PARTICULATE FORMS OF PHENYLALANYL-tRNA SYNTHETASE FROM EHRLICH ASCITES CELLS*

W. K. Roberts and William H. Coleman**

Department of Microbiology, University of Colorado Medical Center, Denver, Colorado 80220

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SUMMARY

Over 80% of the phenylalanyl-tRNA synthetase activity in Ehrlich ascites cell homogenates was found to be associated with the high speed particulate fraction. This enzyme activity occurred in two principle forms: activity bound to the ribosomes, and activity as part of a complex sedimenting at approximately 25S in a sucrose density gradient. The ribosome-associated enzyme was shown to be bound to the 60S ribosomal subunit. Exposure of the ribosomes to RNA resulted in removal of synthetase activity from the ribosomes and the concomitant appearance of activity in a complex sedimenting at 25S.

Aminoacyl-tRNA synthetases have generally been regarded as soluble components of protein synthesizing systems. Isolation of these enzymes commonly involves the pH 5 precipitation of a high-speed supernatant fraction (1). Recently, however, a number of reports have appeared which indicate that the synthetases from animal cells are part of high molecular weight complexes. Bandyopadhyay and Deutscher (2,3) have shown all the aminoacyl-tRNA synthetases and most of the tRNA from liver cells to be present in a non-ribosomal complex with a molecular weight greater than one million. Also, Irvin et al. (4) have reported many of the aminoacyl-tRNA synthetases in lysates of rabbit reticulocytes to be associated with a particulate fraction. However, in contrast to the reports of Bandyopadhyay and Deutscher, these workers found that the particulate synthetases were bound to the ribosomes.

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**Present address: Department of Biology, University of Hartford, West Hartford, Connecticut 06117.

In a previous study concerning the ribosomes from Ehrlich ascites cells, we observed that over 80% of the phenylalanyl-tRNA systhetase activity in cell homogenates sedimented with the ribosomal fraction (5). In the present communication, we report that in this particulate fraction the enzyme is associated with the 60S subunit of the ribosome and also exists as part of a complex which sediments at about 25S.

METHODS

Ribosome preparations. Ehrlich ascites cells were propagated and washed as previously described (6). Ribosomes were prepared according to the procedure of Kerr et al. (7) with the following exceptions. There was no deoxycholate treatment unless indicated in the text. After centrifugation of the low-speed supernatant fluid at 80,000g for 3 hr at 0°, the ribosome pellets were rinsed 3 times vigorously with 0.25 M sucrose in a Vortex mixer. This treatment removed a white precipitate from the top of the transparent ribosome pellets. The pellets were stored under 1.5 ml of 0.25 M sucrose for 16 hr at 4° and resuspended by agitation to give an average concentration of ribosomes of 150 $O.D._{260}$ units/ml.

Ribosomes were washed with 0.5 M NH_4Cl as previously described (5). Heavy sucrose pelleted ribosomes were prepared by layering 3 ml of ribosomes in 0.25 M sucrose on top of 7 ml of 2 M sucrose in TKMD buffer (0.05 M Tris·HCl, pH 7.4, 0.08 M KCl, 0.001 M $MgCl_2$, 0.001 M dithiothreitol) and centrifuging at 230,000g for 40 hr at 0°. Consecutive 1 ml samples were removed from the top of the tube for enzyme assay and the pellet was resuspended in 0.25 M sucrose.

Synthetase assay. Each assay tube contained 0.08 ml Medium A (1); 0.05 ml nucleotide triphosphate solution (12.5 mg ATP, 10.0 mg CTP, 15.0 mg $KHCO_3$ and 10.0 mg $MgCl_2$ in 1.0 ml Medium A); 0.005 ml 1 M Tris·HCl, pH 7.4; 0.01 ml tRNA in water (130 $O.D._{260}$ units/ml, Type I yeast tRNA, Sigma); 0.01 ml L-[^{14}C] phenylalanine (10μCi/ml, New England Nuclear, 2.5 mCi/mg); enzyme and sufficient water to bring the total volume to 0.2 ml (fractions from sucrose

