Further Evidence for a Single Leucyl Transfer Ribonucleic Acid Synthetase Capable of Charging Five Leucine Transfer Ribonucleic Acids in Escherichia coli*

(Received for publication, November 13, 1970)

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SUMMARY

Five components of leucine transfer ribonucleic acid in Escherichia coli separated on a reversed phase column were charged with radioactive leucine. Each labeled leucyl-tRNA was subjected to an exchange reaction with unlabeled E. coli tRNA in which the labeled leucine could be transferred from one leucine tRNA to another. The exchange product, when analyzed on methylated albumin-Kieselguhr columns, suggested that a single leucyl-tRNA synthetase was responsible for the attachment of leucine to all five leucine tRNAs. The existence of a single synthetase specific for all five components of leucine tRNA was further supported by studying the reaction rate of mixed substrates containing two leucine tRNAs under a condition in which the rate was enzyme-dependent.

It is well established that the genetic code is degenerate, i.e. one amino acid can be coded by more than one trinucleotide sequence. In the case of leucine in Escherichia coli, six codons have been assigned to this amino acid (1, 2). Corresponding to code degeneracy, there exists a multiplicity of tRNAs specific to leucine which differ in chromatographic behavior (3–5), species specificity in the aminoacylation reaction (6–8), and coding property (4, 7, 10–12). Berg, Lagerkvist, and Dieckmann (13) further demonstrated that leucine tRNA from E. coli has at least two kinds of terminal sequence, -GpCpApCpCpA and -GpUpApCpCpA. This raises the question as to whether one, or more than one, leucyl-tRNA synthetase is involved in the reaction of attaching leucine to the different leucine-accepting tRNAs.

A number of investigators have shown that the same leucyl-tRNA synthetase is capable of attaching leucine to two fractions of leucine tRNA in E. coli (14–16). On the other hand, Yu (17) has raised the possibility of the existence of more than one leucyl-tRNA synthetase in E. coli. This paper will give additional evidence to support the idea that one leucyl tRNA synthetase can attach leucine to all five leucine tRNAs.

EXPERIMENTAL PROCEDURE

Materials

E. coli B tRNA was obtained from General Biochemicals. This tRNA secured from a commercial source was identical with tRNA prepared in our laboratory as far as leucine tRNA is concerned, when examined by methylated albumin-Kieselguhr columns or reversed phase column chromatography. Leucine tRNA was fractionated into five components on the RPC as previously described (18). 14C-Leucine (247 mCi per mmole) and 3H-leucine (5 Ci per mmole) were obtained from New England Nuclear.

Methods

Preparation of Aminoaql-tRNA Synthetase—A crude synthetase fraction was prepared essentially as previously described (8). E. coli B cells were grown overnight at 37° in a nutrient broth (8 g of Difco nutrient broth and 5 g of NaCl in 1 liter of H2O, pH being adjusted to 7.0 with 1 M NaOH). The culture was transferred to 10 volumes of fresh nutrient broth and the cells were allowed to grow with vigorous shaking at 37°, until the cell concentration reached 5 × 108 per ml. The cells were spun down at 8,000 × g for 20 min and the pellets washed once in a Tris-magnesium buffer (0.01 M Tris-HCl, pH 7.3, and 1 mM MgCl2). The cells were quickly frozen in Dry Ice-ethanol and stored at −80°. Active synthetase can be prepared from cells stored at this temperature for several months. The procedure to be described is for 1 liter of culture. The cells were ground with 4 g of alumina (levigated alumina from Norton Abrasives, Worcester, Massachusetts). This and the succeeding steps were carried out at 0–4°. Tris-magnesium buffer (6.5 ml) was added to the cell paste and the extract was centrifuged at 8,000 × g for 20 min. The supernatant was dialyzed against 1 liter of the Tris-magnesium buffer containing 6 mM 2-mercaptoethanol for 3 hours, changing the buffer every hour. To remove the tRNA, the dialyzed 105,000 × g supernatant was

*This investigation was supported by Grant GM10923 from the National Institutes of Health and Grant GB8160 from the National Science Foundation.
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applied to a column (1 × 6 cm) of DEAE-cellulose previously equilibrated with potassium phosphate buffer (0.02 M, pH 7.7). The charged column was washed with 10 ml of the phosphate buffer, and the enzyme was eluted with the 0.02 M potassium phosphate buffer containing 0.35 M NaCl. Fractions of 1.5 ml each were collected and the tubes with high absorbance were combined. The preparation was divided into small aliquots and stored at −80°C. This crude enzyme fraction, containing all the aminoacyl-tRNA synthetases, is stable for several months.

