The Binding of Monoribosomes, Oligoribosomes and Polyribosomes to Reticular Membranes from Rat Liver

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1. The activity of the enzyme protein disulphide-isomerase (EC 5.3.4.1) was used to measure the binding of ribosomal fractions to reticular membranes from the liver of unstarved male rats. 2. Membranes degranulated by treatment with KCl plus puromycin and washed by centrifugation through 0.5M-KCl bound oligoribosomes but not monoribosomes. 3. Substitution of 0.25M-KCl for 0.5M-KCl in the wash solution produced membranes that bound monoribosomes and 60S subunits in addition to oligoribosomes. 4. The binding of all classes of ribosomes to smooth and 'lightly granulated' rough membranes was activated by oestradiol. The hormone activation was much greater with polyribosomes from fed rats than for monoribosomes or oligoribosomes from starved rats. 5. Exposure of rough membranes to centrifugal forces caused them to undergo partial degranulation, as demonstrated by unmasking of protein disulphide-isomerase activity.

Studies of the control of the binding of ribosomes to membranes are important because of their possible relationship to the control of protein synthesis, particularly in regard to the mechanisms whereby newly biosynthesized proteins find their correct location within the cell. Investigations using direct methods for measuring the binding have provided evidence that both small and large ribosomal subunits, monoribosomes and polyribosomes re-attach to reticular membranes degranulated by LiCl (Scott-Burden & Hawtrey, 1971; Khawaja, 1971, 1977; Schiaffonati *et al.*, 1975; Scott-Burden, 1976), EDTA plus ribonuclease (Shires *et al.*, 1975), citrate plus pyrophosphate (Rolleston, 1972; Ekren *et al.*, 1973) and KCl plus puromycin (Rolleston, 1972; Borgese *et al.*, 1974; Davis & Morris, 1976). Differences in the binding of 'template-depleted polyribosomes', prepared by cell-free protein synthesis and high-salt treatment, and ribosomal subunits, prepared by KCl plus puromycin treatment, have been reported (Shires *et al.*, 1975). There is some controversy in the literature about the binding of small and large ribosomal subunits to membranes degranulated by treatment with KCl plus puromycin (Borgese *et al.*, 1974; Davis & Morris, 1976). The results of these binding studies and other experiments that utilized smooth membranes (Rolleston & Mak, 1973) are difficult to interpret for several

reasons. The methods used to prepare reticular membranes can cause damage that significantly changes their polyribosome-binding properties (J. A. Fielder, unpublished work). The degranulation procedures can cause loss of membrane proteins as well as ribosomes (Lin & Farber, 1977). It is also known that rough membranes can undergo degranulation during centrifugation (Bont *et al.*, 1972; Sarma *et al.*, 1972; O'Toole, 1974). Further, the methods used to separate the putative complexes from the interacting components do not always eliminate artefacts (Vernie *et al.*, 1971; McIntosh *et al.*, 1975a,b). The high-salt treatments often used in the preparation of degranulated membranes and ribosomal fractions may drastically alter the binding properties of both entities. High-salt treatment causes partial degranulation of rough membranes (Adelman *et al.*, 1973; Dani *et al.*, 1976) and destroys membrane-bound protein disulphide-isomerase activity (Dani *et al.*, 1976). High-salt treatment also solubilizes some initiation factors for protein synthesis (Schreier & Staehelin, 1973) and is believed to remove from polyribosomes a factor that appears to mediate detachment of monoribosomes from rough microsomal fractions (Blobel, 1976). Further, it is not at all clear whether the reconstituted complexes obtained after such preparative and separative manipulations are related to native rough membranes (Burke & Redman, 1973; De Groot *et al.*, 1976).

In the present paper we follow our previous studies (Dani *et al.*, 1976) with some new observations on

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the binding of 60S ribosomal subunits, monoribosomes, oligoribosomes and unfractionated polyribosomes (all prepared without high-salt treatment) to reticular membranes, by using a methodology based on the enzyme protein disulphide-isomerase, the activity of which is masked by ribosome-membrane interaction.

Materials and Methods

The source of the materials used, except oestradiol-17 β (Sigma Chemical Co., St. Louis, MO, U.S.A.), and the details of the assay of protein disulphide-isomerase have been described (Dani *et al.*, 1976). TKM (25 mM-KCl, 5 mM-MgCl₂ and 50 mM-Tris/HCl, pH 7.5 at 25°C) buffer solutions used either contained X M-sucrose or were without sucrose as indicated.

