Purification and characterization of ribosomal proteins from the 30 S subunit of the extreme halophile *Halobacterium marismortui*

M. Shoham*, J. Dijk, R. Reinhardt and B. Wittmann-Liebold

*Department of Structural Chemistry, The Weizmann Institute of Science, Rehovot, Israel and Max-Planck-Institut für Molekulare Genetik, Abt. Wittmann, D-1000 Berlin 33 (Dahlem), Germany

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Ribosomal proteins were extracted from 30 S subunits of *Halobacterium marismortui* under native conditions. Their separation was based on gel filtration and hydrophobic chromatography, performed at a concentration of 3.2 M KCl to avoid denaturation. A total of nine proteins were isolated, purified and identified by partial amino-terminal sequences and two-dimensional gel electrophoresis. There is a high degree of sequence homology with 30 S proteins from *H. cutirubrum*, and also some with 30 S proteins of eubacteria. Proton NMR data indicate unfolding of the proteins in low salt. One of the proteins, however, retains its secondary structure at a salt concentration as low as 0.1 M NaCl, and even in 8 M urea. One reason for this outstanding stability could be the high proportion (50%) of β-structure in this protein as determined from circular dichroism measurements. In general, there is a higher β-sheet content than for 30 S proteins from *Escherichia coli*. Measurements of Stokes radii indicate several of the proteins to have a rather elongated shape. One of these is a complex consisting of L3/L4 and L20, similar to the L8-complex from *E. coli*. The presence of this 50 S complex in the preparation of the small subunit suggests a location on the interface between the subunits.

(Halobacterium) *Ribosomal protein* *30 S subunit*

1. INTRODUCTION

Halobacterium marismortui lives in the Dead Sea, the saltiest body of water on earth. The intracellular salt concentration is as high as that of the Dead Sea, and for potassium ions it even exceeds it [1]. Therefore, all the macromolecular constituents of the cell are exposed to extremely high ionic strengths. This requires a special adaptation mechanism expressed at the molecular level. Ribosomal particles from halophilic bacteria have been shown to be very stable in vitro at high concentrations of salt [1–5], in contrast to other ribosomes from which numerous proteins are detached under these conditions. Ribosomes from *H. marismortui* are active at salt concentrations of 3 M KCl or 2 M $(NH_4)_2SO_4$ [6].

A general feature of halophilic proteins is the preponderance of acidic residues [1]. Almost all the ribosomal proteins from halobacteria are acidic [3–5], whereas most of the ribosomal proteins from non-halophilic organisms are basic [7]. This raises interesting questions as to the nature of protein-RNA interactions in halophiles and indicates other than electrostatic contributions to the binding energy.

In this paper, we report the isolation and purification of selected ribosomal proteins from the 30 S subunit of *H. marismortui* under native conditions. For this purpose, the proteins were dissociated from the 30 S particle by removal of magnesium ions, in the presence of 3.2 M potassium chloride. The proteins were purified by a combination of gel filtration and hydrophobic

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chromatography. Physicochemical data for these proteins as well as amino acid sequence information are presented.

2. EXPERIMENTAL

2.1. *Large scale growth of cells*

The original inoculum of *H. marismortui* was a gift from Drs M. and B.Z. Ginzburg [8]. The bacteria were grown according to Mevarech et al. [9]. Ribosomes were isolated and separated into subunits as described in [6].

2.2. *Extraction of proteins from 30 S subunits*

30 S subunits were diluted to 100 A_{260} units/ml with magnesium-free extraction buffer (3.2 M KCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.8) and dialyzed against the same buffer overnight at 4°C. The dialysate was pelleted in a Beckman ultracentrifuge at 30000 rpm, Ti 45 rotor, for 15 h. The supernatant was concentrated in a Millipore Minitan ultrafiltration cell at a rate of 5 ml/min. The pellet was subjected to a second extraction cycle identical to the first one except that the EDTA concentration was raised to 20 mM. The extraction was performed in the ultracentrifuge bottle by resuspending the pellet and stirring it in extraction buffer overnight at 4°C. The extracts were checked for protein content on SDS-polyacrylamide gels.

