

PROTEINS OF ROUGH MICROSOMAL MEMBRANES RELATED TO RIBOSOME BINDING

II. Cross-Linking of Bound Ribosomes to Specific Membrane Proteins Exposed at the Binding Sites

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ABSTRACT

Two proteins (ribophorins I and II), which are integral components of rough microsomal membranes and appear to be related to the bound ribosomes, were shown to be exposed on the surface of rat liver rough microsomes (RM) and to be in close proximity to the bound ribosomes. Both proteins were labeled when intact RM were incubated with a lactoperoxidase iodinating system, but only ribophorin I was digested during mild trypsinization of intact RM. Ribophorin II (63,000 daltons) was only proteolyzed when the luminal face of the microsomal vesicles was made accessible to trypsin by the addition of sublytical detergent concentrations. Only 30–40% of the bound ribosomes were released during trypsinization of intact RM, but ribosome release was almost complete in the presence of low detergent concentrations.

Very low glutaraldehyde concentrations (0.005–0.02%) led to the preferential cross-linking of large ribosomal subunits of bound ribosomes to the microsomal membranes. This cross-linking prevented the release of subunits caused by puromycin in media of high ionic strength, but not the incorporation of [³H]puromycin into nascent polypeptide chains. SDS-acrylamide gel electrophoresis of cross-linked samples a preferential reduction in the intensity of the bands representing the ribophorins and the formation of aggregates which did not penetrate into the gels.

At low methyl-4-mercaptobutyrimidate (MMB) concentrations (0.26 mg/ml) only 30% of the ribosomes were cross-linked to the microsomal membranes, as shown by the puromycin-KCl test, but membranes could still be solubilized with 1% DOC. This allowed the isolation of the ribophorins together with the sedimentable ribosomes, as was shown by electrophoresis of the sediments after disruption of the cross-links by reduction. Experiments with RM which contained only inactive ribosomes showed that the presence of nascent chains was not necessary for the reversible cross-linking of ribosomes to the membranes. These obser-

vations suggest that ribophorins are in close proximity to the bound ribosomes, as may be expected from components of the ribosome-binding sites.

KEY WORDS rat liver · rough microsomes · membrane structure · membrane bound ribosomes · ribosome binding site · chemical cross-linking

In the preceding article (19), we reported the identification of two integral membrane proteins (65,000 and 63,000 daltons) which are characteristic of rough microsomes (RM). These two proteins were isolated together with the membrane-bound ribosomes in residues obtained after extraction of other microsomal components with the nonionic detergent Kyro EOB. Because this association suggested a relationship between the two proteins and the sites of the microsomal membranes which bear the bound ribosomes, the proteins were designated ribophorins I and II (65,000 and 63,000 daltons, respectively) (18).

The possibility has not been ruled out, however, that the association between the ribosomes and the ribophorins observed in Kyro EOB residues is artefactual and occurs during the solubilization of other membrane components because ribosomes are trapped in a collapsed, residual ribophorin network. Indeed, electron microscope observations (19) showed that ribosomes attached to membrane remnants containing the ribophorins were closely packed, and therefore an extensive reorganization in the distribution of ribosome-binding sites in the membranes must have occurred during Kyro EOB extraction.

In this investigation, we have attempted to characterize the relationship of the ribophorins to the ribosome-binding sites in native microsomal membranes, using chemical cross-linking and macromolecular probes. Chemical cross-linkers are useful tools in the study of near-neighbor relationships in biological structures with defined protein arrangements. They were first used (7) to establish the stoichiometric relationship between oligomers of soluble proteins and have since been fruitfully applied to several complex systems including ribosomes (e.g., references 14, 35), red cell membranes (e.g., references 36, 41), mitochondria (39) and chromatin (26). If, in the native microsomal membranes, ribophorins are indeed associated with the binding sites either as constituents of these sites or as part of larger complexes containing ribosome receptors, then it should be possible to detect a close spatial relationship by

covalently linking the ribophorins to the ribosomes. We have cross-linked ribosomes to the microsomal membranes using the irreversible cross-linker glutaraldehyde as well as the reversible reagent methyl-4-mercaptobutyrimidate (MMB). In the former case, we were able to indirectly infer that the ribophorins were cross-linked to the ribosomes, whereas in the latter we could demonstrate directly an association between the ribophorins and the ribosomes.

We have also probed for the exposure of the ribophorins on each face of the membranes, examining their accessibility to LPO and to proteases in intact vesicles and in vesicles opened by treatment with low DOC concentrations (15), as well as in microsomes previously stripped of ribosomes by a nondestructive treatment (2). Lactoperoxidase-catalyzed iodination (23) is a useful method to specifically label proteins exposed on the outer surface of cells and of membrane-enclosed structures without introducing major structural perturbations (for review, see reference 11). It has been shown (16) that rough microsomal vesicles are impermeable to this system, which can be used to selectively label proteins exposed on the cytoplasmic face of the membranes. Our results indicate that the ribophorins are transmembrane proteins which can be labeled from both sides of the membrane (see footnotes 1 and 2).

Accessibility to proteolytic digestion is also a useful test to probe for the surface exposure of membrane proteins in RM. It has been previously shown that microsomal membranes are effective barriers to trypsin which protect the vesicular content even after extensive degradation of proteins on the outer cytoplasmic face (12, 15, 16, 28).¹ We found that ribophorin I is highly susceptible to trypsin digestion. This observation may be correlated with the previous finding that mild

¹ Rodriguez Boulan, E., G. Kreibich, and D. D. Sabatini. 1978. Spatial orientation of glycoproteins in membranes of rat liver rough microsomes. I. Localization of lectin-binding sites in microsomal membranes. *J. Cell Biol.* In press.

² Rodriguez Boulan, E., D. D. Sabatini, B. N. Pereyra, and G. Kreibich. 1978. Spatial orientation of glycoproteins in membranes of rough microsomes. II. Transmembrane disposition and characterization of glycoproteins. *J. Cell Biol.* In press.

trypsinization almost completely abolishes the capacity of RM membranes stripped of ribosomes to rebind 80S ribosomes in vitro (6, 13, 34).

MATERIALS AND METHODS

Sources of animals and reagents, analytical and preparative procedures, isolation and treatments of microsomes (if not described in this manuscript) are given in the preceding paper (19). The same abbreviations were used to designate the microsomal fractions and to describe the centrifugation conditions.

Materials

Chemicals were obtained from the following sources: dithiothreitol (DTT) from Nutritional Biochemicals Corp. (Cleveland, Ohio); [³H]puromycin (sp act 0.84 mCi/mmol) Amersham Corp. (Arlington Heights, Ill.); carrier-free ¹²⁵I-NaI, [³H]choline hydrochloride (sp act 2.3 Ci/mmol) from New England Nuclear, (Boston, Mass); butylhydroxytoluene (BHT) from Eastman Kodak Co. (Rochester, N.Y.); and 3 X crystallized bovine pancreatic trypsin from Worthington Biochemical Corp. (Freehold, N.J.). *N*- α -tosyl-L-lysyl chloromethyl ketone (TLCK), and lactoperoxidase (LPO) purified grade, 100 IU³/mg from Calbiochem (San Diego, Calif.), glucose oxidase (GO) type I 1,000 EU⁴/ml, from Sigma Chemical Co. (St. Louis, Mo.), Ultrapure glutaraldehyde from Tousimis Research Corp. (Rockville, Md.), and methyl-4-mercaptobutyrimidate from Pierce Chemical Co. (Rockford, Ill.).

Cell Fractionation

RM containing labeled ribosomal RNA were prepared from rats (male, 150 g) injected (intraperitoneally) with 200 μ Ci of [³H]thorotic acid. The specific activity of the RNA in the microsomes was about 10⁶ dpm/mg RNA. To prepare RM containing only inactive membrane-bound ribosomes (RM_{ET}), i.e., ribosomes without nascent polypeptide chains, female rats were injected intraperitoneally with 12 ml of an ethionine solution (25 mg/ml) in physiological saline. After 5 h, the animals were sacrificed, and RM were prepared according to Adelman et al. (1). Before use, RM were washed by resuspension and centrifugation (30 min-30K-Ti60) in TKM.

LPO-Catalyzed Iodination of Microsomal Membranes

The procedure used was basically that previously described (10, 16). RM were resuspended (3 mg pro-

tein/ml) in LSB containing 10 mM glucose and 0.001% butylated hydroxytoluene (BHT) to prevent lipid peroxidation (42). The inclusion of BHT in the medium led to a severalfold increase in iodine incorporation. To the undiluted radioactive iodide (100 μ C/ml), nonradioactive iodide was added (10⁻⁶ M). This resulted in a more reproducible level of iodine incorporation. The LPO concentration with incubation mixture was 5-10 μ g/ml.

To follow the kinetics of incorporation, aliquots were pipetted onto Whatman glass filter discs which were extracted with TCA and counted in a gamma counter model 1185 (Searle Diagnostics, Inc.). After 30 min of incubation at room temperature, a plateau was reached. Iodinated microsomes were recovered by sedimentation (20 min-30K-Ti60-4°C) through a 5-ml sucrose cushion (20% SLSB).

