METHYLATION OF PROTEINS IN 60 S RIBOSOMAL SUBUNITS FROM *SACCHAROMYCES CEREVISIAE*

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1. Introduction

Some of the ribosomal proteins of *Escherichia coli* are methylated [1–7] and since the biological significance of protein methylation is not currently understood the phenomenon provides an important field of study. Post-translational methylation of ribosomal proteins may, for example, play a vital role in the assembly of the active ribosome and its subsequent functioning in protein synthesis.

Comb et al. [8] observed that ribosomal proteins from the aquatic fungus *Blastocladiella emersonii* could be methylated in vitro and methylation of ribosomal proteins has now been demonstrated in HeLa cells [9] although the proteins concerned were not identified. Terhorst et al. [1,2] have observed that proteins L7 and L12, in *E. coli* 50 S ribosomal subunits, are methylated and Alix and Hayes [4] have now claimed that in the same organism the ribosomal protein L11 is heavily methylated and contains three methyl groups in a single trimethyllysine residue and an equal number in another unidentified amino acid. Similar results have been reported by Chang et al. [5] and these same authors have shown that at least six and possibly ten proteins from the 50 S ribosomal subunit of *E. coli* are methylated both in vitro [6] and in vivo [5,7].

In the present investigation we have studied the methylation, in vivo, of proteins from the 60 S ribosomal subunit of *Saccharomyces cerevisiae*. Our results indicate that three and possibly six other proteins possess methylated amino acids; the methylation of one protein (L15) is particularly striking.

2. Materials and methods

S. cerevisiae strain A224A (leucine requiring) was maintained on agar plates and grown in complete synthetic medium as described previously for strain Y166 [10]. Ribosomes were labelled by growing cells in 150 ml of medium supplemented with 15 μCi of [1-^{14}C]methionine and 150 μCi of [*methyl*-^{3}H] methionine. When the absorbance at 550 nm had reached 0.8, sodium azide (final concentration 1 mM) was added and the incubation continued for 30 min. Cells were harvested by centrifugation, washed once with distilled water, resuspended in 3 ml of a solution containing 50 mM Tris–HCl buffer, pH 7.4, 50 mM KCl and 5 mM magnesium acetate (low salt buffer) and disrupted (1500 psi) in a French Pressure Cell. The extract was diluted with 5 ml of low salt buffer and centrifuged at 10 000 × *g* for 15 min in a Sorvall SS-34 rotor. The supernatant liquid was centrifuged for 2 h at 49 000 rev/min in a Spinco model SW50.1 rotor to pellet ribosomes and these were then resuspended in 2 ml of a solution containing 50 mM Tris–HCl, pH 7.4, 500 mM KCl, 5 mM magnesium acetate

and 1 mM dithiothreitol (high salt buffer). The sample was layered on top of a 32 ml 10–30% sucrose gradient prepared in high salt buffer and centrifuged for 6 h at 26 000 rev/min at 12°C in a Spinco model SW27 rotor. Gradients were analyzed at 254 nm in an Isco model DUA-2 gradient analyzer. Fractions corresponding to each ribosomal subunit were combined, diluted 1.5-fold with low salt buffer and the particles sedimented by centrifugation for 19 h at 45 000 rev/min in a Spinco model SW 50.1 rotor.

Ribosomal subunits were resuspended in 1 ml of 0.1 M magnesium chloride solution, extracted for 1 h at 0°C with 2 vol. of glacial acetic acid and the resultant precipitate removed by centrifugation at 10 000 × *g* for 15 min. The supernatant liquid was mixed with 5 vol. of acetone to precipitate protein and the precipitates were collected after centrifugation at 10 000 × *g* for 20 min. Protein precipitates were dried under vacuum and dissolved in 100 μl of a solution containing 8 M urea and 10 mM dithiothreitol. The ribosomal proteins (approximately 1 mg) were analyzed by two-dimensional gel electrophoresis essentially as described by Kaltschmidt and Wittmann [11]. Gels were stained overnight in Coomassie Brilliant Blue G250 – the dye being dissolved at 0.04% (w/v) in 3.5% perchloric acid–and protein spots were excised with a scalpel blade. Gel samples were dissolved by soaking overnight in 30% (v/v) hydrogen peroxide [12] and counted for ^{3}H-counts (20% efficiency) and ^{14}C-counts (42% efficiency) in Aquasol (New England Nuclear Corporation, Boston, Massachusetts). [1-^{14}C]Methionine (57 mCi/mM) and [*methyl*-^{3}H]methionine (6 Ci/mM) were obtained from The Radiochemical Centre, Amersham.

