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THE AFFINITY OF RAT LIVER RIBOSOME SUBUNITS FOR DEGRANULATED ROUGH MICROSOMAL MEMBRANES*

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SUMMARY

Potassium and magnesium ion concentrations affected the extent but not the specificity of binding in vitro of 60-S and 40-S ribosome subunits to degranulated rough microsomal membranes from rat liver. Scatchard plots revealed that under ionic conditions most likely to resemble those in vivo, the affinity constants for binding 60-S subunits were approximately four-times greater than those characterizing 40-S subunit binding. Further, the extent to which subunits bound at saturation was close to the level of ribosomes present in intact membranes.

INTRODUCTION

Mammalian liver rough microsomal membranes will, if pretreated with puromycin and high concentrations of KCl to remove attached ribosomes, bind added ribosomes or ribosome subunits in vitro [1–10]. The in vitro binding process is characterized by its sensitivity to inhibition by high concentrations of monovalent cations [3, 7, 9, 11–13] and its facilitation by the inclusion of Mg^{2+} in the medium [3, 5].

Electron micrographs of polysome-membrane complexes within the cell show that the 60-S ribosome subunit (but not the 40-S subunit) is always in close proximity to the membrane [14]. It seems likely that the primary locus of attachment to the membrane lies on the 60-S subunit and evidence to support this idea has come from in vitro experiments which have shown that under certain conditions 60-S subunits [5, 7, 9, 15] are bound to stripped rough membranes more readily than are 40-S subunits. Further support comes from the fact that binding of the 40-S subunit is to some extent contingent upon prior binding of 60-S subunits [7, 10] and that the rate of exchange between free subunits and membrane-bound ribosomes is greater for 40-S than for 60-S subunits [16].

The extent of the contribution of the 60-S subunit to polysome-membrane

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interaction in vitro is, nevertheless, still unknown. Other factors, such as nascent polypeptide chains, may also play a part. As a contribution to the question, we report here the determination of association constants and extents of binding of both 40-S and 60-S ribosome subunits to stripped rough membranes in vitro.

MATERIALS AND METHODS

Unless otherwise specified, all solutions contained 1 mM dithiothreitol and were adjusted to pH 7.6 at 20 °C. Tritium-labeled rough microsomal membranes were isolated from the livers of adult male Wistar rats (Oregon State University, closed colony) sacrificed 18 h after receipt of an intraperitoneal injection of [^{3}H]leucine (0.25 mCi, 40 Ci/mol^{-1}). Livers were homogenized in 0.35 M sucrose-TKM (50 : 25 : 1)* and rough membranes were isolated by isopycnic centrifugation in discontinuous sucrose gradients as described previously [17]. Ribosomes were stripped from the membrane by incubation for 1 h at 37 °C in a solution containing soluble supernatant in 0.25 M sucrose-HKM (25 : 500 : 1)** and puromycin (1 mM). The stripped membranes were isolated by centrifugal flotation in buffered sucrose and freed from contaminating material by sedimentation through 0.8 M sucrose-HKM (25 : 30 : 1) under conditions equivalent to those used for the separation of membranes from unbound subunits in the binding assay described below.

^{32}P-labeled ribosome subunits were derived from polysomes, prepared by Falvey's procedure [18] from rats which had received up to 10 mCi [^{32}P]orthophosphate 18 h previously. Monosomes were prepared by allowing the polysomes to complete protein synthesis in the presence of a mixture of the 20 amino acids necessary (0.9 mM each) plus 1.36 mM ATP, 0.18 mM GTP, 18.2 mM phosphoenolpyruvate, 0.125 mg/ml pyruvate kinase, 0.25 M sucrose-HKM (50 : 94 : 4) and cell sap (33 % v/v) for 1 h at 37 °C. They were pelleted at 150 000 × g for 4 h, suspended in cell sap containing 0.25 M sucrose HKM (25 : 500 : 2) and incubated with 0.1 mM puromycin for 15 min at 37 °C to terminate any remaining nascent peptide chains. The resulting 60-S and 40-S subunits were separated on an isokinetic sucrose gradient (5–31 %) at 95 000 × g for 11 h, precipitated with 0.7 vol. ethanol and 8 mM magnesium acetate, dialysed against 0.25 M sucrose-HKM (25 : 30 : 1), centrifuged briefly to remove insoluble material and stored at −70 °C.

