Proc. Nat. Acad. Sci. USA
Vol. 72, No. 12, pp. 4744–4748, December 1975
Biochemistry

Isolation and characterization of two acidic proteins of 60S ribosomes from *Artemia salina* cysts

(elongation factors/embryology of *Artemia salina*/eukaryotic ribosomes/nucleotide binding)

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Communicated by Severo Ochoa, August 29, 1975

ABSTRACT **60S ribosomes from encysted gastrulae of the brine shrimp *Artemia salina* contain two acidic proteins, which are homologous to the *Escherichia coli* proteins L7 and L12. The proteins were purified and characterized with respect to molecular weight, amino-acid composition, peptide maps, and their functional requirement in the elongation factor dependent binding of aminoacyl transfer RNA to the ribosome.**

The acidic ribosomal proteins L7 and L12, which differ only in that the amino-terminal serine residue of L7 is acetylated, play an important role in bacterial protein synthesis. The two proteins are involved in hydrolysis of guanosine triphosphate, a requirement for peptide chain elongation, and the bulk of evidence indicates that they supply a transient binding site on the ribosome for the elongation factors EF-G and EF-Tu (for a review see ref. 1).

Recently evidence has been presented that rat liver ribosomes possess a protein that is antigenically related to L7 and L12 (2, 3). In yeast the bacterial proteins L7 and L12 can replace the eukaryotic protein in at least some of the partial reactions of protein synthesis (4). The combined findings suggest a universal role for this acidic protein in ribosome function.

In this paper we report on the purification of eukaryotic L7 and L12 (EL7 and EL12) from the dehydrated gastrulae (cysts) of the brine shrimp *Artemia salina*. A number of the structural and functional properties of the protein have been defined, including their functional involvement in the elongation factor-1 (EF-1) dependent binding reaction of aminoacyl transfer RNA to the ribosome.

MATERIALS AND METHODS

80S, 60S, and 40S ribosomes were prepared in gram quantities from dried *Artemia salina* cysts (Metaframe, San Francisco Bay) according to the procedure of Zasloff and Ochoa (5). In a typical preparation starting from 300 g of cysts (dry weight), we obtained 800 mg of 80S ribosomes, 100–150 mg of 40S subunits, and up to 300 mg of 60S subunits.

In order to prepare ribosomes from developed *A. salina*, 300 g of cysts were suspended in 40 liters of 4% artificial sea salt (Weigand, Germany). The organism was allowed to develop for 36 hr at 29° under strong aeration. Free-swimming nauplii were collected and washed with sea salt solution on a nylon screen and subsequently with buffer A as described in ref. 5. Extracts were prepared by grinding at 2°; ribosomes and ribosomal subunits were prepared as described for the cysts.

Proteins of ribosomes and ribosomal subunits were extracted with 67% acetic acid (6). The extract was dialyzed for 48 hr against 0.1% formic acid followed by lyophilization. All isolation operations were performed at 2°. In order to isolate EL7 and EL12, 300 mg of 60S *A. salina* protein (7) were dialyzed against 50 ml of 0.01 M NH_4OAc, pH 6.0, 1 mM 2-mercaptoethanol, 6 M urea followed by application to a carboxymethyl-cellulose column (Serva CM, grade A, 0.6 meq/g, 2.5 × 18 cm), equilibrated with the same buffer in 6 M urea. The column was washed with the equilibration buffer until the acidic protein fraction was completely eluted. The acidic protein fraction was dialyzed against 0.01 M NH_4OAc, pH 5.7, 1 mM 2-mercaptoethanol, 6 M urea and applied to a DEAE-cellulose column (Whatman DEAE 32), equilibrated with the same buffer. The latter column (1.5 × 28 cm) was developed with a linear gradient of 600 ml each of 0.01 M NH_4OAc, pH 5.7, 1 mM 2-mercaptoethanol, 6 M urea, and 0.2 M NH_4OAc, pH 4.5, 1 mM 2-mercaptoethanol, 6 M urea. The molarity of the acetate buffer refers to acetic acid, which was adjusted with concentrated ammonia to pH 5.7 or pH 4.5.

