THE STOICHIOMETRY AND RECONSTITUTION OF A STABLE PROTEIN COMPLEX FROM *ESCHERICHIA COLI* RIBOSOMES

Ingvar PETTERSSON and Anders LILJAS
Department of Molecular Biology, University of Uppsala, Box 562, S-751 22 Uppsala, Sweden

Received 10 November 1978

1. Introduction

The *Escherichia coli* ribosomal protein L12 and its α-aminoacetylated form L7 is the only ribosomal protein present in more than 1 copy per ribosome. It is generally agreed that there are 4 copies of L7/L12 per ribosome [1,2]. L7/L12 is known to play an important role for the binding of all the GTP-hydrolyzing factors in protein synthesis [3]. Ammonium chloride–ethanol extraction of 50 S particles allows the simultaneous removal of proteins L7/L12 and L10 [4] and studies on the rebinding of L7/L12 and L10 to core particles show that L10 is needed for the binding of L7/L12 [5,6]. The existence of an L7/L12–L10 neighborhood in the particle is also supported by crosslinking data [7]. The observation of an L7/L12–L10 complex on urea–acrylamide gels [8] initiated studies on the properties of L7/L12 and L10 in solution. It was found that L7/L12 exists either as a stable, elongated dimer [9] or in a complex with L10 [10].

The molecular weight of this complex suggested that it was made up of 4 copies of L7/L12 and 1 copy of L10. This study provides more precise information about the stoichiometry of the L7/L12–L10 complex extracted from ribosomes as well as the complex formed from the purified proteins in vitro. We conclude that 4 copies of L7/L12 and 1 copy of L10 make up a neighborhood in the ribosome that is very similar to the complex formed from pure components.

2. Materials and methods

2.1. *Extraction and purification of proteins*

50 S ribosomal subunits were prepared from *E. coli* MRE 600 as in [11]. ^{3}H- and ^{14}C-labelled proteins were either obtained from cells grown on media containing labelled lysine or labelled in vitro by reductive methylation [12].

Proteins were extracted by described methods with some modifications [4,13,14]. The resulting supernatant was dialysed against 20 mM Tris–HCl (pH 7.6), 300 mM NaCl, 0.10 mM phenylmethyl sulfonylfluoride (PMSF) and 6 mM β-mercaptoethanol and subsequently concentrated by pressure dialysis on Diaflo UM-2 membranes (Amicon). The concentrated supernatant was chromatographed on Sephadex G-100 columns equilibrated with the above-mentioned buffer. L7/L12 and the L7/L12–L10 complex elute as separate peaks and can be obtained pure after this step. L10 was in some cases further purified essentially by the method in [15]. The identification of the proteins was done by two-dimensional gel electrophoresis [16,17] and by SDS–gel electrophoresis.

2.2. *Determination of the stoichiometry of the L7/L12–L10 complex*

An isotope dilution procedure was used to determine the stoichiometry of complexes. Purified [^{14}C]L10 was added to [^{3}H]L7/L12–L10 complex. Then the mixture was electrophoresed on a 15%

SDS–acrylamide gel, which separates L7/L12 from L10. The gel was stained for protein, the bands cut out and treated with Nuclear Chicago Solubiliser before counting. The counts were corrected for ^{14}C spillover and background. The ratio of ^{14}C-label to ^{3}H-label was measured both in the mixture containing the complex and in the gel band containing L10. The advantage of this procedure is that it is not necessary to recover 100% of the counts from the gel. One assumption is made, namely, that ^{3}H- and ^{14}C-labelled L10 is recovered to the same extent after electrophoresis. The isotope ratios are then used in the following expression which will describe the mass fractions in the complex:

$$A = \frac{^{3}H}{^{14}C} \text{ ratio in the mixture}$$

$$= \frac{[^{3}H]L7/L12+[^{3}H]L10 \text{ in the complex}}{[^{14}C]L10 \text{ added to the complex}}$$

$$B = \frac{^{3}H}{^{14}C} \text{ ratio in the L10 band}$$

$$= \frac{[^{3}H]L10 \text{ from the complex}}{[^{14}C]L10 \text{ from the added reference}}$$

$$\frac{A}{B} - 1 = \frac{[^{3}H]L7/L12 \text{ in the complex}}{[^{3}H]L10 \text{ in the complex}}$$

= stoichiometry of the complex

2.3. *Reconstitution of an L7/L12–L10 complex*

Purified, ^{3}H-labelled L7/L12 and L10 were mixed in 20 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 360 mM NaCl and 15 mM β-mercaptoethanol with about 200 μg protein/ml. The mixture was heated to 37°C for 10 min, cooled on ice and chromatographed on Sephadex G-100 Superfine columns. Fractions were collected and counted. The elution volumes of the complex, L7/L12 and L10 were determined with purified proteins.

