Chapter-3
RESULTS & DISCUSSION
3.1: Isolation of ribosomes and fractionation of r-proteins

Apart from **ultra-centrifugation**, there are a few other methods to isolate ribosomes from bacteria. It was essential for our studies to ensure that the ribosomes contain little or no contamination by proteins of the supernatant fraction. We have employed three methods for the isolation of ribosomes from *S. acidocaldarius*. In the first method, ribosomes were isolated from alumina ground cells by ultra-centrifugation (Minks *et al.*, 1978). In the second method, ribosomes were isolated by gel filtration and ion exchange chromatography, after lysing the cells with low concentrations of Triton X-100 and NP-40. In the third method, ribosomes were isolated by **ultra-centrifugation** of cell lysates obtained by the use of Triton X-100 and NP-40.

**Isolation of ribosomes by different methods and analysis of r-proteins by 2-D gel electrophoresis:**

The table summarises the isolation procedure of *S. acidocaldarius* ribosomes on a medium scale from 10-30 gms of cells using gel filtration and ion-exchange chromatography. The advantage of this technique is that the whole process is completed within a period of 24 hrs (**Table-2**).

Sephacryl S-200 is a very rigid gel filtration medium composed of cross linked **allyl dextran** which allows fast flow rates and excellent resolution even on large columns. Chromatography of S-30 extract on S-200 results in elution of the ribosomes as a sharp peak (**fig. 2**). The peak fractions obtained after S-200 chromatography were diluted to reduce the ammonium chloride concentration to 50 mM, and loaded on the DE-52 column **pre-equilibrated** with the same buffer as described in the materials and methods section. The **non-specifically** bound
Table 2
Purification of *S. acidocaldarius* ribosomes from 10 gms. of cells

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Lysis</td>
<td>1 hour</td>
</tr>
<tr>
<td>S-30 Supernatant</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Sephacryl S-200 Chromatography</td>
<td>8-12 hours</td>
</tr>
<tr>
<td>DE-52 Chromatography</td>
<td>4 hours</td>
</tr>
<tr>
<td>Dialysis</td>
<td>f</td>
</tr>
</tbody>
</table>

Experimental details in materials and methods section.
Fig. 2: Sephacryl S-200 chromatography of *S. acidocaldarius* S-30.
Figure 2
material gets eluted at 0.1 M ammonium chloride while the pure ribosomes elute at 0.7 M ammonium chloride as depicted in fig. 3. The ribosomes were dialysed against $\text{TKM}_{10}\text{Me}_7$ containing 15% glycerol.

It was found that ribosomes isolated under gentle lysis conditions do not contain high molecular weight non ribosomal proteins. This technique is general and simple enough, so that it can be adopted for the isolation of ribosomes from other thermophilic organisms, provided, the concentration of Triton X-100 and NP-40 for the lysis of these organisms are optimised. The ribosomal protein pattern so obtained was almost similar to those published by Schmid and Bock, (1982). Along with ribosomes it is possible to isolate several small basic DNA binding proteins of 7 Kda, 8 Kda and 9 Kda which elute in the later fractions of the S-200 column. Grote et al. (1986) have also described a similar procedure for the isolation of ribosomes from $S. \text{acidocaldarius}$ cell extracts (obtained by means of a french press), employing chromatography on Sephacryl column.

Ribosomes were also isolated by the conventional method viz., from the alumina ground cells as described earlier.

Cell extracts of $S. \text{acidocaldarius}$ were obtained after lysis with non ionic detergents such as Triton X-100 and NP-40 and the ribosomes were isolated by ultra-centrifugation.

Ribosomes isolated by gel filtration and ion-exchange chromatography were compared with ribosomes isolated by ultra-centrifugation of cell lysates obtained by Triton X-100 and NP-40 and also with ribosomes isolated from alumina ground cells using ultra-centrifugation. Ribosomes isolated by the above three methods contain identical ribosomal protein patterns as analysed by two-dimensional gel electrophoresis (fig. 5). But in the case of ribosomes isolated from alumina ground cells and those from ultra-centrifugation of cell lysates, there is a
Fig. 3: DEAE-cellulose chromatography of *S. acidocaldarius* ribosomes.
Fig. 5: Two-dimensional gel electrophoresis of *S. acidocaldarius* r-proteins: (A) Ribosomes isolated from alumina ground cells, (B) Ribosomes isolated from cells lysed with Triton X-100 and NP-40, (C) Ribosomes isolated using ultra-centrifugation of cell lysates obtained by non ionic detergents. In each case, 150 μg of extracted r-proteins were used for electrophoresis.
clear contamination of high molecular weight **non-ribosomal** proteins which are not seen in case of ribosomes isolated by gel filtration and ion exchange chromatography as analysed by **SDS-polyacrylamide** gel electrophoresis (fig. 4).

