DEGRANULATION AND SEX-SPECIFIC REGRANULATION OF RETICULAR MEMBRANES FROM RAT LIVER AS STUDIED USING A SPECTROPHOTOMETRIC ASSAY OF PROTEIN DISULPHIDE ISOMERASE

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Received 1 April 1976
Revised version received 26 April 1976

1. Introduction

One of the important aims of studies of reticular membranes is to further understanding of the mechanisms which control the binding of ribosomes to membranes and thus advance our knowledge of control of protein synthesis at the level of translation. To study ribosome–membrane binding it is first necessary to separate the interacting components in as native a form as possible. A variety of procedures have been used to attempt to achieve this purpose including treatment with puromycin plus KCl [1], citrate plus pyrophosphate [2], EDTA alone [3], or plus ribonuclease [4] and LiCl [5]. A vital consideration in this field is the validity of the methods used for measuring both degranulation and regranulation. The direct methods of study involve determining, by chemical or radiochemical procedures, the quantity of RNA associated with the membranes as a function of either the protein or phospholipid content. In order to make these measurements it is necessary to devise effective methods to separate unbound ribosomes from the membranes. Extensive high speed centrifugation has been used for this purpose and it is now clear that the procedures used, whether they involve sedimentation or flotation, do not always distinguish biologically-relevant binding from artefactual associations [6]. The centrifugal procedures inevitably cause a major perturbation of the system studied by introducing very high hydrostatic pressures at low temperatures and high sucrose concentrations. These effects cannot be controlled for and it is impossible to know whether some of the phenomena reported actually occur at all under physiological conditions.

A second indirect method for studying ribosome–membrane interactions which obviates most of the above difficulties was developed in this laboratory [7]. The method is based on the observation that the catalytic activity of a membrane-bound enzyme, protein disulphide isomerase (EC 5.3.4.1, previously called rearrangease in publications from this laboratory), is related to ribosomal binding. The enzyme exhibits a reversible latency, being suppressed by polysome binding and activated by polysome removal. This indirect method has many advantages: it measures a catalytic parameter related to the terminal stages of protein biosynthesis and is thus much more selective than the direct method; its use eliminates artefacts resulting from centrifugal perturbation of the system; it does not measure artefacts associated with ribosome aggregation at physiological temperatures in vitro [8]. We have used a modified assay for protein disulphide isomerase which is a considerable improvement on our earlier methodology. This methodology has been used to investigate the degranulation of rough endoplasmic reticulum from rat liver by puromycin plus KCl and by LiCl. We show that some of the procedures described in the literature for degranulation using high salt buffers etc. cause serious membrane damage manifested by destruction of membrane-bound protein disulphide isomerase activity. It is not surprising therefore that the reconstituted rough membranes derived from such prepara-

tions fail to discharge newly biosynthesised nascent proteins in the same vectorial fashion as freshly prepared rough membranes [9].

The complete sex-specificity previously reported [10] for the reattachment of polysomes to membranes degranulated using EDTA is confirmed using membranes degranulated by puromycin in the presence of high salt.

2. Materials and methods

2.1. *Reagents used*

Buffer solutions used, containing X molar sucrose (STKM) or without sucrose (TKM), contained 25 mM KCl, 5 mM $MgCl_2$ and 50 mM Tris (Trizma acid and Trizma base from Sigma Chemical Co., USA), pH 7.5 at 25°C. Sucrose, ribonuclease-free, was from Cambrian Chemicals Ltd., Croydon, UK. Randomly reoxidised ribonuclease A was obtained from Miles Laboratories Ltd. Stoke Poges, UK. Puromycin dihydrochloride was from Sigma Chemical Co., USA. Ribonucleic acid, Na-salt ex.yeast, was from Koch Light Laboratories, Colnbrook, Bucks, UK. 2-Mercaptoethanol and lithium chloride were obtained from B.D.H.Chemicals Ltd., Poole.