Preparation of membrane fractions

Membranes were prepared from male albino rats (170–230 g) of the Sprague-Dawley strain fed *ad libitum*. Postmitochondrial supernatant in 0.25 M-sucrose in TKM buffer was prepared from perfused livers as previously described (Dani *et al.*, 1976). It was layered over three separate discontinuous sucrose-gradient systems (preparations A, B and C) to separate the membrane fractions. Preparation A consisted of 10 ml of 2.0 M-sucrose, 15 ml of 1.35 M-sucrose (both in TKM buffer) and 14 ml of post-mitochondrial supernatant from the bottom of the tubes upwards. The gradient was centrifuged in a fixed-angle 8 $\times$ 50 ml rotor for 4 h at 115 000 g_{av} . at 2–4°C. Smooth membranes were collected from the 0.25 M/1.35 M-sucrose interface and rough membranes from the 1.35 M-/2.0 M-sucrose interface. Preparation B consisted of 10 ml of 2.0 M-sucrose, 10 ml of 1.8 M-sucrose, 8 ml of 1.5 M-sucrose, 8 ml of 1.4 M-sucrose and 8 ml of 1.35 M-sucrose (all in TKM buffer) and 20 ml of post-mitochondrial supernatant from the bottom of the tubes upwards, centrifuged in a swing-out MSE 3 $\times$ 70 ml rotor for 4.5 h at 70 000 g_{av} . at 2–4°C. Lightly granulated rough membranes were collected from the 1.4 M-/1.5 M-sucrose interface and heavier rough membranes were from the 1.5 M-/1.8 M-sucrose interface. Preparation C consisted of 20 ml of 2.0 M-sucrose 25 ml of 1.35 M-sucrose (both in TKM buffer) and 20 ml of post-mitochondrial supernatant from the bottom of the tubes upwards, centrifuged in the same rotor under identical conditions as in preparation B. Intermediate rough membranes were collected from the turbid 1.35 M-sucrose layer and heavier rough membranes from the 1.35 M-/2.0 M-sucrose interface. The rough and smooth membranes were harvested through 0.5 M-sucrose/TKM buffer for 1 h in the same rotors and under the same conditions as were used for separation. The membrane pellets were gently rinsed with 0.25 M-sucrose/TKM, the supernatants from the rinsing

discarded and the pellets stored overnight at –20°C. The pellets were gently resuspended in 0.25 M-sucrose/TKM buffer by using a glass homogenizer, to a final membrane protein concentration of 5–9 mg/ml.

Preparation of ribosome fractions

Polyribosomes were prepared from 15–16 Sprague-Dawley rats starved for 18–20 h. Post-mitochondrial supernatant (19 ml) was layered over a discontinuous gradient consisting of 10 ml of 2.0 M-sucrose/TKM buffer under 10 ml of 0.5 M-sucrose/TKM buffer in MSE 8 $\times$ 50 ml fixed-angle rotor tubes. The tubes were centrifuged at 115 000 g_{av} . for 18 h at 2–4°C. The polyribosome pellets were gently rinsed with 0.25 M-sucrose/TKM buffer and resuspended in about 10 ml of the same buffer. The 60S ribosomal subunits, monoribosomes, diribosomes, triribosomes, tetra-ribosomes and pentaribosomes were prepared from the suspension by zonal centrifugation. The polyribosomes were separated on a sucrose density gradient in an MSE B-29 zonal rotor, capacity 1432 ml. The concentration of sucrose was chosen to give a linear increase with respect to radius in the rotor, from 20 to 40% (w/w) from 400 to 1400 ml from the rotor centre. The gradient was generated with a Dialagrad 380 Isco gradient pump. The rotor was loaded at 3000 rev./min in the following sequence: (1) the gradient, volume 1 litre, pumping rate 50 ml/min, to the rotor edge (less-dense end first); (2) underlay of 40% (w/w) sucrose, 50 ml/min to the rotor edge, until liquid appeared from the centre feed tube; (3) sample (5.5 ml) in 15.7% (w/w) sucrose introduced slowly to the centre with a syringe (displacing underlay from the edge); (4) overlay of 0.25 M-sucrose, pumped at 12.5 ml/min for 2 min, then at 25 ml/min until a total of 403 ml of underlay had been displaced. All the rotor contents were in TKM buffer. The rotor was run at 25 000 rev./min at 8.0°C to a total $\int \omega^2 dt$ of 1.234×10^{11} rad²·s^{–1} (about 4 h 30 min at full speed). Unloading was at 3000 rev./min from the centre by displacement with 43% (w/w) sucrose solution, at 48.8 ml/min, and fractions were collected at 15 s intervals. The effluent was monitored continuously at 260 nm with a Gilford 2000 recording spectrophotometer. Fig. 1 shows the absorbance profile of the separated polyribosome mixture. Various ribosomal fractions were collected by diluting the contents of appropriate tubes to about 0.5 M-sucrose/TKM concentration with TKM buffer and sedimenting the suspensions for 14–17 h at 2°C at 135 000 g_{av} . for monoribosomes and at 115 000 g_{av} . for all other fractions. The pellets of various fractions were frozen at –40°C.