Binding assays were carried out by incubating known amounts of subunits and membranes together in 0.25 M sucrose-HKM containing the appropriate levels of KCl and $MgCl_2$. Membrane-bound subunits were removed by centrifugation and assayed for ^{3}H and ^{32}P. Complete details are given in the legend to Fig. 1.

Membrane RNA levels in intact untreated rough membranes were measured by a modified Schmidt-Taunhauser procedure [19] using $E^{1\,\%}_{1\,cm} = 313$. Subunit concentrations were determined from absorbance measurements at 260 nm using [20], $E^{1\,\%}_{1\,cm} = 135$ and molecular weights of $3 \cdot 10^6$ and $1.5 \cdot 10^6$ for the 60-S and 40-S subunits, respectively [21].

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* 0.35 M sucrose, 50 mM Tris, 25 mM KCl, 1 mM $MgCl_2$, pH 7.6 at 20 °C.

** 0.25 M sucrose, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonate, 500 mM KCl, 1 mM $MgCl_2$, pH 7.6 at 20 °C.

RESULTS

In order to determine the number of sites occupied by ribosomes in the intact untreated rough membranes, the RNA content was measured. It was 25.1 % w/w of total protein; a value which corresponds to $7.3 \cdot 10^{-8}$ mol ribosomes per g of membrane protein (assuming a ribosome molecular weight of 4.5×10^6 and correcting the total protein measurement for the ribosomal protein contribution, assumed to be 48 % weight). Any binding values determined in vitro should, in order to be physiologically significant, approach but not markedly exceed this value.

in vitro binding of 60-S subunits was determined at a number of potassium and magnesium concentrations as illustrated by the saturation curves in Fig. 1. In these experiments, membrane recoveries as determined from tritium measurements were uniformly good and ranged around 80 % of input.

The greatest amount of binding was observed at low potassium (30 mM) and high magnesium (5 mM) concentrations. At its greatest, it exceeded the levels at which ribosomes were present in the original unstripped membrane preparation. At