Proteolysis of RM

Conditions of trypsinization are described in the legend to Fig. 2.

Cross-Linking of RM with Glutaraldehyde

RM stored at -70°C in LSB-glycerol (2:1) were diluted five times with LSB-TEA and recovered by sedimentation (30 min-30K-Ti60-4°C) through a cushion of 6 ml of 20% SLSB-TEA. To remove Tris, the microsomes were resuspended in LSB-TEA and washed once by sedimentation and resuspended in LSB-TEA (3 mg protein/ml). Aliquots were cross-linked by adding glutaraldehyde concentrations varying from 0.005 to 0.02%. Controls and samples containing glutaraldehyde were kept for 30 min at 20°C, then diluted fivefold with LSB (containing Tris) to destroy the excess glutaraldehyde. After sedimentation (20 min-20K-Ti60-4°C), the pellets were resuspended in the original volume of the appropriate buffer.

Cross-Linking of RM with methyl-4-Mercaptobutyrimidate (MMB)

The procedure used was essentially that described by Traut et al. (40). RM stored in LSB-glycerol at -70°C were diluted fourfold with LSB-TEA pH 7.5 and recovered by sedimentation (30K-30 min-Ti60-4°C). The RM were resuspended in LSB-TEA, pH 8 and brought to a final concentration of 6 mg protein/ml. Aliquots of RM suspensions were mixed in a ratio of 1:1 with a solution of MMB in LSB-TEA ranging from 3.5 to 0.08 mg MMB/ml, pH 8 (dissolved just before use and titrated immediately to pH 8). Control RM received an equivalent volume of LSB-TEA, pH 8.0. Samples were incubated for 20 min at 0°C and, after addition of H₂O₂ to a final concentration of 40 mM, for another incubation period of 30 min at 0°C. Microsomes were sedimented (30 min-30K-Ti60-4°C) through a 2-ml cushion of 20% SLSB pH 7.5 and resuspended in LSB pH 7.5 (6 mg protein/ml). The extent to which ribosomes were

³ 1 IU catalyzes the conversion of 1 μ mol of tyrosine to moniodotyrosine per minute at 30°C.

⁴ 1 EU catalyzes the oxidation of 1 μ mol of glucose to gluconic acid generating 1 μ mol of H₂O₂.

cross-linked to the ER was determined by sucrose density gradient analysis. RM resuspended in HSB, 2.5 (3 mg protein/ml), were incubated for 20 min at 20°C and 10 min at 37°C with puromycin (10^{-3} M). To reverse the cross-link, DTT (10 mM) or mercaptoethanol (4%) was added to the reaction mixture. Samples for SDS-acrylamide gel electrophoresis were mixed with sample buffer and run either directly or after reduction.

Cross-Linking of RM from Ethionine-Treated Rats

RM isolated from ethionine-treated rats (RM_{Et}) were stored in TKM containing 50% glycerol, diluted four times with LSB-TEA (pH 7.4) before use, and then recovered by sedimentation (30 min-30K-Ti60-4°C). The pellet was resuspended (3.5 mg protein/ml) in LSB-TEA pH 8. The protein concentration was estimated from the OD_{260} of samples solubilized in SDS (1%), assuming that 6.75 OD_{260} units correspond to 1 mg of rough microsomal protein (38).

Aliquots of this microsomal suspension received LSB-TEA, pH 8 (control) or MMB concentrations varying from 0.1 to 1.6 mg/ml final concentration. The suspensions were incubated for 20 min at 0°C and, after addition of H_2O_2 (40 mM final concentration), for an additional period of 30 min, after which the samples were diluted fivefold with LSB containing Tris, pH 7.5, and centrifuged (15 min-30K-Ti50-4°C). Pellets were resuspended in a high salt buffer (HSB-2.5) and analyzed in sucrose density gradients as described in the legend to Fig. 14.

RESULTS

Labeling of Ribophorins on the Cytoplasmic Face of Intact RM

Intact RM were labeled with ^{125}I by the lactoperoxidase-catalyzed iodination reaction under conditions which lead only to labeling of proteins exposed on the outer microsomal surface (16, 43). These microsomes were used to prepare ribosome-ribophorin complexes by the Kyro EOB (2.5×10^{-2} M) extraction procedure (19). This treatment led to the solubilization of more than 70% of the labeled membrane proteins and to the quantitative recovery of the ribophorins together with ribosomes.

Patterns of iodinated proteins of Kyro EOB residues (Fig. 1a) visualized by radioautography (Fig. 1b) of SDS-acrylamide gels showed that both ribophorins (arrows, Fig. 1) had incorporated ^{125}I and were therefore exposed on the cytoplasmic face of the vesicles. Levels of radioactivity incorporated in these proteins were considerably lower than levels incorporated into ribosomal

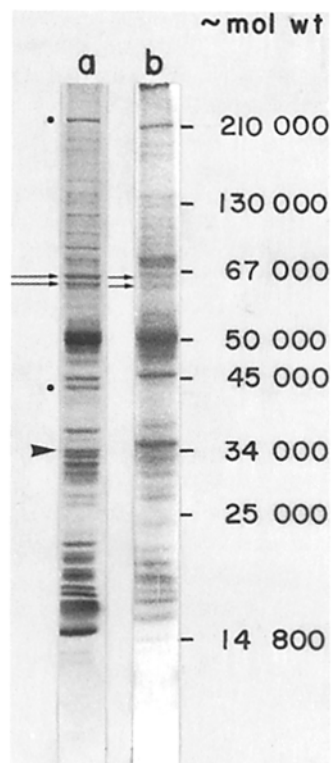


FIGURE 1 Exposure of membrane proteins in intact RM. Intact RM were labeled with ^{125}I by the lactoperoxidase-catalyzed iodination procedure, resuspended in LSB (3 mg protein/ml) and incubated with Kyro EOB (2.5×10^{-2} M). After centrifugation (60 min-40K-4°C), the sediment was resuspended and analyzed on SDS-acrylamide slab gels (1 mm thick, 8-13% acrylamide gradient). After staining, the gel was soaked in 20% glycerol for 30 min, dried onto a sheet of filter paper, and placed on a vacuum drying plate. For autoradiography, the dried gel was exposed for 5 days to an X-ray film. A print of the autoradiograph (b) was made to match the Coomassie blue-staining pattern (a).

proteins, and into other microsomal proteins which are also exposed on the surface of microsomes (16). The Kyro EOB extraction procedure, however, by removing other microsomal membrane components, some of which have molecular weights similar to the ribophorins, allowed the unequivocal demonstration of the exposure of ribophorins on the cytoplasmic surface. In addition to the ribophorins, detergent-insoluble microsomal contaminants such as urate oxidase (mol wt, 34,000) and myosin (mol wt, 210,000), which are sedimentable and are present in Kyro EOB residues obtained by differential centrifugation

(19), were, as expected, labeled with ^{125}I (Fig. 1, arrowhead and dot). This was also the case with some incompletely solubilized membrane proteins found in the Kyro EOB residue, which in intact RM are accessible to LPO (e.g., mol wt, 50,000 and 70,000).

Attempts to determine whether the presence or absence of ribosomes bound to the microsomal membranes affected the extent to which the ribophorins could be iodinated by the lactoperoxidase-catalyzed reaction were carried out. Intact RM were iodinated first with ^{125}I and, after ribosome stripping with puromycin-KCl (2), with ^{131}I . In control RM, not treated for ribosome stripping, both iodinations were performed in succession. Individual polypeptides separated in SDS-acrylamide gels showed similar ratios of ^{131}I : ^{125}I radioactivity throughout the gels. Moreover, ribosome removal did not lead to a significant selective

increase in the labeling of any other protein during the second iodination.