Preparation of unfractionated polyribosomes

Unfractionated polyribosomes consisting of a mixture of monoribosomes and oligoribosomes were prepared under the conditions of preparation A. The

Fig. 1. Absorbance profile of fractionated polyribosomes

The profile has been replotted to show the radial positions of the zones in the rotor; the sloping absorbance scale compensates for radial dilution. The bands A-F under the peaks indicate the regions of the gradient harvested for the preparation of purified fractions. These peaks A-F were shown to represent 60S ribosomal subunits, monoribosomes, diribosomes, triribosomes, tetra-ribosomes and pentaribosomes respectively, by computer analysis of sedimentation rates through the preparation gradient and by direct sedimentation-velocity measurements in an MSE Centriscan analytical ultracentrifuge.

pellets from the discontinuous sucrose-gradient centrifugation were gently rinsed with 0.25M-sucrose/TKM buffer, stored overnight at -20°C , and resuspended in the same buffer before use.

Degranulation with KCl plus puromycin

The method used was a modification of that reported by Borgese *et al.* (1974). Rough membranes of the type stated in the legends were mixed with an equal volume of buffer to final concentrations of 0.5M-KCl, 0.5mM-puromycin, 0.25M-sucrose, 50mM-Tris/HCl and 2.5mM-MgCl₂ (pH 7.5 at 25°C). The mixture was incubated for 1 h at 0°C and then 15 min at 25°C , diluted 4-6-fold with high-salt buffer (0.5M-KCl, 0.25M-sucrose, 50mM-Tris/HCl and 5mM-MgCl₂, pH 7.5 at 25°C) and centrifuged between 25000 and 40000g_{av} for 30 min at 4°C in either an MSE 6×16.5 ml or 6×14 ml swing-out rotor. These membranes are designated high-salt-degranulated membranes. Low-salt-degranulated membranes were obtained by the above treatment including 0.5M-KCl, except that 0.25M-KCl was the salt concentration in the buffer used for final centrifugation. These membranes are designated low-salt-degranulated membranes. The pellets of both high- and

low-salt-degranulated membranes were gently rinsed with 0.25M-sucrose/TKM buffer, the solution was discarded and the pellets were resuspended in the same buffer. The RNA/protein ratios (w/w) of both membranes were similar, and in the range 0.04-0.08.

Membrane-ribosome-binding conditions

All binding incubations (except where stated) were carried out for 1 h at 25°C in a constantly shaking water bath with a membrane concentration of 2-4mg of membrane protein/ml of incubation medium. All observations were confirmed by using different membrane preparations. There were some variations of ratios of added rRNA to membrane protein between experiments. This was because we considered it necessary always to use fresh materials, and the RNA and protein determinations were in most cases subsequent to the binding experiments. These differences in no way affect the general conclusions.

Protein, RNA and phospholipid determinations

Protein was determined by the method of Lowry *et al.* (1951), with bovine serine albumin (Cohn fraction V; Sigma) as a standard protein. RNA concentrations were determined by a slightly modified method of Munro & Fleck (1966) after the original method of Schmidt & Thannhauser (1945). Phospholipids were determined by extracting and purifying membrane lipids by the method of Folch *et al.* (1957), followed by phosphorus determination by a method described by Ames (1966). Values for phospholipid P were converted into phospholipid by using a factor of 25.

