THE IN VIVO ORDER OF ADDITION OF RIBOSOMAL PROTEINS IN THE COURSE OF E.COLI 50S SUBUNIT BIOGENESIS

J.Pichon, J.Marvaldi and G.Marchis-Mouren

Institut de Chimie Biologique
Université de Provence
place V.Hugo - 13 Marseille 3e - France

Received March 20, 1972

Summary

Biogenesis of 50S ribosomal subunits in E.Coli has been studied by following the rate of appearance of pulse labeled ribosomal proteins onto the mature 50S subunit. Cells were pulse labeled for 3, 6 and 12 min. Individual 50S ribosomal proteins, first separated as 2 groups ("split" and "core") were purified by chromatography on CMC and analyzed by bidimensional gel electrophoresis. Unequal labeling was obtained. Proteins have been ordered according to increasing specific radioactivity at 3 min : 5 groups have been defined. These results are interpreted in term of protein addition in the assembly process of the 50S subunit.

Introduction

Biogenesis of the large ribosomal subunit from bacteria has been studied in vivo and in vitro by different approaches. Analysis of in vivo pulse labeled precursor particles indicated the assembly process to be sequential (1) and the rate of appearance of these ribosomal proteins in 50S subunits has been determined using the pulse labeling technique (2). Three groups of 50S ribosomal proteins seem to be added at different rates (2). In addition, information has been obtained from the analysis of natural and artificial particles which are possible intermediates in the biogenesis of the 50S subunit (3). In vitro reconstitution of active B. stearothermophilus and E.Coli 50S ribosomal subunits from their dissociated components has been achieved (4,5). Recently, the number of specific binding sites for proteins on 23S ribosomal RNA was investigated by immunochemical methods (6). A large amount of data concerning the mechanism of assembly of E.Coli 50S subunit is thus available. However, until now, no order of protein addition has been established. In the present work, the order of ad-

Copyright © 1972, by Academic Press, Inc.

dition of 50S ribosomal proteins has been determined by following the rate of appearance of pulse labeled ribosomal proteins in the mature subunit. The same work has been recently carried out for the E.Coli 30S ribosomal subunit (7).

Methods

E.Coli strain 19 ribonuclease I^-_{10} was grown to mid-log phase at 30°C in minimal medium 68 (70 min doubling time). Ribosomes were prepared according to Kurland (8). 70S ribosomes were purified by ultracentrifugation through a 5 - 20% sucrose gradient in T.S.M. buffer (50 mM Tris, pH 7.6, 3 mM succinate, 10 mM $MgCl_2$). 50S ribosomal subunit were prepared from dissociated 70S ribosomes, by the same procedure except that the $MgCl_2$ concentration was 0.1 mM. "Split" proteins were obtained from 50S subunit by the technique of Monier (9) except that the time of incubation was reduced to 4 hours. Proteins were extracted from the 23S core thus obtained according to the technique of Spitnik-Elson (10). "Split" and "core" proteins were purified by column chromatography on CM cellulose (11). After concentration the chromatography peaks were analyzed by bidimensionnal polyacrylamide gel electrophoresis (12). The individual protein spots were cut from the gels, dissolved in 0.15 ml H_2O_2 (30%) at 60°C for 8 hours in sealed vials, then cooled, and counted (13).

In each experiment, cells were labeled with ^3H L-leucine (35 Ci/mM) for at least 2 generations times. Then the cultures were pulse labeled with ^{14}C l-leucine (100 mCi/mM) for either 3, 6 or 12 min. The results were calculated first as specific radioactivities as the ratio of ^{14}C counts/ ^3H counts. Then to allow proper comparison of the amount of radioactivity incorporated at various labeling times, the specific radioactivity of each protein was divided by the corresponding specific activity of the total cellular protein at each time.

