Polyribosomal and Particulate Distribution of Lysyl- and Phenylalanyl-Transfer Ribonucleic Acid Synthetases

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1. Only two aminoacyl-tRNA synthetases from Chinese hamster ovary cells are found associated with ribosomes and polyribosomes. 2. Phenylalanyl-tRNA synthetase activity is found with the 60S subunit, 80S monoribosome and individual polyribosomes. An additional 15S form of the enzyme is also seen. 3. Lysyl-tRNA synthetase activity is found in a form of about 20S and associated with ribosomal subunits and polyribosomes. The ribosomal subunits having lysyl-tRNA synthetase activity are about 6S larger than the bulk of the ribosomal subunits. 4. The lysyl- and phenylalanyl-tRNA synthetases found in different complexes have differential sensitivity to EDTA and centrifugation properties.

Previous reports from our laboratory have described the subcellular distribution of aminoacyl-tRNA synthetases in Chinese hamster ovary cell culture (Hampel & Enger, 1973*a,b*). Significant phenylalanyl- and lysyl-tRNA synthetase activity was found in the microsomal fraction.

Differential distributions of mammalian synthetases have been reported by other workers (Geels *et al.*, 1971; Vennegoor & Bloemendal, 1972; Bandyopadhyay & Deutscher, 1971). Irvin & Hardesty (1972) found arginine, lysine and phenylalanine aminoacyl-tRNA synthetase activities associated with the ribosomal fraction from rabbit reticulocytes. Roberts & Coleman (1972) found phenylalanyl-tRNA synthetase activity to be associated with the 60S ribosomal subunit in Ehrlich ascites cells. In this report we have characterized the aminoacyl-tRNA synthetases for lysine and phenylalanine from Chinese hamster ovary cells in cultures.

A preliminary report of this work has been presented (Moline *et al.*, 1973).

Materials and Methods

Cell culture

A hypodiploid (modal chromosome number of 21) line of Chinese hamster ovary cells (Tjio & Puck, 1958) was grown in suspension culture as described by Hampel & Enger (1973*a*). Cells were grown to a density of 5.0×10^5 cells/ml and collected in batches

of 1×10^9 cells. Cells were washed once in 0.25M-sucrose, resuspended in 18 ml of buffer A (0.1M-KCl, 0.01M-Tris-HCl, pH 7.5 at 25°C, 0.001M-MgCl₂ and 0.1 mM-dithiothreitol) and stored frozen at -90°C.

Cell fractionation

Cells were broken by non-ionic detergent (Nonidet P-40) lysis (Borun *et al.*, 1967). Cells were thawed and the volume adjusted to 20 ml by the addition of 2 ml of a 10% (v/v) solution of Nonidet P-40 in buffer A. This was mixed very gently for 30 min by hand and intermittently placed in an ice-bath (0°C).

The disrupted cell suspensions were centrifuged for 30 min at 1000 g at 4°C. The supernatant fraction was then centrifuged for 2.5 h through 1.5 ml of a 34% (v/v) sucrose pad at 145000 g at 4°C in an IEC A211 centrifuge tube (International Equipment Co.). The resulting microsomal pellet was gently resuspended in 3 ml of buffer B (0.01M-KCl, 0.01M-Tris-HCl, pH 7.5 at 25°C, 0.001M-MgCl₂, 0.1 mM-dithiothreitol).

Ribosomal subunits were prepared by dissolving the microsomal pellet in buffer A or buffer B at a concentration of 18 mg of microsomal protein/ml. This was dialysed for 18 h at 4°C against modified buffer A or buffer B which had no Mg²⁺ and contained 1 mM-EDTA.

Sucrose gradients

Polyribosomes and subunits were run on 10–30% (w/v) linear sucrose gradients in buffer A and buffer B. Samples (1 ml) were layered on to 36 ml gradients and centrifuged in a Spinco SW 27.1 rotor at 27000 rev./min and 4°C at the times indicated in the figure legends. The gradients were monitored for u.v.