density gradients were assayed simply by adding 0.05 ml aliquots to the assay tubes). The assay mixtures were incubated at 37° for 10 min, 0.1 ml non-radioactive phenylalanine (2 mg/ml) and 0.1 ml bovine serum albumin (5 mg/ml) added to each tube and the tubes placed on ice. Cold 10% trichloracetic acid (2 ml) was added to each tube and the samples filtered through Millipore cellulose acetate membranes (FGWP02500), previously washed with 5% trichloroacetic acid containing 0.1 mg/ml phenylalanine. The filters were washed with 2-5 ml portions of cold 5% trichloracetic acid, 2-5 ml portions of cold water, the filter membranes glued to planchets, dried and counted in a Nuclear Chicago windowless gas-flow counter.

RESULTS

Synthetase activity of ribosome preparations. Several methods of preparing and washing ribosomes were investigated to test their effects on the ribosome-associated synthetase activity (Table I). Our standard ribosome preparation

TABLE I

PHENYLALANYL-tRNA SYNTHETASE ACTIVITY

OF VARIOUS RIBOSOME PREPARATIONS

Ribosome preparation	Specific activity X 10^{-3}*
Pelleted and rinsed	40
Pelleted through 2M sucrose	34
Washed with deoxycholate**	45
80S peak from sucrose gradient	26
Washed once with NH_4Cl	6
Washed 3 times with NH_4Cl	1.5

*Defined as cpm [^{14}C] phenylalanyl-tRNA formed/O.D.$_{260}$ unit.

**Ribosome suspension made 0.5% in sodium deoxycholate and the ribosomes centrifuged and resuspended in 0.25M sucrose (7).

was found to be an excellent source of phenylalanyl-tRNA synthetase and contained over 80% of the synthetase activity present in the low speed supernatant fluid. Pelleting the ribosomes through 2 M sucrose solution or treating ribosomes with sodium deoxycholate had little effect on the specific enzyme activity of the ribosome preparations. This suggests that the synthetase activity is not associated with a membrane fraction in the pellet. The 80S ribosomal peak from a zonal centrifugation run retained most, but not all, of the original synthetase activity. Washing the ribosomes with a solution containing 0.5 M NH_4Cl removed approximately 85% of the enzyme activity from the ribosome fraction. A small but significant amount of synthetase activity remained associated with the ribosomes even after three NH_4Cl washes.

The radioactive product from the synthetase assays had the properties expected for [^{14}C] phenylalanyl-tRNA: it sedimented at about 4S in a sucrose gradient, was acid insoluble and radioactivity became acid soluble upon incubation under standard deacylating conditions (pH 9, 37°, 45 min).

Sedimentation properties of ribosome-associated synthetase activity. Ribosomes which had been pelleted through a pad of 2M sucrose were resuspended in 0.25M sucrose and centrifuged through a 20%-40% sucrose gradient. Fractions (1 ml) were collected and analyzed for optical density and synthetase activity (Figure 1A).

The optical density profile shows that the ribosomes in this preparation exist primarily as the 80S monomer, with lesser amounts of aggregates and 60S and 40S subunits also present. Synthetase activity parallels the 80S monomer fractions with a smaller peak of activity coinciding with the 60S ribosomal subunit region. A separate broad peak of activity, which is not associated with significant amounts of optical density, is found in the 20S-30S fractions.

Zonal centrifugation of original ribosome preparations which had not been pelleted through 2M sucrose gave patterns similar to those in Figure 1A. In these preparations, however, very little dissociation into 60S and 40S

