ACIDIC RIBOSOMAL PROTEINS FROM THE EXTREME HALOPHILE, *HALOBACTERIUM CUTIRUBRUM*

The simultaneous separation, identification and molecular weight determination

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1. Introduction

The functions of the ribosome are, to a large degree, invested in the protein components of the macrostructure [1–5]. Ribosomal proteins in most procaryotic and eucaryotic cells are basic in ionic character [6]. Notable exceptions are the acidic ribosomal proteins from the extreme halophilic bacterium, *Halobacterium cutirubrum* [7,8]. While it appears that the transition to halophilism has been accompanied by a drastic shift in the charge of these ribosomal polypeptides, there has been considerable conservation in those structural features which allow for the usual protein–protein and protein–nucleic acid interactions required in the assembly and translation properties of the ribosomal unit [9–11]. The identification of the protein moieties involved in these and other interactions in the halophile ribosome requires a rapid and reproducible screening method for the separation, enumeration and identification of these acidic polypeptides in the complex ribosomal protein mixtures. In this paper we present the resluts of analyses of the halophile ribosomal proteins using a two-dimensional polyacrylamide gel electrophoresis system which incorporates sodium dodecyl sulfate in the second dimension. This system separates the proteins of the 30 S and 50 S ribosomal subunis of *H. cutirubrum*, ascribes 21 proteins to the 30 S and 32 to the 50 S subunit and yields values for the molecular weights which range from 11 800 to 76 000 for the polypeptides of the 30 S and from 11 500 to 48 400 for those of the 50 S subunit. The sum of the molecular weights for the proteins of the 30 S subunit exceeds the expected total molecular weight of the subunit indicating that the 30 S subunit is structurally heterogeneous.

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2. Materials and methods

2.1. *Purification of ribosomal subunits and preparation of ribosomal proteins*

H. cutirubrum cells grown under conditions previously reported [11] were harvested by centrifugation. The 70 S ribosomes and subunits were prepared as described [11] except that the purification by zonal centrifugation in a Spinco BXV Ti Zonal rotor was achieved using an isokinetic sucrose gradient [12] from 2 to 32% (w/v) sucrose in buffer D′ [3.0 M KCL – 500 mM NH_4CL – 10 mM Mg^{2+} – 20 mM Tris–HCL, pH 8.05) of Rauser and Bayley [13]. Freshly prepared subunits in buffer D′ were concentrated to 5 mg/ml in a Amicon Diaflo ultrafilter with an XM50 membrane and then dialysed for 24 hr against 2 changes of TMK buffer (10 mM Tris–HCL –50 mM KCL –0.3 mM Mg^{2+} –6 mM β-mercaptoethanol (BME), pH 7.6). Subsequently, equal volumes of subunit and 7 M lithium chloride –10 mM EDTA were mixed and stirred for 48 hr at 4°C. Solid urea was added to make 4 M. This was allowed to stand for 14 additional hr at 4°C. The suspension was then centrifuged for 1 hr at 165 000 *g*. The supernatant from this centrifugation was dialyzed against 3 changes of 6 litres of distilled water and lyophilized. The protein samples were dissolved in sample buffer containing 0.125 M Tris–HCL, 8 M urea, 20 mM methylamine (MA) and 6 mM BME, pH 9.8.

