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Modification of a Specific Ribosomal Protein Catalyzed by Leucyl, Phenylalanyl-tRNA:Protein Transferase*

(subunits/gel electrophoresis/gradient centrifugation/ *E. coli*)

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ABSTRACT ***Escherichia coli* ribosomes washed with 1 M NH_4Cl were found to function as acceptor for leucine and phenylalanine in the reaction catalyzed by leucyl, phenylalanyl-tRNA:protein transferase. When isolated subunits were acylated with [^{14}C]phenylalanine and reisolated by gradient centrifugation, the recovered 30S particles had a specific radioactivity nearly 30 times that of similarly treated 50S particles. Autoradiography of gels, which contained protein from acylated 30S particles, that had been subjected to electrophoresis in 8 M urea and in sodium dodecyl sulfate, suggested that acceptor activity was largely due to a single protein with a molecular weight of about 12,000. Leucine and phenylalanine residues that had been transferred to ribosomal protein were reactive with fluorodinitrobenzene and were released as leucyl- or phenylalanylarginine after treatment with trypsin.**

The results indicate that leucyl, phenylalanyl-tRNA: protein transferase catalyzes the addition of these amino acids to an NH_2-terminal arginine residue of a specific ribosomal protein on the 30S subunit.

Leucyl, phenylalanyl-tRNA:protein transferase is a soluble enzyme from *Escherichia coli* that catalyzes the transfer of these amino acids from tRNA to certain protein acceptors (1). It is apparently responsible for observations of the incorporation of leucine and phenylalanine into protein in soluble extracts of *E. coli* (2). The transfer reaction is distinguished from that involved in protein synthesis *de novo* by the fact that it does not require ribosomes, magnesium ions, GTP, or template nucleic acids. On the other hand, it does depend upon the presence of a suitable protein acceptor (3). Recently we provided evidence that acceptor proteins have an NH_2-terminal arginine residue to which the amino acids are transferred (4).

It has been suggested that this enzyme may provide a mechanism for regulating the function of those proteins whose modification it catalyzes (3), but little is known concerning such protein acceptors in *E. coli*. In this report, we present evidence that *E. coli* ribosomes possess acceptor activity that is largely due to a single protein located on the 30S subunit; leucine or phenylalanine become attached to the NH_2-terminal arginine residue in this protein.

MATERIALS AND METHODS

L-[U-^{14}C]Leucine and L-[U-^{14}C]phenylalanine were obtained from the New England Nuclear Corp. Materials for disc gel electrophoresis were purchased from the Fisher Chemical Corp. Pancreatic ribonuclease and trypsin were products of the Worthington Biochemical Corp. *E. coli* B was grown and harvested as described (1). Transfer RNA from this organism was purchased from General Biochemicals Inc. and was used to prepare [^{14}C]leucyl-tRNA (1).

Ribosomes were prepared from *E. coli* B by differential centrifugation (5) and were washed three times with 1.0 M NH_4Cl (6). Isolated 50S and 30S subunits were obtained from the washed ribosomes by centrifugation for 17 hr at 24,000 rpm in 15–30% sucrose gradients containing 10 mM Tris·HCl (pH 7.3)–0.5 mM $MgCl_2$–5 mM 2-mercaptoethanol–0.1 mM EDTA. Leucyl, phenylalanyl-tRNA:protein transferase and L-phenylalanyl-tRNA synthetase were purified from *E. coli* B as described (7). [^{3}H]Leucyl- and [^{3}H]phenylalanylarginyl-albumin were prepared by the enzymatic addition of leucine