Protein EL12 eluted at pH 4.8 after about 480 ml of gradient buffer had passed through the column, followed by EL7 starting at an elution volume of about 640 ml. The two peak fractions, monitored at 230 nm, were desalted by extensive dialysis against 0.1% formic acid, and lyophilized. The yield from 300 mg of 60S ribosomal protein was 1.6 mg of EL7 and 0.4 mg of EL12. Estimates of the protein content of the dried samples are based on amino-acid analyses using a Beckman microanalyzer.

Two-dimensional polyacrylamide gel electrophoresis was performed in 6 M urea (8) with the following modification. The first dimension contained 8% acrylamide on the anodic side and 4% acrylamide on the cathodic side. Sodium dodecyl sulfate gel electrophoresis was performed essentially according to Weber and Osborn (9). The method used for isoelectric focussing in polyacrylamide gels has been described (10). Peptide mapping was performed essentially according to Clegg *et al.* (11). In order to wash EL7 and EL12 selectively from the 60S ribosomes of *A. salina* cysts, we applied the method of Hamel *et al.* (12). Their salt-ethanol treatment selectively removes L7 and L12 from the 50S ribosomes of *Escherichia coli*. 60S ribosomes (22 mg) were suspended in 10 ml of 1 M NH_4Cl, 20 mM $Mg(OAc)_2$, 10 mM Tris·HCl, pH 7.4, 1 mM 2-mercaptoethanol. Two 5-ml portions of ice-cold ethanol were added dropwise within 15 min, while the mixture was kept at 0°. The cloudy suspension was centrifuged at 0° for 20 min at 20,000 × *g* and the pellet was dissolved in 5 ml of standard buffer A (5), followed by a second ethanol extraction. The final pellet was dissolved in 0.5 ml of buffer A and dialyzed overnight against this buffer. Prior to use in the transfer RNA binding assay, the 60S P_1-core solution was adjusted to 15 mM

Abbreviation: EF, elongation factor.