Condition for Enzymatic Exchange—Yamane and Sueoka (15) showed that, in the presence of aminoacyl-tRNA synthetase and excess AMP, a rapid exchange of leucine occurs between two components of leucine tRNA discernible on a MAK column through the intermediate leucine AMP-enzyme complex. Essentially the same condition was used here to study the enzymatic exchange of leucine among the five components of leucine tRNA fractionated on the reversed phase column. The exchange was carried out in a 1-ml reaction mixture which contained 100 μmoles of Tris-HCl, pH 7.4; 5 μmoles of magnesium acetate; 4 μmoles of AMP; 4 μmoles of glutathione (reduced); 1 μmole each of 20 12C-amino acids; 3 mg of unfractionated E. coli B tRNA; 10,000 to 20,000 cpm of a fractionated component of 3H- or 14C-leucyl-tRNA; and 80 μg of protein from the crude enzyme fraction. Since the exchange is essentially an equilibrium reaction between various components of leucine tRNA for the labeled leucine, a large excess in the molar ratio of the leucine tRNA present in the unfractionated tRNA to the labeled leucyl-tRNA component in the exchange reaction mixture was essential to demonstrate the exchange. The reaction mixture was incubated at 37°C for 15 min, at which time the reaction is known to be completed. The mixture was shaken with an equal volume of water-saturated phenol. The residual phenol in the aqueous phase was removed by shaking with ether, and the ether was expelled by bubbling nitrogen into the solution. tRNA was precipitated by the addition of 2.5 volumes of absolute ethanol and the precipitate was dissolved in 1 ml of sodium phosphate buffer (0.05 M, pH 6.3). Radioactivity was determined from an aliquot of the solution by trichloroacetic acid precipitation. The precipitate was collected on a glass filter disc, dried, and counted in Omnifluor-toluene (New England Nuclear) with a Packard Tri-Carb scintillation counter. The acid-precipitable counts before and after the exchange reaction determined the percentage of recovery during the exchange reaction.

Methylated Albumin-Kieselguhr Column Chromatography—The product of the exchange reaction was compared on MAK columns with a differently labeled unfractionated leucyl-tRNA. The procedure for MAK column chromatography was as described (19). Generally, the tRNA was eluted with 225 ml of a linear salt gradient from 0.30 to 1.0 M NaCl in 0.05 M sodium phosphate buffer, pH 6.3. Fractions of 2 ml each were collected at the rate of 1 ml per min. Absorbancy at 260 nm was measured and appropriate fractions were assayed for acid-precipitable counts as before.

Rate of Enzymatic Aminoclylation with Mixed Substrates—Preliminary experiments were first carried out to determine the condition in which the initial linear rate of enzymatic attachment of leucine to leucine tRNA was dependent on the amount of enzyme present in the reaction mixture. This was done in a 1-ml reaction mixture which contained 100 μmoles of Tris-HCl buffer, pH 7.4; 1.6 μmoles of ATP; 4 μmoles of glutathione (reduced); 5 μmoles of magnesium acetate; 0.5 μCi of 14C-leucine; 1 mg of E. coli bulk tRNA or its equivalent in the fractionated leucine tRNA component; and varying amounts of the crude enzyme fraction. It was found that the initial rate was enzyme-dependent when the amount of the enzyme was in the range of 10 to 40 μg of enzyme protein. Increasing the amount of tRNA to 2 mg did not alter the initial reaction rate. Consequently, all rate determinations used 20 μg of protein from the crude enzyme fraction in a 1-ml reaction mixture. The reaction was allowed to proceed at 37°C and aliquots of 0.1 ml each were taken at intervals of 0, 1, 2, 3, 5, and 10 min to assay the acid-precipitable counts. The reaction rate was determined for each of the five components of leucine tRNA as well as for mixtures of two of the five components placed in the same tube (mixed substrate experiments).
RESULTS AND DISCUSSION

Analysis of Enzymatic Exchange Product

E. coli B tRNA was fractionated into five leucine tRNA components according to the procedure of Kolmers, Novelli, and Nielberg (5). Furthermore, the leucine tRNA components, once separated on the RPC, could be distinguished from each other when examined on the MAK column (18). The five leucine tRNA components prior to the exchange reaction are shown in Fig. 1, a to e. These components originally appeared as single peaks on the MAK column (18). A slight modification of the column profiles of these components after extended storage (0° for over 1 year) was indicated by the presence of some labeled material in the area of Leu in components Leu, Leu, and Leu. This impurity amounted to 5 to 12% of the fractionated tRNA. This phenomenon, previously observed, could be the result of degradation and aggregation of the degraded products as reported by Zachau (20). The minor impurity, however, should not seriously affect this experiment insofar as one could demonstrate the exchange of leucine. Enzymatic exchange was carried out for each labeled leucyl-tRNA component in a reaction mixture containing enzyme, AMP, and excess unfractionated E. coli B tRNA as described under "Methods." An aliquot of the exchange product containing 6,000 to 10,000 cpm of radioactivity was cochromatographed on the MAK column with a differently labeled bulk E. coli leu-tRNA as a standard. The results are shown in Fig. 2, a to e. The distribution of radioactivity from the exchange product approximated that of the control, indicating that an exchange of leucine had occurred between the various components of leucine tRNA. The profiles of Fig. 2 taken as a whole can best be interpreted on the basis that the labeled leucine freely exchanged among the five leucine tRNA components. Since a labeled leucine tRNA component was added to bulk tRNA in the exchange reaction mixture, the proportion of this component should be higher than that of the unexchanged bulk tRNA when examined on the MAK column. This is best illustrated in the case of Leu in Fig. 2a. The 3H radioactivity from 3H-Leu-tRNA of the Leu component added to the exchange reaction was relatively higher than the 14C label from the unexchanged bulk E. coli Leu-tRNA in the Leu region (Peak 1 of the MAK leucyl-tRNA profile). This was also generally true for the other four leucine tRNA components, although it is difficult to estimate quantitatively...
the relative excess because of the overlapping of the components on the MAK column elution profile.