2.3. *Separation of proteins*

The usual method for separation of ribosomal proteins, ion exchange chromatography, cannot be used in this case, due to the requirement for extremely high ionic strength. The techniques we used were gel filtration and hydrophobic chromatography, both in the presence of high salt concentrations.

2.3.1. Gel filtration chromatography

The mixture of extracted ribosomal proteins was first separated on a Sephacryl S-200 column, of 2.7 l bed volume in the following buffer: 3 M NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.8. The amount of protein loaded onto the column corresponded to 25000 A_{260} units in a volume of 40 ml. The effluent was monitored at 230 nm, and 11 ml fractions were collected at a flow rate of 1 ml/min. Further separation was performed on a Sephadex G-75 Superfine or a Fraktogel TSK HW-50(S) column under similar conditions.

2.3.2. Hydrophobic chromatography

A Sepharose Cl-4B column (5 × 20 cm) was equilibrated with 3.5 M $(NH_4)_2SO_4$, 10 mM Tris-HCl, pH 7.8. The proteins were loaded onto the column at a flow rate of 0.4 ml/min, and were eluted with a linear decreasing $(NH_4)_2SO_4$ gradient from 3.5 to 2.0 M at a flow rate of 1 ml/min. Fractions of 15 ml were collected. To check for remaining proteins on the column at the end of the gradient, the column was subsequently rinsed with water followed by 6 M urea. Aliquots from the effluents were concentrated and analyzed on SDS-polyacrylamide gels.

2.4. *Determination of Stokes radius*

Stokes radii were determined from the elution volumes on an analytical Sephacryl S-200 column (1.7 cm × 193 cm), using upward flow in 3 M NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.8. A sample of 2.0 ml was applied to the column followed by 2.0 ml column buffer which contained 4.0 M NaCl, to keep the protein zone from diffusing backwards. The flow rate was 22 ml/h. The elution was followed on a UV monitor set to the wavelength of maximal absorption for the particular sample. The amount of protein loaded onto the column was at least 1.3 absorbance units at this wavelength, which corresponds to the minimal amount necessary to get a distinct peak in the elution profile with our experimental setup. In general, the proteins could be recovered by pooling the corresponding fractions. The void volume and the total volume of the column were determined with Blue dextran and glycine, respectively. The column was calibrated with the following proteins of known Stokes radius: cytochrome *c*, myoglobin, ovalbumin, bovine serum albumin, catalase, urease, as well as ferredoxin and malate dehydrogenase from *H. marismortui*. The data were analyzed according to Ackers [10].

2.5. *Circular dichroism*

Data were recorded on a Jasco J-500 A spectropolarimeter in the range 190–240 nm with an optical cell of 0.2 ml volume and a path length of 1 mm. The data were analyzed with the aid of the computer program CONTIN [11,12]. The protein

concentration was determined from a quantitative amino acid analysis.

2.6. *Amino acid analysis*

Protein samples were desalted by precipitation with 5% trichloroacetic acid using 50 μg sodium deoxycholate as a carrier [13]. The protein pellets were dissolved in 300 μl of 5.6 M HCl and hydrolyzed for 24 h at 110°C. The dried samples were derivatized with PITC and analyzed on a Waters HPLC chromatograph using a 2-pump gradient system [14].

2.7. *Sequence analysis*

N-terminal sequences were determined in the Berlin liquid-phase sequenator [15] using on-line detection of the PTH-derivatives by an isocratic HPLC system [16].

2.8. *Sequence homology determination*

Each sequence was compared against all ribosomal proteins in the Dayhoff protein sequence data bank. The computer analysis was performed with the aid of programme ALIGN [17]. As scoring matrix the mutation data matrix was used, and a break penalty of 20 was employed.

2.9. *Two-dimensional gel electrophoresis*

Two-dimensional gel electrophoresis was performed according to [18] using an apparatus described in [19,27].

2.10. *Protection against proteolysis*

The following protease inhibitors were added to all buffer systems: 0.1 M phenylmethanesulphonyl fluoride (PMSF) and 0.02 mM benzamidine. In addition, 0.02% (v/v) 2-mercaptoethanol was in all buffer solutions.