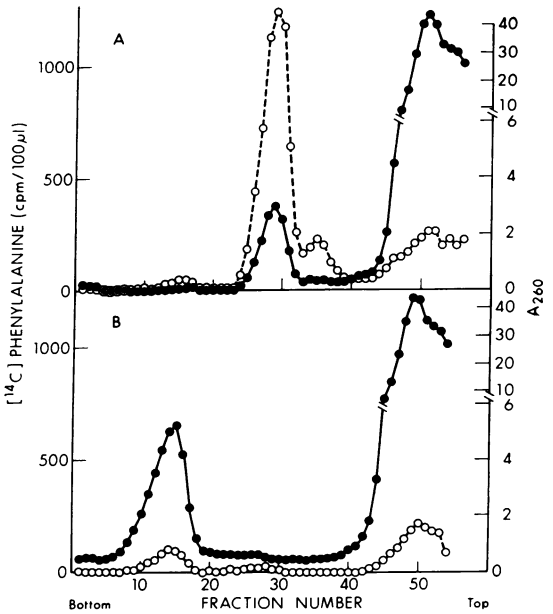

FIG. 1. Reisolation of ribosomal subunits acylated with [^{14}C]phenylalanine. Reaction mixtures (1 ml) similar to those described in Table 1 and containing either 24 A_{260} units (0.66 mg protein) of isolated 30S particles (*A*) or 44 A_{260} units (1.22 mg protein) of isolated 50S particles (*B*) were subjected to centrifugation in 55-ml linear sucrose gradients (15–30%). After centrifugation, the tubes were pierced at the bottom and 1-ml fractions were collected. Absorbance at 260 nm (*closed circles*) was measured with 100-μl aliquots diluted to 1.0 or 4.0 ml with water. Radioactivity insoluble in hot 5% trichloracetic acid (*open circles*) was determined on 100-μl aliquots (9).

* This is paper No. 8 in a series entitled "Enzymatic Modification of Proteins"; the preceding paper in this series is ref. 4.

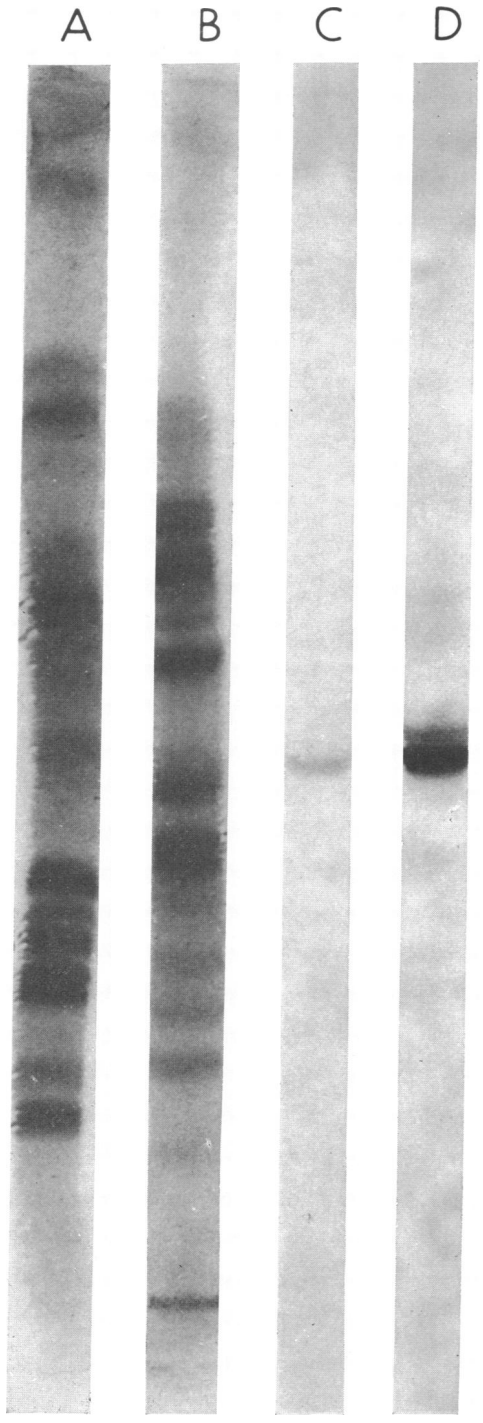

FIG. 2. Disc gel electrophoresis of enzymatically aminoacylated ribosomes. Intact ribosomes (8 mg of protein/ml) were allowed to react with [^{14}C]leucine as described in Table 1 and digested with 2 μg of RNase for 60 min at 37°C. The reaction mixture was then dialyzed exhaustively against 20 mM β-alanine–acetate buffer, pH 4.5, containing 5 mM 2-mercaptoethanol and 6 M urea. An aliquot containing 78 μg of ribosomal protein and 2800 cpm of incorporated leucine was analyzed. Isolated 30S particles that had been acylated with [^{14}C]phenylalanine and recovered as described in Fig. 1 were similarly treated with RNase, and an aliquot containing 50 μg of protein and 34,000 cpm of incorporated phenylalanine was used. Disc gel electrophoresis was performed as described by Traub and Nomura (10). The gels were stained for protein with 0.05% Coomassie Blue, destained with 7% acetic acid, and then sliced and subjected to autoradiography for 29 days with Kodak Medical x-ray films. *A* and *B* are the gels obtained with whole ribosomes and 30S particles, respectively, stained for protein. *C* and *D* are the corresponding autoradiograms.