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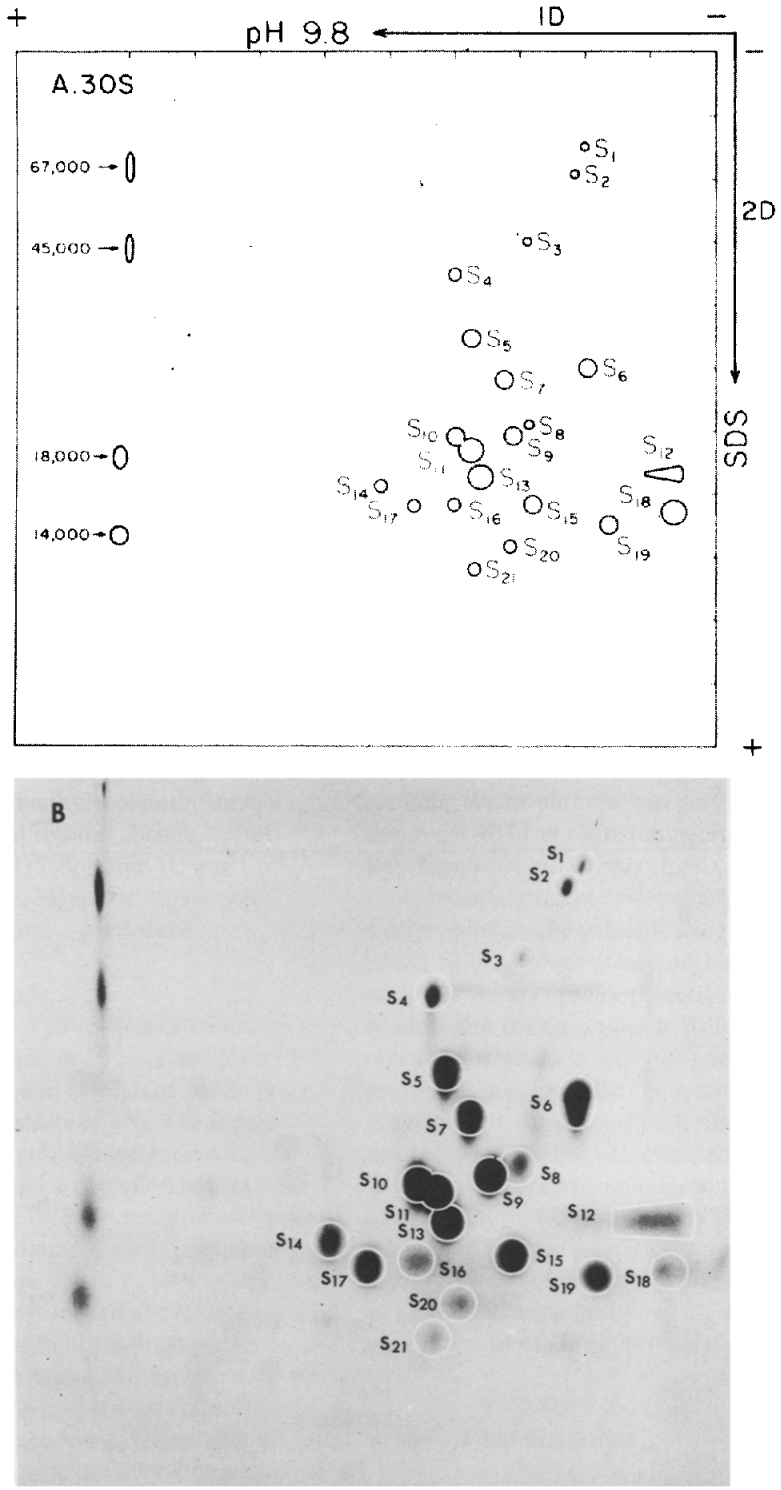

Fig. 1. Two-dimensional electrophoretic pattern of 30 S ribosomal proteins from *H. cutirubrum*. (A) is a schematic representation of the acrylamide gel shown in (B). Migration directions are marked with arrows. The anode was at the left in the first dimension, at the bottom in the second. (For buffer and gel conditions see text.) On the left side of the electrophoretogram are included four proteins of known molecular weight, i.e. bovine serum albumin, ovalbumin, β-lactoglobulin, α-lactalbumin.