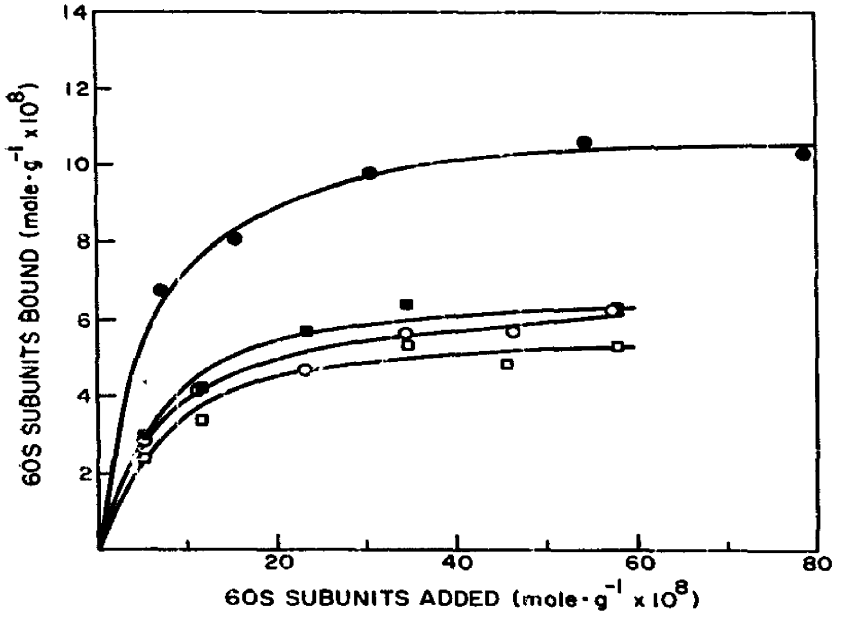

Fig. 1. Saturation curves for binding of 60-S subunits to stripped rough membranes. ^{32}P-labeled subunits were incubated in 0.6 ml with ^{3}H-labeled membranes (200 μg protein) for 30 min at 37 °C in a medium containing dialyzed cell sap from 67 mg rat liver and the appropriate salts and buffers. The sample was layered over a discontinuous buffered sucrose gradient containing 0.8 M (3.2 ml), 1.1 M (0.4 ml), 1.5 M (0.4 ml), and 1.8 M (0.4 ml) sucrose and centrifuged at 104 000 $\times g$ for 20 min. The membrane-bound subunits (which sedimented more rapidly than the excess of free subunits) were collected and the extent of binding was determined from the amounts of ^{3}H and ^{32}P present. Membrane recovery was estimated from the ^{3}H present. It averaged 80 %. Appropriate blanks containing no membrane were run. Incubation mixtures and the sucrose gradients contained 0.25 M sucrose, 25 mM HEPES and one of the following set of concentrations of KCl and $MgCl_2$ (mM): 30 : 5 (●—●); 30 : 1 (○—○); 100 : 5 (■—■); or 100 : 1 (□—□).

more physiological salt concentrations, that is, at high potassium (100 mM) and low magnesium (1 mM), binding levels were lower and approximated those found in intact rough membranes.

In order to compare the relative binding affinities of 60-S and 40-S subunits as a function of ionic conditions, saturation curves were measured at the extremes of potassium and magnesium ion concentration (30 : 5 mM and 100 : 1 mM). The data were plotted by the procedure of Scatchard [22] and are illustrated in Fig. 2. Clearly 60-S subunits have greater affinity than 40-S for the membrane at both salt extremes but the extent of binding of 40-S at 30 : 5 mM is much greater at saturation. There was no evidence for a biphasic component in the data indicative of multiple sites with different affinities.

Association constants and levels of subunits bound at saturation were calculated from Fig. 2 and from other similar experiments. The results are listed in Table I. At a K/Mg ratio of 30 : 5, both 60-S and 40-S subunits bound in excess of the number of sites exposed upon stripping. However at a K/Mg ratio of 100 : 1 the saturation levels were lower and were very close to those observed in an intact rough membrane preparation. At a K/Mg ratio of 100 : 1, equal numbers of 60-S and 40-S subunits were bound but the association constants were notably different. The K_{assoc} for 60-S subunits was at least 4-times greater than that for 40-S subunits.