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absorption at 254nm by using an automatic fractionator from Instrument Specialties Corp. Inc. (Lincoln, Nebr., U.S.A.). Sedimentation values ($s_{20,w}$) were calculated by using the computer program of E. Frampton and J. Zar (personal communication).

Amino acid acceptor assay

The amino acid acceptor assay was a modification of that described by Hampel & Enger (1973a). Heterologous yeast tRNA gave comparable results to homologous Chinese hamster ovary tRNA in these assays. Yeast tRNA was used in the assays. All assays contained 0.02M-Tris-HCl (pH 7.5), 0.015M-MgCl₂, 0.5mM-EDTA (disodium salt, pH 7.5), 1mM-ATP and 0.35mM-CTP. The ATP and CTP had previously been adjusted to pH 7.0 with NaOH.

Assays were done at 37°C in 50 μ l containing the preceding reagents and 15 μ g of yeast tRNA, 17.5 nmol of ¹⁴C-labelled amino acid (specific radioactivity 50 Ci/mol) and aminoacyl-tRNA synthetase (20 μ l of each column fraction which gave product formation linear with time). Incubation was terminated by placing Whatman 3 MM filter discs, which had previously been soaked in 10% (w/v) trichloroacetic acid and dried, on the incubation drops. The discs were then washed at room temperature three times in 10% trichloroacetic acid and three times in 5% trichloroacetic acid, with 4min for each wash. Finally they were quickly swirled for 30s in diethyl ether, dried and counted for radioactivity in a liquid-scintillation counter with 5ml of scintillation fluid (5g of 2,5-diphenyloxazole and 0.3g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/litre of toluene). The efficiency of counting for ¹⁴C radioactivity was 70% as determined by internal standard measurement.

Results and Discussion

The microsomal fraction contains 21% of the phenylalanine activity and 31% of the enzyme activity for lysine. Further analyses of this fraction by sucrose-density centrifugation on 10–30% sucrose density gradients in buffer B show that these activities are associated with the ribosomes and polyribosomes. Fig. 1(a) shows the polyribosome distribution pattern with associated phenylalanyl-tRNA synthetase activity for Chinese hamster ovary cells in culture. Enzyme activity is associated with a 15S form near the top of the gradient. No activity is found with the 40S ribosomal subunit *in vivo*. The majority of the phenylalanyl-tRNA synthetase activity is found associated with the remainder of the polyribosomes. It is shown that the activity follows the 60S subunit and has peaks of activity corresponding to the 80S ribosome and larger polyribosome peaks.

Fig. 1. Aminoacyl-tRNA synthetase activity in polyribosomes

(a) Phenylalanyl-tRNA synthetase activity; (b) lysyl-tRNA synthetase activity. Sucrose-density-gradient centrifugation of the microsomal fraction was on 10–30% sucrose gradients in buffer B for 2h at 27000 rev./min at 4°C. Samples contained 18mg of microsomal protein in 1ml. Amino acid acceptor assays were as described in the text. ●, Radioactivity; —, E_{254} .

An identical gradient was analysed for lysyl-tRNA synthetase activity and is shown in Fig. 1(b). A large amount of enzyme activity is found in a non-ribosome-bound form sedimentating at about 20S.

The rest of the enzyme activity is distributed throughout the microsomal fraction being associated with 40S and 60S subunits as well as the 80S ribosomes and larger polyribosomes.

Sucrose gradients similar to those in Fig. 1(a and b) were run on 10–30% sucrose with buffer A to help eliminate non-specific aggregation or association artifacts. The phenylalanyl- and lysyl-tRNA synthetase activities were comparable with those found in Fig. 1.

EDTA treatment of polyribosomes in low-ionic strength will form 40S and 60S subunits (Roberts &

Coleman, 1972). Fig. 2(a) shows a 10–30% sucrose-density-gradient separation in buffer B of ribosomal subunits and associated phenylalanyl-tRNA synthetase activity. The subunits were prepared by dialysing the microsomal fraction against modified buffer B containing EDTA for 18h. This EDTA treatment completely destroyed the 15S form of phenylalanyl-tRNA synthetase. The enzyme activity associated with the 60S subunit was not affected by dialysis against buffer containing EDTA. In addition, significant activity was found associated with the 40S subunit prepared by EDTA treatment. This is in contrast with the complete absence of activity on 40S subunits *in vivo*.