3. Results and discussion

Yeast ribosomal proteins can be separated using the procedure [11] originally developed for analysis of ribosomal proteins from *E. coli* and the results of such a separation are illustrated in fig.1a. Figure 1b

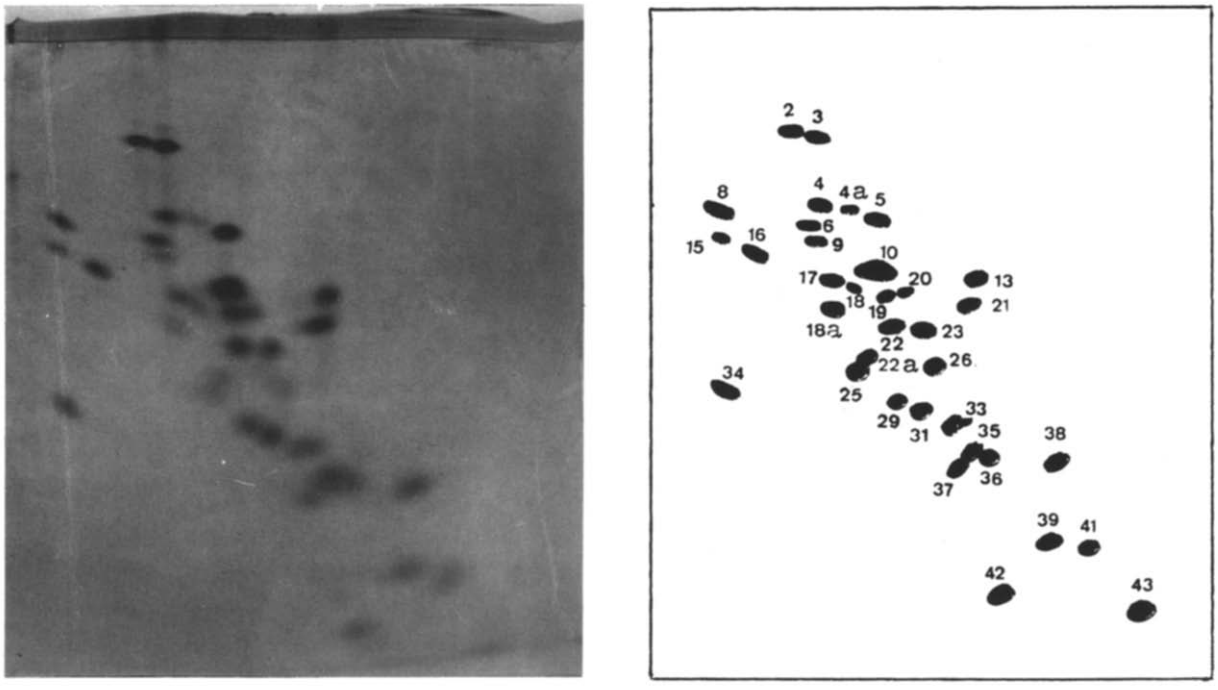

Fig.1. Separation of ribosomal proteins from the large ribosomal subunit of *S. cerevisiae* by two-dimensional electrophoresis. The experimental conditions are described in Materials and methods. (a) Electrophoretic separation of proteins. (b) Diagrammatic representation of the numbering system used to identify individual proteins. Migration in the first dimension was from left to right and in the second from top to bottom.

shows a digrammatic representation of the numbering system employed here to identify the various ribosomal proteins. To date there is no standard numbering system for eukaryotic ribosomal proteins and the one depicted in fig.1b is based upon that already published by Kruiswijk and Planta [13]. In the present work only the basic proteins have been analyzed and the gel pattern shows the presence of at least 35 ribosomal proteins from the large ribosomal unit. This estimate is consistent with results published by other groups [13–15] although there are some differences which may depend upon the yeast strain used. Thus, by comparison with the separation claimed by Kruiswijk and Planta [13] we cannot detect clear spots corresponding to proteins L7, L11, L12, L30, L32 and L40. However, we can identify three protein spots not claimed by Kruiswijk and Planta [13] and we have designated these arbitrarily as L4a, L18a and L22a.

In order to determine whether or not a given ribosomal protein is methylated in vivo we have employed the method developed by Chang et al. [5] in which the $^{3}H/^{14}C$ ratio is compared for each ribosomal protein isolated from cells grown in medium containing both [1-^{14}C]methionine and [*methyl*-^{3}H]methionine. This method is particularly sensitive for proteins, such as ribosomal proteins, which have a low methionine content. Thus, for a given methylated protein, the $^{3}H/^{14}C$ ratio will be higher than that in a protein that is non-methylated [5] the labelled methyl groups being derived from [*methyl*-^{3}H]methionine with *S*-adenosyl methionine as the intermediate methyl donor. To determine the levels of methylation in each case the $^{3}H/^{14}C$ ratio for the total unfractionated ribosomal protein is taken as the control and a significant increase in this ratio for a given protein is taken to indicate methylation.