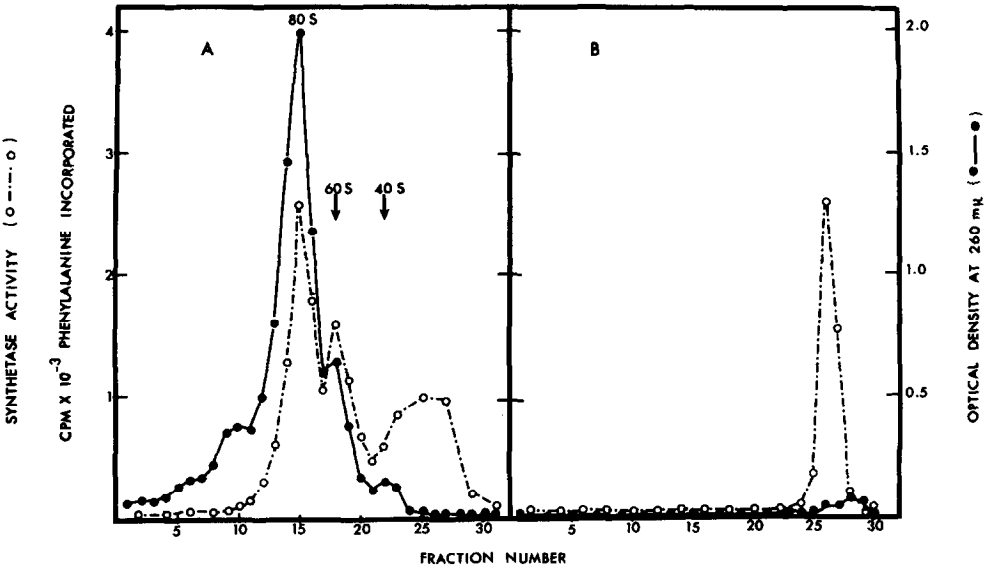

Figure 1. Zonal centrifugation of heavy sucrose pelleted ribosomes (A) and interface material (B). Both centrifugations were carried out by layering 1 ml of material on 31 ml of 20%-40% sucrose in TKMD buffer and centrifuging in a SW27 rotor at 21,000 rpm for 16 hr at 0°. Fractions were collected (1 ml) and 0.05 ml aliquots removed for synthetase assay. A: 12 O.D.$_{260}$ units of ribosomes pelleted through 2 M sucrose were diluted to 1.0 ml with 0.25 M sucrose and centrifuged; B: 0.2 ml of interface fraction was diluted to 1.0 ml with water and centrifuged.

subunits had taken place and the synthetase activity profiles showed only a large 80S peak with a smaller broad peak in the 20S-30S region. Recovery of synthetase activity from the gradient was quantitative in all cases, with approximately 60% of the activity sedimenting in the ribosome (60S-80S) region.

Following the pelleting of ribosomes in 0.25M sucrose through a 2 M sucrose pad, it was found that a small amount of synthetase activity (10%-20% of the total) remained at the interface between the two sucrose solutions. This interface region was removed and analyzed by zonal centrifugation as before. Very little optical density (at 260 mμ) was associated with this region (Figure 1B) However, a significant amount of enzyme activity was observed, all of it sedimenting as a sharp band at about 25S.

Dissociation of synthetase from ribosomes. The possibility that synthetase activity might dissociate from ribosomes under our conditions of zonal cen-