Susceptibility of Ribophorins to Proteolytic Digestion in Intact RM

Several previous reports have shown that digestion with exogenous proteases leads to the release of ribosomes from RM (28) and abolishes the capacity of RMstr to bind 80 ribosomes in vitro (6, 13, 22, 34). When intact RM were subjected to a limited digestion with trypsin (10 $\mu\text{g}/\text{ml}$, 60 min at 0°C ; Fig. 2*f*), a set of microsomal proteins was preferentially digested, as indicated by the disappearance of stained bands from their normal positions in the SDS-acrylamide gels (dots in Fig. 2). Bands representing several membrane polypeptides (dots in Fig. 2*g*) and a polypeptide of mol wt 210,000, which appears to represent con-

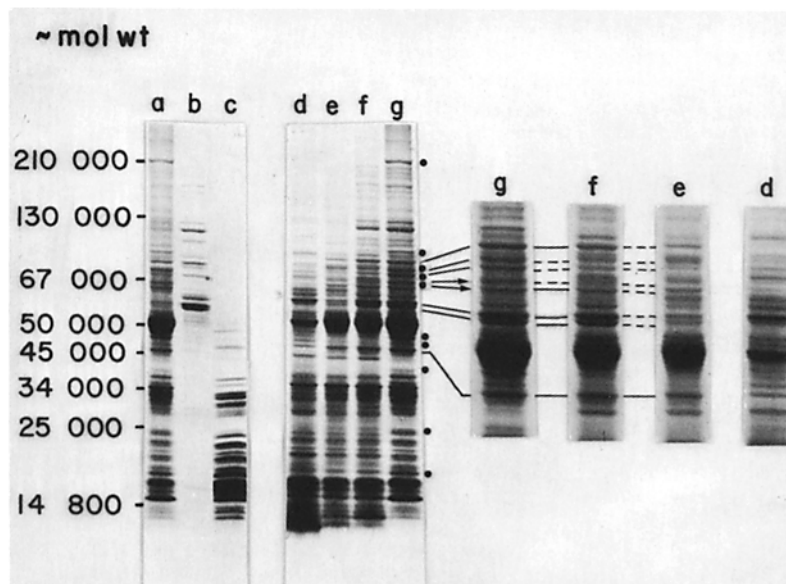


FIGURE 2 Proteolysis of RM. RM were resuspended (3.5 mg protein/ml) in LSB (pH 7.0). 1-milliliter aliquots received trypsin (10 $\mu\text{g}/\text{ml}$) alone (*f*), or trypsin in the presence of two different DOC concentrations, (0.05%) (*e*) and 0.78% DOC (*d*). After 60 min at 4°C , 50 μg of TLCK was added. Samples were diluted $10\times$ with LSB and recovered by sedimentation (30 min-40K-no. 40). Supernates were discarded and the pellets were resuspended in 0.5 ml of LSB (pH 7.0) containing 16 μg TLCK/ml. Aliquots from trypsinized samples (*d*, *e*, and *f*), control RM (*g*), a microsomal content fraction (*b*), the corresponding content-free RM (*a*) as well as the free polysomes (*c*) were prepared for electrophoresis and analyzed in the same SDS-acrylamide gradient gel (8-12%).

The microsomal membrane fraction (*a*) and the corresponding content fraction (*b*) were prepared from RM resuspended in LSB (3.5 mg protein/ml) which were treated with DOC (14). The content-free RM membrane fraction was recovered by sedimentation (30 min-40K-no. 40- 3°C) and resuspended. Equivalent amounts of all samples were loaded onto the gel. Free polysomes were prepared according to Adelman et al. (1). They were resuspended in HSB and washed by sedimentation through a cushion of 2 M SHSB (8 h-40K-Ti60- 3°C).

taminating myosin (see reference 19), were also significantly reduced in staining intensity.

Ribophorin I, but not ribophorin II (arrows), was one of the rapidly proteolyzed proteins. The intensity of other bands corresponding to membrane and content proteins was largely unaffected as was apparent from a comparison of gel patterns from proteolyzed (*f*) and control (*g*) RM with patterns from microsomes devoid of content (*a*) and from the set of purified content proteins (*b*). Similar sets of membrane proteins were digested when intact RM or RM stripped of ribosomes were incubated with trypsin.

Concomitant with the rapid proteolysis of ribophorin I, 30–40% of the bound ribosomes were released from the membranes when intact RM were incubated with trypsin (Fig. 3*c*). This partial release of ribosomes may reflect the resistance to trypsin of ribophorin II or the resistance of a fraction of the nascent polypeptide chains which anchor the ribosomes to the membranes. Trypsinization in the presence of 0.025% DOC, on the other hand, led to an almost complete release of ribosomes (Fig. 3*d*). Under these conditions, the band corresponding to ribophorin II was also absent from its normal position in the gels, indicating that this protein was digested (Fig. 2*e*). It has previously been shown (16) that 0.025% DOC makes RM permeable to trypsin, allowing the digestion of luminal proteins, puromycin discharged nascent chains, and the proteins exposed on the cisternal face of the ER membranes.

Cross-linking of Bound Ribosomes to Microsomal Membranes with Glutaraldehyde

Glutaraldehyde (GA) was used as a bifunctional reagent to cross-link bound ribosomes to microsomal membranes in attempts to recognize membrane proteins which may contribute to the binding sites. It was found that, after treatment of RM with concentrations of glutaraldehyde as low as 0.005–0.02%, a subsequent treatment with puromycin in a medium of high ionic strength was no longer effective in releasing the ribosomes from the microsomal membranes (cf. OD profiles in Fig. 4*a* and *b*). Glutaraldehyde treatment, however, did not abolish the capacity of the bound ribosomes to incorporate puromycin to the same level as controls (Fig. 4*a* and *b*). At the lowest glutaraldehyde concentrations used (0.005%), the puromycin-KCl treatment still caused the release

Proteolysis of RM with and without low concentrations of DOC

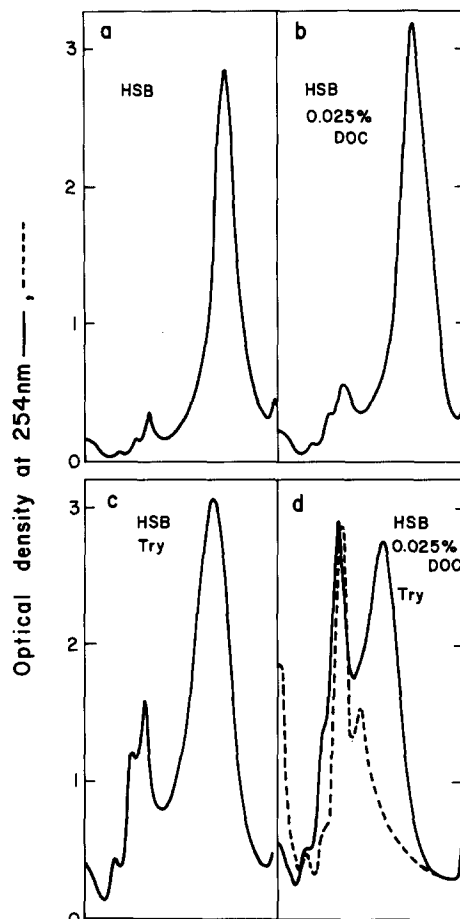


FIGURE 3 Ribosomes released from RM by proteolysis. High salt-washed RM were resuspended (3.5 mg protein/ml) in HSB. Aliquots received: (a) HSB; (b) DOC (0.025% final); (c) Trypsin (10 μ g/ml); and (d) Trypsin (10 μ g/ml) plus 0.025% DOC or 1% DOC (dotted line in panel *d*).

After 60 min, 500- μ l aliquots were loaded onto linear sucrose density gradients (10–50% SHSB). After centrifugation (120 min–40K–SW41–4°C), the OD₂₅₄ profiles throughout the gradients were recorded.

of practically all the 40S subunits, but 60S subunits remained associated with the membranes and therefore appeared to be selectively cross-linked to them (Fig. 5*c*). In this case, the isopycnic density of the microsomes from which the 40S subunits had been removed (Fig. 5*c*) was lower than in control RM (Fig. 5*a*) but higher than the density of totally stripped membranes (Fig. 5*b*).

REACTION OF RM WITH [³H] PUROMYCIN
AFTER FIXATION WITH GLUTARALDEHYDE
(0.02%)

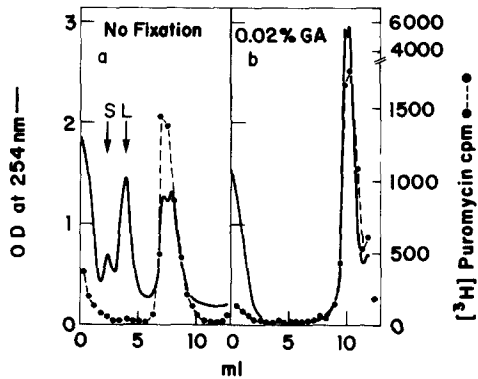


FIGURE 4 Puromycin coupling in ribosomes cross-linked to the membranes of RM. RM extensively washed with LSB-TEA were resuspended (3.5 mg protein/ml) in the same buffer. Samples were incubated with (b) or without (a) glutaraldehyde (0.01%) for 30 min at 20°C, diluted fivefold with LSB, recovered by sedimentation (20 min-30K-Ti60-4°C) and resuspended (3 mg protein/ml) in HSB-2.5. Incubation with [³H]puromycin (100 μ Ci/ml) was carried out for 10 min at 0°C. After addition of nonradioactive puromycin (10^{-3} M) for an additional period of 10 min to complete the reaction, 400- μ l aliquots were loaded on top of linear sucrose density gradients (10-50 SHSB) and centrifuged (120 min-40K-SW41-3°C). The OD profile was recorded (254 nm), and fractions were collected. The radioactivity distribution throughout the gradients was determined by pipetting 100- μ l aliquots onto Whatman 3 M filter disks (2.3 cm diameter) which were processed according to Mans and Novelli (21). In both cases, peptidyl-puromycin molecules remained associated with the microsomes (Fig. 4a and b). The isopycnic density of the fixed RM was unaffected by the puromycin treatment and was therefore significantly higher than that of unfixed puromycin-treated RM, in which an extensive release of ribosomes occurred (cf. Fig. 4a and b).