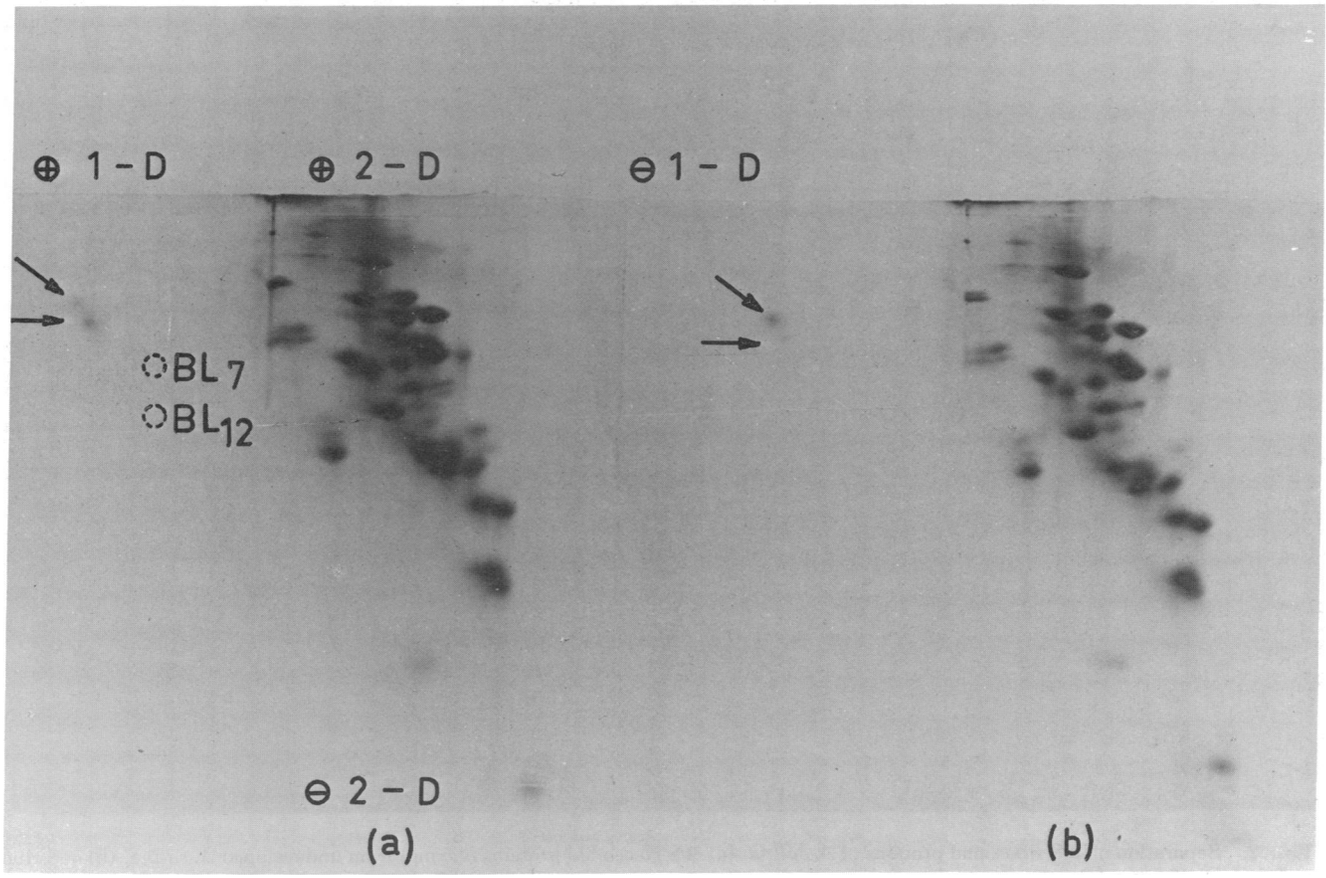

FIG. 1. Separation of 60S ribosomal proteins of *A. salina* by two-dimensional polyacrylamide gel electrophoresis. (a) 60S ribosomal proteins (0.5 mg) from developed *A. salina;* dotted lines indicate corresponding positions of bacterial L7 and L12 (BL7 and BL12) from 50S *E. coli* ribosomes. (b) 60S ribosomal proteins (0.5 mg) from undeveloped *A. salina*. Upper and lower arrows indicate position of EL7 and EL12, respectively.

$Mg(OAc)_2$ and heated for 1 hr at 37°. The supernatants from the ethanol washes were dialyzed against 0.1% formic acid and P_1-protein was recovered by lyophilization. Elongation factor-1 (EF-1) from *A. salina* cysts was prepared according to an unpublished procedure. The purified, intact enzyme gave one band on gel electrophoresis at pH 8.9 (13) and sedimented at the same position as beef liver catalase (molecular weight, 250,000) on a sucrose gradient. This behavior is in agreement with the statement of Nombela and Ochoa (14), who report a molecular weight of EF-1 larger than 200,000.

RESULTS

A comparison between the two-dimensional patterns of ribosomal proteins from developed (Fig. 1a) and undeveloped (Fig. 1b) 60S ribosomes reveals a rather similar pattern. In the acidic region of the gel, one observes two main protein spots which are well separated from the bulk of the proteins. The two proteins migrate to a position of the gel roughly comparable to the acidic proteins L7 and L12 from *E. coli* 50S ribosomes (Fig. 1a). Similar acidic protein spots were seen by us in 60S ribosomes from yeast and rabbit reticulocytes (4).

A comparison was also made between 40S proteins from ribosomes from undeveloped and developed *A. salina*. So far no discernible difference has been observed between the ribosomal proteins from the two states (Fig. 2).