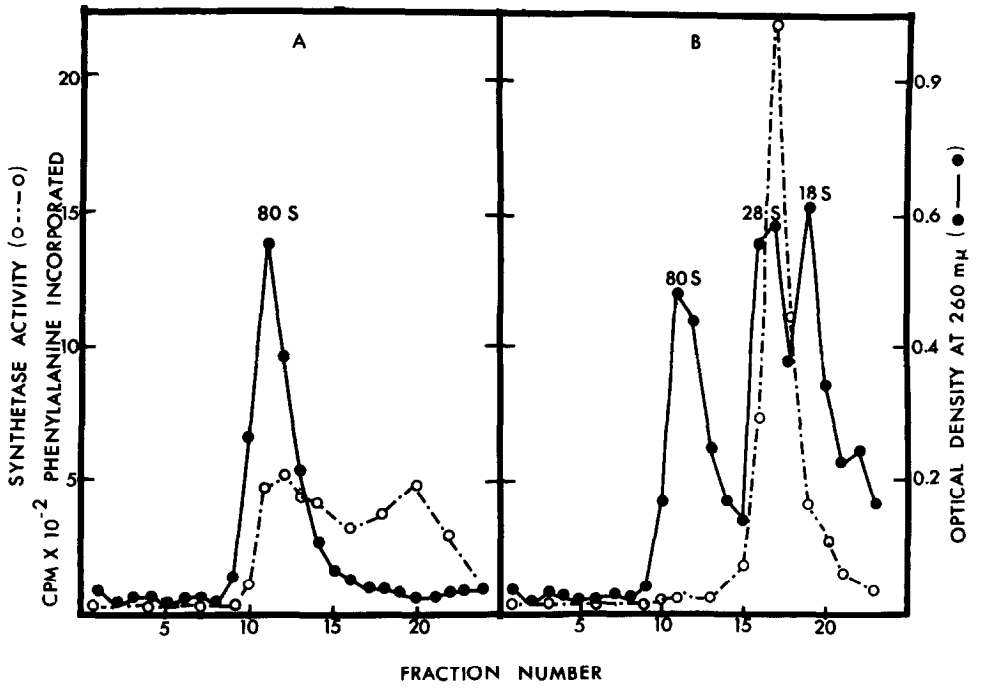

Figure 2. Zonal centrifugation of 80S ribosomes without (A) and with (B) added RNA. Centrifugation of an original ribosome suspension was carried out as in Figure 1A except in a 5%-20% sucrose gradient at 25,000 rpm for 3 hr. The peak 80S fractions were combined, divided into 2 portions (1.8 $O.D._{260}$ units each) and to one portion was added 0.2 ml (4.4 $O.D._{260}$ units) of ascites cell cytoplasmic RNA (8). These were each layered directly onto 26ml of 20%-40% sucrose gradients and centrifuged in a SW25 rotor at 22,000 rpm for 12 hr. Assays were performed as before.

trifugation was investigated by centrifuging ribosomes in a 5%-20% sucrose gradient, removing the 80S fractions, and immediately recentrifuging these in a 20%-40% sucrose gradient. Analysis of this sedimentation run (Figure 2A) shows clearly that the enzyme can dissociate from ribosomes during zonal centrifugation. The washing of the ribosomes by repeated centrifugations may well help promote this dissociation. A peak of dissociated synthetase activity at about 25S can be seen, but the significance of this is uncertain because of the broad band of activity extending across the top two-thirds of the gradient. This band probably results from the enzyme dissociating from ribosomes continually during the sedimentation run.

The complex which we observe sedimenting at 25S is similar in many ways

to the complex of Bandyopadhyay and Deutscher (2,3). Their complex contains tRNA and this suggested to us that RNA might stabilize our complex and might also promote the dissociation of synthetase from the ribosomes. Therefore, a zonal centrifugation identical to that shown in Figure 2A was performed except that cytoplasmic RNA was added to the 80S ribosomes. Analysis of the resulting fractions (Figure 2B) showed a sharp peak of synthetase activity at 25S and no activity remaining in the 80S region. This indicates that the RNA caused the immediate dissociation of synthetase from the 80S ribosomes together with the concomitant formation of a discrete synthetase-containing complex sedimenting at 25S. The exposure of crude ribosome preparations to RNA also resulted in the loss of ribosome-associated synthetase activity and the appearance of a sharp peak of activity at 25S.