Results and discussion

The elution profile of "split" proteins (Fig.1) and "core" proteins

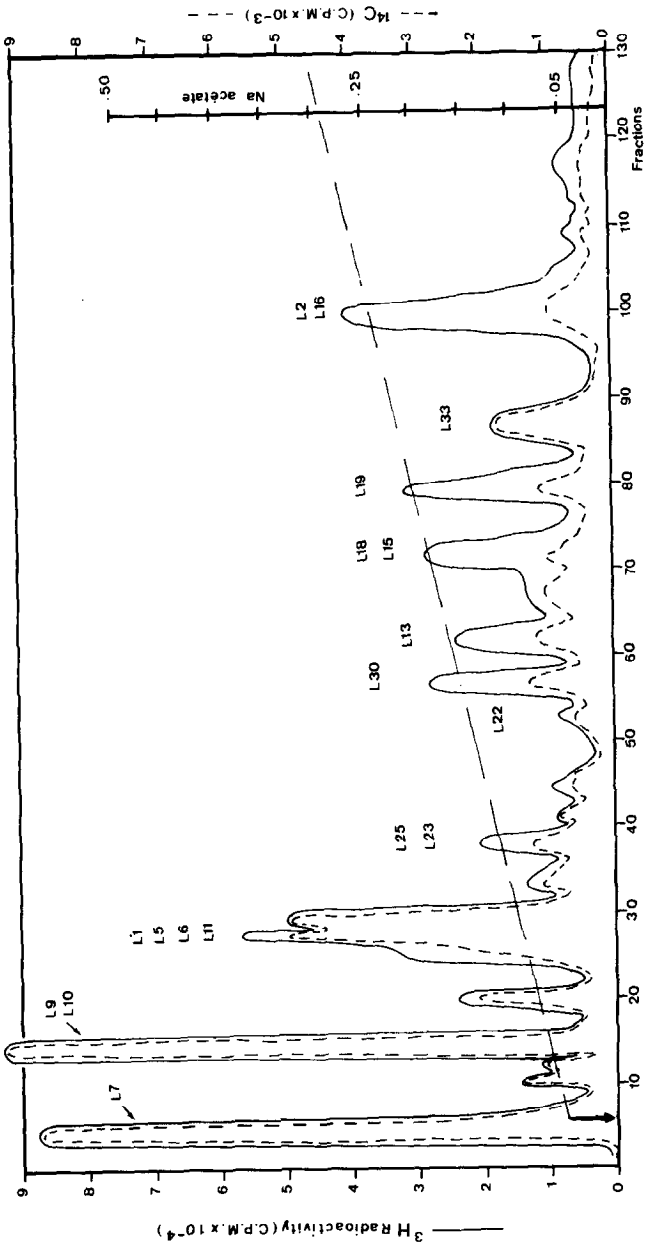

Fig. 1. Carboxymethyl cellulose elution profile of 50S "split" proteins. 10 mg of 3 min pulse labeled 50S protein was chromatographed on a (0.6 x 40 cm) CM 52 Whatman column equilibrated with acetate buffer 5 mM pH 5.6 in 6 M urea. As indicated the elution was carried out by increasing ionic strength (- -), volume of the fraction : 1.8 ml. The proteins in the peak were identified by gel electrophoresis.

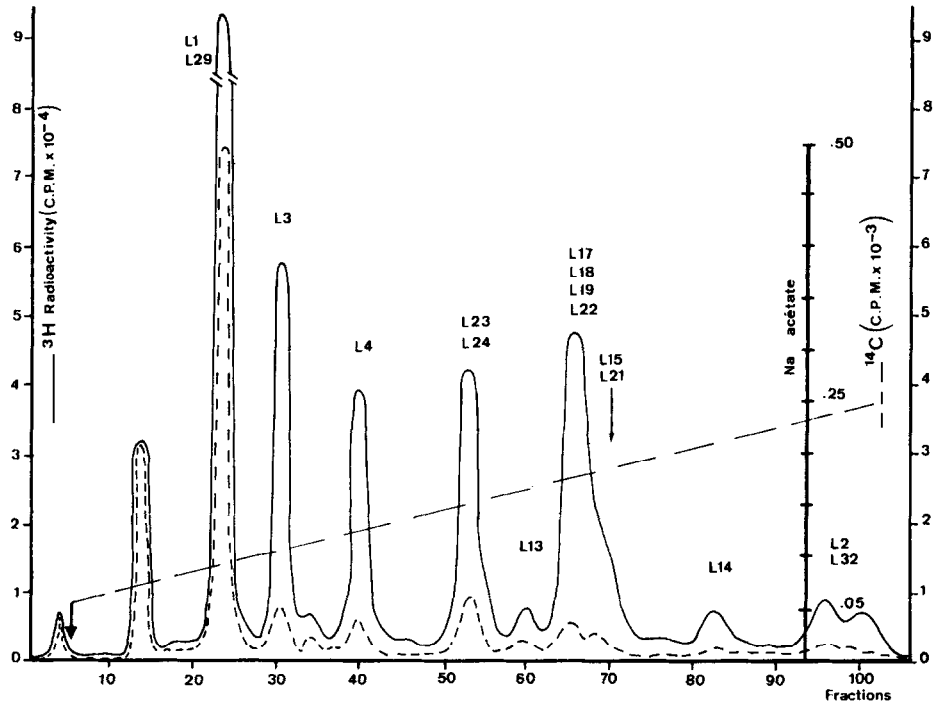

Fig. 2. Carboxymethyl cellulose elution profile of 50S "core" proteins. The conditions are as in Fig. 1.