Aminoacylation of tRNA is known to occur in two steps (21).

**Amino acid + ATP ⇌ (aminoacyl-AMP-enzyme) + PP_i**

(Aminoacyl AMP enzyme) ▶ tRNA ⇌

amiunoayl-tRNA + enzyme + AMP

The fact that one-half to three-quarters of the radioactivity originally present in the labeled leuc-tRNA component in the exchange product was recovered, in spite of the large excess of 1C-amino acids in the exchange reaction mixture, would indicate that the exchange did not involve the first step. Yamane and Sueoka (15), on the basis of such data, originally proposed that the exchange must have occurred through the intermediate complex, the amino acid-AMP-enzyme. Thus, any exchange of amino acid between the two species of tRNA under such a condition implies that these tRNAs share a specificity with the same enzyme. Recently, Bennett (16) studied the coding property of the exchange product of two components of leucine tRNA separated on the MAK column. The conclusion was that only one synthetase was involved in the acylation reaction for both fractions of leuc-tRNA from E. coli. Certainly this interpretation accounts for the present data without evoking the existence of more than one leucyl-tRNA synthetase with a varying degree of specificity toward the different leucine tRNAs. The mixed substrate experiments described below give additional support to this view.

**Rate of Aminoacylation of Leucine tRNA Components**—Preliminary experiments showed that when 1 mg or more of E. coli tRNA was present in a 1-ml reaction mixture, the linear rate of charging was proportional to the amount of enzyme from 10 to 40 μg of protein. Less than 10 μg or more than 40 μg of the enzyme fraction resulted in a somewhat erratic assay. The smaller amount gave a proportionately higher background; the larger amount resulted in such an accelerated reaction that an accurate estimation of the initial rate became difficult, at least at 37°C. Thus, 20 μg of protein per ml of the reaction mixture were used to measure the rate of charging. To charge a leucine tRNA component, 0.7 A260 unit was used in each milliliter of the reaction mixture. In the mixed substrate experiment, each 1-ml reaction mixture contained a 0.7 A260 unit of each of the two leucine tRNA components.

The time course of the 14C-leucyl-tRNA formation of various single and mixed substrates is given in Fig. 3. In one series of experiments, two leuc-tRNA components and a mixture of the two components were charged under identical conditions. The rate of charging in the mixed substrate was either intermediate between the two components or very nearly the same as one of the two components. In no case did the rate approximate a summation of the rate of the two individual leuc-tRNA components, a situation to be expected if the two components were specified by two different enzymes.

This lack of additivity in the rate of acylation of the mixed substrate argues against the possibility of the existence of a synthetase specific for only one leucine tRNA component. A similar result was reported by Keller and Anthony (14) with two fractions of leucine tRNA. To demonstrate conclusively the existence of one leucyl-tRNA synthetase for all five leucine tRNA components with the method of mixed substrate experiments, it is necessary to carry out the acylation reaction for all combinations of two substrates at a time. This was not done mainly because of lack of components LeuG and Leu½. The data presented, nevertheless, provide additional support to the more conclusive evidence obtained from analysis of the exchange products. In this connection, Rouget and Chapeville (22) recently found two interchangeable forms of leucyl-tRNA synthetase in E. coli. However, only one, E½, was able to transfer specifically leucine to leucine tRNA. The other, E¼, catalyzed only the first step of the aminoacylation reactions (the ATP-PPi exchange reaction) with respect to leucine as well as isoleucine, valine, and, to a certain extent, methionine.

A summary of the rate of charging in single and mixed substrates is given in Table I. In one series of experiments, each 1-ml reaction mixture contained a 0.7 A260 unit of each of the two components with the method of mixed substrate experiments, it is necessary to carry out the acylation reaction for all combinations of two substrates at a time. This was not done mainly because of lack of components LeuG and Leu½. The data presented, nevertheless, provide additional support to the more conclusive evidence obtained from analysis of the exchange products. In this connection, Rouget and Chapeville (22) recently found two interchangeable forms of leucyl-tRNA synthetase in E. coli. However, only one, E½, was able to transfer specifically leucine to leucine tRNA. The other, E¼, catalyzed only the first step of the aminoacylation reactions (the ATP-PPi exchange reaction) with respect to leucine as well as isoleucine, valine, and, to a certain extent, methionine.

In conclusion, the evidence presented in this paper indicates that a single leucyl-tRNA synthetase is capable of transferring leucine to all five components of leucine tRNA in E. coli. The existence of a leucyl-tRNA synthetase specific to only one of the five components appears unlikely.

**Acknowledgment**—We would like to acknowledge the valuable assistance of Brenda B. Mihan.

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