Results and Discussion

The results in Table 1 show that diribosomes, triribosomes, tetra-ribosomes, pentaribosomes and mixtures of these with monoribosomes, but not monoribosomes alone, mask the activity of protein disulphide-isomerase on membranes degranulated with KCl plus puromycin and sedimented once through TKM buffer containing 0.5M-KCl. Even at relatively high concentrations in the binding incubations monoribosomes show negligible binding, whereas the binding of a mixture of oligoribosomes to the same membrane causes virtually all the enzyme activity present in the membrane to become masked. Table 1 also shows that the separated fractions of purified oligoribosomes all show comparable binding when incubated under identical conditions. It thus appears that the interaction measured is not dependent on polyribosomal size.

The experiments shown in Table 2 demonstrate that the salt concentration in the final sedimentation

Table 1. *Binding of monoribosomes and oligoribosomes to high-salt-degranulated membranes*

Constant shaking was omitted in Expt. 1. The ratios of added rRNA to membrane protein in the incubation mixtures are given in parentheses. The membranes used in these experiments were prepared from heavy rough membranes obtained from preparation B.

Additions to membrane preparation	Percentage of protein disulphide-isomerase activity masked after 1 h at 25°C		
	Expt. 1	Expt. 2	Expt. 3
Monoribosomes	4 (0.36)	0 (0.31)	0 (1.0)
Diribosomes	31 (0.36)	54 (0.31)	—
Triribosomes	43 (0.36)	60 (0.31)	—
Tettriribosomes	31 (0.36)	65 (0.31)	—
Pentaribosomes	—	60 (0.31)	—
Mixture of the above ribosomes	42 (0.36)	66 (0.31)	—
Mixture of oligoribosomes	—	—	94 (0.8)

Table 2. *Binding of monoribosomes and oligoribosomes to high-salt-degranulated and low-salt-degranulated membranes*

High-salt and low-salt-degranulated membranes in Expt. 1 were prepared by dividing the rough membrane (preparation A) after incubation with KCl plus puromycin into two equal samples. The ratios of added rRNA to membrane protein in the incubation mixtures are given in parentheses. The RNA concentration in Expt. 2 was estimated by direct A_{260} measurement, taking 1 A_{260} unit of unhydrolysed RNA = 42 $\mu\text{g/ml}$.

Interactants	Percentage of protein disulphide-isomerase activity masked after 1 h at 25°C	
	Expt. 1	Expt. 2
High-salt-degranulated membrane + monoribosomes	6 (0.6)	—
Low-salt-degranulated membrane + monoribosomes	45 (0.8)	65 (0.25)
+1:1 mixture of monoribosomes + diribosomes	—	63 (0.75)
+mixture of oligoribosomes	90 (1.5)	—
+60S ribosomal subunits	—	65 (0.34)

buffer has a decisive effect on monoribosome binding. When the salt concentration in this buffer was lowered from 0.5M-KCl to 0.25M-KCl, membranes

were obtained that bound monoribosomes and 60S ribosomal subunits to a considerable extent. These experiments quite clearly show that washing with 0.5M-KCl destroys the ability of degranulated membranes to bind monoribosomes but not oligoribosomes. It is possible that the high-salt treatment may change the binding or extent of binding of oligoribosomes, but such effect, if it occurs, must be small compared with the all-or-none effect on the binding of monoribosomes. It appears that unknown factors on the membrane are required for binding of monoribosomes but not of oligoribosomes, and these are removed or destroyed by sedimenting the membranes through 0.5M-KCl. In this context it is quite likely that high-salt treatment affects the integrity of certain proteins that are thought to have a role in ribosome-membrane interaction (Olsnes, 1971; Jothy *et al.*, 1974; Fujita *et al.*, 1977).

We have also investigated the binding to smooth and lightly granulated rough membranes of monoribosomes, oligoribosomes and total polyribosomes from rats fed *ad libitum*. Smooth membranes as normally prepared show a sex-dependent hormone

Table 3. *Effects of oestradiol on binding of monoribosomes and oligoribosomes from starved rats and unfractionated polyribosomes from fed rats to smooth membranes and lightly granulated rough membranes*

Where appropriate values are presented as means $\pm$ S.E.M. for the numbers of experiments given in parentheses. The RNA/protein ratios of added rRNA to membrane protein in paired experiments (with and without oestradiol) were identical. The RNA/protein ratios of the mixture varied within the range 0.3–0.5. The concentration of oestradiol in the binding mixtures (where added) was 5 $\mu\text{g/ml}$. It was added from a stock solution in Aristar ethanol (1 mg/ml). In control experiments neither oestradiol nor ethanol at the concentrations added had any significant effect on the protein disulphide-isomerase activity in the absence of ribosomes. Ethanol had no effect on the binding.

