ANALYSIS OF RIBOSOMAL PROTEIN CONFORMATION IN *BACILLUS SUBTILIS* BY REDUCTIVE METHYLATION

Identification of Proteins with different Conformation in Monosomes and Polysomes

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1. Introduction

Selective modification of the reactive groups in intact ribosomes or their subunits has been used to study the topography or conformational alteration of their protein components [1–10]. Thus, in *Escherichia coli* striking changes in protein conformation were observed in ribosomal subunits at different states of activity [11–14]. Proteins involved in antibiotic-induced conformational changes were identified by this way [15]. Similarly, in rat liver, proteins responsible for a ribosomal conformation change by ethionine intoxication were identified [16].

To our knowledge, these methods of ribosomal protein conformation analysis were not applied to the spore-forming bacterium *Bacillus subtilis*. Although the surface topography of the ribosomal proteins of the thermophilic bacterium, *Bacillus stearothermophilus* was determined [17], yet it is known [18] that only a few ribosomal proteins of this species can be correlated to those of *B. subtilis*. We, therefore, considered that an analysis of the conformation *in situ* of ribosomal proteins of *B. subtilis* might be useful for understanding their functional role.

We undertook this study to determine the conformation of ribosomal proteins of *B. subtilis* by reductive methylation. We have compared the conformation of individual proteins from monosomes (70 S) and polysomes of exponentially grown cells. We find that 3 of the small ribosomal subunit (30 S) proteins have a different conformation in monosomes and polysomes.

2. Materials and methods

2.1. *Strain and culture condition*

Bacillus subtilis 168M (trpC2) was grown in Schaeffer's double strength Difco medium [19] at 37°C. Exponential cells harvested at A_{650} ~1.5, were washed as in [20] and stored at −20°C.

2.2. *Isotopes and chemicals*

Sodium [^{3}H]borohydride (422 mCi/mM) was purchased from Amersham. [^{14}C]Formaldehyde (56 mCi/mM) was from New England Nuclear. All other chemicals were obtained from Merck.

2.3. *Buffers*

The following buffers were used for different preparations:

Buffer A: Tris (pH 7.8) 30 mM; ammonium chloride 60 mM; magnesium acetate 10 mM; 2-mercaptoethanol 7 mM; phenylmethane sulfonyl fluoride 2 mM; diisopropyl fluorophosphate 0.2 mM.

Buffer B: Tris (pH 7.4) 10 mM; ammonium chloride 60 mM; magnesium acetate 10 mM; 2-mercaptoethanol 7 mM.

Buffer C: Sodium borate (pH 8.5) 100 mM; ammonium chloride 30 mM; magnesium acetate 10 mM; 2-mercaptoethanol 7 mM.

Buffer D: Sodium borate (pH 8.5) 100 mM; urea 8 M; 2-mercaptoethanol 7 mM.

2.4. *Ribosome preparation*

Cells suspended in buffer A were broken, after freezing in an Eaton Pressure cell. DNase was added to the homogenate at 5 μg/ml and centrifuged at 16 000 rev./min for 20–30 min. The supernatant

(300–400 A_{260} unit) was loaded on 15–35% sucrose gradients (50 ml) overlaid on a 56% sucrose cushion (5 ml) dissolved in buffer B (without mercaptoethanol) and centrifuged in a Spinco SW25-2 rotor at 21 000 rev./min for 14 h. The fractions corresponding to 70 S ribosomes (monosomes) and a few fractions of polysomes were collected and centrifuged at 34 000 rev./min for 12 h. Pellets of monosomes and polysomes were suspended in buffer B and stored at −70°C. Occasionally monosome preparations were checked for purity by centrifuging in a 5 ml sucrose gradient (5–20%) in buffer B (Spinco rotor SW39) at 35 000 rev./min for 90 min. Monosome preparations showed <10% disomes. High-salt wash was avoided in order to preserve the conformation intact.

High-salt washed ribosome-derived subunits were prepared as in [21].

2.5. *Protein synthesis in vitro*

Protein synthesizing capacity of the preparations was assayed either by poly(U)-dependent polyphenylalanine synthesis or by in vitro protein synthesis in the presence of natural mRNA extracted from *B. subtilis* exponential cells as in [21]. The assay conditions are given in the legends of the tables.

2.6. *Two-isotope labelling by reductive methylation*

Intact ribosomes were reductively methylated in the presence of Na[^{3}H]borohydride (and formaldehyde) essentially as in [8] except that the incubation was increased to 30 min. Intially the completeness of the reaction was checked as follows: the ribosomal suspension after labelling with Na[^{3}H]borohydride, was dialyzed against buffer C to remove free reactants. An aliquot of the dialyzed preparation was then labelled again with [^{14}C]formaldehyde. Thus we found that 30 min labelling was necessary for completion of the reaction.