The findings that large ribosomal subunits can be cross-linked to the membranes before subunits are cross-linked to each other provides another demonstration of the direct contact between large subunits and the binding sites in the membranes (31).

Most membrane components of RM treated with low concentrations of GA (0.01%) were still effectively solubilized by subsequent treatment with 1% DOC. Analytical sucrose density gradients showed that this led to the release of phospholipids and most membrane proteins, which were found near the meniscus (Fig. 6b).

Ribosomes, however, were present in the form of aggregates (Fig. 6b), much larger than the polyosomes recovered from unfixed RM treated with 1% DOC. Sedimentation profiles of aggregates recovered by solubilization of cross-linked microsomes were similar to those obtained from Kyro EOB-treated, unfixed RM (19). Since the distance between ribosomes in RM is of the order of several hundred angstroms and is unaffected by fixation with even high concentrations of glutaraldehyde (e.g., reference 25), it can be concluded that the aggregates recovered from glutaraldehyde-treated RM after solubilization with DOC do not result from the formation of direct cross-links between ribosomes.

SDS-electrophoretic analysis (Fig. 7) showed that the mobility of most microsomal proteins was unaffected by the glutaraldehyde treatment. After cross-linking, however, bands corresponding to ribophorins I and II were either not present or very weakly represented at their normal positions in the gels. This was also the case for a set of bands representing proteins of low molecular weight, which included several ribosomal proteins and a membrane protein of approx. 14,000 daltons (marked in Fig. 7 by a dot near the bottom of gel). The latter protein was previously found to be present together with the ribophorins and the ribosomal proteins in residues obtained from RM treated with Kyro EOB (19). It should be noted that gel electrophoretic analysis also showed that large heterogeneous aggregates, which did not penetrate the resolving gels, were present after glutaraldehyde cross-linking of RM. In addition, a generalized increase in background staining was observed and a clearly discernible additional band with an apparent mobility corresponding to mol wt 22,000 (marked by an asterisk in Fig. 7) were present in gels of cross-linked samples but not in those of control RM.

The disappearance of the ribophorins from their normal positions in the gel patterns, after glutaraldehyde treatment, suggests that the ribophorins are involved in the cross-linking of large ribosomal subunits to the binding sites which prevents the release of ribosomes by puromycin-KCl. Because complexes of proteins were formed after glutaraldehyde cross-linking which did not penetrate the gels, it could not be established, however, whether, simultaneously with the cross-linking of ribosomes to the membranes, proteins of the binding sites were not also cross-linked to each other or to other membrane components.

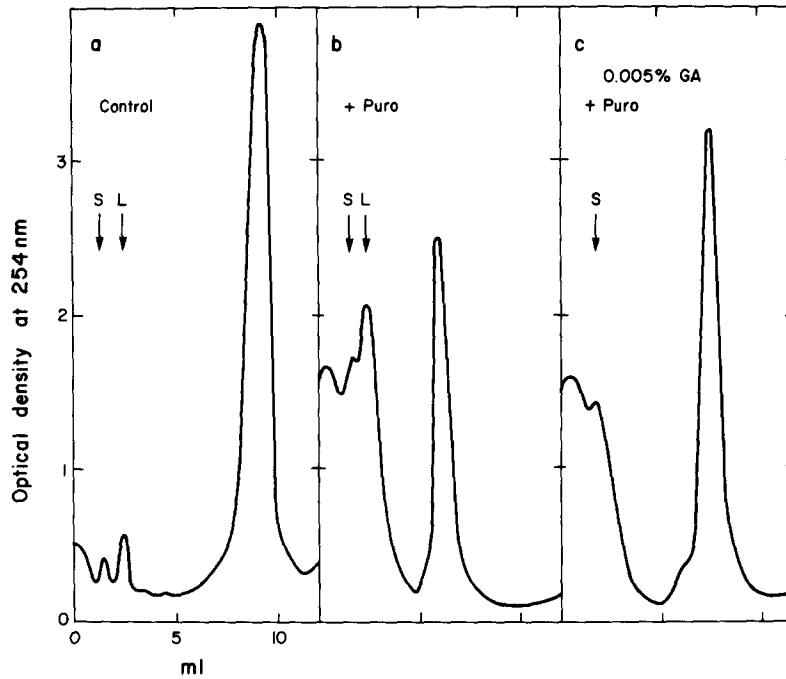


FIGURE 5 Preferential cross-linking of large ribosomal subunit to the membranes of RM treated with 0.005% glutaraldehyde. Control RM were incubated with (c) or without (a and b) 0.005% glutaraldehyde (see Materials and Methods) resuspended in HSB (control RM panel a) or in stripping buffer (HSB-2.5) (b). A sample of control RM (b) and of cross-linked RM (c) received, in addition, puromycin (10^{-3} M final). The samples treated for stripping (b and c) were kept for 60 min at room temperature. 500- μ l samples were layered onto 10-50% SHSB gradients, centrifuged (60 min-40K-SW41-4°C), and the OD₂₅₄ profiles were recorded.

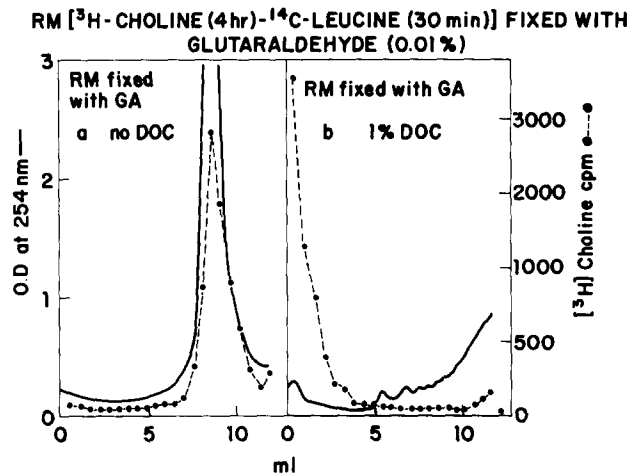


FIGURE 6 Pseudopolysomal aggregates in RM treated with detergent after mild fixation in glutaraldehyde. RM containing phospholipid labeled in vivo ($[^3\text{H}]$ choline, 4 h before sacrifice) were treated with a low concentration of glutaraldehyde (0.005%) which prevents release of large subunits by puromycin-KCl (see Fig. 5). The cross-linked RM were recovered by sedimentation (30 min-30K-Ti60-4°C) and resuspended in LSB (2.5 mg protein/ml). 360- μ l aliquots received 40 μ l of water (a) or 40 μ l of 10% DOC (b). After 30 min of incubation at 0°C, both samples were layered on top of linear sucrose density gradients (10-60% SLSB) and centrifuged (120 min-40K-SW41-4°C). OD profiles throughout the gradients were recorded, and 21 equal fractions were collected. The radioactivity distribution in the gradients was determined in 100- μ l aliquots.

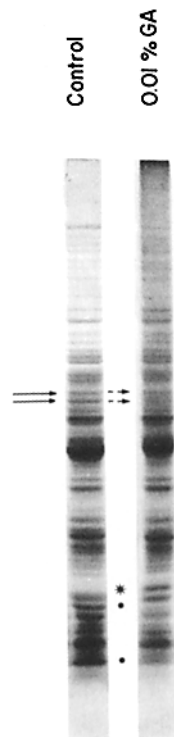


FIGURE 7 Electrophoretic pattern of RM treated with 0.01% concentration of glutaraldehyde. Samples containing 250 μg of protein from microsomal suspensions kept as controls or treated with 0.01% glutaraldehyde were solubilized in the sample buffer and analyzed on SDS-acrylamide gradient (8–12%) gels. Bands representing the two ribophorins in the control sample are indicated by arrows. Broken arrows indicate partial disappearance of the ribophorins in the cross-linked samples. The asterisk in the lower portion of the gel on the right hand side marks a band not found in the staining patterns of control RM which is formed by cross-linking. Dots indicate proteins in the molecular weight range of ribosomal proteins, which are also affected by cross-linking.

Reversible Cross-Linking of Ribophorins to Membrane-Bound Ribosomes

The reversible cross-linking agent methyl-4-mercaptobutyrimidate (MMB) (37, 40) was used to cross-link ribosomes to their binding sites in the membranes of intact RM. In these experiments, we attempted to develop conditions which still allowed solubilization of the membranes with DOC in order to determine which membrane proteins could be recovered with the ribosomes after solubilization of the membranes. Those proteins were identified electrophoretically after dis-

rupting the reversible linkages.

In a first step of the cross-linking process, intact microsomes were incubated with MMB in the presence of mercaptoethanol (ME) to allow a reaction between the imidate moieties of MMB and the primary amino groups of proteins, while the SH groups of MMB remained in the reduced state. In a second step, S-S cross-links between sufficiently close MMB molecules bound to the NH_2 groups were formed by oxidation with H_2O_2 .

