ON THE STOICHIOMETRY OF PROTEINS IN THE SMALL RIBOSOMAL SUBUNIT OF HEPATOMA ASCITES CELLS

P. WESTERMANN, W. HEUMANN and H. BIELKA

Central Institute of Molecular Biology, Academy of Sciences of GDR, Department of Cell Physiology, 1115 Berlin-Buch, GDR

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1. Introduction

For the sum of the protein mol. wts of the small subunit of rat liver ribosomes values of 585 000 [1], 592 000 [2] and 690 000 [3] have been reported. These values and the number of proteins suggest the possibility that the small ribosomal subunit contains one copy of most of the proteins [4,5].

SDS-electrophoresis of proteins from rabbit reticulocyte ribosomes have confirmed also the one copy hypothesis [6]. On the other hand experiments with a cellular slime mold point to different molar amounts of ribosomal proteins [7].

By using an in vivo labelling technique it is possible to show that most of the proteins of the small ribosomal subunit of hepatoma ascites cells are present in equimolar quantities. For two proteins about 2 equivalents were found and two proteins appear in less than one copy per subunit.

2. Materials and methods

2.1. *Preparation of [^{3}H]lysine labelled ribosomal subunits*

1.0 to 1.5 ml of a Zajdela hepatoma ascites cell suspension were injected intraperitoneally (i.p.) into female 8–9 month old rats. After 4 days 0.1 mCi [^{3}H]lysine (270 mCi/mmol; Institute for Isotopes, Budapest) were applied (i.p.); 24 h later the ascites suspension was harvested. The cells were sedimented by centrifugation for 15 min at 10 000 *g*, washed with a buffer containing 140 mM NaCl, 5 mM KCl and 5 mM $MgCl_2$ and lysed according to Schreier and Staehelin [8]. The cells were suspended in 2 vol (w/v) of a 5 mM $MgCl_2$–1 mM dithioerythrol solution and incubated for 10 min at 0°C. Thereafter 1.0 vol of a buffer containing 1.5 M sucrose, 0.2 M Tris·HCl and 0.15 M KCl (pH 7.8) and 0.2 vol of rat liver postmicrosomal supernatant were added and the mixture was homogenized once in a glass–Teflon Potter homogenizer. After two centrifugations for 10 min at 10 000 *g* the ribosomes were sedimented by centrifugation through a cushion of 0.5 M sucrose, containing 50 mM Tris·HCl, 500 mM KCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol (pH 7.8) and incubated for 30 min at 0°C and 10 min at 37°C in the presence of 0.1 mM puromycin [9]. The ribosomal subunits were separated by centrifugation for 16 h at 18 000 rev/min in a SW 25.2 rotor (Beckman Instr.) using a 15 to 30% sucrose gradient in the same buffer at 20°C. The subunits were precipitated by adding 20 mM $MgCl_2$ and 0.7 vol ethanol to the gradient fractions. In a second procedure run-off ribosomes prepared according to Staehelin [10] were used. The KCl concentration in the postmitochondrial supernatant was increased to 240 mM; 1 mM phosphoenolpyruvate and 0.2 mM GTP were added and the mixture was then incubated for 10 min at 37°C. Thereafter 1% Triton X-100 was added, the suspension filtered through DEAE-cellulose, washed with one volume of a buffer of 50 mM Tris·HCl, 260 mM KCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1% Triton X-100 (pH 7.8) and 1.0 vol of this buffer containing 280 mM KCl without Triton. The subunits were eluted with the same buffer containing 460 mM KCl and separated by centrifugation in a B XV zonal rotor (Beckman Instr.) for 195 min at 32 000

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rev/min a 10–50% sucrose gradient in a buffer made of 50 mM Tris·HCl, 500 mM KCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol (pH 7.8). The subunits were pelleted after dilution of the corresponding gradient fractions with 2 vol of a buffer of 50 mM triethanolamine·HCl, 30 mM $MgCl_2$ (pH 7.8) for 16 h at 35 000 rev/min in a rotor type 35 (Beckman Instruments).