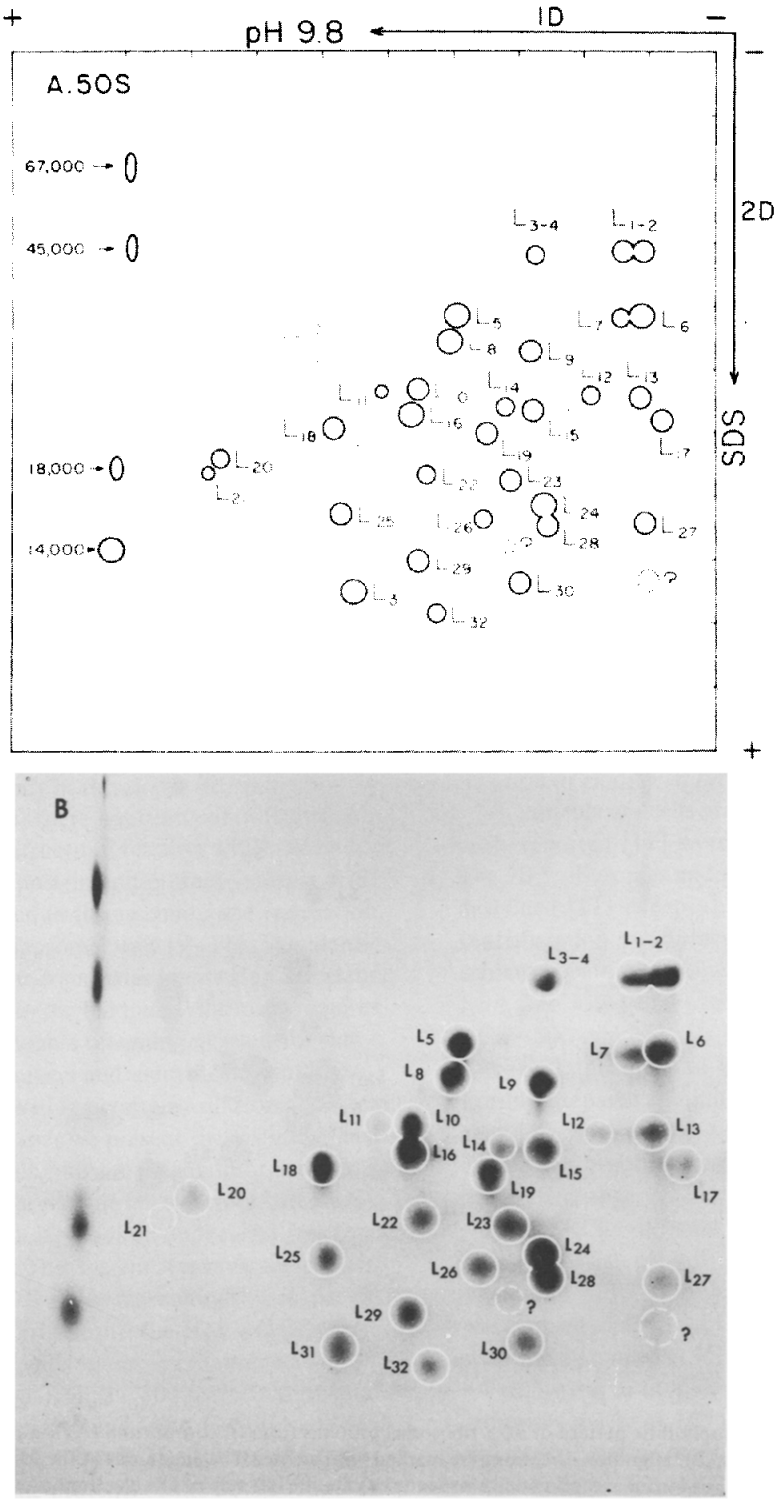

Fig. 2. Two-dimensional electrophoretic pattern of 50 S ribosomal proteins from *H. cutirubrum*. (A) is a schematic representation of the acrylamide gel shown in (B). (See fig. 1.)

2.2. *Electrophoresis: buffers and solutions*

First dimension electrophoresis was a modification of one previously described [14]. All buffers were prepared with distilled and deionized water. Solution for gels of first dimension electrophoresis at pH 9.8 were: Buffer A, which contained 1.0 M Tris, 0.07 N HCL, 0.18% (v/v) *N, N, N', N'*,-tetramethylethylenediamine (TEMED) and 8 M urea; Buffer B, containing 0.25 M Tris, 0.018 N HCl and 8 M urea; Solution C, made up of 15% (w/v) acrylamide, 0.4% (w/v) methylenebisacrylamide and 8 M urea; Solution D, containing 6.7% (w/v) acrylamide, 1.7% (w/v) methylenebisacrylamide and 8 M urea, and Solution E, 0.3% (w/v) ammonium persulfate and 8 M urea. The first dimension electrode buffer was 0.26 M Tris, 0.027 M glycine and 6 M urea.