2.4. *Amino acid analysis*

Samples of ribosome-extracted and reconstituted complex were lyophilised from 1 M HCOOH and then hydrolysed in 6 M HCl for 24 h at 110°C in vacuo. The samples were analysed on a Beckman amino acid analyser 121 M.

2.5. *Statistical analysis of amino acid analysis data*

To express the fit between amino acid analysis data from ribosome-extracted complex and different calculated L7/L12–L10 complexes a reliability factor R was used. R is calculated by the formula:

$$R = 100 \times \sqrt{\frac{\sum_i (X_{\text{obs}\,(i)} - X_{\text{calc}\,(i)})^2}{\sum_i ((X_{\text{obs}\,(i)} + X_{\text{calc}\,(i)})/2)^2}}$$

where $X_{\text{obs}(i)}$ = the mole fraction of residue i in the ribosome-extracted L7/L12–L10 complex and $X_{\text{calc}\,(i)}$ = the mole fraction of residue i in the calculated L7/L12–L10 complex.

The amino acid sequences for L7/L12 and L10 [18–20] were used for calculating the mole fractions of the residues in the calculated L7/L12–L10 complexes.

3. Results

3.1. *Protein extraction and purification*

The proteins extracted at various steps by the

Table 1
Proteins released at various NH_4Cl concentrations

Proteins released	(A)	(B)	(C)
L7/L12	++	++	(+)
L10	–	–	++
L7/L12–L10 complex	+	++	+
Other proteins	–	(+)	+

50 S particles were extracted with NH_4Cl–ethanol as in section 2. (A) 1 M NH_4Cl, (B) 2 M NH_4Cl, (C) 2 M NH_4Cl with particles pre-extracted with 1 M NH_4Cl

Table 2
Determination of the stoichiometry of the ribosome-extracted L7/L12–L10 complex

[^{3}H]L7/L12–L10 complex + [^{14}C]L10 reference				[^{3}H]L10 from complex + [^{14}C]L10 from reference			[^{3}H]L7/L12 in the complex / [^{3}H]L10 in the complex
	^{14}C cpm	^{3}H cpm	$\frac{^3H}{^{14}C}$	^{14}C cpm	^{3}H cpm	$\frac{^3H}{^{14}C}$	$\frac{A}{B}-1$
Exp. 1	992	1236	A_1 = 1.246	1721	431	B_1 = 0.250	3.98
Exp. 2	754	8222	A_2 = 10.90	1113	2416	B_2 = 2.17	4.02

The experiments and calculations are carried out as in section 2

ammonium chloride–ethanol procedure are summarised in table 1. Using 1 M ammonium chloride L7/L12 is washed off and only a very small fraction of the ribosomes lose L10. When salt is raised to 2 M, both L7/L12 and L10 are released, with L7/L12 released to a higher degree. In this case we find most of the released protein as the L7/L12–L10 complex along with some free L7/L12. If particles are extracted with 2 M salt after a 1 M extraction we find the complex and free L10 in the second extract. Thus, by manipulating the salt concentrations it is possible to control the relative amounts as well as purity of the proteins recovered as L7/L12–L10 complex, free L7/L12 or free L10. Other factors influencing the yield and the purity are the number of repeated extractions and the temperature at which the extraction is carried out.

3.2. *Determination of the stoichiometry of the extracted L7/L12–L10 complexes*

Two separate stoichiometry measurements using the isotope dilution procedure are presented in table 2. Experiment 1 used L7/L12–L10 complex extracted from ribosomes of cells grown on [^{3}H]Lys-containing medium. In exp. 2, the L7/L12–L10 complex was labelled in vitro with [^{3}H]HCHO.

In both cases a stoichiometry close to 4 molecules of L7/L12 bound to each molecule of L10 is obtained in accordance with the amounts of these proteins found in the ribosome [1,2].

