Halobacterium Cutirubrum Ribosomes

PROPERTIES OF THE RIBOSOMAL PROTEINS AND RIBONUCLEIC ACID

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1. The 30S ribosomal subunit of the extreme halophile *Halobacterium cutirubrum* is unstable and loses 75% of its ribosomal protein when the 70S ribosome is dissociated into the two subunits. A stable 30S subunit is obtained if the dissociation of the 70S particle is carried out in the presence of the soluble fraction. 2. A fractionation procedure was developed for the selective removal of groups of proteins from the 30S and 50S subunits. When the ribosomes, which are stable in 4M-K⁺ and 0.1M-Mg²⁺, were extracted with low-ionic-strength buffer 75–80% of the 30S proteins and 60–65% of the 50S proteins as well as the 5S rRNA were released. The proteins in this fraction are the most acidic of the *H. cutirubrum* ribosomal proteins. Further extraction with Li⁺-EDTA releases additional protein, leaving a core particle containing either 16S rRNA or 23S rRNA and about 5% of the total ribosomal protein. The amino acid composition, mobility on polyacrylamide gels at pH4.5 and 8.7, and the molecular-weight distribution of the various protein fractions were determined. 3. The *s* values of the rRNA are 5S, 16S and 23S. The C+G contents of the 16S and 23S rRNA were 56.1 and 58.8% respectively and these are higher than C+G contents of the corresponding *Escherichia coli* rRNA (53.8 and 54.1%).

Halobacterium cutirubrum, an extreme halophile, belongs to a select group of bacteria which require near-saturated solutions of NaCl for growth (Larsen, 1967). The intracellular ion concentration in these organisms is also extremely high. For example, *Halobacterium salinarium*, an extreme halophile which is thought to be identical with *H. cutirubrum* (N. E. Gibbons, personal communication), has an intracellular concentration of K⁺ greater than 4M (Christian & Waltho, 1962). The enzymes (Baxter & Gibbons, 1956), cell organelles such as ribosomes (Bayley & Kushner, 1964) and highly organized processes such as protein synthesis (Bayley & Griffiths, 1968) have all adapted to these high internal salt concentrations and require a similar ionic environment *in vitro*.

Bayley & Kushner (1964) have reported that *H. cutirubrum* ribosomes require 4M-K⁺ and 0.1M-Mg²⁺ for stability. These workers also observed that a large amount of protein was released when these ribosomes were suspended in low-ionic-strength buffers commonly used with conventional bacterial ribosomes. This split-protein fraction contained mainly acidic proteins, in sharp contrast to the predominantly basic proteins found in ribosomes from bacteria such as *Escherichia coli* (Waller, 1964). The

ribosomal proteins that remain attached to the RNA in the low-ionic-strength medium contain both acidic and basic proteins (Bayley, 1966).

As part of a project investigating the specific interactions between ribosomal assembly proteins and RNA in certain bacteria that grow under extreme conditions, it was desirable to obtain a more detailed understanding of the RNA and protein components of *H. cutirubrum* ribosomes. In the present paper we describe the isolation of stable ribosomal subunits from *H. cutirubrum* and the general properties of their RNA and protein components.

Methods

Growth of H. cutirubrum

The cells were grown in the complex medium of Seghal & Gibbons (1960) in a 120-litre fermenter with vigorous aeration at 37°C and harvested in early exponential-phase growth. The cells were carefully washed with 4.5M-NaCl–0.03M-KCl–0.1M-MgCl₂ (Bayley & Kushner, 1964) and either used immediately or stored as a pellet in liquid N₂.