2.11. *Concentration of protein solutions*

Protein solutions were concentrated by ultrafiltration using membranes or dialysis bags. For the latter, a dialysis bag was immersed in hygroscopic material, Ficoll-400 (Pharmacia) for small volumes, and refined sucrose for large volumes at intermediate steps in the purification procedure. Ultrafiltration devices Minitan (Millipore), Micro-Pro-Di-Con (Biomolecular Dynamics, for very small final volumes) and Amicon were used occasionally.

2.12. *Proton NMR*

The proteins were transferred into 3 M NaCl, 5 mM phosphate, pH 7 (uncorrected), in ^2H_2O by exhaustive dialysis in Spectra-Por 6 tubing. The protein concentration was between 1 and 3 mg/ml.

Spectra were recorded on a Bruker 270 WH spectrometer operating in the Fourier transform mode. Double-precision accumulation was used for long-term averaging of 5000–20000 transients. A pulse length of 12 μs was used, the pulse repetition interval was 0.9 s and the residual water signal was suppressed by a gated decoupling pulse of 0.3 s immediately before the analytical pulse.

The free induction decay was multiplied by an exponential function equivalent to 2 Hz line broadening in order to improve the signal-to-noise ratio. Chemical shifts were measured relative to 2,2-dimethyl-2-silapentane (DSS).

3. RESULTS AND DISCUSSION

3.1. *Protein purification*

Exposure of individual ribosomal proteins from *H. marismortui* to low ionic strength causes denaturation. Therefore, a high salt concentration of 3 M NaCl or 3.2 M KCl or 2.0 M $(NH_4)_2SO_4$ was maintained at all steps of the isolation and purification procedure for 30 S proteins, to avoid any risk of denaturation. The major tool for separation of ribosomal proteins in general, ion-exchange chromatography, can thus not be used in this case. The methods of separation employed were gel filtration and hydrophobic chromatography, both carried out in the presence of high salt concentrations.

After extraction from 30 S subunits in magnesium-free and high salt buffer the mixture of ribosomal proteins was separated by gel-filtration chromatography on Sephacryl S-200. The proteins were initially separated into a set of nine fractions as outlined in fig.1. The first fraction, as well as the last were satisfactorily purified by this single step. The other fractions contained mixtures which were subsequently concentrated and further separated by gel-filtration chromatography on Sephadex G-75 or Fraktogel TSK HW-50 (S).

Pool E_1 was further fractionated by hydrophobic chromatography on Sepharose CL-4B, using a decreasing gradient of 3.5–2.0 M $(NH_4)_2SO_4$. When phenyl-Sepharose or octyl-

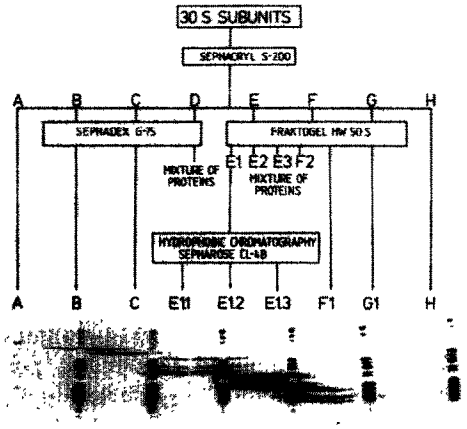

Fig.1. Outline of separation and purification scheme of ribosomal proteins from the 30 S subunit of *H. marismortui*. A high salt concentration (usually 3.2 M KCl) was maintained during all steps to avoid any risk of denaturation. At the bottom the distribution of the proteins, as assayed by SDS gel electrophoresis, is shown.

Sepharose (Pharmacia) were used, the proteins were bound so strongly to the matrix that they could only be eluted by employing denaturing conditions. A total of 9 proteins were purified by non-denaturing techniques.