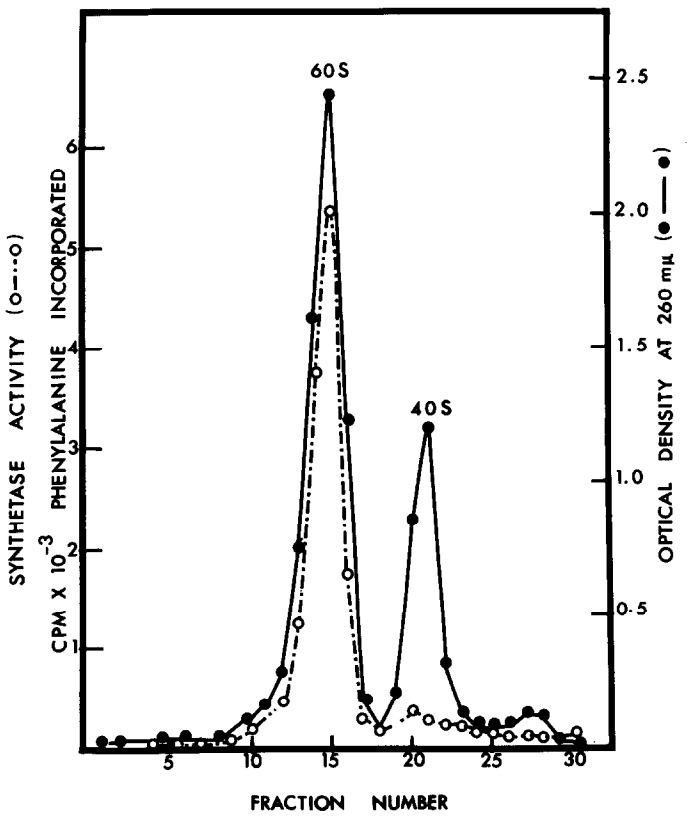

Figure 3. Zonal centrifugation of ribosome subunits. Centrifugation was performed as in Figure 1 except that $MgCl_2$ in the TKMD buffer was replaced with 0.001 M EDTA and centrifugation was at 25,000 rpm for 15 hr. Fractions (1 ml) were collected into tubes containing 0.05 ml of 0.1 M magnesium acetate and analyzed as usual.

Binding of synthetase to the 60S ribosomal subunit. The fate of the ribosome-bound synthetase was examined under sedimentation conditions in which ribosomes dissociate into 60S and 40S subunits. Essentially all of the synthetase activity was found to remain associated with the 60S subunits (Figure 3). Almost no activity remained in the 25S region. This suggests that the complex is destroyed and synthetase activity lost in the presence of EDTA as previously reported (2,3), but that the ribosome-bound enzyme remains bound and active under these conditions. Alternatively, synthetase from the complex might bind to the 60S subunit in the presence of EDTA.

DISCUSSION

The ribosome preparations which we isolate from Ehrlich ascites cells contain phenylalanyl-tRNA synthetase in two discrete particulate forms: one as part of a complex which sediments at about 25S, and one bound to the 60S subunit of the ribosome. Zonal centrifugation of these preparations, therefore, results in a synthetase activity profile which is a composite of 25S complex, ribosome-bound enzyme, and enzyme which dissociates from the ribosomes during the course of the centrifugation. It is not clear whether the entire 25S complex dissociates from a single ribosome or is assembled from synthetases which dissociate from a number of ribosomes. However, it can be postulated that tRNA plays a critical role in the stability of the complex since RNA removes synthetase from ribosomes with resulting complex formation (Figure 2B) and since the complex from liver cells contains tRNA (2,3). tRNA may also be involved in the binding of synthetases to the ribosomes.

The results of Bandyopadhyay and Deutscher (2,3) and Irvin et al. (4), although seemingly contradictory to each other, are both consistent with our observations. It would seem that depending upon the cell type or the methods used for cell homogenization and particulate fraction isolation the aminoacyl-tRNA synthetases from animal cells can be isolated predominately as part of a non-ribosomal complex (2,3) or bound to the ribosomes (4). In common with these workers, we have found a number of aminoacyl-tRNA synthetase activ-

ities associated with our ribosome preparations. The finding of all the aminoacyl-tRNA synthetases together with tRNA in the complex from liver cells has led to the hypothesis that the entire protein synthetic apparatus may exist as highly organized structures in animal cells (2,3). The question of whether these complexes, and/or ribosome-associated synthetases, actually exist *in vivo*, with the important consequences concerning the mechanism and control of protein synthesis that this would imply, or whether they are artifacts of cell disruption is difficult to answer and must await further investigations to be resolved. In any case, these particulate forms should prove valuable as intermediates for the isolation and purification of the aminoacyl-tRNA synthetases.

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