The two main acidic proteins from 60S ribosomes of *A. salina* cysts were purified to homogeneity by means of carboxymethyl-cellulose and DEAE-cellulose chromatography (*Materials and Methods*). Analytical isoelectric focussing and sodium dodecyl sulfate gel electrophoresis of the puri-

Table 1. Amino-acid compositions of EL7 and EL12 proteins of *Artemia salina* cysts

Amino acid	EL7	EL12	L7/L12 (*E. coli*)
Lys	8.0	8.2	10.1
His	0.0	0.0	0.0
Arg	0.7	0.8	0.8
Asp	7.4	7.8	6.6
Thre	2.9	3.1	3.0
Ser	6.4	6.4	5.1
Glu	17.3	16.9	14.9
Pro	4.0	4.2	1.7
Gly	14.0	10.6	6.9
Ala	18.5	19.1	24.0
Val	2.4	2.7	13.5
Met	3.7	4.1	2.6
Ile	3.8	4.2	3.4
Leu	8.1	8.8	5.8
Tyr	1.7	1.3	—
Phe	1.4	1.6	1.5
Cys	0.0	0.0	0.0

The protein was hydrolyzed under reduced pressure under nitrogen for 29 hr in 6 M HCl, 0.02% 2-mercaptoethanol at 110° and the amino-acid composition was determined in a Beckman microanalyzer. Compositions are given in mole percent. Tryptophan was not determined.