Table 1 shows the $^{3}H/^{14}C$ ratio for each of the 60 S ribosomal subunit proteins studied. The ratio for the total unfractionated protein obtained from 60 S ribosomal subunits is 5:1 and after allowing for the difference in counting efficiency between ^{3}H and ^{14}C this ratio indicates that both of the isotopic markers in the culture medium are incorporated equally into protein. It is evident from the data of table 1 that protein L15 is heavily methylated since the $^{3}H/^{14}C$ count ratio is approximately 19:1. Of the other proteins, the one we have numbered L18a is also methylated with a $^{3}H/^{14}C$ count ratio of 8.5:1. The identification of this protein using the Kruiswijk and Planta [13] numbering system is difficult. On our gels there is a distinct, although relatively weak-staining, spot corresponding to this protein just below the positions occupied by proteins L17 and L18. On the gels shown previously [13] there is a faint but definite spot in this same region that was not numbered by the authors. It is of interest that protein L18a, although weak-staining, is very heavily labelled with the isotopes. The yeast ribosomal protein L3, with a $^{3}H/^{14}C$ ratio of 7:1, is also apparently methylated. This protein is very heavily labelled and has more ^{3}H counts incorporated than any other protein separated on the gels. There are also indications that protein L42 could be methylated since the $^{3}H/^{14}C$ ratio is 9:1. However, the radioactive label incorporated in this particular protein is very low and this makes calculation of the count ratio unreliable. Similarly, the apparent absence of ^{14}C counts in protein L41 does not allow an accurate assessment of the $^{3}H/^{14}C$ ratio for this protein even though significant numbers of ^{3}H counts have been incorporated. Protein L41 may be devoid of methionine residues and even slight methylation of such a protein would produce a greatly inflated $^{3}H/^{14}C$ ratio. Several other proteins notably L8, L9, L17, L29, L34, L39 and L43 may also contain methylated amino acids since they have slightly elevated $^{3}H/^{14}C$ ratios. Finally it should be noted that three proteins notably L13, L33 and L38 – all well stained on the gel – apparently contain neither methionine residues nor methylated amino acids.

Although the biological significance of protein methylation is not understood it is of interest to compare our present results, obtained using yeast ribosomes, with those already published for *E. coli* ribosomes. Chang et al. [5] observed that at least six proteins from the large ribosomal subunit of *E. coli* can be methylated in vivo. In particular protein L11 is the most heavily methylated and by analogy the corresponding protein in yeast ribosomes may be L15. In any case the extent of methylation in this yeast ribosomal protein (L15) vastly exceeds that shown by any of the *E. coli* ribosomal proteins. Thus, in *E. coli*, L11 shows an increase of 50% in the $^{3}H/^{14}C$ ratio relative to that of the unfractionated ribosomal protein [5] whereas in our work the corresponding increase for protein L15 is approximately four-fold greater than the control.

Table 1
$^{3}H/^{14}C$ Ratio in proteins from 60 S ribosomal subunits

Protein	^{3}H Incorporated (cpm)	^{14}C Incorporated (cpm)	$^{3}H/^{14}C$ Ratio
L2	5963	1325	4.5
L3	18 487	2553	7.2
L4	7705	1545	5.0
L4a	1467	348	4.2
L5	4550	968	4.7
L6	7184	1261	5.7
L8	10 169	1659	6.1
L9	3521	572	6.2
L10	4006	826	4.9
L13	–	–	–
L15	13 241	692	19.1
L16	11 926	2807	4.3
L17	1015	150	6.8
L18	1274	301	4.2
L18a	13 114	1536	8.5
L19	10 047	2092	4.8
L20	1542	410	3.8
L21	3673	813	4.5
L22	14 824	2843	5.2
L22a	2664	522	5.1
L23	199	55	3.6
L25	3017	579	5.2
L26	1454	297	4.9
L29	6134	929	6.6
L31	1549	277	5.6
L33	–	–	–
L34	3364	525	6.4
L35	3744	716	5.2
L36	1082	246	4.4
L37	2399	501	4.8
L38	–	–	–
L39	5584	879	6.4
L41	3093	–	–
L42	560	60	9.3
L43	1079	170	6.4

S. cerevisiae was grown in the presence of both [1-^{14}C]methionine and [*methyl*-^{3}H]methionine and protein from 60 S ribosomal subunits prepared as described in Materials and methods. The $^{3}H/^{14}C$ ratio for total unfractionated ribosomal protein was 5:1. Ribosomal proteins were separated and counted for both ^{3}H and ^{14}C as described in Materials and methods.

At this stage of our investigations it is possible to make two further comparisons. Chang et al. [5] observed extensive methylation of protein L18 in *E. coli* ribosomes and the corresponding protein in yeast ribosomes may be L18a (again heavily methylated). Finally it should be noted that both protein L33 from *E. coli* ribosomes [5] and protein L41 from yeast ribosomes appear to be methylated seemingly because of the absence of methionine residues in their structures.

A number of post-translational and post-transcriptional modifications of ribosomes have been described, including acetylation, phosphorylation and methylation of proteins and methylation of RNA.

Some of these modifications have been shown to have physiological significance; in particular resistance in bacteria to the antibiotic kasugamycin is related to the phenomenon of ribosomal RNA methylation [16]. It will be of interest to analyze a variety of yeast mutants for their content of methylated ribosomal proteins.

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