Isolation of ribosomes

H. cutirubrum cells were disrupted either in an Eppenbach colloid mill or by grinding with alumina in the presence of deoxyribonuclease in solution D

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(3.4M-KCl-0.1M-Mg²⁺-0.1M-tris-HCl, pH 7.6) of Bayley & Griffiths (1968). Cell debris was removed by centrifugation at 12000g for 10min, followed by 60000g for 30min to give the supernatant 'S-60 fraction'. The 70S ribosomes were obtained by centrifuging the S-60 fraction at 250000g for 4h in a Spinco Ti60 rotor at 4°C. If 50S and 30S subunits were required the S-60 fraction was dialysed against solution D' (3.0M-KCl-0.5M-NH₄Cl-0.001M-Mg²⁺-0.02M-tris-HCl, pH 8.05) of Rauser & Bayley (1968), and the subunits were separated in a Spinco BXV Ti zonal rotor at 29000 rev./min for 16h at 10°C by using a linear gradient of 2-35% (w/v) sucrose in solution D'. The contents of the rotor were removed with a cushion of 60% (w/v) sucrose in solution D' and 20ml fractions were collected. The fractions containing the 30S and 50S subunits were pooled, dialysed against solution D' and concentrated in an Amicon Diaflo ultrafilter with a PM-10 membrane.

The purity of the subunits was checked on a Spinco model E analytical ultracentrifuge and if further purification was required the subunits were centrifuged again in the BXV Ti zonal rotor by using the same conditions as described above. The purified subunits were stored in liquid N₂.

Preparation of RNA

The RNA was isolated by the phenol-sodium dodecyl sulphate method (Knowland & Miller, 1970) and fractionated either on polyacrylamide gels

(Loening, 1969) or by sucrose-density-gradient centrifugation (Sendecki *et al.*, 1971). RNA from *E. coli* Q-13, isolated by the phenol-sodium dodecyl sulphate method, was used as marker. The polyacrylamide gels were scanned at 260nm in a Gilford model 240 spectrophotometer equipped with a model 2410 linear transport.

The nucleotide composition of the RNA was determined by the method of Blattner & Erickson (1967).

Fig. 1. Separation by sucrose-density-gradient centrifugation of *H. cutirubrum* ribosomal subunits labelled with [¹⁴C]lysine

(a) and (b) 70S ribosomes dialysed at 4°C against solution D' and centrifuged in a BXV Ti zonal rotor as outlined in the Methods section. (c) 70S ribosomes in S-60 fraction dialysed at 4°C against solution D'. —, E₂₆₀; ----, radioactivity (c.p.m.). Peaks (I) and S are discussed in the text.

Scheme 1. Fractionation of ribosomal proteins

For experimental details and TMK buffer see the text.

Protein determination

Protein was measured by the method of Lowry *et al.* (1951) with bovine plasma albumin as standard.

Total ribosomal protein was extracted with freshly prepared 2M-LiCl–4M-urea for 18 h at 4°C and the protein was fractionated on 7.5% polyacrylamide gels at either pH 8.7 or 4.5 (Leboy *et al.*, 1964). The gels were fixed with 12.5% (w/v) trichloroacetic acid for 30 min at 65°C, stained in 0.2% Coomassie Brilliant Blue for 30 min at 65°C and destained at 65°C, first in ethanol–acetic acid–water (5:2:13, by vol.) and then in 10% (v/v) acetic acid as outlined by Ortec (1970).

Molecular weights of the proteins were determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (Weber & Osborn, 1969).

To gain some insight into the location of the acidic and basic proteins in *H. cutirubrum* a fractionation scheme was developed for stepwise removal of protein from the ribosome (Scheme 1). This scheme was based on the observation of Bayley & Kushner (1964) that a large proportion of the ribosomal protein was removed from *H. cutirubrum* ribosomes by low-ionic-strength buffers, and the report by Itoh *et al.* (1968) that *E. coli* proteins could be selectively removed by various concentrations of Li⁺–EDTA.

The 30S or 50S ribosomal subunits (5 mg/ml) of *H. cutirubrum* were resuspended in 10 mM-tris–HCl–50 mM-KCl–0.3 mM-Mg²⁺–6 mM-β-mercaptoethanol, pH 7.6, buffer (TMK buffer) and dialysed overnight against the same buffer. The ribosomal suspension was centrifuged for 24 h at 165 000g in a Spinco Ti50 rotor at 4°C to give supernatant S₁ and pellet P₁. Pellet P₁ was resuspended in TMK buffer and tris was added to make the tris concentration 50 mM. An equal volume of 7M-LiCl–10 mM-EDTA was added and the suspension stirred for 72 h at 4°C. It was then centrifuged at 165 000g for 24 h to give supernatant S₂ and

pellet P₂. Pellet P₂ was resuspended in TMK buffer and an equal volume of 4M-LiCl–8M-urea was added. The suspension was stirred at 4°C for 24 h and then centrifuged for 30 min at 165 000g to give supernatant S₃ and pellet P₃.