It should be recognized that cross-linking experiments based on the formation of S-S bridges would be difficult to interpret if membrane proteins in RM were naturally extensively cross-linked via disulfide bridges or if during the oxidation step they would become cross-linked through their native SH groups. The extent of natural cross-linking in microsomes was therefore assessed by comparing electrophoretic patterns of proteins from untreated microsomes with those from microsomes in the oxidized (Fig. 8*Ia-IVa*) or reduced state (Fig. 8*Ib-IVb*). In general, unreduced patterns (Fig. 8*Ia*) were unaffected by subsequent oxidation (Fig. 8*Ila*), indicating that formation of new disulfide bridges by this treatment was negligible. Moreover, when compared with native microsomes, only small differences (see dots in Fig. 8*IVa*) were noted when samples were reoxidized after reduction (cf. *Ia* with *IIIa* in Fig. 8), showing that mostly the same groups were involved in the reformed bridges as in the native microsomes. SDS-acrylamide gels from unreduced (*Ia*) oxidized (*Ila*) or reduced and reoxidized (*IIIa*, *IVa*) samples had, however, a higher staining background than those from samples analyzed after reduction with ME (Fig. 8*Ib-IVb*). Moreover, in untreated or oxidized samples (Fig. 8*Ia-IVa*), some aggregates were present at the interface between the spacer gel and the separation gel, and many bands had different mobilities than when electrophoresis was performed after reduction with ME (Fig. 8*Ib-IVb*). However, ribosomal proteins (bands in the lower third of the gels) seemed to be unaffected by reduction. These observations show that S-S bridges are abundant in microsomal proteins, making it difficult to identify corresponding proteins in gel patterns from reduced and oxidized microsomal samples.

To determine whether intermolecular or intramolecular S-S bridges were present in the unreduced microsomes (Fig. 8*Ia*), S-S bridges were first broken *in situ* by reduction with ME and allowed to reform by oxidation in intact micro-

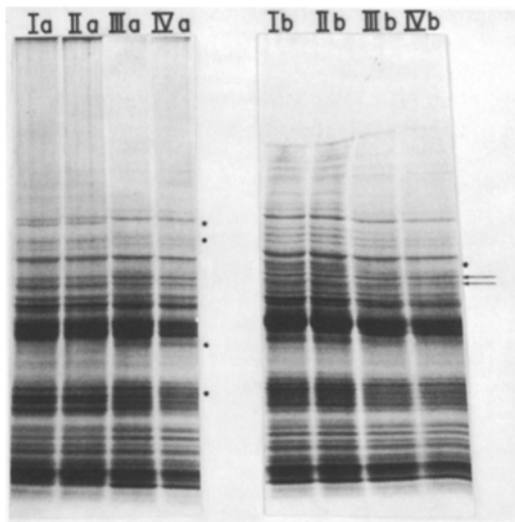


FIGURE 8 Changes in the electrophoretic pattern after reduction of disulfide bonds with mercaptoethanol. High salt-washed RM were resuspended in LSB (3.5 mg protein/ml). Samples (I-IV) (1 ml each) received: (I) 40 μ l H_2O ; (II) 40 μ l H_2O_2 (1 M); (III) 40 μ l mercaptoethanol (14.6 M); and (IV) 40 μ l mercaptoethanol. After 30 min of incubation at room temperature, 10 μ g of catalase was added to sample (II) to destroy excess H_2O_2 . All four samples (I-IV) were sedimented (60 min-30K-Ti60-4°C) and resuspended in 1 ml of LSB buffer. Sample IV received 100 μ l of DOC (10%). All samples were divided into two (*a* and *b*). Samples in one set (I *a*, II *a*, III *a* and IV *b*) received 20 μ l of H_2O while the remaining samples received either 20 μ l of mercaptoethanol (I *b* and II *b*) or 20 μ l of H_2O_2 (III *a* and IV *a*). All samples were prepared for electrophoresis in SDS-acrylamide gradient (5-12%) gels without further addition of mercaptoethanol. Coomassie blue-stained bands which disappeared or changed in their mobility due to the various treatments are indicated by dots in gels IV *a* and IV *b*. The position of the ribophorin is marked by arrows in gel IV *b*.

some or after solubilization of the proteins with DOC, to disrupt near-neighbor relationships. There was no detectable difference between electrophoretic patterns from these two types of samples (Fig. 8III*a* and IV*a*). These findings suggest that natural and reformed S-S bridges involve similar groups, even after proteins are separated from each other by solubilization, and therefore that most disulfide bridges are intramolecular. The minor differences (indicated by dots in Fig. 8) between native samples and reduced-reoxidized samples are most probably due to the formation of different pairs of intramolecular disulfide bridges, which do not lead to drastic shifts in the

position of the bands. It is well known that intrachain S-S linkages affect the conformation and therefore the mobility of polypeptides in SDS-acrylamide gel electrophoresis (9, 27).

The preceding observations do not totally exclude, however, the possibility that some protein complexes linked by disulfide bridges exist in RM but are not disrupted by the detergent treatment which follows reduction. The predominant intramolecular nature of most S-S bridges in microsomal proteins was, however, clearly demonstrated by two-dimensional electrophoresis. As expected, when microsomal proteins were run twice in the reduced state, they had similar mobilities in both dimensions and lay in an approximately diagonal position (Fig. 9*a*). When proteins were run during the first dimension in the unreduced state, reduction with ME before the second dimension did not result in vertically aligned new spots separated from the diagonal, as might be expected from the disruption of intermolecular cross-links. Instead, minor displacements of spots from the diagonal position were observed. The major change was the appearance of a streak revealing the polymerization, in the oxidized state, of a protein which in the reduced state had a mobility similar to that of cytochrome P-450 (Fig. 9*b*). These observations do not rule out the possibility that some of the intramolecular cross-links leading to the small horizontal separation of spots in the second dimension were formed by random pairing of S-S through oxidation during preparation of microsomes. They indicate, however, that after MMB is introduced into microsomal proteins, the major intermolecular cross-links resulting from oxidation should be those formed through the MMB moieties. These observations are in agreement with those made with erythrocyte membrane proteins (36, 41), although in this case it was shown that oxidizing agents stronger than H_2O_2 led to the formation of interprotein cross-links.

The lowest concentration of MMB needed to cause a significant cross-linking of ribosomes to the membranes while still allowing solubilization of the microsomes in DOC was then determined. RM containing ribosomes labeled *in vivo* with 3H -orotic acid were processed for cross-linking with a series of MMB concentrations (from 0.04 to 1.71 mg/ml). Cross-linked samples were recovered and treated with puromycin-KCl (Fig. 10*a-c*) with or without simultaneous reduction of the cross-links with DTT. The extent of ribosome release which

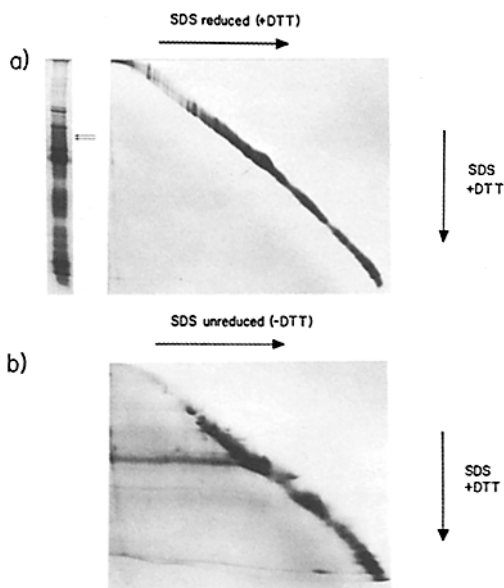


FIGURE 9 Effect of reduction of disulfide bonds on the electrophoretic mobilities of microsomal protein demonstrated by two-dimensional SDS-acrylamide gel electrophoresis of microsomal proteins with reduced or unreduced disulfide bridges. Samples containing 500 μg of RM protein were either kept in the unreduced state (*b*) or treated with DTT (*a*) (50 mM for 2 min at 90°C) before analysis on SDS-acrylamide gradient (5–10%) gels (first dimension). The gel strips of both samples were cut out from the first dimension gel and incubated at room temperature under mild agitation in sample buffer containing 10 mM DTT. The reducing sample buffer was replaced after 30 min and incubation continued for a total of 60 min. For the second dimension, the gel strips were attached on top of a slab gel (spacer gel: 3% acrylamide; resolving gel 8–12% acrylamide) using 0.5% agarose dissolved at 80°C in sample buffer. After electrophoresis in the second dimension, gels were fixed and stained with Coomassie blue. In (*a*) the one-dimensional gel pattern of reduced RM was added for comparison. The position of the two ribophorins is indicated by arrows.

