Phenylalanyl Transfer Ribonucleic Acid Synthetase Activity Associated with Rat Liver Ribosomes and Microsomes†

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ABSTRACT: Of the total phenylalanyl-tRNA synthetase (Phe-synthetase) activity in a rat liver homogenate, only about 50% is present in the postribosomal supernatant fraction. The microsomal pellet was found to have most of the remaining activity and considerable amounts of aminoacyl-tRNA synthetases for several other amino acids. Nonidet P-40 treatment of this pellet indicates that the Phe-synthetase is associated both with ribosomes and with the membranous components of the smooth and rough endoplasmic reticulum. This bound enzyme appears to be considerably more resistant to thermal inactivation than the purified "soluble" enzyme. The Phe-synthetase activity can be released from ribosomes by treatment with 1 M NH_4Cl or 0.5% protamine sulfate and appears to be identical with the "soluble" enzyme we have previously purified. A poly(U)-directed poly(phenylalanine)-synthesizing system using deoxycholate-treated ribosomes is dependent upon added $tRNA^{Phe}$ for maximal activity. The isolated ribosomes, however, contain sufficient quantities of Phe-synthetase and transfer factors to synthesize poly(phenylalanine) without the addition of a "pH 5 fraction," or additional amounts of purified Phe-synthetase. All these findings suggest that Phe-synthetase may exist *in vivo* in a stable matrix with other components of the protein-synthesizing system.

We previously described the purification and initial characterization of the Phe-synthetase[1] from rat liver (Lanks *et al.*, 1971). During the course of these studies we found that under the conditions of our isolation procedure a considerable portion of the total cellular content of the Phe-synthetase remains bound to microsomes and ribosomes. The presence of aminoacyl-tRNA synthetases in high molecular weight aggregates is becoming fairly well established.

An aggregate from rat liver of several aminoacyl-tRNA synthetases with a sedimentation coefficient of 28 S has been purified (Vennegoor and Bloemendal, 1972). Bandyopadhyay and Deutscher (1971) have isolated a large lipid-containing complex from rat liver cells. They report that it contains 18 aminoacyl-tRNA synthetase activities as well as tRNA and has a molecular weight of at least 10^6. Other groups (Roberts and Coleman, 1972; Irvin and Hardesty, 1972) have reported the presence of large amounts of Phe-synthetase and activities for a number of other amino acids present on partially purified mammalian ribosomes.

Here, we will examine the functional role of the ribosome-bound fraction of the rat liver Phe-synthetase and describe some properties of the complex between this enzyme and the endoplasmic reticulum. We feel that the further study of such associations will lead to greater appreciation of the mechanisms by which protein synthesis is regulated in higher organisms and to a general understanding of the structure of higher order molecular complexes which exist *in vivo*.

Materials

All centrifugations were performed in a Beckman L2-65B ultracentrifuge, and all absorbance readings were measured using a Zeiss PMQ spectrophotometer. Sodium deoxycholate was purchased from Sigma. Protamine sulfate and Casamino acids were from Difco, and Nonidet P-40 was a gift of the Shell Chemical Co. Crude yeast tRNA was purchased from Plenum Scientific Corp. [^{14}C]Amino acids were obtained from Schwarz BioResearch at the following specific activities (Ci/mol): phenylalanine (455), tyrosine (450), threonine (120), valine (260), and serine (103).

Methods

Preparation of Microsomes. These procedures are shown schematically in Figure 1. Fasted male Holtzman rats were used for the preparation of all cell fractions. These rats were decapitated, and their livers were removed and placed on ice. All subsequent operations were performed at 0–4°; 5 g of liver/22 ml of TKM–0.25 M sucrose buffer or PMM–10% glycerol buffer were minced with scissors and homogenized by 7 strokes in a Teflon-glass Potter-Elvehjem homogenizer operated at 2000 rpm. After removal of mitochondria, nuclei, and cell debris by centrifugation at 12,000*g* for 20 min, ribosomes were pelleted from the 12,000*g* supernatant fraction (S-12) by centrifugation at 230,000*g* for 1 hr (60,000 rpm in a Beckman Type 65 rotor). The middle two-thirds of the supernatant fraction from the 230,000*g* spin was designated the S-230, and the pellet the P-230. The surface of this pellet was rinsed with 1 ml of buffer. In most experiments, except a few ribosome preparations where only the ribosomes themselves were of interest, the P-230 was resuspended by gentle homog-

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[1] Abbreviations used are: Phe-synthetase, phenylalanyl-tRNA synthetase; TKM buffer, 0.01 M Tris-HCl (pH 7.8), 0.005 M magnesium acetate, 0.006 M mercaptoethanol, and 0.06 M KCl; PMM buffer, 0.01 M potassium phosphate (pH 7.5), 0.01 M magnesium acetate, and 0.005 M mercaptoethanol; NP-40, Nonidet P-40; ER, endoplasmic reticulum.