(Fig. 2) from cells labeled for 3 min. indicate that some peaks contain very little or no ^{14}C radioactivity, while others are highly labeled. Analysis of the peaks by gel electrophoresis reveals those fractions containing ribosomal proteins. Most of the 50S proteins obtained by chromatography of the "split" proteins (SP) and the "core" proteins (CP) have been identified, some proteins are present in both groups (L1, L22, L23). However, 8 proteins are missing, most of them are "split" proteins (P.Gray personnal communication) : L8 (SP), L11 (SP), L14 (CP), L26 (?), L27 (SP), L28 (SP-CP), L31 (?), L34 (?),.At this time pulse (3 min), although unequal labeling is already apparent in both the CP and the SP, more label is found in the "split" proteins. After final purification and characterization of proteins from the peaks by bidimensional gel electrophoresis, the amounts of labeled individual proteins were counted.

Differential labeling is obtained at each time pulse. At 3 min very lar-

ge differences in the specific radioactivity ratios are found (0-645), at 6 min unequal labeling is still obtained (97-570) while at 12 min most of the protein ratios are in the same range (400-500). The proteins are arranged in Table I according to increasing incorporation at 3 min from the lowest to the highest value. At this time many of the proteins are poorly labeled (0-118) although a few are highly labeled proteins (204-645). Moreover within these 2 main categories some proteins appear with identical or very similar close labeling. From these data one may then define 5 groups : 1) L21- L5, 2) L32- L2, 3) L25 and L20, 4) L1 and L6, 5) L12- L33. One should note the high increase in labeling of the first 2 groups observed at 6 min, while the specific radioactivity ratios of the proteins of the last group remain unchanged.

According to sequential assembly, unlabeled or poorly labeled proteins are added early in ribosome assembly and remain there ; on the other hand

TABLE I - Specific radioactivity ratio (X 1000) of 50S ribosomal protein to the total protein of the cell (left) and comparison with the composition of particles (right) obtained from 50S subunits by LiCl treatment (reference 3).

Proteins	Specific radioactivity ratio at the indicated labeling time			Protein composition at different LiCl concentration			
	3 min	6 min	9 min	0.6 M	1 M	2 M	4 M
L21	0 -	-	481 ± 34	+	+	+	+
L22	9 ± 1	97 ± 8	307 ± 4	+	+	+	+
L3	9 ± 1	260 ± 6	488 ± 6	+	+	+	+
L4	18 ± 1	250 ± 3	529 ± 5	+	+	+	+
L13	23 ± 2	200 ± 10	511 ± 25	+	+	+	+
L23	36 ± 2	260 ± 10	477 ± 14	+	+	+	±
L24	45 ± 3	170 ± 8	411 ± 8	+	+	+	-
L18	68 ± 3	140 ± 4	411 ± 7	+	+	-	-
L5	63 ± 7	232 ± 21	340 ± 18	+	+	-	-
L32	90 ± 5	311 ± 49	514 ± 41	+	+	+	-
L17	90 ± 9	340 ± 18	533 ± 10	+	+	+	±
L19	90 ± 4	268 ± 11	522 ± 10	+	+	+	-
L15	86 ± 3	330 ± 6	570 ± 13	(+)	±	-	-
L29	109 ± 3	200 ± 7	352 ± 6	+	+	+	+
L16	109 ± 3	142 ± 5	326 ± 7	-	-	-	-
L30	118 ± 4	348 ± 8	452 ± 18	+	+	-	-
L2	118 ± 8	367 ± 28	518 ± 23	+	-	-	-
L25	204 ± 9	296 ± 9	400 ± 24	+	+	-	-
L20	245 ± 32	-	-	-	-	-	-
L1	350 ± 7	500 ± 5	503 ± 5	+	±	-	-
L6	363 ± 19	614 ± 27	585 ± 18	(+)	-	-	-
L12	522 ± 16	450 ± 18	-	-	-	-	-
L10	554 ± 9	540 ± 14	574 ± 12	+	-	-	-
L7	591 ± 16	527 ± 12	555 ± 54	-	-	-	-
L9	591 ± 8	510 ± 9	570 ± 13	+	+	-	-
L33	645 ± 14	570 ± 35	592 ± 56	±	±	-	-

counting errors were calculated by classical way (Packard operation manual 2018/1 p III-3).

+ present in normal amount.
(+) present in reduced amount.
± present in traces.
- not detectable.

highly labeled proteins are late proteins in the assembly sequence.