Materials added	Percentage of protein disulphide-isomerase activity masked after 1 h at 25°C	
	Smooth membranes (preparation A)	Light rough membranes (preparation B)
Monoribosomes	37 $\pm$ 9 (3)	15 (2)
Monoribosomes + oestradiol	59 $\pm$ 5 (3)	56 $\pm$ 10 (3)
Oligoribosomes	30 $\pm$ 6 (3)	30 (1)
Oligoribosomes + oestradiol	40 $\pm$ 8 (3)	67 $\pm$ 8 (3)
Unfractionated polyribosomes (fed rats)	7 $\pm$ 5 (4)	5 $\pm$ 3 (4)
Unfractionated polyribosomes (fed rats) + oestradiol	47 $\pm$ 11 (3)	41 $\pm$ 6 (3)

Table 4. *Relative specific protein disulphide-isomerase activities of some membrane fractions prepared by different methods*

Where appropriate values are presented as means $\pm$ S.E.M. Each individual ratio was derived from membrane fractions prepared from the same post-mitochondrial supernatant. The actual activities of any one membrane fraction from different preparations varied with S.E.M. values as much as 40% of means, but the ratios of activities varied considerably less. The numbers of experiments are given in parentheses. Degranulated heavy rough membranes and degranulated rough membranes refer to membranes assayed after preincubation with KCl plus puromycin.

	Type of preparation	Ratio of specific protein disulphide-isomerase activities	
		On protein basis	On phospholipid basis
Intermediate rough membrane/heavy rough membrane	C	3.8 (2)	3.8 (2)
Light rough membrane/heavy rough membrane	B	3.3 $\pm$ 0.4 (4)	—
Light rough membrane/heavy rough membrane	B + further centrifugation	2.2 (2)	1.9 (2)
Degranulated heavy rough membrane/heavy rough membrane	B	2.9 $\pm$ 0.9 (8)	—
Degranulated rough membrane/rough membrane	A	1.6 $\pm$ 0.6 (7)	—

requirement in their binding of polyribosomes, when the interactants were prepared from fed rats (Sunshine *et al.*, 1971). Oestradiol promotes the binding in the male and testosterone in the female. As shown in Table 3 smooth and lightly studded rough membranes from fed male rats bind monoribosomes, and oligoribosomes from starved rats and unfractionated polyribosomes from fed rats. The binding is activated in all cases by the addition of oestradiol. In the absence of oestradiol, negligible binding of unfractionated polyribosomes from fed rats to smooth and lightly granulated membranes occurs. In sharp contrast, significant binding of both monoribosomes and oligoribosomes from starved rats to the same membranes is observed in the absence of added hormone. Clearly starvation of the animals has a major effect on ribosome-membrane interactions. It is worth noting here that overnight starvation of the rats produces lightly granulated membranes that bind polyribosomes from the same animals with great avidity. This binding is completely inhibited by incubation with known carcinogens (J. A. Fielder & B. R. Rabin, unpublished work).

The lightly granulated membranes that we have used in these studies were shown by electron microscopy to be less heavily studded with ribosomes than are the heavier rough fractions. Table 4 shows that these lighter membranes have a considerably higher protein disulphide-isomerase activity than the heavier fractions. The difference in specific activities of the two fractions is greatly decreased by further centrifugal manipulation of the membranes. These experiments show very clearly that damage can occur to the membranes in the centrifuge. Whether this damage is

caused by the centrifugal *g* forces, or hydrostatic pressure, or both is not yet known. The changes shown in Table 4 are caused by an increase in activity of the heavy fraction rather than a decrease in that of the lighter fraction. It could well be that the light fraction used in our experiments has been generated from heavier fractions by loss of a class of polyribosomes in the centrifuge. The methodology is not available at the present time to test this possibility. It is also clear from Table 4 that the different preparative methods have a marked effect on the latent protein disulphide-isomerase activity. It seems clear that the masking of protein disulphide-isomerase in the rough membranes is accomplished by a sub-population of ribosomes that can be removed by centrifugation. This is an important consideration when devising methods for the preparation of rough membranes for studies of degranulation by carcinogens.

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