TABLE 1. *Acceptor activities of E. coli ribosomal particles*

Addition	Ribosomal protein (mg/ml)	Incorporation of leucine* (pmol/ml)	(pmol/mg)	Incorporation of phenylalanine† (pmol/ml)	(pmol/mg)
None	—	15	—	87	—
Ribosomes	0.6	213	330	456	615
Ribosomes	1.2	432	342	849	636
Ribosomes minus enzyme	1.2	3	3	33	28
50S particles	1.2	213	165	285	165
30S particles	0.6	483	807	837	1251

* Reaction mixtures (150 μl) contained 50 mM Tris·HCl (pH 7.8), 10 mM 2-mercaptoethanol, 0.15 M KCl, 5 mg of leucyl-tRNA containing 4.5 nmol of [^{14}C]leucine (240 Ci/mol)/ml, 93 mU (1) of leucyl, phenylalanyl-tRNA:protein transferase per ml, and unfractionated ribosomes or isolated ribosomal subunits as indicated. After incubation for 30 min at 37°C, incorporation into material resistant to RNase and insoluble in cold 5% trichloracetic acid was determined on 50-μl aliquots by the filter-paper disc technique (4).

† Reaction mixtures (150 μl) contained 50 mM Tris·HCl (pH 7.8), 10 mM 2-mercaptoethanol, 0.15 M KCl, 10 mM magnesium acetate, 6 mM ATP, 50 μM L-[^{14}C]phenylalanine (382 Ci/mol), 1.0 mg chloramphenicol/ml, 10 mg tRNA/ml, 50 μg L-phenylalanyl-tRNA-synthetase/ml, 70 mU of leucyl, phenylalanyl-tRNA:protein transferase/ml, and ribosomes as indicated. Incorporation into protein was determined on 50-μl aliquots after incubation for 4 hr.

or phenylalanine to bovine serum albumin that had been arginylated at its NH_2-terminal position in the reaction catalyzed by arginyl-tRNA:protein transferase (7). Radioactive leucylarginine and phenylalanylarginine were isolated from tryptic digests of [^{14}C]leucyl- or [^{14}C]phenylalanyl[^{3}H]arginylalbumin by chromatography on Dowex 50 (7).

Protein concentrations were estimated by the method of Lowry *et al.* (8), using bovine serum albumin as a standard.

RESULTS

Acceptor activity of *E. coli* ribosomes

The addition of ribosomes or their subunits was found to increase the extent of incorporation into protein of leucine or phenylalanine, catalyzed by leucyl, phenylalanyl-tRNA:protein transferase (Table 1). The greater incorporation observed for phenylalanine as compared with leucine was probably due to the use of the generating system for phenylalanyl-tRNA. This system was used to overcome the limitation of the transfer reaction known to result from depletion of aminoacyl-tRNA (1). Qualitatively similar results were obtained with both amino acids. In each case, the smaller ribosomal subunit was found to account for most of the acceptor activity. The difference between the two particles was considerably more pronounced when the isolated subunits were first acylated with [^{14}C]phenylalanine and then reisolated by sedimentation through a sucrose gradient (Fig. 1).