2.2. *Extraction of ribosomal proteins*

The small ribosomal subunits were suspended in a buffer of 50 mM triethanolamine·HCl, 50 mM KCl, 5 mM $MgCl_2$ (pH 7.8) and clarified by centrifugation for 10 min at 10 000 *g*. After addition of 1 vol of a solution containing 4.0 LiCl and 8 M urea [11], or 1/3 vol of 1 M HCl [12] or 2 vol of acetic acid containing 100 mM $MgCl_2$ [13], the samples were stored for 4 h at 0°C, centrifuged for 10 min at 10 000 *g* and the supernatants dialysed against 6 M urea.

2.3. *Two-dimensional polyacrylamide gel electrophoresis*

Two-dimensional polyacrylamide gel electrophoresis and numbering of protein spots were performed as described by Welfle et al. [14]. The gel slabs were stained with 0.0005% Coomassie Brilliant Blue G250 (Serva, Heidelberg) in 3% trichloroacetic acid for 24 h. Thereafter the gel pieces containing the stained proteins were cut out, squashed using a syringe with an 1 mm aperture, incubated for 16 h with 1.0 ml 0.4 M

Table 1
Distribution of lysine residues of proteins of the small ribosomal subunit of rat liver calculated from their mol. wts, and amino acid compositions

Subunit proteins	Molecular weight ($\times 10^{-3}$)	Lysine content (per cent)	Number of lysine residues per protein	Percentage of lysines related to the total number of lysines
S 1	26.7	9.0	24.0	4.2
2	29.7	7.0	20.8	3.7
4	28.6	11.8	33.7	6.0
5	22.0	8.6	18.9	3.4
7	25.8	12.1	31.2	5.5
9	30.5	14.2	43.3	7.7
10	26.0	13.5	35.1	6.2
11	26.8[a]	6.5[b]	17.4	3.1
12	17.3	11.6	20.1	3.6
13	23.3	8.1	18.9	3.4
14	19.7	12.2[b]	24.0	4.2
15	17.3	10.6	18.3	3.2
16	13.7	9.2	20.7	3.7
17	17.1	8.3	14.2	2.5
18	19.0	17.2	32.7	5.8
19	17.6	11.5	20.2	3.6
20	14.8	10.4	15.4	2.7
21	13.0	10.4	13.5	2.4
22	14.0	11.5	16.1	2.8
23	18.2	5.8	16.6	1.9
24 + 25	17.3	12.4	42.8	7.6
26	18.2[a]	16.5[b]	30.0	5.3
27	16.0	11.5	18.4	3.3
28	10.6	7.6	8.1	1.4
29	10.0	9.4	9.4	1.7
30	8.0[a]	7.7	6.9	1.2

[a] The mol. wts of S11, 26 and 30 are taken from Welfle [15].
[b] The amino acid compositions of S11, 14 and 26 are given by Goerl [16].

hyamine hydroxide (Packard Instruments, Ill.) in toluene and counted after addition of 10 ml scintillation mixture (0.4% 2.5-diphenyloxazole, 0.01% 1.4-bis(5-phenyl-2-yl)-benzene, 10% Triton X-100, 0.3% acetic acid in toluene) in a Wallac Scintillation Counter 81 000 (LKB, Sweden) to an error of less than 7%.

3. Results

Using the mol. wts and amino acid compositions estimated for ribosomal proteins from rat liver [2,15,16] the number of lysine residues of 25 proteins can be calculated (see table 1). The number of lysines ranges from 8.1 to 43.3 residues per molecule, and a total number of 565 lysines was calculated for the proteins specified in table 1.

For S24 and S25 the amino acid composition of the mixture is known only, and a value of 12.4 mol lysine per 100 mol amino acids was used for both proteins. Proteins S3, S6 and S8 are not included because their lysine content is not yet known. For protein S31 a number of 25.7 lysine residues was computed, but the radioactivity could not be estimated because the protein spot had been enlarged during electrophoresis by diffusion so that an exact localisation was not possible.

Comparing the three protein extraction methods described under Materials and methods it becomes obvious that the most effective extraction of proteins was obtained by using LiCl–urea.