2.2. *Preparation of rough endoplasmic reticulum and polysomes*

Male or female albino rats (170–230 g) of the Sprague-Dawley strain were fed ad libitum and killed by cervical dislocation. The livers were quickly perfused with 0.25 M STKM, finely chopped and washed several times with the same buffer. To each gram of liver, 2.25 volumes of 0.25 M STKM was added and the mixture homogenised in a Potter-Elvehjem apparatus at 0–4°C using a motor driven Teflon Pestle rotated at 2700 rev/min in a glass vessel with a clearance of 0.18 mm. 10–12 passes were used to achieve homogenisation. Post-mitochondrial supernatant (PMS) was prepared by centrifuging the homogenate in a fixed angle MSE 8 X 50 ml rotor at 10 000 rev/min (11 500 g_{av}) for 20 min at 4°C. Rough endoplasmic reticulum was prepared both by using fixed angle and swing-out rotors. For preparing membranes using an M.S.E. 8 X 50 ml fixed angle rotor, 14 ml PMS was layered over a discontinuous gradient consisting of 10 ml 2.0 M STKM below 15 ml 1.35 M STKM. The tubes were centrifuged for 4 h at 105 000 g_{av} at 2–4°C. Experiments demonstrating sex-specificity of polysomal binding were done on membranes prepared by fixed angle MSE 8 X 50 ml rotor by using a discontinuous sucrose gradient consisting of 10 ml of 1.7 M STKM under 15 ml of 1.35 M STKM. The separation was done at 70 000 g_{av} for 3 h at 4°C. The preparations using swing out rotors were separated using 70 000 g_{av} for 4.5 h in a more complex gradient system. Complete separation of the rough membranes from unbound ribosomes is not achieved nor is it required in the subsequent experiments. The rough membranes were harvested through 0.5 M STKM for 1 h in the same rotors and under the same conditions as used for separation. The pellets of membrane were frozen overnight at −20°C. The pellets were gently resuspended in 0.25 M STKM using a glass homogeniser to final membrane protein concentration ranging from 5–9 mg/ml for enzyme assays.

The polysomal pellets obtained from the discontinuous spin were very gently washed by decantation in the centrifuge tubes with small volumes of 0.25 M STKM and gently resuspended by hand in 0.25 M STKM using a glass homogeniser. The resuspended polysomes were frozen at −20°C in small aliquots.

2.3. *Determination of protein and RNA*

Protein concentrations were estimated by the method of Lowry et al. [11], using bovine serum albumin as a standard protein. RNA concentrations were assayed according to a slightly modified method of Munro and Fleck [12] after the original method of Schmidt and Thannhauser [13].

2.4. *Protein-disulphide isomerase assay*

The modified assay used for these studies was essentially designed according to details documented by Williams [14] but employing a double wavelength Perkin-Elmer (356) spectrophotometer. A brief workable methodology of the assay is described below. The primary incubation consisted of TKM at 25°C to a final volume of 1 ml, 25 µl of 2-mercaptoethanol (10 µl/7 ml double distilled water), 50 µl of randomly reoxidised ribonuclease (1 mg/ml in double distilled water). The reaction was started by the addition of the membranes (0.1–0.4 mg protein) and incubated at 25°C. Five or six samples were removed at timed intervals over a period of 35 min for the

determination of ribonuclease in the secondary incubation. The secondary incubation was started by adding 10 μl aliquots from the primary incubation to a cuvette containing 3 ml of 0.25 M STKM and 50 μl of 5 mg/ml RNA and was monitored in the spectrophotometer at 25°C. The measuring and reference wavelengths were 260 and 280 nm respectively. The 0.1 absorbance scale of the instrument was used. The initial slopes of the progress curves were measured and plotted against the time of the primary incubation. Examples of such plots are shown in fig.1. The slope of the linear plot of ribonuclease activity against the time minus the spontaneous rate of isomerisation in the absence of membranes was used as a measure of protein-disulphide isomerase activity. One arbitrary unit of ribonuclease is equivalent to 4.8×10^{-8} μmol