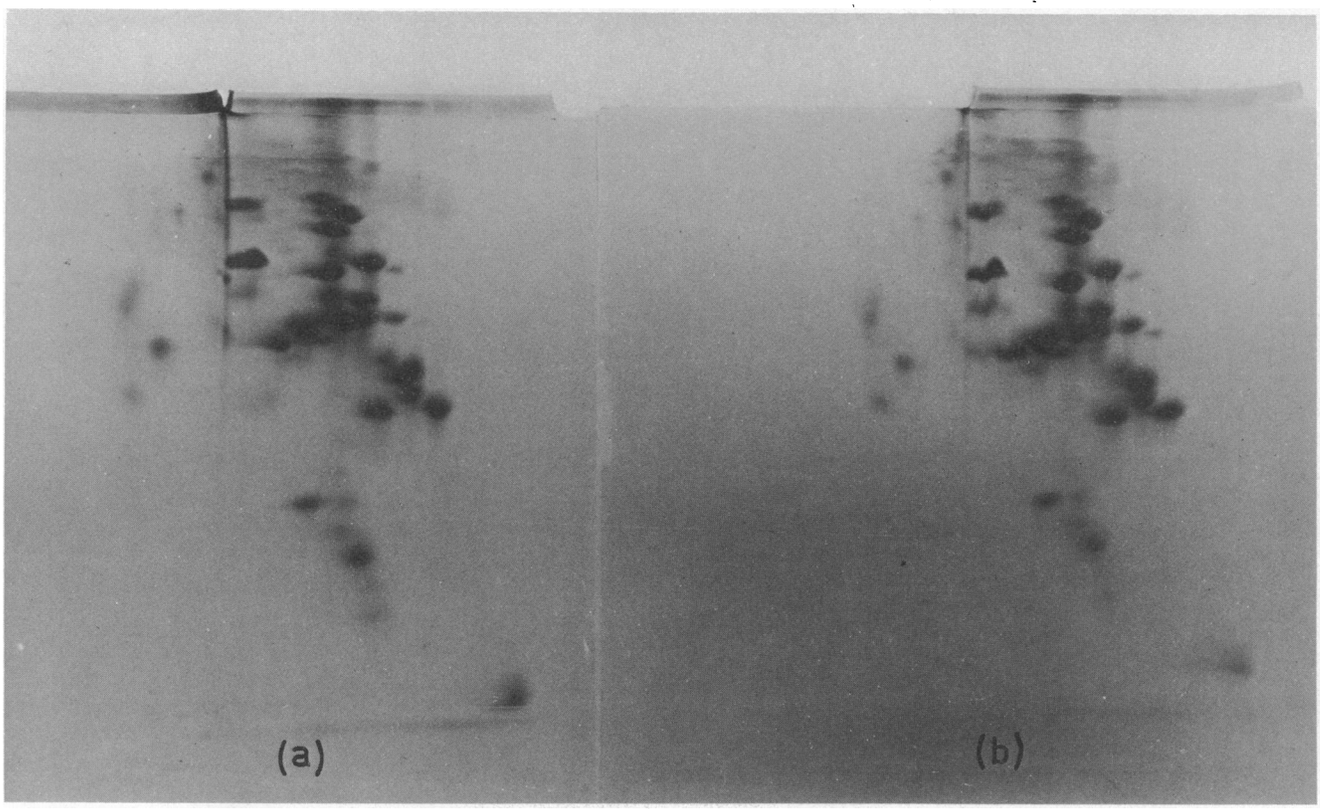

FIG. 2. Separation of 40S ribosomal proteins of *A. salina*. (a) 40S ribosomal proteins (0.5 mg) from undeveloped *A. salina*. (b) 40S ribosomal proteins (0.5 mg) from developed *A. salina*.

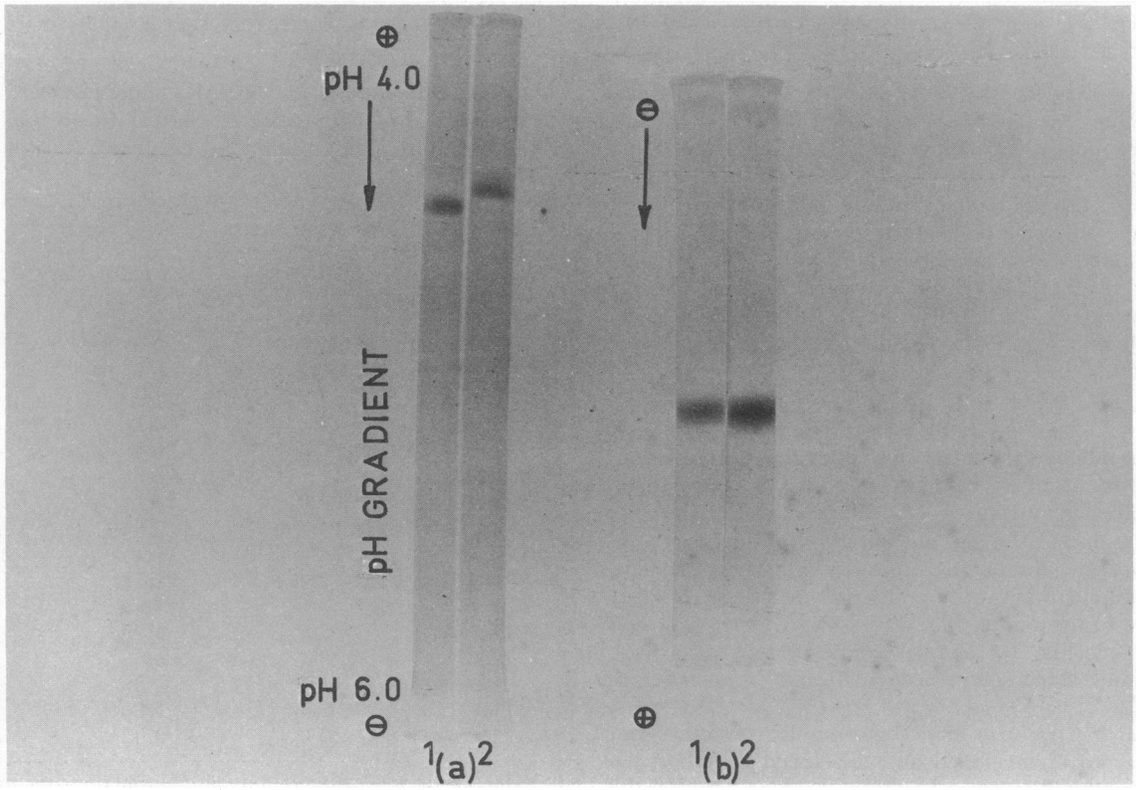

FIG. 3. (a) Isoelectric focussing patterns of EL12 (*1*) and EL7 (*2*) in a 5% polyacrylamide gel, containing a pH 4–6 gradient of Ampholine; L.K.B. Instruments; 6 M urea. (b) Sodium dodecyl sulfate gel electrophoresis of EL12 (*1*) and EL7 (*2*). Concentration of polyacrylamide gels 10% in 0.1% sodium dodecyl sulfate, 50 mM sodium tetraborate, pH 9.2.