The amino acid composition of the various protein fractions was determined by hydrolysing the protein

Fig. 2. Separation of *H. cutirubrum* rRNA on polyacrylamide gels

(a) Total RNA extracted from 70S ribosomes and run on 3% gels; (b) RNA extracted from P₁ fraction of the 50S subunit and run on 2.4% gels; (c) RNA extracted from the 30S subunit and run on 2.4% gels; (d) RNA extracted from S₁ fraction of the 50S subunit and run on 5% gels. Arrows and *s* values indicate location of *E. coli* rRNA markers. o, Origin. Samples, dissolved in buffer containing 0.036M-tris–0.03M-NaH₂PO₄–0.001M-sodium EDTA–0.2% sodium dodecyl sulphate, pH 7.7, were mixed with glycerol and layered on to polyacrylamide gels containing the same buffer. Electrophoresis was carried out at 23°C in this buffer at 3 mA/tube. Total running time was 90 min.

for 18 h at 110°C in 6M-HCl, and the hydrolysate was analysed on a Technicon sequential multi-sample amino acid analyser supplied with type C-4 Chromo-Bead resin. The amino acid compositions are expressed as mol/100mol. No attempt was made to correct the results for the destructive hydrolysis of certain amino acids. The cysteine and tryptophan contents of the proteins were not measured.

Radioactivity studies

In experiments involving ^{14}C -labelled ribosomes, *H. cutirubrum* was grown in a modified synthetic medium of Onishi *et al.* (1965) and labelled with [^{14}C]lysine (Ducharme *et al.*, 1972). The cells were disrupted with alumina and the ribosomal subunits isolated by zonal centrifugation as described above. The radioactivity in the various fractions was measured by adding the sample (0.5 ml) to Aquasol (13 ml) (New England Nuclear Corp., Boston, Mass., U.S.A.) and measuring the ^{14}C in a Beckman model 250 liquid-scintillation spectrometer.

Results

Instability of the 30S ribosomes

When ^{14}C -labelled 70S ribosomes were dissociated into the 30S and 50S subunits by dialysing against solution D' a very small amount of 30S ribosomes was obtained after sucrose-density-gradient centrifugation (Fig. 1a). Most of the 30S material was present in a fraction with a lower *s* value (peak I). Both the 30S subunit and peak (I) contained intact 16S RNA but little protein. The specific radioactivity (c.p.m. of ^{14}C -labelled protein/ E_{260} unit) was only 0.25 of that found in the 50S subunit, indicating that about 75% of the 30S protein had been removed. This protein was found at the top of the gradient.

In certain experiments the ratio of 30S to 50S subunits was the normal 1:2 value (Fig. 1b). However, the 30S subunits again had only 0.25 the specific radioactivity of the 50S subunits, indicating that a large amount of protein could be removed from the 30S subunit without a change in *s* value.

If, however, the 70S ribosomes were dissociated in the presence of the soluble fraction (S-60), stable 30S subunits were obtained and the specific radioactivity of the 30S particle was identical with that of the 50S subunit (Fig. 1c).

The above experiments suggest that a soluble factor(s) was required to stabilize the 30S subunit on dissociation of the 70S ribosomes. An energy-requiring step may also be involved, as suggested by the following experiments. If the soluble fraction, from the top of the sucrose-density-gradient centrifugation of S-60 fraction (peak S, Fig. 1c), was added to 70S ribosomes, which were then dialysed against solution D' at 37°C, stable 30S particles were obtained. Unstable 30S subunits were obtained, however, if the dialysis was done at 4°C. The temperature of dialysis had no effect on the results obtained with the S-60 fraction (stable 30S subunits) or when 70S ribosomes *per se* were dialysed (unstable 30S subunits).

Once stable 30S subunits were formed they remained stable and could be dialysed against solution D or D'.