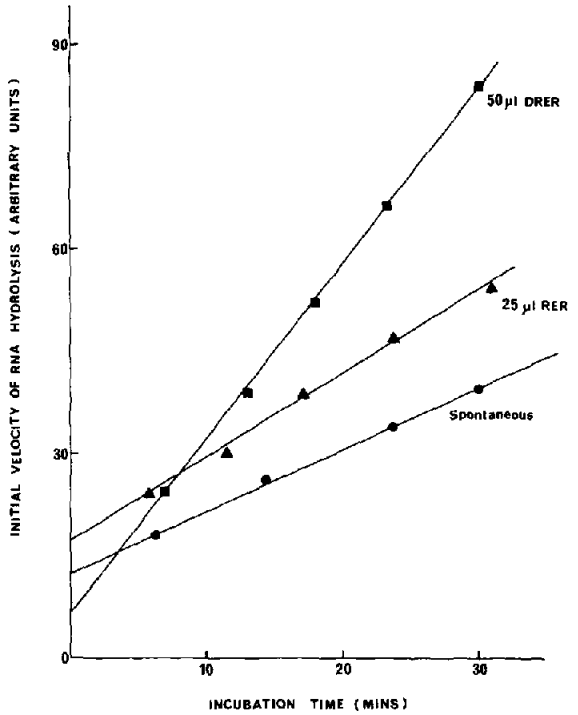

Fig.1. Initial velocity of RNA hydrolysis by refolded ribonuclease with and without (spontaneous) the membranes. Enzyme assays were carried out as described under Materials and methods. 25 μl rough membrane (7.0 mg protein per ml) (▲—▲). 100 μl of rough membrane were mixed with 100 μl of puromycin + KCl and incubated at 0°C for 1 h followed by 15 min at 25°C and 50 μl of the mixture was assayed (■-■-■). Spontaneous rate of refolding of inactive ribonuclease (●-●-●).

of the enzyme in the secondary incubation. One arbitrary unit of protein-disulphide isomerase produces 4.8×10^{-6} μmol of ribonuclease in the primary incubation in 30 min. The spontaneous rate was 25–30. The rate was proportional to membrane concentration over the range studied. As an example of the precision of the method, in an experiment involving a four-fold variation of membrane concentration the standard error of the parameter, isomerase activity/membrane concentration was 3% of its measured value. No change in the activity of protein-disulphide isomerase was observed on incubating the membranes alone for several hours at 0°C and up to 1 h at 25°C. None of the materials used at the concentrations employed in any of the experiments in this paper significantly affected the assay. Enzyme activities in figs.2–5 have been expressed in arbitrary units calculated for 1 mg membrane protein.

2.5. *Membrane degranulation and reconstitution*

Puromycin + KCl degranulation was by the method of Borgese et al. [15] with slight modifications as described in individual experiments. Lithium chloride degranulation was according to the method of Scott-Burden [5] with modifications as described in the individual experiments. All reconstitution experiments were conducted by mixing degranulated membranes with polysomes in such proportions that the ratio of the contents of RNA in the polysomes to the quantity of protein in degranulated membranes was 0.2 to 0.4. In all experiments controls were run to test the effects of carry over of the reagents on the spectrophotometric assay. No detectable effects were observed. RER and DRER refer to rough endoplasmic reticulum and degranulated rough membranes in this paper.

3. Results and discussion

All the experiments we report below were carried out with at least three different membrane preparations and are qualitatively completely reproducible. The two separate experiments illustrated in fig.2 show that incubation of rough membranes with puromycin + KCl under the recommended conditions [15] causes considerable increase in the activity of protein-disulphide isomerase. The variable activity of the different

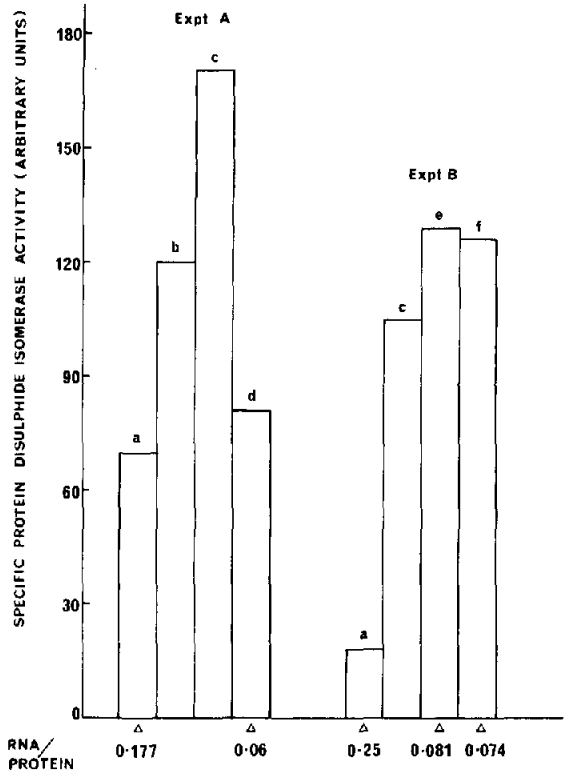

Fig.2. Degranulation of RER by puromycin + KCl. Two separate experiments (A and B) are illustrated employing different samples of rough membrane. (a) RER, no treatment; (b) RER, treated with puromycin + KCl for 1 h at 0°C; (c) RER, treated as in (b) and incubated for further 15 min at 25°C; (d) DRER, obtained after two washes with high salt buffer followed by one wash using 0.25 M STKM; (e) DRER, obtained after one wash with high salt buffer; (f) DRER, obtained as in (e) followed by 1 wash with 0.25 M STKM.