Figure 1 shows the result of a statistical analysis of data obtained from an amino acid analysis of

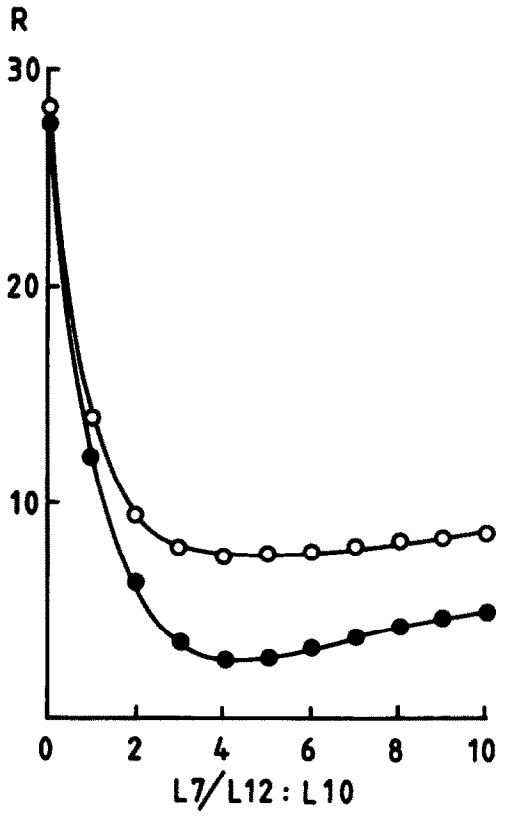

Fig.1. The R value as a function of the L7/L12–L10 ratio. R values were calculated as in section 2. (○) R values for ribosome-extracted L7/L12 complex. (●) R values for a calculated 4:1 L7/L12:L10 complex assuming ± 0.002 mol fraction error in the determination of each amino acid.

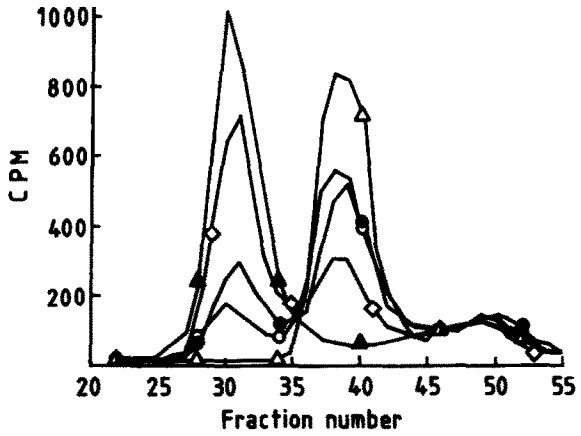

Fig.2. Reconstitution of the L7/L12–L10 complex in solution. [^{3}H]L7/L12 was incubated as in section 2 with increasing amounts of L10 and chromatographed on Sephadex G-100 Superfine columns. Reconstitution mixtures: (△) 500 pmol L7/L12 dimers; (○) 500 pmol L7/L12 dimers + 75 pmol L10; (●) 500 pmol L7/L12 dimers + 125 pmol L10; (◇) 500 pmol L7/L12 dimers + 250 pmol L10; (▲) 500 pmol L7/L12 dimers + 400 pmol L10.

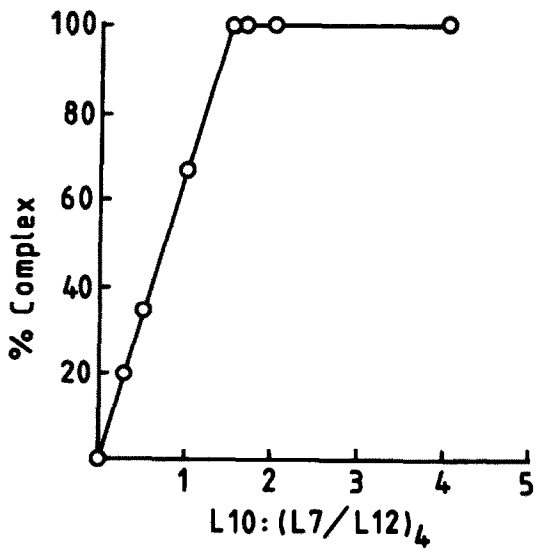

Fig.3. The amount of reconstituted L7/L12–L10 complex as a function of amount of added L10. The numbers are obtained from experiments like those described in fig.2.

$$\%\ \text{complex} = \frac{[^3\text{H}]\text{L7/L12 in complex} \times 100}{[^3\text{H}]\text{L7/L12 in complex} + [^3\text{H}]\text{L7/L12 as free dimers}}$$

L10 is plotted as pmol L10/pmol L7/L12 tetrameter.

ribosome-extracted complex. The fit between the real and the calculated L7/L12–L10 ratio is expressed in the reliability factor *R*. According to this criterion the best fit to the observed values is obtained by assuming a 4:1 L7/L12–L10 ratio in the ribosome-extracted complex.