Ribosomal RNA

The mobility on polyacrylamide gels of the *H. cutirubrum* RNA is identical with that of the *E. coli* rRNA markers (Fig. 2). This would indicate that the *H. cutirubrum* 30S subunit contains 16S RNA whereas the 50S subunit contains 23S and 5S RNA.

The nucleotide composition of the 5S, 16S and 23S *H. cutirubrum* RNA is shown in Table 1. The com-

Table 1. Comparison of nucleotide composition of *H. cutirubrum* and *E. coli* ribosomal RNA species

For experimental details see the text. Values are the means derived from five independent analyses of *H. cutirubrum* RNA.

	<i>s</i> (RNA component)	Nucleotide content (mol/100mol)				G+C A+U	Purine Pyrimidine	G+U A+C
		GMP	AMP	CMP	UMP			
<i>H. cutirubrum</i>	23S	32.8	24.4	23.3	19.5	1.28	1.34	1.10
	16S	34.7	22.0	24.1	19.2	1.43	1.31	1.17
	5S	30.1	18.3	33.5	18.1	1.75	0.94	0.93
<i>E. coli</i> (Spirin & Gavrilova, 1969)	23S	32.8	25.5	21.0	20.6	1.17	1.40	1.15
	16S	31.6	24.5	22.5	21.3	1.18	1.28	1.12
	5S	33.8	19.2	30.0	17.0	1.76	1.13	1.03
<i>H. salinarium</i> (Larsen, 1967)	Total RNA	33.4	24.0	22.0	20.0	1.26	1.37	1.16

EXPLANATION OF PLATE I

Separation of ribosomal proteins from E. coli and H. cutirubum on 7.5% polyacrylamide gels

(a) pH 8.7 gels, 7.5%, at 3mA/tube for 90min at 4°C. At pH 8.7 samples were dissolved in 8M-urea buffer containing 0.025M-tris-glycine buffer-0.012M-methylamine-0.2% mercaptoethanol, pH 8.7. Electrophoresis was carried out in 0.025M-tris-glycine buffer, pH 8.7. (b) pH 4.5 gels, 7.5%, at 3mA/tube for 150min at 4°C. At pH 4.5 protein samples were dissolved in 8M-urea containing 0.07M-β-alanine-acetic acid-0.012M-methylamine-0.2% mercaptoethanol, pH 4.5. Electrophoresis was carried out in 0.07M-β-alanine-acetic acid buffer, pH 4.5. The arrow indicates the direction of electrophoresis.

EXPLANATION OF PLATE 2

Polyacrylamide-gel profiles of protein fractions obtained from stepwise extraction of the ribosomal subunits (Scheme 1
Conditions were as described for Plate 1. (a) pH 8.7; (b) pH 4.5.

position of *E. coli* RNA as well as the total RNA from *H. salinarium* are given for comparison.

Ribosomal protein

The protein profiles of the total 70S, 50S and 30S ribosomes obtained on polyacrylamide gels at pH 8.7 and 4.5 are shown in Plate 1. As noted by Bayley (1966), the *H. cutirubrum* ribosomes contain a large number of acidic proteins, much more than is normally found in bacterial ribosomes such as those from *E. coli*.

When the ribosomal proteins are removed in a stepwise manner as outlined in Scheme 1, about 75–80% of the 30S protein and 60–65% of the 50S protein is released into supernatant S_1 , when the ribosomes are treated with TMK buffer. This protein fraction contains mainly acidic proteins as shown in Plate 2. The pellet P_1 core particle from 30S ribosomes had an s value of 16S whereas pellet P_1 from 50S ribosomes had an s value of 28S. When the pellet P_1 core particle is treated with LiCl–EDTA an additional 15–20% of the 30S proteins and 30–35% of the 50S proteins are released into fraction S_2 . The S_2 proteins are more basic than those found in supernatant S_1 . The remaining protein (about 5%), which is tightly bound to the pellet P_2 core particle, is removed by treating pellet P_2 with LiCl–urea to give fraction S_3 . The protein profiles on polyacrylamide gels of S_1 , S_2 and S_3 are shown in Plate 2.