For the second dimension the stock solutions were the same as those used by [15] for single dimension sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis: Solution F, was 22.2% (w/v) acrylamide and 0.6% (w/v) methylenebisacrylamide; Solution G, contained 0.2 M sodium phosphate buffer pH 7.2 and 0.2% (w/v) SDS; Solution H, was 1.5% (w/v) ammonium persulfate. Electrode buffer consisted of 1 part Solution G and 3 parts water.

2.3. *Electrophoresis methods*

For the first dimension a 150–300 μg 30 S or 50 S ribosomal protein sample in 50 μl of sample buffer was placed on a 5 X 180 mm column of polyacrylamide gel (separation gel + staching gel). The separation gel [7.5% acrylamide (w/v)] was prepared by mixing 3 parts Solution A and 4 parts C. The mixture was deaerated and cooled before the addition of 1 part Solution E. A 170 mm separation gel was polymerized in 5 mm i.d. acid washed glass tubes. A 10 mm stacking gel was subsequently polymerized above the separation gel by mixing 2 parts Solution B, 3 parts Solution D, 1 part 8 M urea, 2 parts Solution E and 1 μl β-dimethylaminopropionitrile per 1 ml solution. Electrophoresis was conducted toward the anode at 1.5 ma per gel column for approximately 4 hr at room temperature. Bromphenol blue was used as a tracker dye. Buffer (6 litres) was circulated from the upper to lower reservoir via a mixing vessel by means of a peristaltic pump at 3 litres hr.

For the second dimension, first dimension gels were removed and placed for a period of 30 min at 25°C in an equilibrating solution consisting of 1% (w/v) SDS, 6 mM BME, 20 mM MA, 0.1 mM dithiothreitol (DTT), and 6 M urea.

The second dimension electrophoresis was performed using slab cells similar to that described by Kaltschmidt and Wittmann [16], except that the slab thickness was reduced to 3 mm to facilitate radioautography. The cells were filled with cooled second dimension gel solution prepared by mixing 10 parts Solution G, 9 parts Solution F, 1 part Solution H, and 1.5 μl of TEMED per ml of solution [15]. The first dimension gels were then placed at the upper end of the cells together with a 20 mm gel column containing 4 marker proteins, i.e. bovine serum albumin (67 000), ovalbumin (45 000), β-lactoglobulin (18 000), and α-lactalbumin (14 000). The marker gels were prepared in the same manner as the first dimension gels in that the proteins were subjected to electrophoresis at pH 9.8 (1.5 mA per column for 30 min).

After polymerization at room temperature, descending electrophoresis was performed toward the anode for 40 hr at room temperature at 50 mA/gel slab. Electrode buffer was circulated at 3 litres/hr from the upper to the lower chamber. Following electrophoresis, the gel slabs were removed and total length of the gel was measured. Gels were then fixed with 12.5% (w/v) trichloroacetic acid for 45 min, stained with 0.2% Coomassie Brilliant Blue for 30 min and destained in 10% (v/v) acetic acid. The total length of the gel was then measured again as well as the migration distance of each ribosomal protein spot and the 4 calibration proteins. Mobility as a function of molecular weight was determined essentially as described by Weber and Osborn [15]. (Mobility = distance of protein migration X length of gel before staining/length of gel after staining). A standard curve of mobility of standard proteins against log molecular weight was plotted for each gel slab.

3. Results and discussion

Fig. 1 and 2 show the second dimension gel slabs with the four calibration proteins and the fingerprint patterns for the 30 S and 50 S ribosomal proteins. Based on these fingerprints we propose to establish a nomenclature for the *H. cutirubrum* ribosomal proteins, analagous to that previously established for the

Table 1
Molecular weights of 30 S and 50 S ribosomal proteins of *Halobacterium cutirubrum*.

