REDUCTIVE METHYLATION: A METHOD FOR PREPARING FUNCTIONALLY ACTIVE RADIOACTIVE RIBOSOMES *

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1. Introduction

Radioactive ribosomal proteins are widely used in many studies on ribosome topography and function. The standard preparative procedure involved growing bacteria on a radioactive amino acid source; a method which is expensive and not readily applicable to higher organisms.

This paper describes a simple method for labelling all the ribosomal proteins of 30 S, 50 S and 70 S particles without affecting their functional activities. The method involves reductive methylation of lysine residues using formaldehyde and sodium borohydride [1]. Ribosomes with extremely high specific radioactivity can be prepared in this manner.

2. Materials and methods

2.1. *Materials*

Samples of [^{3}H] sodium or potassium borohydride with specific activities ranging from 0.31 to 7.4 Ci/mmole were purchased from Amersham–Buchler, Braunschweig. Biochemicals used in functional activity assays were from Boehringer (Mannheim) with the exception of [^{14}C] valine and [^{14}C] phenylalanine which were from New England Nuclear, Dreieichenhain. TMA buffer was 10 mM Tris–HCl, 10 mM magnesium chloride, 20 mM ammonium chloride and 6 mM β-mercaptoethanol, pH 7.8. BMK buffer was 100 mM sodium borate, 10 mM magnesium chloride, 20 mM potassium chloride and 6 mM β-mercaptoethanol, pH 8.5.

2.2. *Ribosome preparation*

70 S ribosomes from *E. coli* strain A19 harvested in late log phase of growth were isolated by standard procedures [2]. 30 S and 50 S subunits were separated by sucrose density gradient centrifugation, isolated by alcohol precipitation and stored at −80°C in 5 mM magnesium acetate.

2.3. *Reductive methylation*

The reaction was carried out in a fume hood as follows: to 30 S, 50 S or 70 S ribosomes (10 A_{260} units) in 0.3 ml of BMK buffer at 0°C, 50 μl of 0.075 M formaldehyde was added, followed after 30 sec by 20 μl of [^{3}H] sodium borohydride solution (10 mg/ml). After 1 min the treatment with formaldehyde and sodium borohydride was repeated. The reaction mixture was left at 0°C with occasional shaking for 10 min, and then dialysed against several changes of TMA buffer. The number of methyl groups incorporated into the sample was calculated on the assumption that one hydrogen atom derived from sodium borohydride is incorporated per methyl group per lysine residue[1].

2.4. *Two-dimensional polyacrylamide gel electrophoresis*

Unlabelled ribosomes (40 A_{260} units of 30 S; 80 A_{260} units of 50 S) were added to each labelled sample, the RNA was removed by 67% acetic acid treatment and the proteins separated by two-dimensional gel electrophoresis [3]. Spots on the gel were cut out and counted after solubilising with hydrogen peroxide [4]. The recorded counts for each protein

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were corrected for rich medium fractionality [5] and for the percentage of material remaining at the origin of the polyacrylamide plates using curves kindly provided by H.J. Weber [6]. The position of the spots on the plates is unaffected by reductive methylation.

2.5. *Functional activity*

Functional activities of 30 S, 50 S and 70 S ribosomes in poly-U dependent polyphenylalanine synthesis and R-17 phage messenger RNA directed polypeptide synthesis were determined by the methods of Traub et al. [2] and Nierhause et al. [7] respectively.

2.6. *Total reconstitution of methylated 30 S proteins and 16 S RNA*

30 S proteins and 16 S RNA were isolated by treating 30 S ribosomes with 4 M urea 2 M LiCl [8]. The protein solution was dialysed into BMK buffer, pH 8.5, containing 4 M guanidinium hydrochloride, and reductive methylation was performed as described in section 2.3. The solution was dialysed against reconstitution buffer and reconstituted with 16 S RNA as described by Schreiner and Nierhaus [9]. The reconstituted 30 S particles were then assayed for activity in poly-U dependent polyphenylalanine synthesis [2].