3.2. *Identification and amino-terminal sequences of the isolated proteins*

The amino-terminal sequences are presented in fig.2. The proteins were identified by comparison of the amino-terminal sequences to 30 S proteins from *H. cutirubrum* (table 1). However, this approach is difficult because of the limited amount of sequence information available on *H. cutirubrum* 30 S proteins [20], so that it is not surprising that for protein C no homology could be found. This protein, as well as a protein complex obtained in very small amounts (E1.1), were identified by comparison of the two-dimensional gel-electrophoresis pattern with that of both *H.*

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                    5         10        15        20
3           A D Y Q Y F I E D G L Q R?T Q I D N?F F A . . .
dc-S4       S D E L E F I E E G L Q R S Q I D E F F A . . .

C           V E M I V K S K V K E A V K A I D P E M R V N . . .

E1.3        A T L Y D V P P E E L E?E A I T E T I A D . . .
Hm-S12      A T L Y D V P P E E L L E A I T L X T L . . .
Hc-S13      A T L Y D A P V D E L I D E L A A B L Z T . . .

E1.2        M D I D I I E E D E N P M L H R T D V R F E V V X D E A T . .
Hm-S15      M D I D I I E E D E N P M L H R T D V R F E V V H D E A T . .
Hc-S17      M E I E I L G Q E D B P L L H R T N V Z F K I V H N D A . . .

F1          V T N T H?G K K K T A V A R A T V R E G E?G R V X I . . .
Hm-S17      V T N T S G K K K T A V A R A T V R E G E G R V R I . . .

G1          A I N D A F A N A L X A L N . . .
Hc-S20      T A N D P L S D E L S Z I D . . .

H           P G N K Y Y N D E G X L D P X T . . .
Hm-S19      P L N E Y V R D . . . . . .
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Fig.2. Sequence homology of ribosomal proteins from the 30 S subunit of *H. marismortui* (Kimura, M., personal communication) and *H. cutirubrum*. Amino acid sequences for the latter are taken from Yaguchi et al. [20].

Table 1

Comparison of ribosomal proteins from various bacteria

Protein[a]	*H. marismortui*[b]	*H. cutirubrum*[c]	Sequence homology to *E. coli*
A	–	Hc-(L3/4 + L20)	–
B	Hm-S1	Hc-S4	Ec-S19
C	–		–
H	Hm-S19		–
E1.1	–	Hc-(S14 + S18)	–
E1.2	Hm-S15	Hc-S17	–
E1.3	Hm-S12	Hc-S13	–
F1	Hm-S17		Ec-S9
G1	–	Hc-S20	Ec-S21

[a] Described in this paper
[b] Arndt et al. [21]
[c] Yaguchi et al. [20]

cutirubrum [18] and *H. marismortui* [21]. A spot corresponding to protein C is absent in the *H. cutirubrum* pattern and is labeled S2 in the *H. marismortui* proteins. The nomenclature used by Matheson and co-workers [20] and Kimura [21], although both based on 2D gels, are different from each other. For comparison, we list the names of the isolated proteins in both systems of nomenclature (table 1).

We also checked for homology of the N-terminal sequences of *H. marismortui* against all the sequences of ribosomal proteins from *Escherichia coli* and *Bacillus stearothermophilus*. Significant homology, above two units of standard deviation, was found for three of the proteins (table 2). For the other proteins, no such homology could be found.

Two of the isolated protein fractions are complexes. Fraction A is a complex between a relatively large and a small protein. Its electrophoretic mobility, Stokes radius, circular dichroism spectrum, and proton NMR spectrum are identical to those for the complex between L3/L4 and L20, isolated from preparations of the large subunit of *H. marismortui* (G. Nasioulas, personal communication). The R_s value (54 Å) is nearly the same as that found for the complex from the 50 S subunit (53 Å) and indicates that the stoichiometry for both is probably 4:1 as in the case of the L8-complex of *E. coli* [22]. Unfortunately, the poor staining properties of protein L20 on gels did not allow precise measurements. The fact that the same complex is found in preparations of both the small and large subunit seems to indicate that this complex is located on the interface between the two subunits. Proteins from the 50 S subunit have occasionally been found associated with the 30 S subunit [23]. Fraction E1.1 is an equimolar complex of a 14 and 16 kDa protein. This complex elutes as a single peak on the analytical S-200 column, corresponding to a molecular mass of 30 kDa. Protein H is a dimer of molecular mass 2 × 11 kDa.