The standard deviations for the relative labels of the electrophoretically separated proteins presented in table 2 range from 3–27%. With regard to these deviations an error range of 30% was taken for comparison of the calculated distribution of lysines with the in vivo labelling.

The comparison shows that for 20 proteins (S1,2, 5,7,9,10,11,12,13,14,16,17,18,20,22,23,27,28,29,30) the labelling values correspond within an error range of 30% to the calculated lysine contents.

Proteins S4 and S26, however, show lower labelling values of about 50% in all three sets of experiments.

For proteins S15 and S19, the observed label was about twice as high as calculated on the basis of the lysine contents, as well as by using different extraction methods as by labelling times of 24 and 48 h. The values for the sum of S24 and S25 also exceed significantly the calculated value and give first evidence for the occurrence of more than two protein equivalents. The high value for S21, however, could not be confirmed by the other extraction methods.

Table 2
Distribution of [^{3}H]lysine label in proteins of the small ribosomal subunit

Subunit proteins	Calculated lysine percentage	Estimated percentage of label in electrophoretically separated protein spots
S 1	4.2	3.6 ± 0.5
2	3.7	2.6 0.5
4	6.0	3.6 0.2
5	3.4	2.7 0.1
7	5.5	4.3 0.7
9	7.7	5.4 0.5
10	6.2	7.1 0.2
11	3.1	3.1 0.5
12	3.6	3.1 0.2
13	3.4	2.5 0.2
14	4.2	4.9 0.1
15	3.2	7.9 0.4
16	3.7	3.9 0.4
17	2.5	2.2 0.2
18	5.8	4.4 0.3
19	3.6	5.8 0.5
20	2.7	3.0 0.2
21	2.4	3.8 0.3
22	2.8	2.6 0.3
23	1.9	1.5 0.2
24+25	7.6	11.4 1.0
26	5.3	3.3 0.7
27	3.3	3.1 0.3
28	1.4	1.5 0.4
29	1.7	1.2 0.3
30	1.2	1.5 0.3

The proteins were extracted by LiCl and urea [11] and electrophorized as described in Materials and methods.

4. Discussion

Besides unit proteins the small ribosomal subunit of E. coli possesses fractional and repeated proteins [17]. On the contrary, first evidence was reported that most of the proteins in the small subunit of eukaryotic ribosomes are present on the average as

one copy per subunit [2,4–6,18] using protein mol. wts. estimated by SDS-electrophoresis. The stoichiometry of proteins can also be estimated by using [^{3}H]lysine labelling of ribosomes in ascites cells. This method excludes additional errors caused by different protein staining intensities. The use of ascites cells for in vivo labelling of ribosomal proteins is advantageous because administration of 0.1 mCi [^{3}H]lysine per animal is sufficient to obtain a ribosomal protein mixture with 6 to $10 \cdot 10^4$ dpm per mg protein. The two-dimensional electrophoretic pattern of ribosomal proteins of Zajdela hepatoma ascites cells is identical with that of rat liver. Furthermore, ribosomes of several hepatomas have been compared with ribosomes of normal liver by immunological methods and no differences were found [19]. Therefore, we assume that the lysine contents of the corresponding ribosomal proteins of liver and hepatoma are very similar if not identical.

By the procedure described here, for 20 proteins relative labels were observed corresponding to about one protein molecule per small ribosomal subunit. The lower values for the proteins S4 and S26 were observed in all experiments using different protein extraction methods. For these two proteins we suggest that they appear in the subunit preparations used in amounts of less than one copy per subunit. It is possible that they are partially washed off by using high cation concentration during the subunit preparation. For S4 it is also possible that lysine containing peptides are lost by proteolytic cleavage during preparation [20].

On the other hand the values for the proteins S15 and S19 are higher than calculated in all three cases. On the average 2.2 equivalents were observed for S15 and 1.8 equivalents for S19. If we take the overlapping of both protein spots in the gel slabs into consideration, it seems justified to conclude that about two equivalents of both these proteins are present. The same results as described here for the 24 h labelling experiments were found after a labelling time of 48 h. This confirm the idea that the amount of the proteins and not differences in their rate of biosynthesis or incorporation into ribosomes cause the higher labelling values.

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