3. Results and discussion

In an experiment using sodium borohydride of specific activity 770 mCi/mmole (prepared by adding a solution of 4.4 mg of unlabelled sodium borohydride in 0.5 ml of water at 0°C to 100 mCi of sodium borohydride of specific activity 6.4 Ci/mmole), 40 A_{260} units each of 30 S, 50 S and 70 S ribosomes of specific activity 2×10^6 dpm per A_{260} unit were prepared. By using sodium borohydride of specific activity 6.4 Ci/mmole, incorporations of greater than 10^7 dpm per A_{260} unit of ribosomes are possible.

Reductive methylation can be used to prepare mixtures of radioactive ribosomal proteins of high specific activity for use as markers in chromatographic separation of the individual proteins. In this connection the highest incorporations are obtained by carrying out the reductive methylation on mixtures of proteins, isolated by 67% acetic acid treatment of ribosomal subunits, dissolved in BMK buffer, pH 9, containing 4 M guanidinium hydrochloride.

In comparison to the 30 S subunit, twice as many methyl groups are incorporated into the 50 S subunit and three times as many are incorporated into 70 S ribosomes. The degree of labelling is dependent upon the paraformaldehyde content of the formaldehyde sample used. Highest incorporations are obtained when the formaldehyde is heated under reflux for 1 hr prior to use. In general about fifty methyl groups are incorporated into the 30 S subunit. There are about 200 lysines per 30 S particle, 400 lysines per 50 S particle, and 600 lysines per 70 S particle. Assuming that the principal product of the reaction is ϵ-*N*-dimethyllysine [1], 10–15% of the lysines in ribosomes are methylated.

When ribosomal RNA obtained from 67% acetic acid treatment is solubilised by digestion with a mixture of pancreatic ribonuclease and ribonuclease T_1, it is found to contain about 5% of the total radioactivity of the ribosomes. By performing the reaction with labelled formaldehyde and unlabelled sodium borohydride, it can be shown that this radioactivity is not due to methylation of RNA but derives from a non-specific reaction of RNA with sodium borohydride.

The extent of methylation of a lysine ϵ-amino group in intact ribosomes is dependent upon its accessibility to the reagents and its pK_a in the microenvironment. Residues on the surface of the ribosome which are exposed to the solvent will tend to be methylated more rapidly than internal residues or lysines having an abnormally high pK_a. It is unlikely that at pH 8.5 the ϵ-amino groups of lysine residues involved in salt linkages would be readily methylated, and therefore it might be expected that RNA-binding proteins in both 30 S and 50 S subunits would display a relatively low incorporation of label in comparison to proteins not involved in RNA-binding.

Examination of the data in table 1 shows that this is generally the case for the known RNA-binding proteins S4, S7, S8, S13, S15, S20, L2, L6, L16, L17, L18, L19, L20, L23, L24 and L25 [10–14]. It is interesting that all of the RNA-binding proteins are methylated to some degree – probably indicating that in no case does an RNA-binding protein have all its lysines involved in salt linkages to phosphate groups on RNA. In agreement with this, it is possible to methylate protein L24 when bound in a trypsin-resistant complex with 23 S RNA, the extent of methy-

Table 1
Reductive methylation of lysines in intact ribosomes.

Protein	30 S or 50 S subunit		70 S ribosomes[c]	
	% of Total incorporation[a]	% of Total protein lysine methylated[b]	% of Total incorporation[a]	% of Total protein lysine methylated[b]
S1[d]	8	29	2.5	32
S2	6	14	2	15
S3	10	12	4	14
S4	6	4	3	5
S5	10	12	3	11
S6[e]	5	50	–	–
S7	6	8	1	4
S8	2	4	1	5
S9	7	12	2	11
S10	8	50	1.5	30
S11	3	6	0.5	3
S12	1.5	11	0.5	13
S13	4	11	1.5	13
S14	2	11	0.5	8
S15	3.5	8	1.5	10
S16	1	4	0.3	4
S17	1.5	3	0.5	3
S18	1.5	10	0.5	10
S19	5	14	1.5	12
S20	4	10	1	8
S21	3	11	1	12
L1	11	30	8	34
L2	1.5	4	1	4
L3	5	14	4	16
L4	4	20	3	26
L5	4	10	2	8
L6	3	14	2	14
L7	6	26	6	34
L8+L9	7	36	4	32
L10	3	18	2	18
L11	5	26	5	34
L12[e]	5	34	–	–
L13	2.5	10	1.5	8
L14	1	10	1	12
L15	3	10	1	6
L16	2	12	1	10
L17	1.5	8	0.5	6
L18	4	14	2	12
L19	5	10	2.5	8
L20[f]	0.5	–	0.2	–
L21	1.5	10	1	10
L22	5	16	3.5	18
L23	2	8	1.5	10
L24	4	10	2	8
L25	3.5	14	2	12
L27	1.5	10	1	10
L28	0.3	8	0.2	8
L29	2	12	2	16
L30	1.5	14	1	14