was still possible after cross-linking was determined by fractionation in sucrose density gradients. Aliquots of the samples were also analyzed by SDS-acrylamide gel electrophoresis to assess the pattern of cross-linking of membrane proteins (Fig. 11). Sedimentation profiles showed (Fig. 10*c*) that ribosome release by puromycin-KCl was abolished by previous treatment with a high concentration of MMB (1.71 mg/ml). Simultaneously, however, membranes were rendered insoluble in DOC, and large protein aggregates were present in the SDS-treated samples which did not

penetrate the gels (Fig. 11). After treatment with lower MMB concentrations (0.04–0.26 mg/ml), 60S subunits were preferentially cross-linked to the membranes (Fig. 10*a* and *b*), as expected from the observations with glutaraldehyde and the fact that these subunits bear the proteins responsible for binding to the membranes (31). It is interesting to note that at all MMB concentrations, even after complete cross-linking of the ribosomes to the membranes, disruption of the cross-links by reduction with DTT restored the response to puromycin, allowing detachment of all the bound ribosomes and complete solubilization of the membranes in DOC (cf. ●—● and ▲—▲ in Fig. 12). The full reversibility of the cross-linking was also reflected in the gel patterns, which after reduction of cross-linked samples with DTT became identical to those from control RM (Fig. 11). Fig. 11 also shows that concentrations of MMB higher than 0.25 mg/ml produced extensive cross-linking of membrane proteins with one another. In samples treated with 0.66 and 1.71 mg/ml MMB, the number and intensity of many gel bands, especially in the high molecular weight range, decreased dramatically. On the other hand, microsomes cross-linked with 0.25 mg/ml MMB produced patterns similar to those of native non-reduced samples (cf. control (0) with 0.25 mg/ml MMB in unreduced samples, Fig. 11). An analysis of Fig. 10*b* shows that 0.25 mg/ml MMB cross-linked a significant fraction of the ribosomes to the membranes. This concentration still allowed solubilization of the membranes in DOC and did not produce extensive cross-linking between membrane proteins (Fig. 11). This concentration was therefore chosen for the final experiments.

RM were processed for cross-linking with 0.25 mg/ml MMB, and ribosomes not cross-linked to the membranes were released by treatment with puromycin-HSB. Microsomal membranes with the cross-linked ribosomes were then solubilized with 1% DOC, and the ribosomes were recovered by differential centrifugation. All fractions (Fig. 13*a–d*) were then treated with ME to reduce the cross-links, and the sets of protein in different samples were characterized by SDS-acrylamide gel electrophoresis.

Fig. 13*c* shows that ribophorins I and II (indicated by arrows) were the only major membrane proteins cross-linked to the ribosomes and were therefore recovered with them in the DOC residues. It should be noted, however, that, in spite of the reduction before electrophoresis, not all the

Reversible crosslinking of ribosomes to the microsomal membrane with
4-methyl-thiobutyrimidate

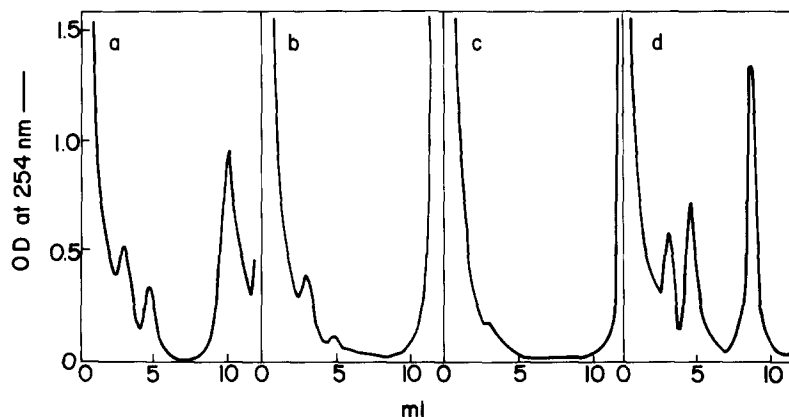


FIGURE 10 Reversible cross-linking of ribosomes to microsomal membranes with methyl-4-thiobutyrimidate (MMB). High salt-washed RM were suspended in LSB-TEA (pH 7) (3.5 mg protein/ml) and incubated (0°C) with (a) 0.04 mg/ml, (b) 0.26 mg/ml, and (c) 1.7 mg/ml. After 30 min, H₂O₂ was added, samples were diluted 10 × with LSB, and the microsomes were recovered by sedimentation (30 min-30K-Ti60-4°C). For ribosome stripping, all samples were resuspended in the original volume of HSB-2.5 containing puromycin (10⁻³ M final concentration). Sample (d) received, in addition, DTT (10 mM) to reverse the disulfide cross-links. After incubation for 10 min at 37°C, aliquots of the sample (1.5 mg protein) were layered onto sucrose density gradients (10-45% SHSB). After centrifugation (120 min-40K-SW41-4°C), OD₂₅₄ profiles were recorded.

material penetrated the gels which might have led to a lower intensity of ribophorin II. Since in control (not cross-linked) RM ribophorins are completely solubilized by DOC (19), it was concluded that in intact RM ribophorins are sufficiently close to the bound ribosomes to be cross-linked to them, as may be expected for components of the binding sites.

Since nascent polypeptide chains in the large ribosomal subunits provide a link between active ribosomes and microsomal membranes (2, 29), we examined the possibility that MMB cross-links do not involve the large subunits directly but are mediated by the nascent polypeptide chains. A preparation of RM lacking nascent chains in the bound ribosomes was obtained from rats injected with ethionine, a drug which leads to extensive ribosome run-off and polysome disassembly (32). RM prepared in a medium of low salt concentration from ethionine-treated rats have been shown to retain a large number of inactive ribosomes attached to the binding sites in the membranes through a direct interaction with the 60S subunits (20). Fig. 14 shows the effect of increasing MMB concentrations in cross-linking the inactive ribosomes to the membranes. At each concentration, the extent of cross-linking was determined from

the release of ribosomes caused by raising the ionic strength without the need of puromycin. Throughout the concentration range examined, the effect was parallel to that obtained when active bound ribosomes from untreated rats were cross-linked to the membranes. The comparable effect of MMB in both preparations (cf. Fig. 14 and Fig. 10) demonstrates that nascent polypeptide chains are not required for the cross-linking of ribosomes to their binding sites using MMB.

DISCUSSION

Several observations made in this investigation reinforce the suggestion (19) that ribophorins I and II, two integral membrane proteins which are found in rough but not in smooth microsomes, are components of the ribosome-binding sites in RER membranes. Cross-linking experiments suggested the proximity of both of these proteins to the ribosomes bound to membranes of native RM.

Components of the ribosome-membrane junction were shown to be very sensitive to cross-linking with glutaraldehyde or with the reversible bifunctional reagent MMB. Very low concentrations of glutaraldehyde (0.02%) completely prevented the release of ribosomes from the mem-