30 S protein	Average molecular weight*	Range of mol.Wt	50 S protein	Average molecular weight*	Range of mol. Wt
HS_1	76 000	73–79 000	HL_1	48 400	47–50 000
HS_2	67 000	65–70 000	HL_2	48 400	47–50 000
HS_3	50 000	48–52 000	HL_3	47 300	45–49 000
HS_4	41 800	40–43 000	HL_4	47 300	45–49 000
HS_5	31 800	31–33 000	HL_5	37 200	35–39 000
HS_6	29 000	27–30 000	HL_6	36 600	35–38 000
HS_7	27 300	26–28 000	HL_7	36 400	35–38 000
HS_8	22 600	21–24 000	HL_8	32 800	31–34 000
HS_9	21 300	20–22 000	HL_9	32 000	31–33 000
HS_{10}	20 900	20–22 000	HL_{10}	27 300	26–28 000
HS_{11}	19 900	19–21 000	HL_{11}	27 000	26–28 000
HS_{12}	18 800	18–20 000	HL_{12}	27 500	26–29 000
HS_{13}	17 700	17–18 000	HL_{13}	27 300	26–28 000
HS_{14}	16 800	16–17 000	HL_{14}	25 900	24–27 000
HS_{15}	15 900	15–17 000	HL_{15}	24 900	23–26 000
HS_{16}	15 500	14–16 000	HL_{16}	24 200	23–25 000
HS_{17}	15 100	14–16 000	HL_{17}	23 800	22–25 000
HS_{18}	15 400	14–16 000	HL_{18}	22 600	21–24 000
HS_{19}	15 000	14–16 000	HL_{19}	22 500	21–23 000
HS_{20}	13 300	12–14 000	HL_{20}	21 000	20–22 000
HS_{21}	11 800	11–12 000	HL_{21}	19 500	18–20 000
			HL_{22}	19 300	18–20 000
			HL_{23}	18 900	18–12 000
			HL_{24}	16 900	16–18 000
			HL_{25}	16 700	16–17 000
			HL_{26}	16 300	15–17 000
			HL_{27}	16 200	15–17 000
			HL_{28}	15 300	14–16 000
			HL_{29}	13 800	13–14 000
			HL_{30}	13 800	13–14 000
			HL_{31}	11 600	11–12 000
			HL_{32}	11 500	11–12 000

* Average molecular weight data derived from 4 independent preparations and 8 molecular weight determinations for each spot.

ribosomal proteins of *E. coli* [16, 17]. Hence as shown in Fig. 1 and 2 we have designated the proteins of the small subunit with the prefix S and those of the large subunit as L. To indicate the genus we propose to identify our proteins as 'H' designating *Halobacterium* (see table 1).

Using this classification system, and enumerating the spots from left to right, beginning at the top and proceeding to the bottom, we ascribe 21 distinct spots to the S series and 32 to the L group; a total of 53, not unlike the total of 54–55 assigned to *E. coli* ribosomes [17]. Not seen in fig. 2B but displayed schematically in fig. 2A are two minor spots located below L_{26} and L_{27} respectively. These might not be true 50 S proteins as their appearance in 50 S preparations is variable and may be related to either the method of purification of the subunits or the media and/or temperatures at which the bacteria are grown (unpublished observations). Similarly proteins S_1 and S_2, though present in every 30 S preparation tested so far may be cytoplasmic proteins adsorbed to the smaller subunit as is thought to be the case by some authors for a 67 000 mol. wt. protein in the *E. coli* 30 S subunit designated S_1 [3].