(Table 1) continued

Protein	30 S or 50 S subunit		70 S ribosomes	
	% of Total incorporation	% of Total protein lysine methylated	% of Total incorporation	% of Total protein lysine methylated
L31[g]	0.1	–	0.1	–
L32[g]	1.5	–	1	–
L33	3	16	1	10

[a] Distribution of radioactivity corrected for percentage of material remaining at the origin of the polyacrylamide plate. Each value is an average determined from between 5 and 9 polyacrylamide plates; standard errors vary from 10 to 30%.
[b] Data from an experiment in which 44, 96 and 138 methyl groups were incorporated into 30 S, 50 S and 70 S ribosomes respectively. Calculated as dimethyllysine after correction for [1] fractionality and [2] percentage of each protein moving on the polyacrylamide plate, and using amino acid compositions [15] and molecular weights [16] for the individual proteins.
[c] 70 S ribosome preparations contained significant amounts of 30 S and 50 S subunits.
[d] Data for S1 was extremely variable. The values given represent an average.
[e] Data from 70 S ribosomes not available because of overlap of proteins on two-dimensional plate.
[f] Fractionality of L20 is not known.
[g] Molecular weight and amino acid composition data not available.

lation being less than for the protein alone (R.R. Crichton, unpublished observation).

Several preparations of ribosomes were treated under conditions designed to produce different degrees of labelling. In all but one case the poly-U dependent polyphenylalanine synthesizing activities and the phage messenger RNA dependent polypeptide synthesizing activities of 30 S, 50 S and 70 S ribosomes were unchanged by the methylation treatment (table 2). In one experiment the phage messenger dependent activities of 30 S and 50 S subunits were reduced to about half the activity of the controls, whereas the poly-U dependent activities were unchanged. Huang and Cantor [17] have shown that flurescein-treated ribosomes maintain full biological activity (poly-U assay).

Table 2
Functional activity of methylated ribosomes.

Ribosomes	No. of methyl groups	cpm in assay[a] poly-U	R-17
30 S	0	10300	2940
30 S	10	9770	3160
30 S	37	9690	2660
50 S	0	6250	5530
50 S	24	6100	6390
50 S	64	6950	5110
70 S	0	11280	6850
70 S	33	9850	5700
70 S	114	12120	5960
Reconstituted 30 S[b]	0	31 560	–
Reconstituted 30 S[b]	101	31 170	–

[a] Refer to section 2.5.
[b] Refer to section 2.6.

Reductive methylation of ribosomes offers a quick and simple method for producing fully functional radioactive ribosomes of high specific activity. The technique can also be applied to individual proteins or core particles, and has many potential uses in reconstitution experiments or in determinations on protein exchange in ribosomes. *N*-methylation of protein L24 does not prevent it from binding to 23 S RNA, and this finding has been used in the determination of the binding stoichiometry of the reconstituted RNA–protein complex [18].

Total reconstitution of methylated 30 S proteins and 16 S RNA is also possible. Using the conditions described in section 2.6., about a hundred methyl groups are incorporated into a mixture of 30 S proteins. These modified proteins can be reconstituted with 16 S RNA to give 30 S particles which have the same poly-U-dependent polyphenylalanine synthesising activity as the unmodified control (table 2). This finding suggests that methylation of lysines has very little effect on the binding of proteins to RNA, or indeed on the interactions between all components of the 30 S subunit.

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