3.3. *Stokes radius and molecular mass*

Non-halophilic as well as two halophilic proteins were used to generate a calibration curve on an analytical Sephacryl S-200 column. 2Fe-2S ferredoxin and malate dehydrogenase from *H. marismortui* are not hydrodynamically distinct from non-halophilic proteins (fig.3). It is therefore legitimate to use non-halophilic proteins for calibration of the column. The Stokes radii of the individual isolated proteins range from 13 to 54 Å

Table 2

Sequence homology to 30 S proteins from *E. coli*

Protein from *H. marismortui*	Length of N-terminal sequence of *H. marismortui* protein	Homology to *E. coli* protein	Number of identical residues	Alignment with line-up residues to *E. coli* protein	Score (in units of standard deviation)
B	27	S19	3	55–81	3.3
G1	14	S21	4	6–19	2.5
F1	22	S9	6	4–25	2.5

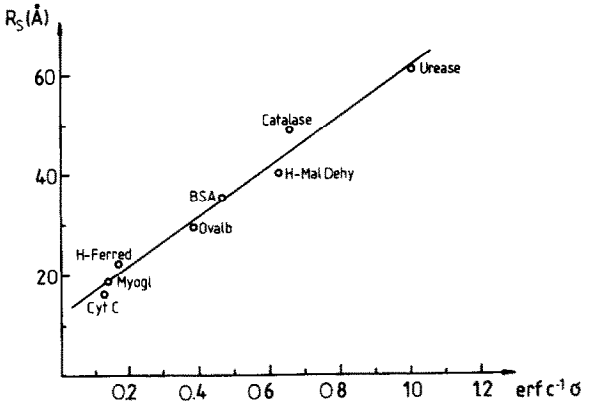

Fig.3. Calibration curve of Stokes radii (R_S) for the proteins on an analytical Sephacryl S-200 column vs the inverse error function complement of the partition coefficient according to Ackers [10]. The following values for R_S were used: cytochrome *c* (Cyt C) 16.4 Å, myoglobin (Myogl.) 19.4 Å, ovalbumin (Ovalb.) 30.0 Å, bovine serum albumin (BSA) 36.0 Å [25,26], catalase 49.8 Å (calculated from diffusion coefficient given by Agner [27]), urease 61.9 Å (calculated from diffusion coefficient given by Sumner et al. [28]). For malate dehydrogenase (H-Mal.Dehy.) and ferredoxin (H-Ferred.) from *H. marismortui* 41.1 Å and 22.6 Å were calculated, respectively, from the radii of gyration ([29]; E. Wachtel and H. Eisenberg, personal communication).

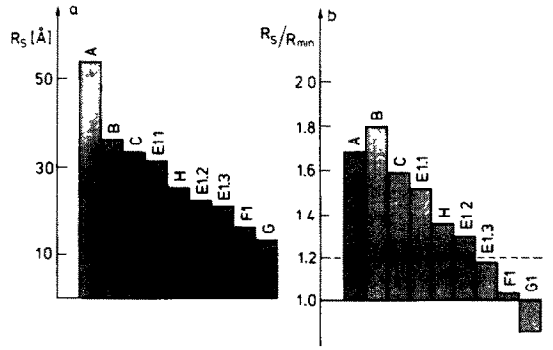

Fig.4. (a) Stokes radii (R_s of the isolated proteins, as determined on an analytical Sephacryl-200 column) (see text). (b) R_{min} is the radius of the equivalent sphere, calculated from a partial specific volume of 0.735 ml/g and the molecular mass of the protein, as determined on an SDS-polyacrylamide gel. The Stokes radius R_s is higher than R_{min}, because it represents the radius of a hydrodynamic sphere, including a shell of hydration. For globular proteins, the ratio of R_s/R_{min} is around 1.2, as indicated by the broken line. A very high value of R_s/R_{min} is indicative of an elongated shape. Accordingly, proteins B, C and E1.1 appear to have an axial ratio far from 1.0.