How significant is an assembly sequence obtained in this manner ? There are at least 2 variables which must be considered. First the technical precision and second the protein order obtained with pulses of longer times. The three technical problem one can encounter and must account for are the presence of ribosomal precursors to be removed during the purification of mature ribosomes, the elimination of non-ribosomal radioactivity and the problems involved in measuring the radioactivity (of proteins) in polyacrylamide gels. The presence of ribosomal precursors was eliminated by using only those subunits associated with 30S subunit as 70S ribosomes. Contamination of proteins with non-ribosomal radioactivity was effectively removed during chromatography (Fig. 1 and 2) and subsequent electrophoresis. The elution profiles of "split" and "core" proteins show a few non-identifiable peaks containing highly labeled non-ribosomal material which were thus eliminated. Counting of radioactivity in the gel slab did not reveal any contaminating material, radioactivity was only found localized at the protein spots. This of course does not exclude the possibility of radioactive contaminating material which has identical behaviour on the column and on the gel as the ribosomal proteins.

For the measurement of radioactivity, two questions were studied. One, the amount of quenching due to the polyacrylamide and H_2O_2 ; and two, the possible degradation of ^{14}C labeled protein as CO_2 in H_2O_2 (14). Quenching was limited to 25% and ^{14}C loss as CO_2 was reduced to a minimum by appropriate conditions as indicated in the techniques.

The significance of the protein order obtained in this work is complicated by changes in the sequences obtained after 3 min. These differences are especially apparent at 6 min where there are the following changes : L16, L29 and L23, L24, L18, L5 and L4, L3. At 12 min there are also changes between L29, L16 and L13, L23, L25 and L4, L5 and L3, L21. However, the gross order of the

3 min pulse remains largely unchanged. These changes may be due to prior differences in pool size and or biosynthetic cycling during cell growth.

We have compared our data with the protein patterns of "core" particles from 50S subunits obtained after CsCl equilibration centrifugation or incubation with increasing LiCl concentrations over the molarity range 0.4 to 4 M. As shown in Table I our results are in a fairly good agreement with the results obtained by these workers (3), with the exception of L16 and L9 which differ slightly when one compares their rate of labeling to their behaviour in 0.6 to 1 M LiCl. However, no specific correlation appears between our results and the 8 specific binding sites on the 23 S RNA demonstrated by Stöffler et al (6). We therefore conclude that a complete sequence for assembly may be represented by the 3 min pulse and that the proteins are added by groups : we propose 5 main categories of protein addition from early to late :

1) L21, L22, L3, L4, L13, L23, L24, L18, L5

2) L32, L17, L19, L15, L29, L16, L30, L2

3) L25, L20

4) L1, L6

5) L12, L10, L7, L9, L33

We are presently attempting to confirm this order more precisely and to determine the appearance rates for the eight 50S proteins not reported here. The kinetic aspect of this question will be also more carefully studied.

Acknowledgements

We are very much indebted to Dr H.G.Wittmann for training one of us (J.Pichon) in the gel electrophoresis technique. We are very grateful to Dr Gray for his advices in the preparation of the manuscript. We thank Miss J.Secchi for her skillfull technical assistance. This work has received a financial support from the CNRS (ERA 173) and from the CEA (For the furniture of radioisotopes).

References

1. Mangiarotti, G., Apirion, D., Schlessinger, D. and Silengo, L., Biochemistry 7, 456 (1968).

2. Sells, B.H. and Davis F.C., J. Mol. Biol., 47, 155 (1970).
3. Homann , M. and Nierhaus, K.H., Euro. J; Biochem., 20, 249 (1971).
4. Erdmann, V.A., Fahnestock, S., Higo. K. and Nomura, M., Proc. Natl. Acad. Sci. U.S., 68 , 2932 (1971).
5. Maruta, H., Tsuchiya, T., and Mizuno, D., J.Mol. Biol. 61, 123 (1971).
6. Stöffler, G., Daya, L., Rak, K.H. and Garrett, P.A., J.Mol.Biol.62, 407 (1971).
7. Marvaldi, J., Pichon, J, and Marchis-Mouren G., Biochim. Biophys. Acta (submitted).
8. Kurland, C.G. J. Mol. Biol., 18, 90 (1966).
9. Marcot-Queiroz, J. and Monier. R., Bull. Soc. Chim. Biol. 49, 477 (1967).
10. Spitnik-Elson, P., Biochem. Biophys. Res. Comm., 18, 557 (1965).
11. Otaka, E., Itoh, T., and Osawa, S., J.Mol. Biol., 33, 93 (1968).
12. Kaltschmidt, E. and Wittmann, H.G. Anal. Biochem., 36, 401 (1970).
13. Bray, G.A., Anal. Biochem., 1, 279 (1960).
14. Tishler, P.V. and Epstein, C.J., Anal. Biochem, 11, 389 (1965).