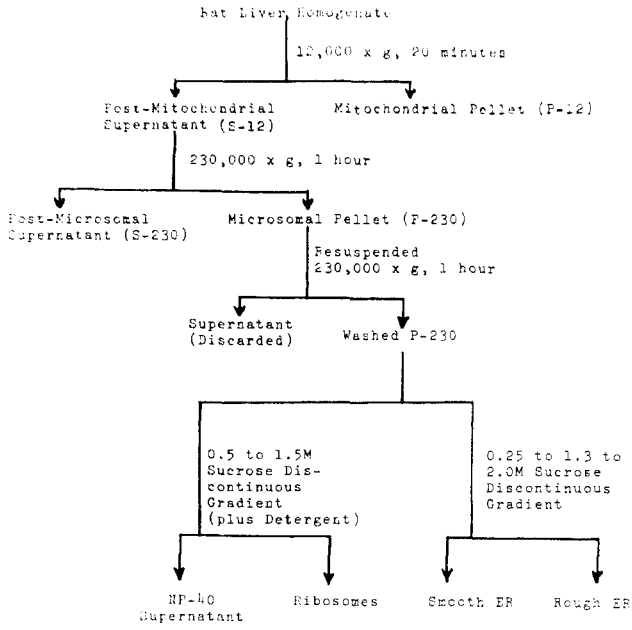

FIGURE 1: Preparation procedure for microsomes and ribosomes.

enization in the original volume of the S-12 using either PMM–10% glycerol buffer or TKM–0.25 M sucrose buffer and recentrifuged at 230,000*g* for 1 hr. The pellet was then resuspended in a small volume of the same buffer. (This washing procedure did not release a significant amount of Phe-synthetase from the P-230 fractions.)

Preparation of Smooth and Rough Endoplasmic Reticulum. The microsomes were further fractionated by equilibrium density centrifugation into smooth and rough endoplasmic reticulum by a modification of the methods of Dallner *et al.* (1966) and P. Siekevitz (personal communication). A discontinuous sucrose gradient was prepared in TKM buffer containing 15 mM CsCl as follows: 1 ml of 2.0 M sucrose in buffer was overlayered with 1.3 M sucrose in buffer. Then a 4-ml sample consisting of either the S-12 fraction, the recentrifuged P-230 fraction, or the supernatant fraction obtained from the gradient used to prepare ribosomes with NP-40 (the "NP-40 supernatant" as described below) was made 15 mM in CsCl and layered on top of the gradient. The gradients were centrifuged for 2 hr at 60,000 rpm in a Beckman Type 65 rotor at 0° and the successive layers were removed from the top with a pipet. The material above the 1.3 M region was clear and contained whatever free enzyme was present in the original sample as well as some material of very low density. A fraction, operationally called "smooth ER," was a densely opalescent layer which banded at the interface between the 0.25 and 1.3 M sucrose layers, corresponding to a density between 1.034 and 1.174 g per cm^3. "1.3 M" was the designation given to the 1.3 M sucrose layer itself, which was faintly opalescent and corresponded to a density of 1.174 g/cm^3. This layer appeared to contain ER of an intermediate density, as well as some ribosomes and rough ER which had not completely equilibrated. "Rough ER" corresponded to a densely opalescent layer at the interface between the 1.3 and 2.0 M sucrose layers, corresponding to a density between 1.174 and 1.264 g per cm^3. There was also a pellet of free ribosomes which had sedimented through the 2.0 M sucrose layer and, therefore, had a density of greater than 1.264 g/cm^3. Each of the sucrose layers except the ribosomes was then diluted to 10 ml with TKM buffer and centrifuged for 1 hr at 49,000 rpm in a Beckman Type 50 rotor. This step was included to separate enzyme activity which was firmly bound to a particulate cell component from either contaminating or loosely bound soluble enzyme. It was an especially important step when the starting material was the S-12 fraction or the P-230 treated with NP-40 since these fractions contained considerable amounts of soluble protein.

For the determination of RNA, protein, and phospholipid content, the fractions were differentially extracted according to the method of Schneider (1945). The RNA content was determined by the orcinol reaction (Volkin and Cohn, 1954), and the protein content according to the method of Lowry *et al.* (1951). The method of Bartlett (1959) was employed to determine the amount of phosphorus and subsequently converted to the phospholipid concentration (Lee *et al.*, 1969).

Preparation of Ribosomes. Total cellular ribosomes were obtained by the method of Wettstein *et al.* (1963), using the P-230 (microsomal pellet) and 1% sodium deoxycholate (see Figure 1). In later experiments 2% NP-40 was used instead of sodium deoxycholate since the NP-40 preserved the activity of the Phe-synthetase more effectively. Ribosomes obtained in this way were resuspended in TKM–0.25 M sucrose buffer by trituration and undispersed material removed by centrifugation at 12,000*g* for 10 min. For the thermal stability experiments, the ribosomes were resuspended in PMM–10% glycerol buffer.