The molecular-weight distribution of the proteins in the various fractions was determined by using the polysodium dodecyl sulphate–acrylamide gel system of Weber & Osborn (1969). The results are shown in Figs. 3 and 4.

The amino acid compositions of the various protein fractions are given in Table 2.

Fig. 3. Molecular-weight distribution of the various protein fractions obtained by stepwise extraction of the 50S ribosomal subunit as outlined in Scheme 1

The proteins were fractionated on sodium dodecyl sulphate–polyacrylamide gels by the method of Weber & Osborn (1969). The gels were stained with Coomassie Brilliant Blue and the gels were scanned at 570nm in a Gilford model 240 spectrophotometer equipped with a model 2410 linear transport. The arrows and numbers indicate location and molecular weight of the protein markers: cytochrome c (12400), myoglobin (17800), ovalbumin (45000) and bovine serum albumin (67000). (a) Total ribosomal protein from 50S subunit; (b) protein from supernatant S_1 ; (c) protein from supernatant S_2 ; (d) protein from supernatant S_3 . For experimental details see the text.

Discussion

One of the unusual properties of the *H. cutirubrum* ribosome has been the instability of the 30S subunit when the isolated 70S monomer is dissociated into its subunits in solution D' . As much as 75% of the 30S ribosomal proteins are released from the 30S subunit during this dissociation. However, stable 30S subunits are obtained if the dissociation is carried out in the presence of the soluble fraction and once a stable 30S particle is formed it is then completely stable in solution D' . These observations suggest that consider-

Fig. 4. Molecular-weight distribution of the various protein fractions obtained by stepwise extraction of the 30S ribosomal subunit as outlined in Scheme 1

See Fig. 3 for details. (a) Total ribosomal protein from 30S subunit; (b) protein from supernatant S_1 ; (c) protein from supernatant S_2 ; (d) protein from supernatant S_3 .

able rearrangement of the 30S subunit may occur during dissociation of the 70S ribosome, and factors in the soluble fraction are required to stabilize this new conformation. It would also appear that these

factors may be involved directly in the conformational change. This is suggested from the experiments in which the soluble fraction from sucrose-density-gradient experiments, i.e. 'used' soluble fraction (peak S, Fig. 1c) was used again with isolated 70S ribosomes. Under these conditions stable 30S subunits were formed only at 37°C, suggesting that a factor(s) was limiting and higher temperatures were required to reorganize the dissociated 30S subunits into a stable configuration.

Other evidence to suggest that the 30S subunit may be a less-stable structure than the 50S subunit was obtained when the subunits were treated with TMK buffer (Scheme 1). About 80% of the ribosomal protein of the 30S subunit was released under these conditions, compared with only 60–65% of the 50S ribosomal protein.

The proteins released in buffers of low ionic strength are predominantly acidic proteins, as previously shown by Bayley & Kushner (1964). This 'split-protein' fraction presumably contains the 'external' proteins, which not only must cope with the high ionic environment *in situ* but also are involved in the complex interactions required for protein synthesis. It is possible, by careful manipulation of the Mg^{2+} and K^+ concentrations, to remove selectively certain supernatant S_1 proteins (S. Hasnain, unpublished work), and competitive labelling experiments (L. P. Visentin, unpublished work) also suggest that certain supernatant S_1 proteins are more exposed than others. It should also be noted that 5S rRNA is completely removed in the S_1 fraction (Fig. 2d).

The relative basicity of the various fractions (Scheme 1) increases as the stepwise extraction continues. For example, the ratio of basic to acidic amino acids in fractions S_1 , S_2 and S_3 from the 50S subunit increases in the order 0.38, 0.62 and 0.79 respectively (Table 2). However, the total aspartate and glutamate values have not been corrected for the asparagine and glutamine content so that one cannot assume that the S_3 proteins are the most basic. Indeed, Plate 2 indicates that the S_3 proteins migrate more slowly than some of the S_2 proteins, and since the S_3 proteins have a molecular weight equal to or smaller than those of the S_2 proteins (Fig. 3) this would indicate that the S_3 proteins are less basic than some of the S_2 proteins. The S_3 proteins are of special interest since experiments indicate they may be assembly proteins (C. Chow, unpublished work).