^{3}H-Labelled ribosomal proteins were then extracted from intact ribosomes (0.1 M Mg^{2+}) by 2 vol. glacial acetic acid [22]. An aliquot (0.1 A_{260} unit equiv.) was precipitated in 10% trichloroacetic acid to determine the labelling efficiency.

Extracted proteins were dialyzed, lyophilized and then dissolved in buffer D. The protein solution was methylated again by [^{14}C]formaldehyde (and Na borohydride) for 30 min. The solution was acidified with 0.2 vol. acetic acid before dialysis against 5% acetic acid and lyophylized.

Methylation of ribosomes (60 A_{260} units) in the absence of isotopes was carried out on a preparation dialyzed against buffer C and increasing the reaction time to 60 min. The methylated ribosomes were either extracted with acetic acid for proteins or were dialyzed for assays against buffer B (3 changes) for ~24 h. To check the completeness of reaction, ~1 A_{260} unit of ribosomes was methylated again in the presence of Na[^{3}H]borohydride.

2.7. *Two-dimensional polyacrylamide gel electrophoresis*

The two dimensional electrophoresis was done as in [23] adapted to 10 × 10 cm 2nd-dimension gel as in [24]. However, two minor modifications were introduced:

(i) Sample gel was photopolymerized in the absence of ammonium persulfate;

(ii) The upper 10% acrylamide gel in 1st dimension contained 0.18% bis (instead of 0.375%). For electrophoresis ~5 A_{260} unit equiv. of double-labelled ribosomal protein was mixed with 200 μg cold ribosomal proteins and dissolved in sample gel solution. Electrophoresis in the 1st dimension was at 75 V for 18 h at 4°C and the 2nd-dimension at 50 V for 24 h at room temperature. After electrophoresis, gels were stained [24]. The spots corresponding to identified proteins were cut out from the gel and mixed, by crushing, with 5 ml scintillation mixture. The scintillation mixture contained 3% Protosol (New England Nuclear) and 0.4% Omnifluor (New England Nuclear) in toluene as in [25]. The gel suspension was shaken overnight at 37°C and cooled to 4°C for 8–16 h before counting.

Adjustment of channels for ^{3}H and ^{14}C counting was carried out with several concentrations of known amount of isotopes adsorbed on gel fragments. Relative quenching and spillover of one isotope in the channel of the other isotope was determined with or without gels. ^{14}C spillover in tritium channel was 8% and was subtracted from the ^{3}H counts. ^{3}H spillover in ^{14}C channel was always <0.5% and was neglected.

The ^{3}H/^{14}C ratio for each protein was calculated. The ratio for total subunit proteins was then determined and the latter ratio was normalized to 1.00, from which the relative ratio for each protein was determined. These calculations were carried out in a Wang calculator with the help of a program for double-label counting. The relative ^{3}H/^{14}C ratio for each protein was taken as an indication of exposure

of reactive groups. The terms 'more exposed' or 'less exposed' proteins are explained in section 3.

The nomenclature of the proteins is as in [8,24]. The letter S preceeding the protein number indicates that the protein belongs to the 30 S subunit while the letter L signifies the large (50 S) subunit protein.

3. Results

3.1. *Electrophoretic pattern of methylated ribosomal proteins*

In order to utilize the method of protein labelling by reductive methylation to analyze the conformation of proteins in the ribosome, it has to be assumed that methylation does not alter electrophoretic mobility of the ribosomal proteins. In fact, it was claimed [8] that the ribosomal protein profile of *E. coli* remains unchanged after methylation. To assure ourselves that *B. subtilis* ribosomal proteins also remains unaltered we have compared the protein mobility pattern by 2-dimensional acrylamide gel electrophoresis of normal and methylated ribosomes. The mobility of all the detectable proteins remained unaltered after methylation (not shown).

3.2. *Protein synthesizing capacity*

Methylated ribosomes were compared with normal ribosomes for their poly(U)-dependent polyphenylalanine synthesizing capacity and for natural mRNA (*B. subtilis* exponential mRNA)-dependent protein synthesis. Table 1 shows that the methylated ribosomes retain 99% polyphenylalanine synthesizing capacity and 91% of natural mRNA-dependent protein synthesizing capacity of the control.