When ribosomes were isolated from smooth and rough endoplasmic reticulum, the fractions (prepared as described above) were diluted with TKM buffer, NP-40 was added to 2%, and the samples (9 ml) were layered on 3 ml of 1.5 M sucrose in 0.05 M Tris-HCl (pH 7.8), 0.005 M $MgCl_2$, 0.025 M KCl, and 0.006 M mercaptoethanol (Wettstein *et al.*, 1963). After centrifugation at 230,000*g* for 2.6 hr, the ribosomal pellets were rinsed and resuspended in TKM–0.25 M sucrose buffer.

Isolation of Ribosomal-Bound Phe-synthetase. Ribosomal-bound Phe-synthetase was removed from ribosomes by incubation of the ribosomes at 0° for 30 min with an equal volume of either 1% protamine sulfate or 2 M NH_4Cl in 0.02 M Tris-HCl (pH 7.4), 0.02 M $MgCl_2$, and 0.01 M mercaptoethanol. The ribosomes were removed by centrifugation for 2 hr at 60,000 rpm in an SW65 rotor, and the supernatant fraction was assayed for Phe-synthetase activity as described below. The Phe-synthetase released from ribosomes was dialyzed against 0.01 M potassium phosphate (pH 7.0), 0.005 M magnesium acetate, 0.005 M merceptoethanol, and 10% glycerol, and applied to a 2.3 × 10 cm hydroxylapatite column equilibrated with the dialysis buffer and maintained at −5°. The column was eluted with a 200-ml linear phosphate gradient from 0.01 to 0.5 M potassium phosphate in the column buffer.

Rebinding of Phe-synthetase to Salt-Washed Ribosomes. For those experiments in which the binding of the synthetase to salt-washed ribosomes was measured, the ribosomes prepared in the usual manner with sodium deoxycholate and resuspended in TKM buffer were made 1 M in KCl and stirred for 30 min at 0°. The resulting suspension was centrifuged at 60,000 rpm in a Beckman Type 65 rotor for 1 hr at 0°. The pellet of salt-washed ribosomes was resuspended in TKM buffer. The binding experiments were performed by incubating 1–2 A_{260} units of salt-washed ribosomes for 30 min at 0° with 0.002 mg of a partially purified preparation of Phe-synthetase in 4.0 ml of PMM–10% glycerol buffer. The mixture was then centrifuged for 2 hr in an SW65 rotor at 56,000 rpm. The supernatant fraction and the pellet, resuspended in a small amount of PMM–10% glycerol buffer, were assayed for Phe-synthetase activity as described below.

Poly(phenylalanine) Synthesis by DOC-Treated Ribosomes. Protein synthesis (Nirenberg and Mattaei, 1961), using ribosomes prepared as described above, was carried out in a 0.4-ml system. Each reaction contained 0.625 mM ATP, 0.125 mM GTP, 3.1 mM phosphoenolpyruvate, 10 mM magnesium acetate, 0.03 mg of pyruvate kinase, 0.5 μCi of [^{14}C]phenylalanine, and the remaining 19 [^{12}C]amino acids each at a concentration of 30 mM. When present, poly(U) was at a concentration of 250 μg/ml, "pH 5 fraction" (Hoagland *et al.*, 1958) from rat liver at 3.75 A_{260}/ml, ribosomes at 12.5 A_{260}/ml, $tRNA^{Phe}$ (purified by benzoylated and silicylated benzoylated DEAE-cellulose, Wimmer *et al.*, 1968) at 2.5 μg/ml, and Phe-synthetase purified by hydroxylapatite chromatography (Tscherne *et al.*, 1973) at 15 μg/ml. Incubation was for 30 min at 37° followed by precipitation with 2.0 ml of 7% trichloroacetic acid containing 3% Casamino acids. Any aminoacyl-tRNA was hydrolyzed by digestion at 90° for 30 min in trichloroacetic acid, and the precipitates were collected and washed three times on Whatman No. 3MM filters with ice-cold 5% trichloroacetic acid containing 1% Casamino acids, and once with 70% ethanol. The filters were dried under an infrared spot lamp, and radioactivity was measured in a toluene-based scintillation fluid using the Nuclear-Chicago Mark II scintillation spectrometer.

Sucrose Gradient Velocity Sedimentation. Continuous sucrose gradient (5–20% in TKM buffer) analysis was performed using a Beckman SW25.1 rotor for 2 hr at 25,000 rpm. Gradients were collected from the top using the Buchler Autodensiflow apparatus. The pellet of each gradient was suspended in 1.5 ml of TKM–20% sucrose buffer and is plotted as "pellet" in Figure 2.