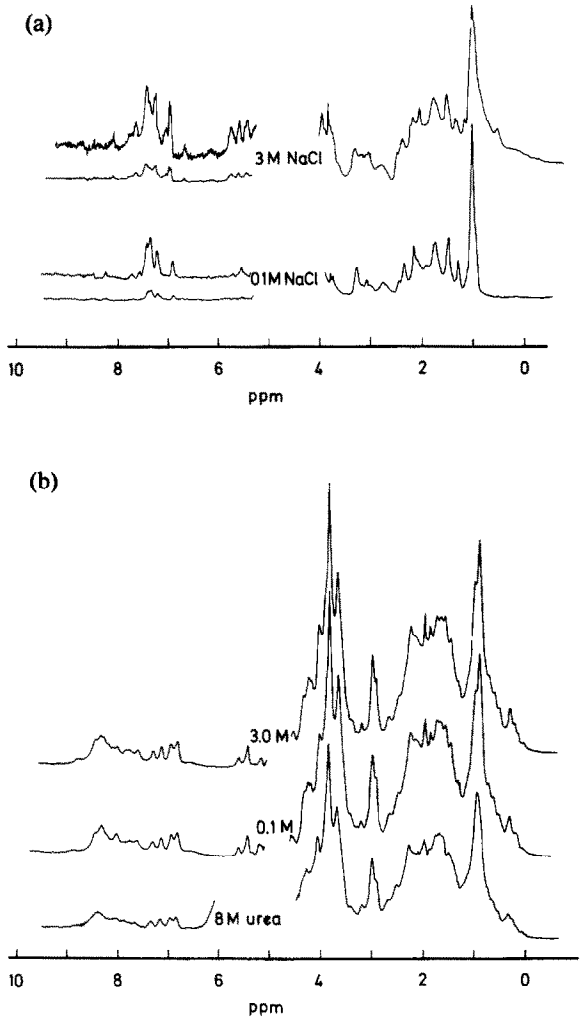

Fig.5. Protein NMR spectra, recorded on a Bruker 270 MHz spectrometer. Chemical shifts are measured relative to 2,2-dimethyl-2-silapentane (DSS). (a) Protein F. Most of the evidence for folding derives from amide protons at 9 ppm, the perturbed aromatic resonances of phenylalanine (7.4 ppm), tyrosine (7.1 and 6.9 ppm) and poorly resolved ring current shifted methyl resonances (below 1 ppm). The latter disappear to a large extent in 0.1 M NaCl. The same applies to resonances between 5 and 6 ppm, which arise from interactions between neighbouring strands of a β-sheet [30]. (b) Protein C. There is a very large number of broadened amide resonances between 8 and 10 ppm, some perturbed aromatic resonances and an unusually large number of ring current shifted methyl resonances. The spectrum barely changes in low salt or even in 8 M urea. In the latter many of the amide resonances survive, which is another indication for an unusually stable structure.

(fig.4a). Four of the isolated proteins have unusually high Stokes radii relative to their molecular mass (fig.4b). This means that a fair proportion of their mass is located far from the center of mass. Such a behaviour is expected for complexes, such as A and E1.1. The individual proteins B and C with Stokes radii of 33 and 31 Å, respectively, thus appear to have a rather elongated shape.

3.4. *Conformational changes on transfer to low salt*

For most of the isolated proteins there is evidence, derived from NMR, for unfolding of the proteins on transfer from 3 to 0.1 M NaCl. For example, some of the ring current shifted methyl resonances of protein F vanish on lowering the salt concentration to 0.1 M NaCl (fig.5a). On the other hand, protein C behaves differently. Its NMR spectrum remains unchanged in 0.1 M NaCl and even in 8 M urea (fig.5b). Protein C is thus an exceptionally stable protein.

3.5. *Secondary structure*

The secondary structure content was analyzed by circular dichroism measurements in the range 190–240 nm. Some of the isolated proteins have a rather high percentage of β-structure, e.g., 67 and 50% for proteins B and C, respectively. This may be one of the reasons for the unusually high stability of protein C in solutions of low salt and urea. These two proteins have more β-structure than any of the 30 S proteins from *E. coli* [24]. On the other hand, protein E1.3 has very little β-structure and 75% α-helical content.

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