3.3. *Relative methylation of intact ribosomes and their proteins*

In intact ribosomes only the exposed reacting groups of proteins (ϵNH_2 of lysine) are methylated, while the unexposed groups are methylated only in denaturing conditions. We found that both in monosomes and polysomes ~20% methylable sites are exposed while the remaining 80% is labelled only after denaturation. As every ϵNH_2 of lysine in proteins is dimethylated, each mole of ribosome should have ~100 mol lysine available for in vitro methylation.

3.4. *Relative methylation of individual ribosomal proteins*

Incorporation of methyl groups into individual proteins relative to total incorporation was determined by labelling total proteins from isolated ribosomal subunits obtained by sucrose gradient centrifugation of high salt-washed exponential phase ribosomes. Extracted proteins from each subunit were methylated in the presence of [^{14}C]formaldehyde and separated by 2-dimensional electrophoresis. Each protein spot was dissolved in 0.5 ml hydrogen peroxide and counted. Results are presented in table 2. The relative proportion of methylation for each protein coincides approximately with what can be computed from total 70 S proteins. The only exception is the small subunit protein S1, whose methylation here is relatively low. The

Table 1
Protein synthesis in vitro by methylated ribosomes

Ribosomes	Poly(U)-dependent [^{14}C]phenylalanine incorp. (cpm)	% Activity in methylated ribosomes	mRNA-dependent [^{14}C]leucine incorp. (cpm)	% Activity in methylated ribosomes
Normal	7495	100	6268	100
Methylated	7418	99	5716	91

Poly(U)- and natural mRNA-dependent assays were done as in [21]. The assay in presence of poly(U) contained 0.45 A_{260} unit of ribosome, 185 μg S150 protein and 25 μg poly(U) and [^{14}C]phenylalanine (0.1 μCi/0.6 nmol). Incubation was for 30 min at 30°C. In presence of natural mRNA the assay contained 0.9 A_{260} unit of ribosomes, 25 μg crude initiation factor and 350 μg S150 protein. The mixture was preincubated for 15 min at 30°C to remove endogenous mRNA and the incubation (30 min at 30°C) was started by the addition of mRNA (1.8 A_{260} unit obtained from uninfected *B. subtilis* exponential cells), [^{14}C]leucine (0.2 μCi/nmol) and sufficient buffer to compensate the volume change. Counts obtained in the absence of mRNA was subtracted from each assay

Table 2
Relative methylation of ribosomal proteins

	30 S subunit			50 S subunit	
	cpm mean	Relative %		cpm mean	Relative %
S1	1 645	1.90	L1	32 366	12.96
2	2 854	3.28	2	16 763	6.49
3	1 023	1.18	3	902	0.33
4	7 382	8.65	4	7 443	3.00
5	6 078	6.97	5	5 965	2.26
7	10 940	12.51	6	23 873	9.64
8	5 276	6.11	7	22 548	8.83
10	5 910	6.81	8	18 150	7.16
11	842	0.97	11	4 039	1.47
12	4 155	4.71	12/13	22 732	9.14
13	3 948	4.55	14	18 342	7.23
14	1 480	1.74	15	4 010	1.53
16	8 765	10.20	16	8 717	3.45
17	5 256	6.02	17	9 778	3.92
18	5 280	6.05	18	13 305	5.31
19	7 080	8.04	20	9 952	3.79
20	7 768	9.10	21	3 225	1.27
21	1 055	1.21	22	9 144	3.52
			24	6 384	2.50
			27	2 214	0.92
			28/30	16 391	6.42
			29	8 091	3.22

Proteins were extracted from subunits obtained from high salt-washed ribosomes and were methylated in urea (8 M) containing buffer in presence of [^{14}C]formaldehyde. Separated proteins from 2-dimensional gel were recovered and dissolved in 0.5 ml hydrogen peroxide [26] and counted in 10 ml Triton–toluene-based scintillation mixture. The counts for each protein are the mean value from 3 different gels

reason for this is that the high salt washing of the ribosomes causes a partial loss of protein S1.

3.5. *Protein synthesis in vitro by monosomes and polysomes*

Table 3 shows that the polysomes can incorporate, in the absence of added mRNA, 4 times more [^{14}C] leucine than the monosomes of the same cell extract, thus indicating the presence of bound mRNA in polysomes.