3.3. *Reconstitution of a L7/L12–L10 complex from purified L7/L12 and L10*

Results from a set of reconstitution experiments are described in fig.2. In these experiments a constant amount of [^{3}H]L7/L12 was incubated with increasing amounts of L10. The mixture was chromatographed on a Sephadex G-100 Superfine column. When L10 is added to the reconstitution mixture the [^{3}H]L7/L12 starts to appear at the position of the L7/L12–L10 complex. No complex eluting at an intermediate position is seen. When the amount of complex formed is plotted against the amount of added L10 as in fig.3, a linear dependence on added L10 is seen up to about 1.5 copies of L10 per 4 copies of L7/L12. Beyond this point, all available L7/L12 molecules are part of the complex. The shape of the curve suggests a very strong complex formation. If the reconstituted complex is a pentamer also, maximum reconstitution at 1.5 copies of L10 per 4 copies of L7/L12 indicates that all L10 molecules are not able to complex with L7/L12.

Indeed, we have observed during the purification of L10 that it is unstable and easily converted to an insoluble form. This could explain why all the purified L10 is not competent for complex formation.

The stoichiometry of the reconstituted complex was determined by comparing its amino acid composition with the composition of the ribosome-extracted complex, fig.4. The near-identity in amino acid composition suggests that the complex formed in solution is also a pentamer with a 4:1 L7/L12:L10 ratio.

4. Discussion

The data presented here as well as in [22–24]

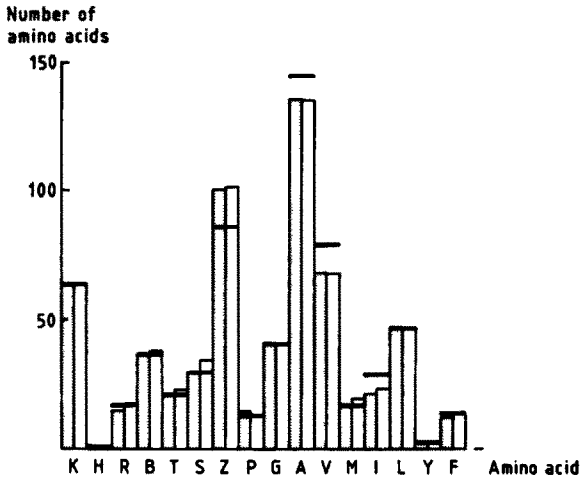

Fig.4. The amino acid composition of ribosome-extracted and reconstituted L7/L12–L10 complex. Open bars: ribosome-extracted complex. Filled bars: reconstituted complex. The horizontal line indicates the theoretical numbers that would be obtained from a 4:1 complex. Alanine, valine and isoleucine are underestimated due to incomplete hydrolysis after 24 h. The amino acids are denoted with the one-letter code of [21].

show that L7/L12 and L10 represents a self-organizing protein complex. The stability of this protein–protein interaction makes it reasonable to assume that the isolated complex retains at least some of the features of the L7/L12 domain in the ribosome, especially as rebinding of the complex of 50 S core particles restores Tu-dependent GTPase activity [25].

The stability of the complex is in fact such that we have not observed any dissociation unless strong denaturing reagents such as SDS or guanidinium hydrochloride are used (I.P., unpublished observation). The necessity of applying denaturing steps [22,24], or in our case heating to 37°C, in the reconstitution hints that some perturbation of the protein structures as they exist outside the ribosome is needed at least to initiate complex formation in vitro.

The complex is remarkable in that L10 seems to bind precisely 4 copies of L7/L12. No intermediates in the formation of the complex have yet been observed. It is relevant here that the amino acid sequence of L10 [19,20] has no obvious repeat structure. Since L7/L12 alone does not form tetramers, and since L10 does not produce infinite aggregation with L7/L12, it seems that the two L7/L12 dimers in the complex must be arranged asymmetrically, i.e., they have somewhat different conformations. What this might mean with regard to interaction with factors, GTP hydrolysis [25] and the possible interaction with the 30 S particle [26] is discussed in [27].