rough membrane preparations is mainly due to the heterogeneity of the membrane preparations which contain variable amounts of contaminating hybrid smooth/rough vesicles. These can be removed by a more complex separation protocol but their presence in no way affects the conclusions drawn from these experiments. The recommended method for preparing the membranes degranulated by puromycin + KCl involves two subsequent centrifugations through buffers containing 0.5 M KCl [15]. As shown in fig.2A this is highly damaging treatment which destroys the membrane-bound protein disulphide isomerase activity. The damage appears to occur in the centrifuge in the presence of high salt and membranes prepared by this method are clearly not suitable for reconstructing the protein synthetic apparatus of rough membranes in a native form. A single high salt wash, which can be followed by washing with 0.25 M STKM, seems to cause very little loss of enzyme (fig.2B) and produces a membrane which is considerably, but not completely, degranulated.

It was of interest to test whether the KCl treatment in the absence of puromycin caused any exposure of protein-disulphide isomerase activity. Fig.3B shows that an increase is observed, in agreement with similar experiments of Adelman et al. [1] using a direct methodology. Fig.3 also shows that membranes degranulated by puromycin + KCl rebind polysomes

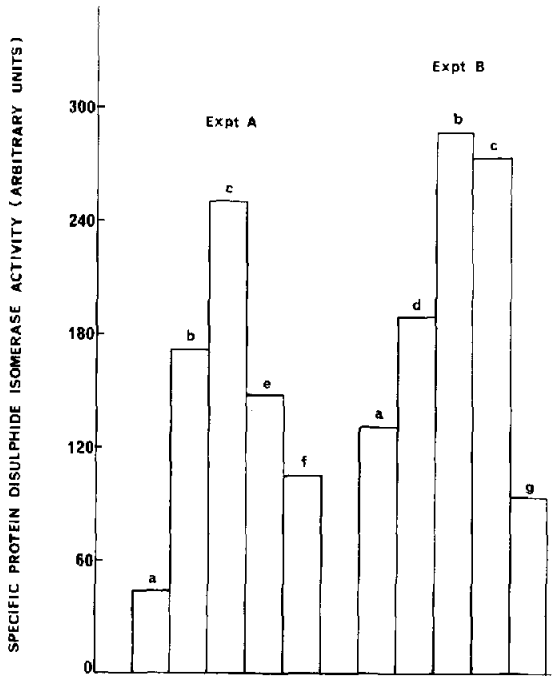

Fig.3. Rebinding of polysomes at 0°C and 25°C to membranes degranulated by puromycin + KCl. The two experiments illustrated employed different preparations of RER. (a) RER, no treatment; (b) RER, treated with puromycin + KCl for 1 h at 0°C and 15 min at 25°C; (c) DRER, separated after one wash with high salt buffer and one wash with 0.25 M STKM; (d) RER, treated with 0.5 M KCl for 1 h at 0°C followed by 15 min at 25°C; (e) and (f) DRER + polysomes (RNA/protein ratio of mixture about 0.2) incubated at 0°C for 30 and 90 min respectively; (g) DRER + polysomes (RNA/protein ratio of mixture about 0.2) incubated for 1 h at 25°C.