As mentioned previously and implicit in our use of a first dimension gel at pH 9.8 is the difference in

ionic character between the ribosomal proteins of *H. cutirubrum* and any other procaryotic ribosomal proteins. As a consequence of this ionic difference, attempts to fingerprint the ribosomal proteins of this halophile by other published two dimensional methods [16, 18] were unsuccessful. Indeed if second dimension electrophoresis was carried out at low pH (pH 4–6) most of the polypeptides were insolubilized or retarded in their movement from the first dimension gel disc into the second dimension slab. This was not unexpected as Bayley had indicated earlier that the majority of ribosomal proteins from this organism had isoelectric points around 3.9 [7]. At the pH utilized herein, all of the 30 S and 50 S proteins migrate through the first dimension gel column and subsequently into the second dimension slab. This is crucial to the utilization of this procedure for the future analyses of stoichiometry and topography of these proteins in the halophile subunits. Indeed, in the two dimensional procedures currently in use for these analyses in *E. coli* there is a differential retention of proteins in the sample gel of the first dimension [19, an our own unpublished observations]. As a result numerous repeated analyses and subsequent statistical extrapolations are necessary. These difficulties are not encountered with our method. The calculated molecular weights from run to run were reproducible and within allowable experimental error (for the results presented herein the average standard error was ± 3.8%). The fingerprint pattern was not altered through the range of acrylamide concentrations from 8 to 18% in second dimension. In addition, because no detectable amounts of protein were retarded in the first dimension and because of the very sensitive staining method used we have been able to employ very small quantities of 50 S or 30 S protein mixtures, down to 150 μg.

With respect to the variability of protein spots in our fingerprints, we note particularly the variance of 50 S proteins L_{20} and L_{21} . Both of these proteins are unusually rich in alanine and identical in almost every respect. The isolated, purified, variants differ in size as is indicated for the mixture in fig. 2. This molecular variation is related to the temperature at which the cells are grown (manuscript in preparation).

Table 1 shows the molecular weight for each of the small and large subunit proteins. The average molecular weights of the proteins from the 30 S subunit vary from 11 800 to 76 000 and those of the 50 S subunit lie between 11 500 and 48 400. The average molecular weights for the 30 S and 50 S proteins are about 27 000 and 26 000 respectively. These differs substantially from those of the small and large subunits of *E. coli* reported to be 19 000 and 16 300 respectively [20]. The sum of the molecular weights for the 30 S and 50 S ribosomal proteins of *H. cutirubrum* is 563,000 and 880,000 respectively. Based on an average standard deviation of 3.8% for these determinations, the values may range from 798 000 to 862 000 for the total 50 S proteins and from 542 000 to 584 000 for the 30 S. Bayley [21] determined the molecular weight for the 50 S and 30 S subunit of *H. cutirubrum* as being 1.9×10^6 and 0.9×10^6 respectively. A protein content of 40% as reported by Bayley [21] and verified in our laboratory would indicate that the total molecular weight of the 50 S and 30 S proteins should be around 760 000 and 360 000. Since the sums of the individual molecular weights of the 30 S proteins exceed the expected total molecular weight, we conclude, as did others, [19,23] for the 30 S particle of *E. coli*, that the ribosomal 30 S subunit in *H. cutirubrum* is structurally heterogeneous.

The correlation between the total molecular weight for the halophile 50 S proteins and the estimated total molecular weights is remarkably analogous to the data obtained for the *E. coli* 50 S unit [20] and could indicate that in both these procaryotes the proteins of 50 S subunit are present in near stoichiometric amounts. Strict adherence to stoichiometry may not be true as Weber [19] has shown that there is some heterogeneity in the *E. coli* 50 S subunit proteins and our unpublished data for HL_{20}, HL_{21} indicates that this may be the case in *H. cutirubrum* as well. Our data for another very different procaryote would further strengthen Kurland's original contention that the ribosome is structurally and functionally, a heterogeneous organelle [22,23]. The apparent size difference between the average ribosomal protein from *H. cutirubrum* in contrast to those from *E. coli*, may be a result of some structural compensation in the halophile ribosomal proteins manifest in their unique ionic character (for ribosomal proteins) and related to the 4 molar salt environment in which the proteins must exist. These aspects of halophile ribosome protein structure, topography and stoichiometry are now being investigated.

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