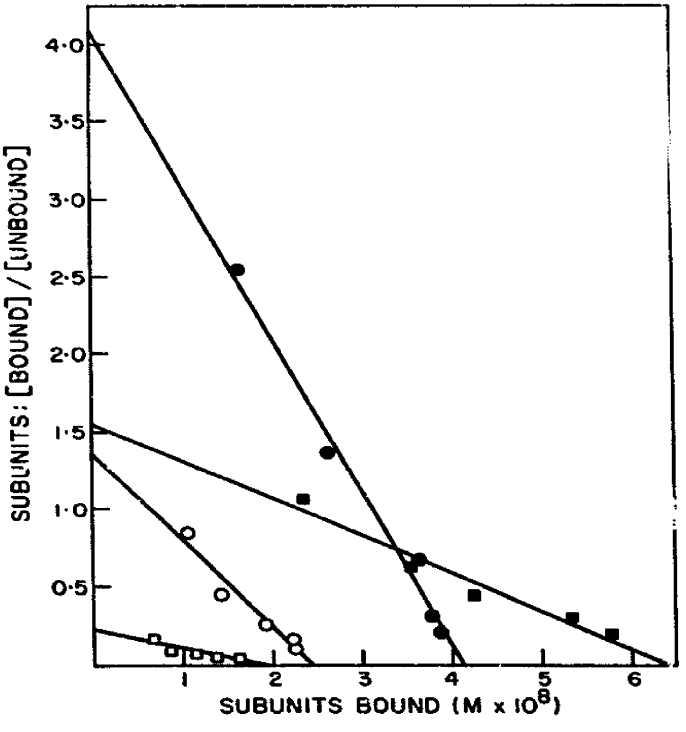

Fig. 2. Scatchard plots: effect of salt concentrations on binding of 60-S and 40-S subunits to stripped rough membranes. Assays were performed as in Fig. 1. Regression lines were fitted by a linear least-squares procedure. Samples contained the following subunit, KCl and $MgCl_2$ concentrations: 60-S at 30 : 5 (●—●); 60-S at 100 : 1 (○—○); 40-S at 30 : 5 (■—■); 40-S at 100 : 1 (□—□).

TABLE I

PARAMETERS CHARACTERISTIC OF BINDING OF SUBUNITS TO STRIPPED ROUGH MEMBRANES

Experiments were performed as described under Figs. 1 and 2. Binding parameters are averages (except where indicated*) derived from two or more experiments.

Subunit	Salt concentration		Subunits bound at saturation ($mol \cdot g^{-1}$ protein $\cdot 10^{5}$)	Association constant ($M^{-1} \cdot 10^{-9}$)
	KCl (mM)	$MgCl_2$ (mM)		
60-S	30	5	12.1	8.5
60-S	100	1	6.3	6.3
40-S	30	5	19.2*	2.4
40-S	100	1	5.8*	1.2

DISCUSSION

The binding assay described here capitalizes upon the large differences in sedimentation velocity between microsomal membranes and free ribosome subunits. The technique is similar to that described by Rolleston [7] and Khawaja and Raina [23] with the major difference being that membrane preparations were labeled with [^{3}H]leucine in order to permit calculation of recoveries.

The data indicate that the relative affinities of subunits for membranes are not particularly dependent upon the ionic environment but that the extent of binding at saturating levels of subunits is very dependent upon the medium. Thus, at either high potassium : low magnesium (100 : 1 mM) or low potassium : high magnesium (30 : 5 mM), 60-S subunits had greater affinity for stripped rough membrane than did 40-S subunits. These findings are certainly consistent with the model for in vivo assembly of membrane-bound polysomes in which attachment of the large subunit to the membrane is a first step followed by interaction with messenger RNA and 40-S subunits [10]. The difference in affinity constants is, however, not particularly large and probably may not be sufficient in itself to account for the exclusive 60-S attachment noted in vivo. Indeed, from the observed salt effects it seems likely that rather non-specific ionic forces drive the interaction and that other factors must contribute to specificity of binding in vivo.

The one parameter which was salt dependent was the extent of subunit binding. Under ionic conditions close to those supposed to exist in the cell, the membranes bound levels of subunits ($5.8–6.3 \cdot 10^{-8}$ $mol \cdot g^{-1}$) comparable to those existing on unstripped rough preparations ($7.3 \cdot 10^{-8}$ $mol \cdot g^{-1}$). However, at low potassium : high magnesium, binding was much greater, perhaps due to non-specific interaction or to binding of dimers.

This dependence of binding upon the magnesium concentration at low potassium ion levels may be contrasted with the findings of Borgese et al. [10]. These workers found no change in the subunit binding to stripped membranes upon lowering the magnesium concentration from 5 mM to 1 mM at any potassium concentration between 25 and 250 mM. Since the only major difference in experimental protocol appears to be the incubation temperature (37 °C used here vs. 0 °C by Borgese et al. [10]), the reasons underlying the difference are not obvious.

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