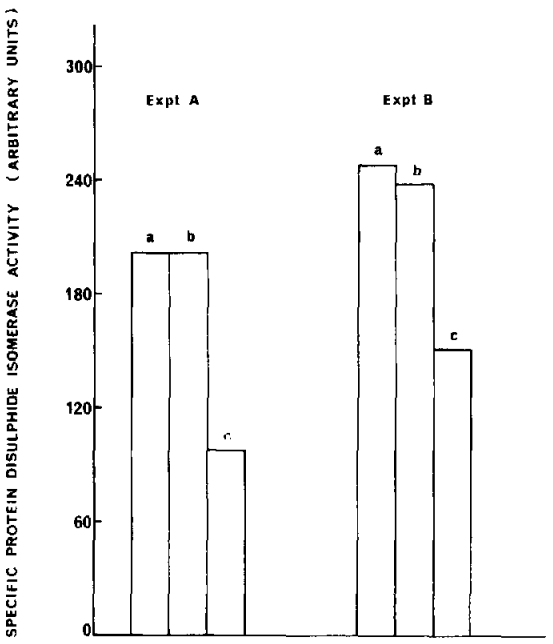

Fig.4. Sex-specific binding of polysomes to membranes degranulated with puromycin + KCl. The degranulated membranes were prepared as described in legend to fig.2 (Expt.B). Membranes were incubated with polysomes for 1 h at 25°C (RNA/protein ratios in final mixtures was 0.35–0.39). Corrections were made for the enzyme activity accompanying polysomes. The same polysomes were used in the two experiments. (a) DRER, no polysomes; (b) DRER + polysomes of opposite sex; (c) DRER + polysomes of the same sex. Experiments A and B were done using membranes from female and male rats respectively.

of the same sex. In fig.4 we demonstrate that this rebinding is completely sex-specific for membranes degranulated by the procedure stated, which retains the membrane bound protein-disulphide isomerase activity. No such sex-specificity was observed by a direct methodology (H. M. Dani, unpublished information) using membranes degranulated by the procedure of Borgese et al. [15] which causes loss of membrane-bound protein-disulphide isomerase. This is a further example of the failure of the direct methodology to measure biologically relevant processes.

The results of some experiments to investigate the effects of LiCl on rough membranes are shown in fig.5. It can be seen that the standard procedure recommended for degranulation by LiCl [5] destroys most of the membrane bound protein-disulphide isomerase (fig.5C). The binding experiments reported by Scott-Burden et al. [16,17] employed membranes degranulated by this procedure and the biological relevance of the conclusions drawn can be seriously questioned. A further reservation concerning these experiments is the use of deoxycholate to prepare the polysomes since detergent can affect their binding properties [18]. The data in fig.5 show that it is possible to degranulate membranes with LiCl with retention of protein-disulphide isomerase activity.

It is clear from the results reported here that the measurement of the activity of protein-disulphide isomerase can provide information on reticular

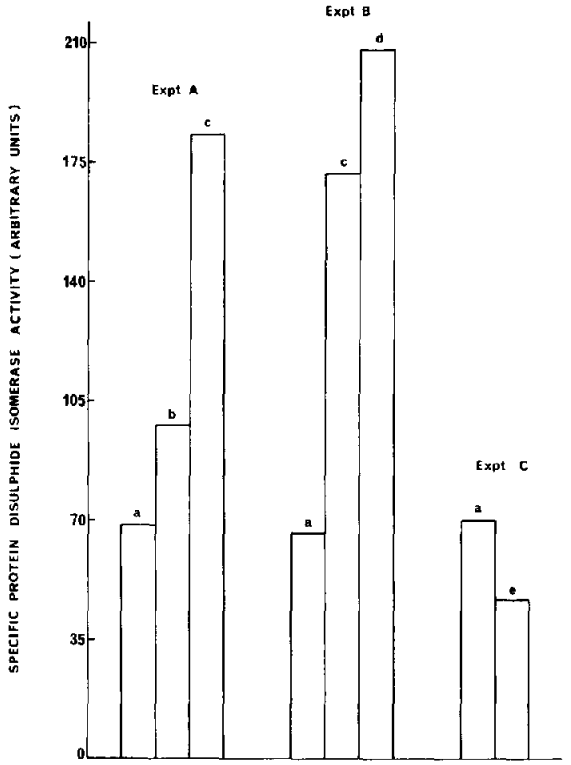

Fig.5. Degranulation of RER with lithium chloride. Equal volumes of RER suspended in 0.25 M STKM were mixed with LiCl solutions to give the final concentration of LiCl stated below. Mixtures were incubated at 0°C for the time stated and assayed for protein-disulphide isomerase activity. (a) RER, no LiCl; (b) RER + 0.5 M LiCl for 45 min; (c) RER + 1.0 M LiCl for 45 min; (d) RER + 1.0 M LiCl for 90 min; (e) DRER, obtained by treating RER with 2.0 M LiCl for 16 h at 4–5°C as recommended by Scott-Burden et al. [5].

membranes which cannot be obtained by any other methodology. The modified assay described can be used for quantitative studies of polysome binding to a particular set of membrane sites and as a sensitive probe for damage to degranulated and smooth membranes.

Acknowledgements

We wish to thank Ms Rosemary Clark for technical assistance. We are grateful to the following grant-giving bodies: The Medical Research Council and Cancer Research Campaign for grants in support of this work; The Jawaharlal Nehru Memorial Trust, London, for financial support to H.M.D.

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