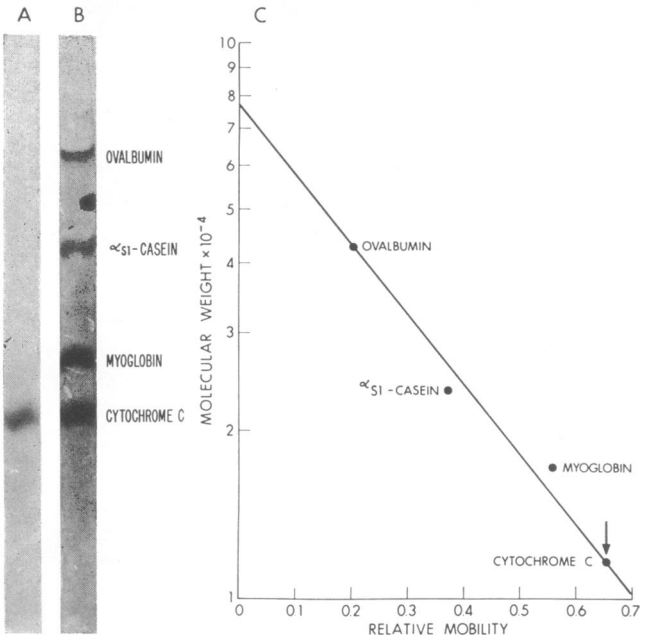

FIG. 3. Molecular weight of the ribosomal acceptor protein. A sample (7000 cpm) of 30S particles that had been reisolated after acylation with [^{14}C]phenylalanine, as described in Fig. 1, was subjected to disc gel electrophoresis in sodium dodecyl sulfate as described by Shapiro *et al.* (11), The autoradiogram (*A*) was obtained after exposure for 14 days to Kodak Medical x-ray film. A gel for calibration (*B*) was run simultaneously, using a mixture containing 5 μg (each) of chicken ovalbumin, α_{s1}-casein, sperm whale myoglobin, and cytochrome *c* from horse heart. This gel was stained for protein with 0.05% Coomassie Blue. The molecular weight of the ribosomal protein acceptor was estimated (*C*) from its mobility (indicated by the *arrow*) relative to those of the marker proteins. The molecular weights of these proteins were those cited by Weber and Osborn (12), except for that of α_{s1}-casein, which was taken from Grosclaude *et al.* (13).

The recovered 30S particles had a specific radioactivity almost 30 times that of the 50S particles.

Characterization of the acceptor protein

Whole ribosomes acylated with [^{14}C]leucine, as well as 30S particles purified after acylation with [^{14}C]phenylalanine, were subjected to disc gel electrophoresis in 8 M urea (10), and the distribution of radioactivity was determined by autoradiography (Fig. 2). Most of the radioactivity was located in a single band, the mobility of which was identical in the two preparations. The radioactive product obtained with 30S particles also migrated as a single component during disc gel electrophoresis in sodium dodecyl sulfate (Fig. 3). Its mobility in this system corresponded to that of cytochrome *c*, which indicates a molecular weight of about 12,000 for the reduced, denatured protein.

Characterization of the incorporated amino acids

Acylation of whole ribosomes with [^{14}]leucine or [^{14}C]phenylalanine was performed under conditions in which incorporation was more than 95% dependent upon the presence of ribosomes. Almost all (>90%) of both of the incorporated amino acids were found to retain a free alpha-amino group, as determined by the fluorodinitrobenzene reaction (14).

Samples of the acylated proteins were treated with trypsin

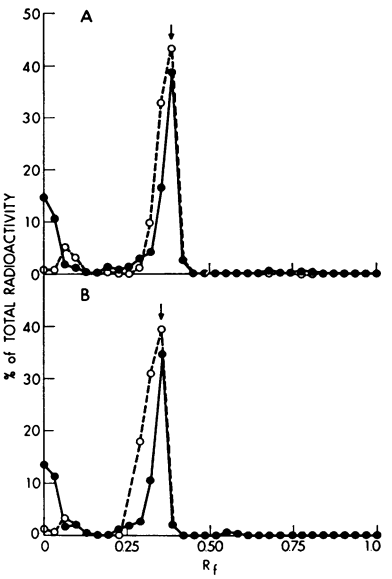

FIG. 4. Analyses of tryptic digests from ribosomal protein acylated with [^{14}C]leucine or [^{14}C]phenylalanine. Whole ribosomes (5 mg of protein/ml) were acylated in 1-ml reaction mixtures (Table 1). Under these conditions, 0.3 nmol of leucine and 0.5 nmol of phenylalanine were incorproated per mg of ribosomal protein; the reaction was more than 95% dependent on the presence of ribosomes. RNase (250 μg) was added, and incubation was continued for 60 min. Protein was precipitated by the addition of 1 ml of 10% trichloracetic acid, and the pellet was dissolved in 1 ml of 0.2 N NaOH and dialyzed exhaustively against water. [^{3}H]Leucylarginylalbumin (53 μg) or [^{3}H]phenylalanylarginylalbumin (21 μg) was then mixed with 700 μg of ribosomal protein acylated with the corresponding [^{14}C]amino acid and digested with 200 μg of trypsin for 16 hr at 37°C in 50 mM Tris·HCl (pH 8.2). Aliquots containing 4000 cpm of ^{14}C and 13,000 cpm of ^{3}H from the leucyl-proteins (*A*) or 4000 cpm of ^{14}C and 7000 cpm of ^{3}H from the phenylalanyl-proteins (*B*) were subjected to descending paper chromatography in *n*-butanol–acetic acid–water 4:1:5. The positions of marker leucyl- and phenylalanylarginine (7), which were run simultaneously, are indicated by the *arrows* in *A* and *B*, respectively. *Open circles* represent ^{14}C, *closed circles* represent ^{3}H.