Acknowledgements

We thank Professor C. G. Kurland for continuous support and interest, Dr Dieter Donner for providing labelled subunits and for valuable suggestions and Professor Kirk C. Aune for computing the statistical analysis. Mr J. Ericson is thanked for running the amino acid analyses. The technical assistance of Mrs B. Wiklund, Mr B. Karlsson and Mr S. Eriksson is gratefully acknowledged. This work was supported by the Swedish Natural Science Research Council and the Magnus Bergvall Foundation.

References

[1] Hardy, S. J. S. (1975) Molec. Gen. Genet. 140, 253–274.
[2] Subramanian, A. R. (1975) J. Mol. Biol. 95, 1–8.
[3] Möller, W. (1974) in: Ribosomes (Nomura, M. et al. eds) pp. 711–731, Cold Spring Harbor Press.
[4] Hamel, E., Koka, M. and Nakamoto, T. (1972) J. Biol. Chem. 247, 805–814.
[5] Schrier, P. I., Massen, J. A. and Möller, W. (1973) Biochem. Biophys. Res. Commun. 53, 90–98.
[6] Highland, J. H. and Howard, G. A. (1975) J. Biol. Chem. 250, 831–834.
[7] Expert-Bezançon, A., Barritault, D., Millet, M. and Hayes, D. H. (1976) J. Mol. Biol. 108, 781–787.
[8] Pettersson, I., Hardy, S. J. S. and Liljas, A. (1976) FEBS Lett. 64, 135–138.
[9] Österberg, R., Sjöberg, B., Liljas, A. and Pettersson, I. (1976) FEBS Lett. 66, 48–51.
[10] Österberg, R., Sjöberg, B., Pettersson, I., Liljas, A. and Kurland, C. G. (1977) FEBS Lett. 73, 22–24.
[11] Hardy, S. J. S., Kurland, C. G., Voynow, P. and Mora, G. (1969) Biochemistry 8, 2897–2905.
[12] Rice, R. H. and Means, G. E. (1971) J. Biol. Chem. 246, 831–832.
[13] Sander, G., Marsh, R. C. and Parmeggiani, A. (1972) Biochem. Biophys. Res. Comm. 47, 866–873.
[14] Glick, B. R. (1977) FEBS Lett. 73, 1–5.

[15] Hernandez, F., Vazquez, D. and Ballesta, J. P. G. (1977) Eur. J. Biochem. 78, 267–272.
[16] Kaltschmidt, E. and Wittmann, H. G. (1970) Proc. Natl. Acad. Sci. USA 67, 1276–1282.
[17] Kaltschmidt, E. and Wittmann, H. G. (1970) Analyt. Biochem. 36, 401–402.
[18] Terhorst, C., Möller, W., Laursen, R. and Wittmann-Liebold, B. (1972) FEBS Lett. 28, 325–328.
[19] Dovgas, N. V., Vinokurov, L. M., Velmoga, I. S., Alakhov, Yu. B. and Ovchinnikov, Yu. A. (1976) FEBS Lett. 67, 58–61.
[20] Heiland, I., Brauer, D. and Wittmann-Liebold, B. (1976) Hoppe-Zeyler's Z. Physiol. Chem. 357, 1751–1770.
[21] IUPAC–IUB Commission on Biochemical Nomenclature (1968) J. Biol. Chem. 243, 3557–3559.
[22] Gudkov, A. T. (1976) Dokl. Akad. Nauk SSSR 230, 979–980.
[23] Chu, F., Caldwell, P., Samuels, M., Weissbach, H. and Brot, N. (1977) BBRC 76, No. 2, 593–601.
[24] Gudkov, A. T., Tumanova, L. G., Venyaminov, S. Yu. and Khechinashivili, N. N. (1978) FEBS Lett. 93, 215–218.
[25] Donner, D., Villems, R., Liljas, A. and Kurland, C. G. (1978) Proc. Natl. Acad. Sci. USA 75, 3192–3195.
[26] Bäumert, H. G., Sköld, S-E. and Kurland, C. G. (1978) Eur. J. Biochem. 89, 353–360.
[27] Donner, D., Kurland, C. G., Liljas, A. and Pettersson, I. (1978) Proc. 12th FEBS Meet., Dresden, Pergamon, in press.