Bayley (1966) has shown that the total ribosomal protein fraction of *H. cutirubrum* has a much higher content of glutamate and, to a smaller extent, aspartate than *E. coli* ribosomal proteins. It also has a much lower content of lysine and, to a lesser degree, arginine. Our results indicate that this will be a general property of most of the *H. cutirubrum* ribosomal proteins as this generalization holds for all the ribosomal protein fractions from both the 30S and

Table 2. Amino acid composition of the ribosomal protein fractions from *H. cutirubrum*

For experimental details see the text. Values are expressed as mol/100 mol of amino acid recovered. Values for 70S and 50S fractions are averages from five determinations and those for 30S fraction are from three determinations. The glycine value for S₃ (30S) was omitted because of RNA contamination.

	70S fraction			30S fraction				50S fraction			
	<i>E. coli</i> (Spahr, 1962)	<i>H. cutirubrum</i> (Bayley, 1966)	<i>H. cutirubrum</i> (present work)	Whole	S ₁	S ₂	S ₃	Whole	S ₁	S ₂	S ₃
Aspartic acid	9.2	13.5	12.7	10.9	11.4	10.9	10.5	12.9	14.2	11.8	11.7
Threonine	5.8	9.6	5.9	6.2	6.4	5.3	6.5	5.9	5.8	6.1	4.8
Serine	4.8	7.6	5.3	5.6	5.7	6.7	9.6	5.3	5.2	6.9	6.6
Glutamic acid	4.1	13.4	13.9	13.9	15.8	16.4	14.6	13.6	14.8	12.3	9.4
Proline	4.1	4.5	4.0	3.5	3.3	3.2	5.6	4.2	4.0	4.0	2.8
Glycine	9.0	—	9.8	11.1	10.2	14.9	—	10.8	8.4	11.2	18.7
Alanine	12.1	11.7	11.2	10.3	10.2	12.6	9.3	11.2	12.9	10.7	8.1
Half-cystine	0.6	0	0.7	0.5	0.3	0.4	0.9	0.9	0.4	1.1	0.8
Valine	10.6	10.0	8.6	7.4	7.4	6.3	7.7	7.8	9.1	7.7	6.9
Methionine	2.6	1.3	1.3	1.1	1.3	0.2	1.2	1.1	1.0	1.0	1.0
Isoleucine	6.1	4.4	3.4	3.9	4.0	3.5	4.4	3.0	3.1	2.9	2.9
Leucine	8.2	7.0	6.4	6.3	6.7	4.7	7.6	5.8	6.8	5.7	5.9
Tyrosine	2.0	1.9	1.8	2.4	2.3	0.8	3.2	1.7	1.5	1.8	2.0
Phenylalanine	3.3	2.2	1.9	2.0	2.2	1.3	3.9	2.0	1.9	2.0	1.8
Histidine	2.1	2.2	1.9	2.0	1.9	2.2	3.0	2.0	1.6	1.9	1.1
Lysine	9.9	4.4	4.4	3.9	3.9	4.8	5.6	4.7	3.6	4.6	5.0
Arginine	8.1	7.1	6.8	7.1	6.9	5.8	6.6	7.4	5.7	8.7	10.5
Ratio:											
His + Lys + Arg	1.60	0.50	0.49	0.49	0.47	0.47	0.61	0.53	0.38	0.62	0.79
Asp + Glu											

50S ribosomal subunits (Table 2). The amino acid compositions of the individual ribosomal proteins vary considerably (M. Yaguchi, unpublished work) and some show unusual amino acid compositions. For example, one 'external' protein on the 50S ribosome has aspartate, glutamate and alanine making up 66% of the total amino acids.

The rRNA of the *H. cutirubrum* ribosomes shows no unusual properties. The *s* values of the three rRNA molecules are identical with those of *E. coli* rRNA, i.e. 5S, 16S and 23S. The C+G content of the 16S and 23S rRNA from *H. cutirubrum* appears to be significantly higher than that for *E. coli* rRNA and may reflect the tighter binding between the rRNA and ribosomal protein required to stabilize the *H. cutirubrum* ribosome in the high-ionic-strength environment of the cell.

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