Fig. 2(b) shows the results from an identical gradient analysis for lysyl-tRNA synthetase activity. A large amount of activity is found in the 20S region unassociated with ribosomal subunits. The enzyme activity in this region was completely unaffected by EDTA treatment in contrast with the phenylalanyl-tRNA synthetase particulate activity which is EDTA-sensitive. This along with the different sedimentation coefficients of these two activities indicates they are different and unrelated forms of enzyme activity.

Lysyl-tRNA synthetase activity is also found in the region of the 40S and 60S subunits. Fig. 2(b) shows that the enzyme activity occurs in a form about 6S larger than the majority of the 40S and 60S subunits respectively.

Fig. 2. Aminoacyl-tRNA synthetase activity in EDTA-dissociated microsomal fractions

(a) Phenylalanyl-tRNA synthetase activity; (b) lysyl-tRNA synthetase activity. The microsomal fraction in buffer B was dialysed for 18 h at 4°C against modified buffer which contained no Mg²⁺ and 1 mM-EDTA. Sucrose-density-gradient centrifugation was on 10–30% sucrose gradients in buffer B for 5 h at 27000 rev./min at 4°C. The sample contained 9 mg of microsomal protein in 1 ml. Amino acid acceptor assays were as described in the text. ●, Radioactivity; —, E₂₅₄.

Fig. 3. Lysine-tRNA synthetase activity in EDTA-dissociated microsomal fractions

●, Enzyme radioactivity; —, E₂₅₄. The procedure was as described in the legend to Fig. 2 except that buffer A was used.

Fig. 3 shows the results obtained for lysyl-tRNA synthetase activity when subunits were prepared by using higher ionic strength buffers. Ribosomal subunits were prepared by dialysis against modified buffer A and separated on 10–30% sucrose gradients in modified buffer A. Lysyl-tRNA synthetase activity is found in a 20S form and in material sedimenting slightly faster than the bulk of the 40S and 60S ribosomal subunits. The distribution of lysyl-tRNA synthetase activity at the higher ionic strength was similar to that at low ionic strength in the presence of Mg^{2+} as shown in Fig. 2(b). Phenylalanyl-tRNA synthetase activity was lost under these conditions.

Gradients identical with that shown in Fig. 3 were run, except that the subunits were first dissociated with EDTA, dialysed against buffer A containing $MgCl_2$, and separated on a sucrose gradient containing $MgCl_2$ (buffer A). The increased ionic strength in the presence of Mg^{2+} gave the same pattern of dissociated subunits and lysyl-tRNA synthetase activity as in Fig. 3. Again the stability of the lysyl-tRNA synthetase association with particles and subunits in the presence of EDTA was observed.

These results indicate the polyribosomal association of phenylalanyl- and lysyl-tRNA synthetase activities. Sucrose-gradient analyses of the microsomal fraction shown in Fig. 1 were done at low ionic strength. Because of the low ionic strength there was a possibility of non-specific nucleic acid-protein aggregation (Warner & Pene, 1966). This was minimized by preparing microsomal fractions and running sucrose gradients at higher ionic strength, which gave the same results. In addition, this possible artifact is unlikely because of the fact that no other aminoacyl-tRNA synthetases were associated with the isolated polyribosomes indicating a specificity for lysyl and phenylalanyl-tRNA synthetases.