**2-D gel electrophoretic analysis of *S. acidocaldarius* ribosomal subunits:**

Ribosomes isolated from Sephacryl S-200 and DE-52 were dialysed against low \( \text{Mg}^{++} \) buffer and dissociated into subunits by **ultra-centrifugation** in vertical rotors. Sucrose density gradient profiles of *E. coli* and *S. acidocaldarius* ribosomal subunits are shown in fig. 6A and 6B respectively. Ribosomal subunit proteins were extracted and analysed by two-dimensional gel electrophoresis (fig. 7A & 7B). The 30S subunit and 50S subunit of *S. acidocaldarius* contain 27 and 34 electrophoretically distinguishable proteins respectively (fig. 7A). Three additional proteins are present in both 30S and 50S subunits. There was no detectable cross contamination of the subunits. It is interesting to note that there was no 30S protein with a molecular weight higher than 30,000 Da. In the case of both 30S and 50S subunits there are three proteins called a, b, c which have migrated to identical positions. They may represent the ribosomal proteins present on both subunits like protein S20 and L26 of *E. coli* ribosomes (Wittman and Wirtman-Liebold, 1974; Stoffler, 1974). For comparison, 2-D gel patterns of *E. coli* 30S and 50S subunit proteins are given in fig. 7B.

The 2-D gel patterns of the subunits from *S. acidocaldarius* are very similar to those obtained by Schmid and Bock, (1982) indicating that the present isolation procedure yielded ribosomal subunits with the usual number of proteins.
Fig. 4: SDS-gel electrophoresis of ribosomes isolated by three different procedures:

(A) Ribosomes isolated from alumina ground cells,
(B) Ribosomes isolated from cells lysed with Triton X-100 and NP-40,
(C) Ribosomes isolated using ultra-centrifugation of cell lysates obtained by non ionic detergents. In each case, 60 μg of extracted r-proteins were used for electrophoresis.
Fig. 6: Sucrose density gradient centrifugation profile of

(A) *E. coli* ribosomal subunits, (B) *S. acidocaldarius* ribosomal subunits.
Figure 6
Fig. 7A: Two-dimensional gel electrophoretic pattern of *S. acidocaldarius*.

(1) 30S subunit proteins (100 μg), (2) 50S subunit proteins (150 μg).
Fig. 7B: Two-dimensional gel electrophoretic pattern of *E. coli*

(1) 30S subunit proteins (100 μg), (2) 50S subunit proteins (150 μg).
Fractionation of ribosomal proteins using novobiocin:

The ribosomal proteins from *S. acidocaldarius* and *E. coli* were fractionated by novobiocin as described in the materials and methods. Since higher concentrations of urea (> 4 M) and the presence of SDS (>0.1%) inhibits the precipitation of proteins by novobiocin, ribosomal protein solutions at a concentration of 0.2 mg/ml were dialyzed against 20 mM Tris-Cl (pH 8.0), 7 mM 2-mercaptoethanol buffer containing 1 M ammonium chloride. Low concentrations of ribosomal protein were chosen to ensure better solubility. It was also reported that precipitation by novobiocin was more efficient in higher ionic strength buffers (Brand and Toribara, 1976). Novobiocin (sigma) was dissolved in water so as to get a concentration of 10 mg/ml and stored at -20 °C in the dark (the antibiotic is susceptible to light induced cleavage that appears to interfere with its ability to precipitate histones). Precipitation of proteins by novobiocin was performed in 20 mM Tris-Cl (pH 8.0) and 1 M ammonium chloride at 4 °C as described in materials and methods chapter.