in the presence of [^{3}H]leucyl- or [^{3}H]phenylalanylarginyl-albumin. The latter was included as a measure of the adequacy of digestion, since it is known to yield leucyl- or phenylalanylarginine as the major tryptic peptide (7). The digests were analyzed by paper chromatography (Fig. 4). For each digest, the major fraction of both isotopes was found in a position corresponding to that of leucyl- or phenylalanylarginine. These results were confirmed by paper electrophoresis in 1.5 M formic acid and establish that both leucine and phenylalanine had been transferred to an NH_2-terminal arginine residue of the ribosomal protein acceptor.

DISCUSSION

The present results indicate that leucyl, phenylalanyl-tRNA: protein transferase catalyzes the addition of these amino acids to an NH_2-terminal arginine residue of a protein located on the 30S subunit of the *E. coli* ribosome. This conclusion is in accord with other evidence that suggests that acceptor protein specificity in this reaction is due to an NH_2-terminal arginine moiety (4), but is at variance with analysis of the NH_2-terminal amino acids of *E. coli* ribosomal proteins in which no

dinitrophenylarginine was detected after reaction with fluorodinitrobenzene (15).

The total mass of 30S ribosomal protein has been estimated at 330,000 daltons (16), and the observed incorporation of phenylalanine was 1.25 nmol/mg of 30S protein. If transfer was largely to a single protein, as suggested by our data, and if there is one molecule of this protein per subunit, then it can be calculated that about 40% of the acceptor protein molecules participated in the reaction. This figure may represent incomplete acylation of potential acceptor molecules, complete acylation of proteins that had been partially acylated *in vivo*, or complete acylation of a protein present in amounts less than one molecule per ribosome. It seems unlikely that this amount of acceptor activity can be explained by a soluble protein contaminating the preparations of salt-washed ribosomes, particularly since most of the incorporated phenylalanine remained associated with the particles when they were isolated from the reaction mixture.

Interpretation of the small quantity of acceptor activity of the 50S subunit is more difficult. The fact that radioactivity incorporated in its presence was largely dissociated during reisolation leads us to believe that a contaminating soluble protein may account for this activity. In preliminary studies on leucine transfer (3), we found approximately equal stimulatory activities for the two ribosomal subunits isolated from *E. coli* Q13. This discrepancy with our present results may be related to the strain difference or to the fact that the acylated particles were not reisolated in the previous experiments. We cannot, however, exclude the possibility that acceptor activity of the 50S subunit is due to a 50S ribosomal protein that is dissociated from the particle under our reaction conditions, either before or after the putative protein has been acylated.

The acceptor activity described in this report is probably responsible for the observation of Momose and Kaji (17) that leucine and phenylalanine incorporated onto *E. coli* ribosomes in the absence of added template RNA remain reactive with fluorodinitrobenzene. It may also be related to the finding of Otaka and Osawa (18) that these amino acids are specifically incorporated onto *E. coli* chloramphenicol particles *in vitro*. In this context it should be emphasized, however, that soluble acceptor activity is also present in *E. coli*, as originally shown by Momose and Kaji (17). Preliminary experiments in our own laboratory suggest that the number of different soluble protein acceptors is small, although they account for the bulk of the total acceptor activity of the cell. The possible relationship of the soluble acceptor activity to the ribosomal acceptor protein remains to be investigated.

Many aspects of the leucine, phenylalanine-transfer reaction (1, 4, 7), and of the reaction catalyzed by the corresponding mammalian enzyme, arginyl-tRNA:protein transferase (19, 20), are now understood. However, the naturally occurring protein substrates for these reactions, as well as the factors determining the extent of acylation of these proteins *in vivo*, are largely unknown and the function of this class of enzymes is not understood. The existence of a specific acceptor on the 30S subunit of *E. coli* ribosomes suggests that leucyl, phenylalanyl-tRNA:protein transferase may play a role in regulating the translation from mRNA to protein in *E. coli*. The possibility that acylation of the ribosomal protein acceptor may alter the function of the 30S particle in protein synthesis is currently being examined.

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