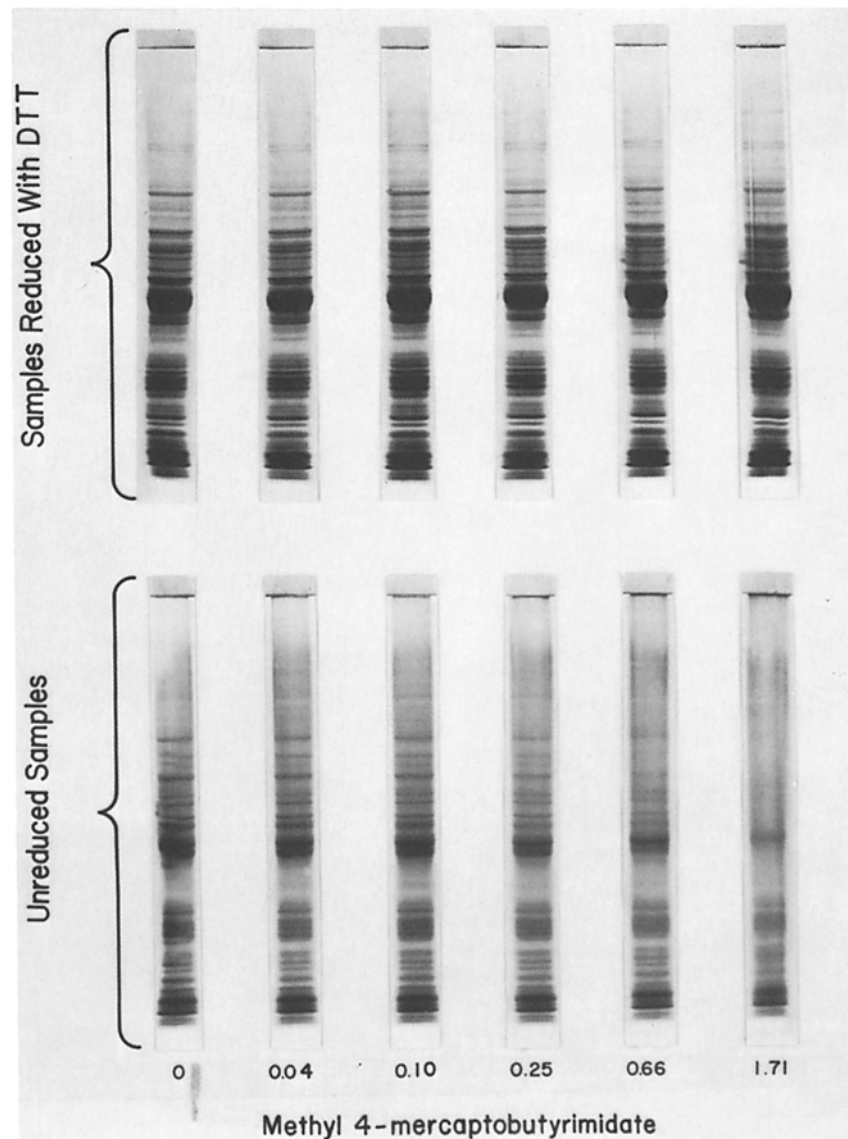


FIGURE 11 SDS-acrylamide gel patterns of RM samples processed for cross-linking with different concentrations of the reversible cross-linker MMB. High salt-washed RM were resuspended in LSB-TEA and processed for cross-linking with various concentrations of MMB as indicated in the figure. After formation of the cross-links by oxidation with H_2O_2 , the treated microsomes were recovered by sedimentation (30 min-30K-Ti60-4°C) and resuspended for analysis in SDS-acrylamide gradient gels (5-10%). Samples were either reduced with DTT (10 mM) (upper half of the figure) or loaded onto the gel in the unreduced state (lower half).

branes which is produced by puromycin in a medium of high salt concentration. At even lower concentrations (0.05%), only large subunits remained associated with the membranes, while small ribosomal subunits were released by the puromycin-KCl treatment. Thus, cross-linking of

the ribosomes to the membranes preceded both the cross-linking of ribosomal subunits to each other and the impairment of the puromycin coupling which might be expected from a reaction of GA with components of the peptide synthesis site in the large subunit. SDS-acrylamide electropho-

resis of proteins from glutaraldehyde-treated RM revealed the disappearance from their normal positions of the two bands representing the ribophorins and the formation of aggregates which did not penetrate into the gels. Under these conditions, few other changes in the gel patterns were detected. The exquisite sensitivity of components of the ribosome-membrane junction to cross-linking with glutaraldehyde contrasted with the rela-

tive resistance of other membrane proteins to be cross-linked under the same conditions. This was also evident from the fact that membranes remained soluble in DOC after complete ribosome cross-linking. Moreover, ribosomes in glutaraldehyde cross-linked RM retained the capacity of coupling tritiated [^3H]puromycin to their nascent polypeptides, although this was not accompanied by ribosome release. The incorporation of

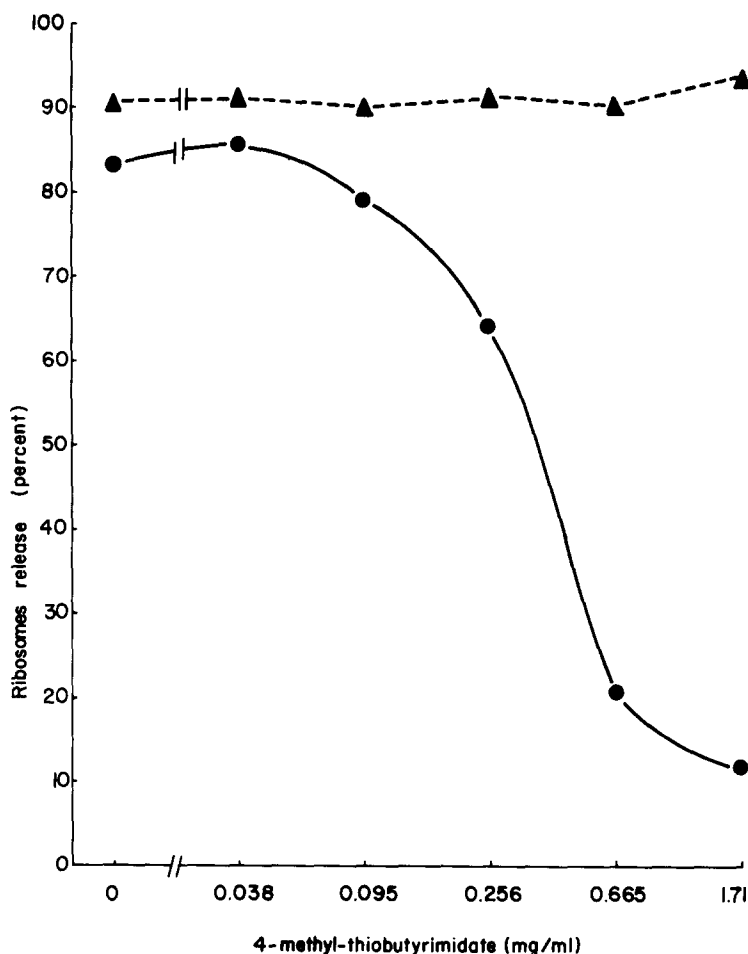


FIGURE 12 Reversible cross-linking of ribosomes to RM membranes with MMB prevents the release of ribosomes by puromycin in HSB. RM containing RNA labeled in vivo (^3H)orotic acid 40 h before sacrifice) were processed for cross-linking with various concentrations of MMB, recovered by sedimentation and resuspended in stripping buffer (3 mg protein/ml, HSB 2.5) containing puromycin (10^{-8} M). An aliquot (400 μl) was taken from each sample which received DTT (50 mM final) to reverse the cross-links. After incubation (30 min at room temperature and 10 min at 37°C), all samples were analyzed by sucrose density gradient (10–50% SHSB) centrifugation (120 min-40K-SW41- 4°C). OD profiles (254 nm) were recorded and 15 identical fractions were collected from each gradient. Aliquots were counted for [^3H]RNA radioactivity. The release of ribosomes from reduced (▲—▲) and unreduced samples (●—●) was calculated from the radioactivity under the small and large ribosomal subunit peaks and expressed as percent of the radioactivity found in the whole gradient.

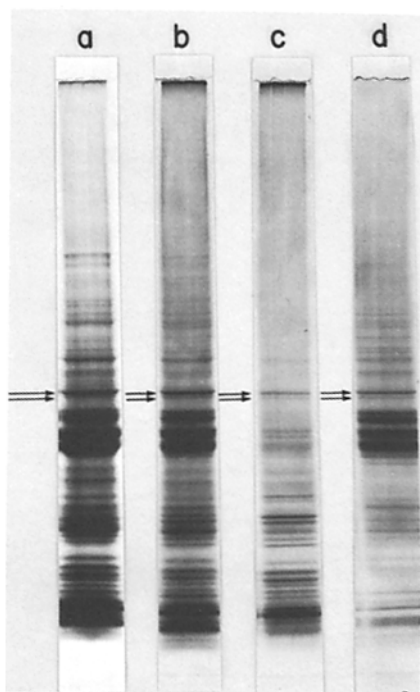


FIGURE 13 Cross-linking ribophorins to membrane-bound ribosomes. RM were processed for cross-linking with MMB (0.2 mg/ml) (a) and ribosomes not cross-linked by this treatment were removed with puromycin-KCl (2). The microsomes with the remaining cross-linked ribosomes were recovered by sedimentation (30 min-40K-no. 40-15°C) (b), resuspended in LSB (3 mg protein/ml) and treated with 1% DOC to solubilize the membranes. The released ribosomes, with the membrane proteins cross-linked to them, were recovered by centrifugation (60 min-40K-no. 40-4°C) through a 2-ml cushion of 1.5 M SHSB (c). The corresponding amount of dissolved membrane proteins remaining in the supernatant was loaded in slot d, so that the total protein loaded in gels c + d corresponds to that in loaded gel b. All samples were reduced with mercaptoethanol before analysis in SDS-acrylamide gels (5-13%). The position of bands corresponding to ribophorins is indicated by arrows.

[³H]puromycin demonstrates the functional preservation of the active sites and indicates that the lack of ribosome release is not due to an impairment in the puromycin reaction. Sedimentation analysis of glutaraldehyde cross-linked RM after solubilization of the membranes with DOC showed pseudopolysome profiles which resembled those observed after RM are treated with Kryo EOB (19), a neutral detergent which does not disrupt ionic bonds and appears to preserve the ribosome-ribophorin association in intact RM.

Since Kryo EOB residues are normally dissolved by DOC, the formation of DOC-resistant aggregates by GA cross-linking suggests that cross-links were also formed directly or indirectly between the ribophorins themselves and therefore that these proteins are likely to be in close proximity to each other within the membranes of intact RM.

A similar approach using the reversible cross-linking agent MMB permitted the isolation of complexes containing ribosomes and ribophorins from which these proteins could, upon reduction of the disulfide bridges, be released for electrophoretic identification. This reagent was also able to cross-link ribosomes to the membranes without abolishing the reaction with the puromycin. It is noteworthy that reversal of the cross-linking in RM also restored the puromycin-dependent ribosome release, showing that lack of release was not due to the blocking of NH₂ groups in proteins caused by the reagent but most likely to the formation of disulfide bridges between modified residues in ribosomes and membrane proteins or membrane phospholipids. As was the case with glutaraldehyde, low MMB concentrations also led to selective cross-linking of large subunits to the membranes, providing another striking demonstration that these subunits are in direct contact with the binding sites (6, 8, 31, 33).