Assay of Aminoacyl-tRNA Synthetase Activity. The standard assay mixture contained: 0.1 M Tris-HCl (pH 7.5), 0.01 M magnesium acetate, 0.002 M ATP, 0.1 μCi of the appropriate [^{14}C]amino acid, 0.2 mg of unfractionated yeast tRNA (which contains 2 μmol of $tRNA^{Phe}$ as determined by complete acylation using purified rat liver Phe-synthetase), and 5–30 μl of the cell fraction to be assayed for enzyme activity. The samples (100 μl) were incubated at 37° for 10 min. If the samples were to be filtered on Millipore filters, the reaction was stopped by the addition of 1 ml of ice-cold 5% trichloroacetic acid containing 1% Casamino acids, and the precipitate was washed and counted as described previously (Lanks *et al.*, 1971). Alternatively, an 80-μl aliquot of the reaction mixture was removed and precipitated on Whatman No. 3MM paper filters, and treated as described previously (Nishimura and Weinstein, 1969).

Thermal Stability of the Phe-synthetase Present in the Subcellular Fractions. To determine the thermal stability of the Phe-synthetase present in various subcellular fractions, the fraction was incubated at 37° in 0.01 M Tris-HCl (pH 7.5) in the presence of 2.5% glycerol. At the indicated times, an aliquot was withdrawn and added to 80 μl of the standard mixture, incubated for 10 min at 37°, and precipitated as described above.

Results

Distribution of Aminoacyl-tRNA Synthetases in Postmicrosomal Supernatant and Microsomal Pellet. The distribution of several aminoacyl-tRNA synthetases between the S-230 and the P-230 is shown in Table I. (The preparation of these fractions is summarized in Figure 1.) The data are expressed as the amount of activity in the fraction compared with that present in the S-12. While assay conditions used may not have been optimal for some of the synthetases, the ratio of soluble to particulate associated activity has been assumed to be a valid measure of enzyme distribution. The low-speed centrifugation pellet, the P-12, contained less than 5% of the total Phe-synthetase activity present in the crude cellular homogenate, indicating that little or no Phe-synthetase is associated with either the nuclei or mitochondria.

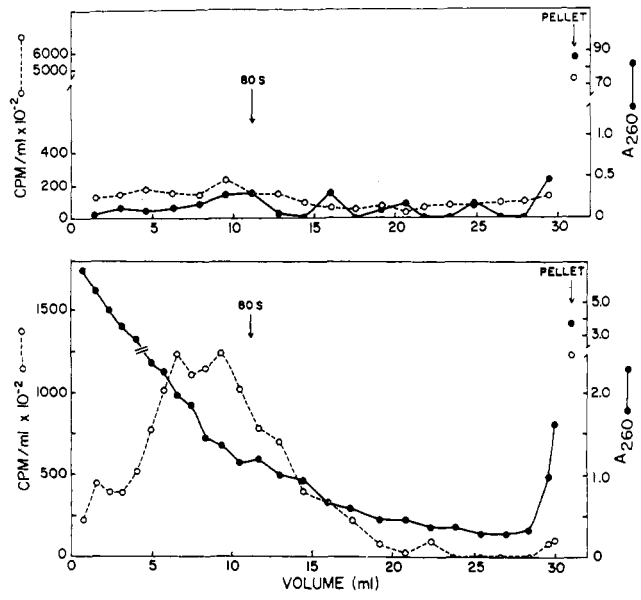

FIGURE 2: Sucrose gradient analysis of the phenylalanyl-tRNA synthetase activity present in the P-230 before and after treatment with NP-40. (Upper) A washed P-230 (see Figure 1) was resuspended to its original volume of S-12 in TKM buffer. Two milliliters of this material was layered on a linear 5–20% sucrose gradient and centrifuged in an SW25.1 rotor at 25,000 rpm for 2 hr at 0°. (Lower) P-230 prepared as above was exposed to 2% NP-40. Two milliliters of this material was layered and centrifuged as above. In each case 1.5-ml fractions were collected. The absorbance at 260 nm of each fraction was measured, and a 10-μl aliquot of each was assayed for 10 min in the standard assay system for Phe-synthetase activity.

It can be seen from Table I that, while recovery of the activity is not complete, under these conditions of preparation the microsomal pellet (P-230) contains a significant portion of the total cellular aminoacyl-tRNA synthetase activity for phenylalanine and valine. In these experiments resuspension and repelleting of the P-230 resulted in no loss of the associated enzyme activities, indicating that these enzymes are firmly bound to particulate elements. On the other hand,

TABLE I: Distribution of Aminoacyl-tRNA Synthetases in S-230 and P-230 Fractions of Rat Liver.

Synthetase	Activity (% of Homogenate)	
	S-230[a]	P-230[a]
Phenylalanine	49.3	29.7
Valine	50.0	21.8
Tyrosine	99.0	5.3
Threonine	87.4	3.8
Serine	97.0	3.6

[a] These values represent the number of units present in the indicated fraction relative to the number of units present in an equivalent amount of the S-12 × 100.