3.6. *Two isotope labelling pattern of proteins*

From the data obtained from intact ribosomes and total proteins, we have assumed that for a protein the normalized $^{3}H/^{14}C$ ratio of 1.000 is equivalent to 20% exposed methyl at able groups. Any protein showing this ratio equal or superior to 1.333 (equiv. 25% exposed groups) is considered as 'more exposed'

Table 3
In vitro protein synthesis by monosomes and polysomes

Ribosome	[^{14}C]leucine incorp. (cpm)	
	− mRNA	+ mRNA
Expt 1		
Monosome	1776	6195
Polysome	6892	10 911
Expt 2		
Monosome	2362	10 341
Polysome	9610	14 623

Monosomes and polysomes were obtained from the same cell free extract. Each assay contained 1.0 A_{260} unit of monosomes or polysomes, 25 μg initial factor, 350 μg S150 protein, 1.8 A_{260} unit of mRNA (*B. subtilis* exponential cells). Incubation was for 30 min at 30°C

Table 4
Normalized $^{3}H/^{14}C$ ratios of 30 S ribosomal subunit proteins

	30 S subunit proteins		
	Monosome	Polysome	p
S1	2.367 ± 0.069	1.888 ± 0.125	<0.02
2	1.604 ± 0.053	1.461 ± 0.073	
4	0.641 ± 0.037	0.553 ± 0.060	
7	1.162 ± 0.091	1.011 ± 0.070	
8	1.198 ± 0.079	0.894 ± 0.039	< 0.02
10	0.688 ± 0.053	0.555 ± 0.071	
11	0.678 ± 0.038	0.817 ± 0.057	
12	0.583 ± 0.121	0.617 ± 0.051	
13	0.896 ± 0.068	0.917 ± 0.050	
14	1.386 ± 0.121	2.156 ± 0.178	<0.01
16	0.506 ± 0.058	0.461 ± 0.025	
17	0.586 ± 0.043	0.570 ± 0.055	
18	0.410 ± 0.039	0.395 ± 0.043	
19	1.001 ± 0.087	1.068 ± 0.045	
20	0.719 ± 0.077	0.579 ± 0.082	
21	1.555 ± 0.051	1.990 ± 0.210	

Results are expressed as normalized $^{3}H/^{14}C$ ratio ± SEM. The enumeration of proteins is as in [8,24]. Only the proteins showing a $p < 0.05$ are considered as significantly different. n (no. gels analyzed) = 5. Proteins which were not definitely identified or comigrated with 50 S proteins are not listed here

and when this ratio is equal or inferior to 0.710 (equiv. 15% exposure), the protein is considered as 'less exposed'. Comparison of monosomal and polysomal proteins shows that 3 proteins of the 30 S subunit have their $^{3}H/^{14}C$ ratio altered in the polysomes. The proteins S1 and S8 are relatively less exposed and S14 is more exposed in polysomes than in monosomes (table 4). No protein of the 50 S subunit has a significantly different conformation in polysomes (not shown).

4. Discussion

We have shown here that all the identified ribosomal proteins of *B. subtilis* are methylated in vitro to some degree. The extent of methylation is not correlated with the charge or relative molecular mass of a protein. It should however be noted that some ribosomal proteins of *B. subtilis*, as in *E. coli* [27], can be highly methylated in vivo, offering, thus, only a few groups available for methylation in vitro. In the absence of any information on in vivo methylated lysine of ribosomal proteins of *B. subtilis* we can only take into account, in these experiments, the in vitro methyl at able groups.

We have preferred the method of reductive methylation by two-isotope labelling for several reasons. This method allows a determination of the ratio of exposed and unexposed groups even if the total amount of the protein to be analyzed is not recovered from the gel, while with the single isotope labelling an accurate determination of the amount of protein requires a quantitative migration in the gel. We have observed that some proteins do not migrate quantitatively in the gel and may appear then undermethylated. Moreover, in analysing surface topography with a single isotope it is assumed that proteins highly labelled in intact ribosomes are surface proteins [10]. However our results indicate that some proteins, though poorly labelled in vitro, might be 'more exposed' (e.g., S14) while others with a relatively high incorporation of label are 'less exposed' (e.g., S16, S20).

Comparison of proteins from monosomes and polysomes show that only 3 proteins of the 30 S subunit and none of the 50 S subunit are altered in their conformation. The conformational alteration in the 30 S subunit of polysomes is not surprising since this subunit is the site of binding of mRNA. It is to be noted that two (S1, S14) out of the 3 altered proteins in the 30 S subunit are 'more exposed', which may be due to their surface location and might thus participate directly in the binding of mRNA and/or aminoacyl tRNA. Another altered protein is S8 and its role is yet to be determined. None of the 'less exposed' proteins are altered in their conformation in polysomes.

In *Escherichia coli*, protein S1 has an important role in the attachment of mRNA to the 30 S subunit. This protein, in *E. coli* as in *B. subtilis*, is susceptible to removal by high-salt washing. Moreover the S1 protein has a similar electrophoretic mobility in both of these organisms. It is possible then that this protein in *B. subtilis* has an identical role to that in *E. coli*.

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