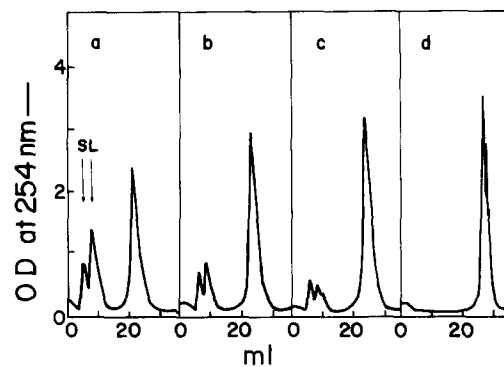


FIGURE 14 Cross-linking of inactive ribosomes to the membranes of RM from ethionine-treated rats. RM obtained from ethionine-treated rats (RM_{EI}, see Materials and Methods) were treated for cross-linking in a low salt buffer with various concentrations of MMB. (a) Control; not cross-linked RM_{EI}; (b) 0.1 mg/ml MMB; (c) 0.4 mg/ml MMB; and (d) 1.6 mg/ml MMB. After raising the salt concentration to HSB-2.5 and a brief incubation (10 min-37°C), the samples were analyzed by sucrose density gradient (10-55% SHSB) centrifugation (120 min-40K-SW41-4°C). OD profiles at 254 nm were recorded.

Large ribosomal subunits are known to be linked to sites on the microsomal membranes by two types of bonds (2): (a) direct electrostatic interactions between the subunits and components of the binding sites, which can be disrupted by an increase in the ionic strength, by anionic detergents, such as DOC, and by treatment with the anionic dye aurintricarboxylic acid (ATA) (6); (b) indirect links provided by sufficiently long nascent polypeptides, which are deeply embedded in the ER membrane and anchor the ribosomes to the binding sites. The nascent polypeptide chain is known to be the substrate for co-translational modifications, carried out by enzymatic systems associated with the membranes in the region containing the binding site, where a peptidase which removes a signal sequence is also thought to be located (3, 4). It was shown, however, that the presence of the nascent polypeptide chains is not necessary for the reversible cross-linking of the ribosomes to the membrane receptors caused by MMB, since cross-linking was equally efficient in microsomes from ethionine-treated rats. After ethionine treatment, which prevents initiation of protein synthesis and leads to disassembly of polyosomes (32), RM contain ribosomes which lack nascent polypeptides and are only bound through ionic interactions with components of the binding sites. It may therefore be assumed that the important effect of MMB cross-linking was to establish S-S bridges which stabilized an interaction between large subunits and binding sites, which is normally mediated by ionic bonds.

The possibility should be considered that MMB also caused the formation of S-S bridges between ribosomes or nascent polypeptide chains and lipid moieties of the membranes, such as phosphatidyl ethanolamine and phosphatidyl serine which contain amino groups. Such linkages may be sufficient to retain the ribosomes on the membranes during the puromycin reaction in high salt medium, but could not account for the presence of large pseudopolysomal aggregates after DOC treatment of MMB cross-linked microsomes.

Using macromolecular probes such as trypsin and LPO, we were able to obtain information concerning the disposition of the ribophorins in the ER membranes and their relationship to each of the membrane faces of the microsomes. Enzymatic iodination experiments indicated that in intact RM (more than 95% right-side-out vesicles) both ribophorins are accessible to LPO and therefore are exposed on the cytoplasmic, ribosome-

bearing face of the membranes. Since it has been shown that ribophorins are glycoproteins, which bind to Con A sepharose columns² and since most, if not all, glycoproteins in rough microsomal membranes have their carbohydrate moieties located exclusively on the luminal face of the microsomal membrane,² this finding suggests that both proteins have a transmembrane disposition. A direct demonstration of this disposition has been provided by double-labeling experiments using lactoperoxidase² in which intact RM were first labeled on the outer face with ¹³¹I and then labeled again with ¹²⁵I, after opening the vesicles with a low DOC concentration to allow access of lactoperoxidase to both faces. The results of these experiments showed that, while ribophorins will accept ¹³¹I atoms on the outer face, even higher levels of iodination are obtained when the luminal face is also exposed, in the presence of low DOC concentrations.

In attempts to detect whether bound ribosomes mask ribophorin segments exposed on the outer face of the membranes, double-labeling experiments with the lactoperoxidase system were also carried out. Labeling with the two different iodine isotopes was performed before and after ribosome release with puromycin-KCl. The results did not show, however, higher levels of iodination of the ribophorins or of any other membrane protein, after ribosome removal. These results indicated that, if the ribophorins provide amino acid sequences for ribosome binding, these sequences either do not contain a significant number of tyrosine residues or, even before stripping of ribosomes, are placed in a region of the ribosome-membrane junction which is accessible to lactoperoxidase. The possibility that the direct interaction of the ribosomes with the receptors is a loose one, and that a periodic detachment of ribosomes still held on the membranes by their nascent polypeptide chains allows accessibility of lactoperoxidase to the ribosome receptors is unlikely. Previous observations have demonstrated that, even after release of the nascent polypeptide chains, large subunits do not spontaneously detach from the binding sites or exchange with other added large subunits (5).

Trypsin was also used as a probe to test for the exposure of ribophorins on each membrane surface. It was found that ribophorin I was rapidly degraded before other substantial changes in the protein electrophoretic patterns were detected, indicating that this protein contains lysine and/or

arginine residues exposed on the cytoplasmic surface of the membranes. Previous studies have shown that similar digestion conditions also rapidly destroy the binding sites for ribosomes which are exposed on the surface of RM after ribosome detachment (22). Ribophorin II, on the other hand, was digested only when RM were opened by treatment with sublytical concentrations of DOC, so that trypsin had access to the luminal face of the membranes. It is interesting to note that, while partial ribosome release (approx. 30-40%) occurred during digestion of ribophorin I in a medium of low salt concentration, an almost complete release of ribosomes followed the digestion from both faces, which caused the degradation of ribophorin II. Release of ribosomes by proteolysis implies degradation of components of the binding sites and cleavage of the nascent polypeptide chains (or of membrane components with which they interact). Therefore, the partial release of ribosomes which accompanies digestion from the outer face may be due to the fact that the nascent polypeptides of the remaining ribosomes are only sensitive to proteolysis in segments which are exposed on the luminal side of the membranes, or to a residual role of ribophorin II in maintaining binding. This observation suggests that at least ribophorin I plays a direct role in ribosome binding. It cannot be excluded that ribophorin II is also a direct component of the binding site which becomes largely incompetent for binding when ribophorin I is degraded.

A differential accessibility of nascent polypeptides to trypsin may correlate well with the earlier finding that approx. 50% of the nascent chains synthesized on bound ribosomes are, after puromycin discharge, transferred to the vesicular lumen while the rest of nascent chains remain associated with the membranes and are accessible to trypsin added from the outside (17, 30). These differences in the fate of puromycin discharged polypeptides have also been correlated with the secretory or membrane character of the nascent chains synthesized in bound polysomes (24). Alternatively, receptors for nascent transmembrane polypeptides may exist on the luminal face of the ER membranes and therefore only be accessible to trypsin in open vesicles. It may be speculated that ribophorin II, which rapidly disappears during trypsinization from the inner face, may perform a receptor role for nascent chains. It cannot be excluded, however, that nonspecific effects of the detergent treatment account for the larger release of ribosomes caused by trypsin from open vesicles.

Sublytical detergent concentrations may alter the configuration of the membranes, allowing a more effective digestion of nascent polypeptide receptors on the cytoplasmic face, resulting in a more complete release of ribosomes.

The exposure of the ribophorins on the outer face of the intact RM demonstrated by the experiments with LPO labeling suggests that these proteins have an extended configuration within the ER membranes. This may lead to interactions between ribophorins of neighboring binding sites and to the formation of an extensive network of these integral membrane proteins to which the ribosome binding sites may be attached. It has been suggested that a protein network exists within the membranes which could account for the spatial segregation of ribosome-binding sites within the continuous ER system and the morphological characteristics of the rough cisterna (19).

Although the observations reported in this paper are compatible with the notion that ribophorins directly interact with ribosomes, the possibility has not been ruled out that another, as yet, unrecognized protein of small molecular weight mediates the binding between the ribophorins and the ribosomes. Such a protein may be difficult to recognize in gels of RM because of overlap with ribosomal proteins. No protein compositional difference was observed, however, in the low molecular weight region when gels of membrane proteins from smooth microsomes were compared with gels of proteins from RM stripped of ribosomes by puromycin-KCl. A proof of the direct relationship between ribophorins and ribosomes may have to await the isolation of ribophorins directly cross-linked to one or a small number of ribosomal proteins.

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