Table 2. Dependency of EF-1 linked Phe-tRNA binding on 60S P_1-core particles, 40S ribosomal subunits, and purified 60S acidic proteins in an *Artemia salina* cell-free system

Additions	% EF-1 dependent Phe-tRNA binding Exp. 1	Exp. 2
40S + 60S	100	100
60S	5	—
60S core	—	4
60S EL7	3	2
40S	—	25
40S + 60S P_1-core	34	32
40S + 60S P_1-core + 50S L12 (*E. coli*)	70	—
40S + 60S P_1-core + 60S P_1-protein	55	—
40S + 60S P_1-core + 60S EL12	67	77
40S + 60S P_1-core + 60S EL7	74	—

One hundred percent activity corresponds to experiments where 60S (0.5 A_{260} unit) and 40S (0.7 A_{260} unit) were used. EF-1 directed Phe-tRNA binding to ribosomes was carried out in 50 μl of reaction mixture with 0.7 A_{260} unit of 40S, 10 pmol of [^{3}H]Phe-tRNA (specific activity, 1000 pmol/mg of tRNA), 10 μg of poly(U), 2 μg of purified 60S acidic protein (with exception of 5 μg of EL12 in experiment 2), 5 μg of bacterial L12, 2 μg of EF-1, and 0.5 A_{260} unit of 60S P_1-core particle. One hundred percent activity corresponds to 4 pmol of [^{3}H]Phe-tRNA bound per A_{260} unit of ribosomes. Assays were performed in 20 mM Tris·HCl, pH 7.4, 100 mM KCl, 5 mM $Mg(OAc)_2$, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.25 mM GTP. Incubation was for 20 min at 30°. Values reflect extent of reaction within 20 min.

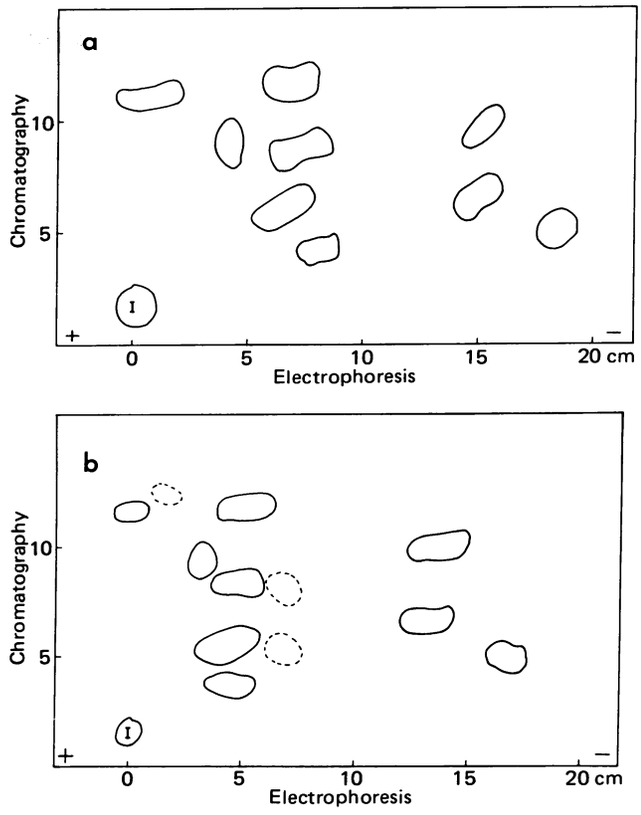

FIG. 4. Tryptic peptide maps of EL7 (a) and EL12 (b). EL7 or EL12 (120 μg) was dissolved in 0.25 ml of 1.2% (wt/vol) $(NH_4)_2CO_3$ (pH 8.5) and digested at 37° for 2 hr with 5 μl of TPCK-trypsin (1 mg/ml). The digest was then lyophilized, dissolved in water, and relyophilized. High-voltage electrophoresis (40 V/cm, 75 min) was performed in a Camag cooled-plate apparatus; chromatography was at room temperature for 5 hr. The peptide map was dipped into a solution prepared by mixing equal volumes of 2.4 mg of fluorescamine (4-phenylspiro[furan-2(3),1′-phtalan]3,3-dione) in 100 ml of acetone, and of 0.2 ml of pyridine in 100 ml of acetone. After drying, the peptides were located on the map under a UV light source.

