protease action could be explained by selective release of protein, resulting in an increase in the ratio of lipids to protein.

Analysis of Vesicles by Dextran Density Gradient Centrifugation

Wallach and Kamat (39) have pointed out that a vesicle equilibrated in a density gradient composed of a solute to which the membrane is permeable will locate at a density equal to that of the membrane matrix. On the other hand, in a density gradient composed of a solute to which the membrane is impermeable, the equilibrium density will be determined not only by the matrix density but also by the density and volume of the fluid inside the vesicle. We employed this principle to see if macromolecules such as proteases can enter into the smooth vesicles of liver microsomes. In practice, we adopted dextran (weight average molecular weight, 40,000) to construct the density gradient, because proteases were inadequate as solutes to form gradients. It was thus found that the untreated smooth vesicles showed a buoyant density of 1.05–1.10 in the dextran density gradient, as can be seen in Fig. 4. This value was definitely smaller than the value of 1.14 obtained in sucrose. Although the value in sucrose was always fairly constant, the value in dextran varied, from one experiment to another, in the range between 1.05 and 1.10. This variation seemed to be caused by uncontrollable alterations in the volume of the vesicular content. At any rate, the observed difference in equilibrium densities in sucrose and dextran gradients was consistent with the view that the smooth vesicles were closed sacs which were impermeable to dextran though they permitted free entry of sucrose. This finding suggested that proteases, being macromolecules like dextran, were also unable to pass through the membranes of the vesicles.

Fig. 4 also indicates a reduction in equilibrium density of the vesicles in dextran after the action of the bacterial protease. In the experiment with the protease-treated vesicles, a small shoulder was seen at a density of about 1.13, a value which corresponded to the density of the protease-treated vesicles in sucrose. This seemed to indicate that a small portion of the vesicles had been broken during the proteolytic attack so to permit the entry of dextran molecules. A comparison of Figs. 3 and 4 shows further that the equilibrium density of the protease-treated vesicles in the dextran density gradient was smaller than the corresponding value in sucrose. Thus, it could be concluded that most of the vesicles still retained the semipermeable property even after proteolytic digestion.
from each other. Although cytochrome $b_5$ and the NADPH-flavoprotein could be solubilized with comparable efficiency by $B. subtilis$ protease, the flavoprotein was much more susceptible than the cytochrome to the actions of trypsin and chymotrypsin. Especially in the case of chymotryptic digestion, no solubilization of cytochrome $b_5$ was observed at a concentration of chymotrypsin (3 $\mu$g/mg vesicular protein) which caused almost complete release of the NADPH-flavoprotein. In the suboptimal concentration range, $B. subtilis$ protease was most effective in solubilizing the flavoprotein.

**Susceptibility of Cytochrome $b_5$ and NADPH-Specific Flavoprotein to Proteolytic Solubilization**

As mentioned above, two microsomal proteins, i.e. cytochrome $b_5$ and NADPH-specific flavoprotein, could be selectively and almost quantitatively released from the vesicles when proteolytic digestion was carried out nearly to the 30\% limit. It was, therefore, of interest to investigate the behavior of these two proteins on incomplete proteolysis of the vesicles with different proteases. For this purpose, the vesicles were digested with various, suboptimal concentrations of $B. subtilis$ protease, trypsin, or chymotrypsin. Incubation was performed at 0°C for 15 hr to avoid inactivation of NADPH-specific flavoprotein, which was assayed by its NADPH-cytochrome $c$ reductase activity. As shown in Fig. 5, the responses of the two proteins to the three proteases were very different from each other.
DISCUSSION

In such studies as reported in this paper, the homogeneity of membrane material is of importance. This is especially so in view of the fact that liver microsomes are considerably heterogeneous both morphologically and biochemically (40-42). The material used in the present study, namely smooth-surfaced vesicles from liver microsomes, is advantageous in that complications arising from the presence of nonmembranous elements such as ribosomes and ferritin particles can be avoided. The results of chemical analyses and electron microscopic observations indicate that this material is not appreciably contaminated by rough vesicles and free ribosomes. The smooth vesicles are also essentially homogeneous in both sucrose and dextran density gradient centrifugations. Moreover, all the vesicles in the preparation are susceptible to the protease action to practically the same extent.

The finding that proteolytic solubilization of protein from the smooth vesicles never exceeds a limit of about 30%, regardless of the specificity and concentration of the protease used, seems to suggest the presence, in the structure of each vesicle, of a barrier against the protease action. The possibility of the occurrence of various vesicles differing in susceptibility to proteolysis can be ruled out from the results of sucrose density gradient centrifugation which indicate that all the vesicles have equally been attacked. It should also be noted that the proteolytic attack causes no solubilization of lipid substances, which are the major constituents of the membranes.