TABLE II: Distribution of Phenylalanyl-tRNA Synthetase Activity in Rat Liver Microsomes.

Fraction	S-12		P-230		P-230 + NP-40	
	Particulate[a]	Soluble[b]	Particulate[a]	Soluble[b]	Particulate[a]	Soluble[b]
Smooth ER	9.1[c] (15.3%)[d]	34.9[c] (58.7%)[d]	4.9[c] (44.6%)[d]	2.2[c] (20.0%)[d]	3.3[c] (23.2%)[d]	4.9[c] (34.5%)[d]
1.3 M region	1.7 (2.9%)	5.7 (9.6%)	2.0 (18.2%)	0	0	4.0 (28.2%)
Rough ER	5.5 (9.2%)	1.2 (2.0%)	1.7 (15.4%)	0	0.9 (6.3%)	0
Free ribosomes	1.4 (2.4%)		0.2 (1.8%)		1.1 (7.8%)	

[a] Phe-synthetase activity which is recovered in the pellet after centrifugation for 1 hr at 165,000*g*. [b] Phe-synthetase activity which is recovered in the supernatant after centrifugation for 1 hr at 165,000*g*. [c] cpm $\times 10^5$/fraction. [d] Figures in parentheses represent the per cent of total activity recovered from all fractions obtained from either the S-12, P-230, or P-230 + NP-40.

nearly 100% of the activity for tyrosine, threonine, and serine is found in the supernatant fraction (S-230).

Characterization of Phe-synthetase in Microsomes. To determine whether the Phe-synthetase activity present in the P-230 was bound to membranes, ribosomes, or both, a P-230 was fractionated on a linear 5–20% sucrose gradient in the presence and absence of NP-40. The enzyme activity and absorbance profiles are shown in Figure 2. The large amount of absorbance at the top of the gradient (Figure 2) is due to the fact that NP-40 absorbs strongly at 260 nm.

In the absence of NP-40 (Figure 2) most of the enzyme activity was pelleted, and no definite peaks were found in other regions of the gradient. This further demonstrated the firm association of enzyme with extremely high molecular weight material. When, however, the P-230 was treated with NP-40, or with sodium deoxycholate (data not shown), only a small fraction of the activity appeared in the pellet. A broad peak of activity was now observed in the top third of the gradient. While most of this material had too low a sedimentation coefficient to be associated with intact ribosomes, it was quite heterogeneous in nature. Probably this was due not only to a distribution of membrane fragment size, but also to dissociation with time of the enzyme from the membrane. This was suggested by the fact that prolonged treatment with NP-40 seemed to completely solubilize the enzyme. Smaller amounts of rapidly sedimenting material were found in the region of 80–120 S, and these could represent ribosomal-bound material (see below).

The above results, as well as studies with isolated ribosomes (see Table V), suggested that the bulk of the enzyme present in the P-230 was not associated with ribosomes and therefore was apparently bound to the membranous fraction of the microsomes. To further clarify this, the microsomes (P-230) were subfractionated into predominantly smooth and rough endoplasmic reticulum according to the methods of Dallner *et al.* (1966) and P. Siekevitz (personal communication). Modifications of this procedure, as well as the composition of the various fractions which were obtained, are given in Methods and Table III.

The distribution of the Phe-synthetase in the various fractions is shown in Table II. Since the S-12 contains the soluble enzyme as well as the particulate enzyme, the large amount of nonsedimenting activity in each of the fractions resulting from separation of this material probably represents contamination by the supernatant or soluble enzyme. It is, therefore, more informative to consider the particulate enzyme in each fraction. The results indicate that approximately twice as much particulate enzyme is present in the smooth ER than in the rough ER. A small but significant amount of enzyme is found in the free ribosome fraction. Since these ribosomes were obtained in the absence of detergent, this suggests that those ribosomes which are not attached to the ER of liver *in vivo* also contain bound Phe-synthetase.

In order to study the release of enzyme from the microsomes, it was necessary to avoid contamination with the enzyme present in the cell supernatant (soluble enzyme). Therefore, similar studies were performed starting with the P-230 (microsomal pellet) in the presence and absence of NP-40 (Table II). With the P-230 it was again apparent that, although there was a significant amount of particulate enzyme associated with the rough ER, there was two to three times more activity with the smooth ER. Some particulate activity was also observed at a density intermediate between the two (1.3 M fraction). Following treatment of the P-230 with NP-40, there was a loss of enzyme both from the rough ER fraction and the particulate portion of the smooth ER fraction. There was also a relative increase in the amount of enzyme present in the free ribosome fraction. This was probably due to an increase in the number of free ribosomes which were released from the membranes by NP-40. In addition, a large amount of nonsedimentable enzyme (referred to as soluble in Table II) now appeared in the smooth ER and 1.3 M fractions. This suggests that enzyme released from the membranes by NP-40 came from two sources: enzyme attached to membrane-bound ribosomes, and a separate fraction of enzyme associated with the membranes themselves.