fied EL7 and EL12 demonstrated that both proteins had similar molecular weights while possessing a slight difference in isoelectric point (about pH 4.5). As seen from Fig. 3a, EL12 is more basic than EL7, in agreement with their relative elution positions on DEAE-cellulose. The molecular weights of EL7 and EL12 were estimated from sodium dodecyl sulfate gel electrophoresis (Fig. 3b) to be approximately 13,000. The similarity in structure of EL7 and EL12 is further indicated by the results of amino-acid composition (Table 1) and peptide mapping (Fig. 4) of the two proteins. Possibly with exception of glycine, the amino-acid contents of EL7 and EL12 differ by less than one residue on the basis on their estimated molecular weights. Moreover, the peptide maps of the two proteins show a similar pattern of spots (Fig. 4).

The role of EL7 and EL12 in the EF-1 dependent binding of aminoacyl tRNA to ribosomes was examined. As is the case with 50S ribosomes from *E. coli* (12), ethanol-salt extraction of 60S ribosomes from *A. salina* led to the detachment of the acidic protein from the 60S ribosomal core. On the other hand the P_1-protein showed the presence of EL7 and EL12 together with a number of more basic components (results not shown). The 60S P_1 core particles, when supplemented with 40S ribosomes, supported the EF-1 dependent binding of Phe-tRNA only to a low level (Table 2). Addition of purified EL7 and EL12 to these cores largely restored the EF-1 dependent binding. The 50S ribosomal protein L12 from *E. coli* is also capable of restoring part of the activity. A similar result was obtained earlier with a yeast core supplemented with L7 and L12 from *E. coli* (4).

During our studies we observed variations in the extent of binding of Phe-tRNA to 60S reconstituted particles. Heating of the 60S P_1 core particles in a buffer, containing 15 mM magnesium acetate, prior to the reconstitution (see *Materials and Methods*) improved the binding efficiency of aminoacyl tRNA. The mechanism and minimum requirements of the heat activation step is presently under investigation.

DISCUSSION

From a comparison of the total number of ribosomal proteins and the sizes of their constituent RNAs, it appears that the structure of the ribosome is different in prokaryotes and eukaryotes (2). Nevertheless our studies support the earlier observations (2–4) that the general structure and function of L7 and L12 are conserved during evolution, indicating a vital role for these proteins in protein biosynthesis. The ability of bacterial L7 and L12 to replace their eukaryotic counterpart in the EF-1 dependent binding of Phe-tRNA suggests the existence of a conservative domain in the protein such as to assure a universal recognition site for the elongation factors. The limited interchangeability between the factors from prokaryotes and eukaryotes suggests that other components of the ribosome besides L7 and L12 are also involved in the recognition of the elongation factors (4, 15).

We have attempted to determine the number of copies of EL7 and EL12 in the 60S ribosome. In this regard it should

be mentioned that both acidic proteins usually stain lightly with Coomassie blue (see also ref. 16). However, so far our chromatographic procedure (uncorrected for losses) gave less than one copy of EL7/EL12 per ribosome. Therefore, more work is required to determine the stoichiometry of EL7 and EL12. As is the case with bacteria, two related forms of a 60S acidic protein were found in *A. salina*. The small charge difference could well be due to a side chain modification, as is the case with the bacterial L7 and L12 (17), although a more complicated relationship cannot be excluded presently.

We thank Mr. J. Kriek for his perseverance in the fractionation work, Mr. W. J. M. Pluijms for his amino-acid analyses, and Mrs. A. Rutten for the two-dimensional gel electrophoresis. The help of Dr. J. A. Maassen in the early phase of this work is gratefully acknowledged. This investigation was supported in part by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) and a grant from the National Science Foundation, U.S.A.

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