On the other hand, the data obtained in the dextran density gradient centrifugation experiments suggest that the vesicles are closed, semipermeable sacs, which do not permit the entry of macromolecules such as dextran into the space enclosed by the vesicular membranes. Although dextran differs from proteases in molecular shape and electric charge, it does not seem unreasonable to assume that the membranes of vesicles are also impermeable to the protease molecules. If this is the case, a protease added to the suspension can attack the vesicles only at the outside surface of the membranes. This attack, which liberates and decomposes the protein components situated on the surface area of the vesicular membranes, becomes eventually limited by the barrier. It should be realized that most of the vesicles still retain the impermeability to dextran and probably also to proteases, even at this stage of proteolysis. In support of this finding are the observations of Lust and Drochmans (13) and Omura et al. (7) that tryptic digestion of liver microsomes does not cause the rupture of the vesicular membranes.

It is tempting to speculate that the barrier preventing further proteolysis is the lipid bimolecular layer postulated in the membrane models of...
Danielli and Davson (43) and Robertson (44). If so, maximum solubilization of protein could be expected to approach a 50% level,1 a value which is considerably higher than the actual extent of solubilization (about 30%). Furthermore, as mentioned above, Omura et al. (7) have reported the persistence of a triple-layered (unit-membrane) appearance in the membranes of trypsin-treated microsomal vesicles. Therefore, it is likely that considerable quantities of protein components still remain in the outside layer of the unit membranes. However, it should be emphasized that there is as yet no decisive evidence that the lipid layer is actually acting as the barrier against proteolysis. In this situation, it may be worthwhile to try to interpret the present results on the basis of other membrane models such as those proposed by Benson (45) and Green and Purdue (46). At any rate, it is very likely that only certain specific protein components residing on the outside surface area of the membranes are susceptible to the action of proteases.

A necessary consequence of the above interpretation is the conclusion that both cytochrome b5 and microsomal NADPH-specific flavoprotein are located exclusively in the outside layer of the vesicular membranes, because these two proteins can be almost quantitatively liberated into solution by the proteases. It is also certain that cleavage of peptide bonds is somehow involved in their solubilization. We have recently reported that cytochrome b5 occurs in microsomes as a hydrophobic protein having a molecular weight of about 25,000, whereas the cytochrome solubilized by proteases is hydrophilic in nature and has a molecular weight of the order of 12,000.2 It is, therefore, likely that

1 Since there is evidence for the presence of a certain amount of protein in the space enclosed by the membrane of a vesicle, even complete removal of the protein constituting the outside layer of the unit-membrane structure may not lead to protein solubilization to such a high extent as 50%. However, the present study has indicated that 5–8% of the protein in the preparation is the cytoplasmic components which had been adsorbed on the vesicular surface and can also be removed by the proteolytic attack. In the absence of our knowledge concerning the precise amount of enclosed proteins, it seems plausible to assume a tentative value of about 50% for apparent protein solubilization under the postulated conditions.


the protein is attached to the membranes by hydrophobic interactions with the other membrane constituents, and that proteolytic decomposition of the hydrophobic moiety of the cytochrome molecule causes the detachment from the membranes of a heme-containing, protease-resistant core (or cores) having a molecular weight of about 12,000. The presence of a large amount of peptide fragments in the supernatant of the digest may, at least partly, have been due to such decomposition. Although nothing is as yet known of the mode of binding of the NADPH-specific flavoprotein, a situation similar to the case of cytochrome b5 may also be considered for this flavoprotein.

The proteolytic attack on the vesicles causes the solubilization of only a small portion of the NADH-cytochrome b5 reductase activity. Takesue and Omura (47) have, however, reported that this reductase can be selectively and almost completely solubilized upon incubation of microsomes with lysosomes at pH 5.7. This observation may suggest that the NADH-cytochrome b5 reductase flavoprotein is also located at the outer surface of the vesicles. This flavoprotein seems to be bound to the membranes by a linkage which differs from those involving cytochrome b5 and the NADPH-flavoprotein, because the lysosomal attack results in only slight solubilization of the latter proteins. The intramembranous localization of P-450, Mg2+-ATPase, and glucose-6-phosphatase is still unknown.

The postulated localization of cytochrome b5 and NADPH-specific flavoprotein exclusively in the outside surface layer of the vesicular membrane asymmetry of biological membranes. Since there are indications that the original “inside-outside” relationship is preserved even after the conversion of the endoplasmic reticulum into microsomal vesicles (39), it may be concluded that cytochrome b5 and the NADPH-flavoprotein are located on the cytoplasmic surface of endoplasmic reticulum membranes in intact hepatocytes. The significance of this localization is to be explored in future.

Finally, it is of interest that proteases with widely different substrate specificity can cause protein membranes provides another example of the functional solubilization to almost the same extent. Since the action of two different proteases at the same time does not result in increased solubilization, it appears that the same species of protein components
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A. ITO and R. SATO  Protolytic Microdissection of Smooth-Surfaced Vesicles 189