The results obtained with both the S-12 and the P-230 (see Table II) suggested that the smooth ER fraction contained two to three times as much enzyme as the rough ER fraction. One trivial explanation could have been that what was presumed to be smooth ER actually contained more ribosomes than the fraction we called rough ER. To eliminate this possibility, the chemical composition of the fractions obtained from the S-12 was determined and is shown in Table III. It is apparent that although the smooth and rough ER fractions contain approximately the same amount of phospholipid, the rough ER contains almost twice as much RNA. As shown in the last column of Table III, three to four times as many ribosomes could be recovered from the rough ER as from the smooth ER following treatment with NP-40.

Taken together, the results given above indicate that the large amount of enzyme activity present in the smooth ER cannot be attributed to that bound to ribosomes. The activity can be at least partially solubilized with NP-40. This suggests that it is a distinct component associated with membranes.

As further evidence that Phe-synthetase is actually bound to the membranous components of the ER, the supernatant fraction, obtained by NP-40 treatment of the microsomes, was separated by the discontinuous sucrose gradient method

TABLE III: Composition of Cell Fractions.

Fraction	RNA (μg/g of Liver)	Phospholipid (μg/g of Liver)	Protein (μg/g of Liver)	Ribosomes (A_{260}/g of Liver)
Smooth ER	354 (26.8%)[a]	6500 (38.0%)[a]	14000 (84.1%)[a]	9.7 (13.0%)[a]
1.3 M region	46 (3.5%)	2200 (13.6%)	445 (2.7%)	8.3 (11.2%)
Rough ER	615 (46.2%)	7600 (45.1%)	1850 (11.1%)	35.4 (47.5%)
Free ribosomes	309 (23.3%)	560 (3.3%)	372 (2.2%)	21.1 (28.4%)

[a] Figures in parentheses represent the per cent of the total component present in S-12.

used above (Dallner *et al.*, 1966; P. Siekevitz, personal communication). This supernatant fraction should contain any Phe-synthetase released from ribosomes or membranes by NP-40, plus any synthetase which might remain bound to lower molecular weight fragments of membrane.

Table IV indicates that nearly all of the above enzyme activity banded at approximately the same density as the smooth ER (1.034–1.174 g/cm^3), suggesting that it remains bound to membranes. Some of the enzyme, however, was found in the 0.25 M sucrose layer of the gradient (density 1.034 g/cm^3). Separate studies had indicated that the soluble Phe-synthetase from the S-230 fraction of rat liver remains in the 0.25 M region. It appears, therefore, that following NP-40 treatment of the P-230 a portion of the bound enzyme is completely solubilized.

Ribosome-Bound Aminoacyl-tRNA Synthetases. In these studies ribosomes were obtained in two ways. When an S-12 was applied to the discontinuous gradient used above (Dallner *et al.*, 1966; P. Siekevitz, personal communication), those ribosomes which existed free in the cell were obtained in the pellet. Prior treatment of the S-12 with 2% NP-40 to solubilize the membranes yielded total cellular ribosomes. This procedure indicated that about 80% of the ribosomes in rat liver are membrane bound. This is in agreement with previously published data (Blobel and Potter, 1967), and therefore suggests that the isolation procedure was minimally traumatic. Moreover, ribosomes thus prepared were found to have a considerable amount of Phe-synthetase activity associated with them. The specific activity (units of Phe-synthetase/A_{260} units of ribosomes) of the ribosomes released from the membranes was almost the same as the specific activity of the small amount of ribosomes obtained in the absence of detergent. This eliminated the possibility that the binding of Phe-synthetase to ribosome was related to the presence of NP-40 during preparation.

Alternatively, larger quantities of ribosomes were prepared by the method of Wettstein *et al.* (1963) using NP-40 and sodium deoxycholate as described in the Methods section. These ribosomes were assayed for several aminoacyl-tRNA synthetases. Table V lists the proportion of synthetase activities for a number of different amino acids which were detected bound to ribosomes. It can be seen, by comparison with Table I, that those synthetases (phenylalanyl and valyl) which were present in large amount in the P-230 were found in appreciable levels bound to the ribosomes. Those which were only present as minor components in the P-230 (threonyl, tyrosyl, and seryl) were apparently bound mainly to the membranous fraction. This is supported by the observation that their activities appear in the supernatant of the discontinuous sucrose gradient used to prepare the ribosomes (see Table V, column labeled NP-40 supernatant). It should be pointed out that NP-40 enhanced the acylating activity of the enzymes about 20%, but the data in Table V have not been corrected for this. Hence, they may appear high.