Phenylalanyl-tRNA synthetase activity is present on the 60S subunit and on all larger ribosomal forms containing the 60S subunit. The consistent absence of phenylalanyl-tRNA synthetase activity from the 40S subunit *in vivo* supports a previous report that this activity is not found on the 40S subunit in Ehrlich ascites cells (Roberts & Coleman, 1972). However, when subunits are prepared in buffers containing EDTA we find significant phenylalanine activity associated with the 40S subunit. This is true even when subunits were prepared by (1) dialysis of microsomal fractions against modified buffer B containing EDTA and separation on 10–30% sucrose gradients in buffer B; (2) dissolving the microsomal pellet in modified buffer B and immediately layering on to a sucrose gradient in buffer B, or (3) dissolving the microsomal pellet in modified buffer B followed by dialysis against buffer A overnight and separation on a sucrose gradient in buffer A. This is convincing evidence

that the association of the enzyme with the EDTA-prepared 40S subunits is very rapid. Conditions (2) and (3) cited above were adequate to prepare subunits, but the EDTA treatment was too short to destroy the 15S form of phenylalanyl-tRNA synthetase. This form remained intact and active under these conditions. This supports the notion that the artificially generated phenylalanyl-tRNA synthetase-40S ribosomal subunit complex is not the result of association between an intact 40S subunit and a smaller form of the enzyme artificially generated by EDTA destruction of the 15S particle. The explanation for this anomaly may be due to the artifact subunits generated by EDTA treatment of ribosomes and polyribosomes as shown by Schreier & Staehelin (1973a) who found a large class of inactive mammalian ribosomal subunits generated by EDTA treatment.

Lysyl-tRNA synthetase is distributed throughout the microsomal fraction and is associated with both subunits *in vivo* and those generated by EDTA treatment under all conditions. EDTA-treated subunits were generated by (1) 18h dialysis against modified buffer B; (2) dissolving the microsomal pellet in modified buffer B and immediately layering it on to a sucrose gradient in buffer B; (3) dissolving the microsomal pellet in modified buffer B, dialysis overnight against buffer A and separation on a sucrose gradient in buffer A; (4) 18h dialysis against modified buffer A and separation on a sucrose density gradient in modified buffer A; (5) 4h dialysis against modified buffer A followed by 11h dialysis against buffer A and separation on a sucrose density gradient in buffer A. These conditions were designed to eliminate artifacts of reassociation. All of these conditions gave results the same as Figs. 2(b) and 3, a 20S form of enzyme and forms about 6S larger than the bulk of the 40S and 60S ribosomal subunits respectively.

The possibility of free lysyl- or phenylalanyl-tRNA synthetases existing in discrete aggregated forms of such a large size as ribosomal subunits is unlikely. We have checked this possibility by preparing ribosomal subunits from cells grown at 42°C, which gives a microsomal pattern composed of 40S and 60S subunits as well as 80S monoribosomes (McCormick & Penman, 1969). The distribution of lysyl- and phenylalanyl-tRNA synthetase activities on sucrose gradients is the same from these ribosomal subunits generated *in vivo* as from those *in vitro*, making a real association between these two synthetases and ribosomal subunits most likely.

Differences in the properties of non-ribosomally associated phenylalanyl- and lysyl-tRNA synthetase particles are also shown by our results: (1) they have different sedimentation coefficients, 15S and 20S respectively; (2) phenylalanyl-tRNA synthetase is EDTA-sensitive, whereas under the same conditions

lysyl-tRNA synthetase activity is EDTA-resistant; (3) lysyl-tRNA synthetase activity is associated with all ribosomal subunits and particles, whereas phenylalanyl-tRNA synthetase activity is not associated with the 40S subunit *in vivo*.

This makes the process of protein synthesis in a mammalian cell a much more complicated phenomenon. Why are such large complexes necessary? Why is there a heterogeneity in ribosomes with respect to associated synthetases? Recent reports have shown the protein synthesis factor If-E3 to be particulate and associated with the 40S subunits in a form slightly larger (about 48S) than the bulk of the 40S subunits (Schreier & Staehelin, 1973b; Parilla *et al.*, 1973). The existence of a large protein complex involved in initiation of translation becomes more intriguing when one considers the evidence available suggesting that translational factors (e.g., the aminoacylation of tRNA) are involved in the regulation of initiation of translation (Adamson *et al.*, 1972; Vaughan & Hansen, 1973).

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