Treatment of ribosomes with either 1.0 M NH_4Cl or 0.5% protamine sulfate released 60 and 40% of the bound Phe-synthetase, respectively. The proteins released by protamine sulfate treatment were chromatographed on an hydroxylapatite column under conditions identical to those used during the purification of the soluble synthetase (Tscherne *et al.*, 1973). Although the absorbance profile shown in Figure 3 is different from that of the soluble fraction containing Phe-synthetase (Tscherne *et al.*, 1973), the peak of enzyme activity in each case occurs at 0.35 M phosphate. Sucrose gradient analysis (data not shown) suggests that they both have a sedimentation coefficient of approximately 10 S. Taken together, these data indicate that the soluble enzyme and that bound to ribosomes are similar, if not identical.

An attempt was made to bind purified rat liver Phe-synthetase back to sodium deoxycholate treated and salt-washed

TABLE IV: Density of Membrane Bound Component of Phenylalanyl-tRNA Synthetase.

Fraction	Cpm/ml	% of Total Act.
A (p = 1.03)	598.0	19.0
B (p = 1.03–1.17)	1944.0	62.1
C (p = 1.17)	86.1	2.7
D (p = 1.17–1.26)	72.2	2.3
E (p > 1.26)	395.0	12.6

TABLE V: Distribution of Aminoacyl-tRNA Synthetases in the Microsomal Pellet.

	Activity (% of Homogenate)		
Amino Acid	DOC Ribosomes[a]	NP-40 Ribosomes[a]	NP-40 Supernatant[a]
Phenylalanine	3.4	9.2	19.0
Valine	0.5	3.1	27.4
Tyrosine	1.8	0.3	3.7
Threonine	0.3	0.1	6.1
Serine	0.0	0.1	6.8

[a] These values represent the enzyme activity present in the indicated fraction relative to enzyme activity present in an equivalent volume of S-12 × 100. DOC = sodium deoxycholate.

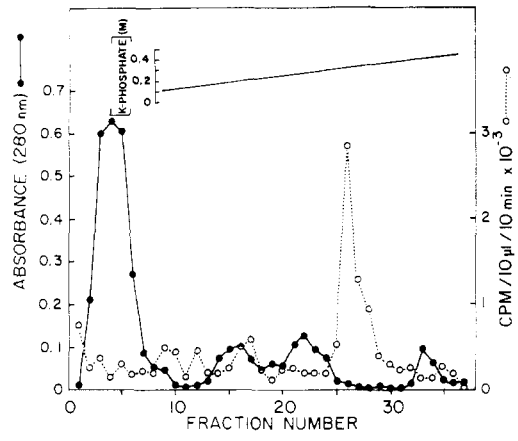

FIGURE 3: Hydroxylapatite chromatography of ribosome-bound phenylalanyl-tRNA synthetase. Ribosomes prepared by sodium deoxycholate were resuspended in TKM buffer and treated with protamine sulfate as described in Methods for the removal of Phe-synthetase from ribosomes. The released protein was layered on an hydroxylapatite column and eluted according to Methods. Fractions (5.0 ml) were collected and the absorbance at 280 nm was measured for each (●). Aliquots (10 µl) were assayed for 10 min in the standard assay system for Phe-synthetase activity (○).

ribosomes, prepared as described in Methods. The results indicated that the KCl-washed ribosomes could, in fact, rebind Phe-synthetase and the specific activity of the resulting particles was actually somewhat higher than before KCl treatment and was similar to that of ribosomes prepared with NP-40. It has not been possible to obtain isolated ribosomal subunits on which Phe-synthetase activity is preserved, and thus far the binding of purified synthetase to isolated subunits obtained from KCl-treated ribosomes has not yielded clear-cut results.

The ability of ribosomes prepared with sodium deoxycholate to carry out poly(phenylalanine) synthesis in an *in vitro* system is shown in Table VI. No attempt was made to obtain high molecular weight polysomes. However, the low level of endogenous activity in the system containing only

TABLE VI: Poly(phenylalanine) Synthesis by Sodium Deoxycholate Treated Rat Liver Ribosomes.

Addition to System	pmol of [^{14}C]Phe Incorp/30 min	% of Max. Incorp
None	0	0
Ribosomes (R)	8.1	16
R + pH 5	8.7	17
R + pH 5 + poly(U)	43.1	86
R + poly(U)	6.0	12
R + poly(U) + tRNA^{Phe}	47.0	94
R + poly(U) + Phe-synthetase	5.3	10
R + poly(U) + tRNA^{Phe} + Phe-synthetase	50.6	100
R + pH 5 + poly(U) + tRNA^{Phe}	45.0	90
R + pH 5 + poly(U) + Phe-synthetase	38.8	78
R + pH 5 + poly(U) + tRNA^{Phe} + Phe-synthetase	45.1	90

TABLE VII: Thermal Stability of Several Subcellular Fractions.

Fraction	$k_i \times 10^3$ (min^{-1})
Purified Enzyme	24.0
S-230	4.3
P-230	3.3
Ribosomes	1.2

ribosomes suggests that some fragments of native mRNA and at least small amounts of several tRNAs and synthetases are present on these ribosomes. The most noteworthy finding is that the synthesis of poly(phenylalanine) using poly(U) as a template is enhanced more by the addition of tRNA^{Phe} than by the addition of a "pH 5 fraction" (Hoagland *et al.*, 1958). Moreover, additional Phe-synthetase actually depresses poly(phenylalanine) synthesis.

Apparently, ribosomes contain sufficient Phe-synthetase and transfer factors to synthesize protein, and it is mainly tRNA and mRNA which is limiting in this system. The low but significant incorporation obtained with endogenous messenger without the addition of a "pH 5 fraction" (Hoagland *et al.*, 1958) suggests that at least small amounts of most of the other synthetases and tRNAs are present in the ribosome preparations even though they could not be detected by other means. Similar observations have been made by other investigators (Webster, 1957; Cohn, 1959; Ochoa and Weinstein, 1964). Early work showed that detergent-treated ribosomes were very active in the incorporation of amino acids into protein in the absence of a "pH 5 fraction" (Hoagland *et al.*, 1958). However, the limiting components were not determined, and aminoacyl-tRNA synthetase activity was not demonstrated in the preparations of mammalian ribosomes.

Thermal Stability of Phe-synthetase Present in Subcellular Fractions. The first-order thermal inactivation constants (k_i) for hydroxylapatite-purified Phe-synthetase (Tscherne *et al.*, 1973) and that present in the S-230, P-230, and ribosomal fractions are shown in Table VII. The enzyme in the subcellular fractions is considerably more stable to thermal inactivation than the purified synthetase. The enzyme bound to ribosomes is 20 times more so.

We have previously shown that the enzyme present in the cell supernatant, represented by the S-230 in the inactivation experiments, is probably all bound to tRNA (Lanks *et al.*, 1971). Since there was also evidence (Tscherne *et al.*, 1973) that tRNA protected against thermal inactivation, an attempt was made to determine the amount of tRNA present in the S-230, P-230, and ribosomes both by kinetic analyses and extraction procedures. Preliminary results indicated that the tRNA concentration in any of these partially purified fractions was not sufficient to give detectable protection against thermal inactivation. This was particularly true in the case of the very stable synthetase present in the P-230 and ribosomes. Thus, stabilization of the synthetase in subcellular fractions probably involves the effect of some substance other than tRNA.

Discussion

The aminoacyl-tRNA synthetases which we found bound to ribosomes differ somewhat from those which have been observed by other investigators. Irvin and Hardesty (1972) found considerably more ribosomal-bound Phe- and Val-synthetase in rabbit reticulocytes than was found in this study

on rat liver. Roberts and Coleman (1972) found approximately 80% of the Phe-synthetase in ascites tumor cells bound to ribosomes. This difference, however, may be due to the fact that in that system there are almost no membrane-bound ribosomes; therefore, a relatively greater proportion of Phe-synthetase is ribosome bound.

The possibility that the association of certain aminoacyl-tRNA synthetases with ribosomes is simply an artefact of the method of preparation cannot be rigorously eliminated by the data available at the present time. However, it seems reasonable to assume that the less traumatic the procedure the more likely the product will resemble its *in vivo* state. This is borne out by reports from other laboratories. Norton *et al.* (1965) found that with increasingly vigorous cell disruption increasing amounts of aminoacyl-tRNA synthetase activities were found in the supernatant fraction following high-speed centrifugation. Bandyopadhyay and Deutscher (1971) also found that their complex was quite fragile and was dissociated by prolonged homogenization. Presumably what they have observed existed as a larger complex in the cell. Depending on the severity of the isolation method, the ribosomes and microsomes retain more or less of their bound synthetase.

It also seems likely that the aminoacyl-tRNA synthetases are actually bound to ribosomes *in vivo* for the following reasons: (1) a significant, although somewhat variable, fraction of the total cellular Phe-synthetase is found associated with ribosomes whether these ribosomes are prepared with NP-40 or sodium deoxycholate and after they have been centrifuged through 1.5 or 2.0 M sucrose; (2) the specific activity (units of enzyme/A_{260}) of the ribosomes which exist free in the cell and those which are liberated by NP-40 are similar. This indicates that the association of synthetase is not an artefact induced during the NP-40 treatment.

It is likely, therefore, that *in vivo* at least some of the aminoacyl-tRNA synthetase exists in a complex with each other, membranes, and ribosomes. Henriksen and Smulson (1972) have isolated from HeLa cells complexes of aminoacyl-tRNA synthetases and elongation factors with sedimentation coefficients between 5 and 28 S. These supramolecular complexes in association with ribosomes may provide a compact organized structure on which the cell efficiently carries out protein synthesis. At the same time we must emphasize that the data which we and other laboratories have obtained with disrupted cells do not actually prove that these complexes actually exist *in vivo*.

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