*S. acidocaldarius* ribosomal proteins were fractionated into four groups, corresponding to those precipitated with 200 µg/ml (fig. 8B), 500 µg/ml (fig. 8C), 1000 µg/ml novobiocin (fig. 8D) and the proteins soluble at 1000 µg/ml novobiocin (fig. 8E). The proteins precipitated at each stage was dissolved in 8 M urea and 7 mM 2-mercaptoethanol and subjected to 2-dimensional gel electrophoresis. The same procedure was also successful for the fractionation of ribosomal proteins from *E. coli* (fig. 9B-E). In case of *E. coli*, only three groups of proteins were obtained, those corresponding to 500 µg/ml (fig. 9C), 1000 |µ|g/ml (fig. 9D) and those proteins soluble at 1000 µg/ml novobiocin (fig. 9E). Unlike *S. acidocaldarius*, in the case of *E. coli* no proteins were precipitated at 200 µg/ml novobiocin (fig. 9B). Proteins precipitated at each stage contained specific proteins.
Fig. 8: Two-dimensional gel electrophoretic patterns of novobiocin fractions of *S. acidocaldarius* ribosomal proteins:
Fractionation of ribosomal proteins with novobiocin and electrophoresis were carried out as described in the text.
(A) Total ribosomal proteins 125 µg, (B) to (D) 200, 500, 1000 µg novobiocin precipitated fractions respectively and (E) 1000 µg novobiocin supernatant fraction. 60 µg protein of each of the novobiocin fraction was electrophoresed.
Fig. 9: **Two-dimensional** gel **electrophoretic** patterns of novobiocin fractions of *E. coli* ribosomal proteins:
Fractionation of ribosomal proteins with novobiocin and electrophoresis were carried out as described in the text.
(A) Total ribosomal proteins *125 μg*, (B) to (D) *200, 500, 1000 μg* novobiocin precipitated fractions respectively and (E) 1000 (μg novobiocin supernatant fraction. 60 μg protein of each of the novobiocin fraction was electrophoresed.
which are present in large amounts. However, in each group there were small amounts of cross contaminating proteins belonging to other groups. This may be partly due to the contamination by small amounts of supernatant fluids at each step of the novobiocin fractionation and incomplete precipitation of some proteins at that novobiocin concentration. Different proteins precipitated at each novobiocin concentration were identified after comparing the two-dimensional gel patterns with those of 70S ribosomes and subunits of _S. acidocaldarius_ and _E. coli_. The results are summarised in Tables 3 and 4.

**Discussion:**

_Ultra-centrifugation_ has been widely used to isolate ribosomes from a variety of organisms. In the literature, isolation of highly functionally active ribosomes without the use of ultra-centrifuge has been reported for _E. coli_ ribosomes by Jelenc, (1980). We have modified this procedure for the isolation of ribosomes from _S. acidocaldarius_. We found that ribosomes isolated by ultra-centrifugation from alumina ground cells were contaminated with large amounts of high molecular weight non-ribosomal proteins as indicated by SDS-poly acrylamide gel electrophoresis. Like eukaryotic cells, _S. acidocaldarius_, can be lysed with non ionic detergents to prepare cell extracts. Earlier in our laboratory, _S. acidocaldarius_ nucleoid was successfully isolated from detergent lysed cell extracts (Reddy and Suryanarayana, 1988; 1989). Ribosomes isolated by the procedure developed here, contain all the r-proteins as reported earlier for _S. acidocaldarius_ ribosomes, isolated by a lengthy procedure involving ultra-centrifugation three times (Schmid and Bock, 1982).

Several techniques have been developed for the fractionation and purification of proteins. These include separation by precipitation with high salt
<table>
<thead>
<tr>
<th>Novobiocin fraction</th>
<th>Proteins present in large amounts</th>
<th>Proteins present in reduced amounts</th>
<th>Proteins present in trace amounts</th>
</tr>
</thead>
</table>

Only the proteins whose spots are notably visible in the gel electropherograms are listed in the Table. Certain protein spots were faintly visualised and were exclusive to the group in which they are present. Faint spots may be due to weak staining by Coomassie blue, e.g., low molecular weight basic proteins such as L.32, L.33, L.34, S.25, S.26 and S.27.
Table - 4

_E. coli_ ribosomal proteins present in different novobiocin fractions

<table>
<thead>
<tr>
<th>Novobiocin fraction</th>
<th>Proteins present in large amounts</th>
<th>Proteins present in reduced amounts</th>
<th>Proteins present in trace amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 μg/ml precipitate</td>
<td>---</td>
<td>---</td>
<td>L2, L4, L6, L16, S2, S3, S4, S5, S7.</td>
</tr>
</tbody>
</table>

Only the proteins whose spots are notably visible in the gel electropherograms are listed in the table. Certain protein spots were faintly visualised and were exclusive to the group in which they are present. Faint spots may due to weak staining by Coomassie blue, eg., L29, L30, L32, S20, S21 etc.
concentrations (salting out), organic solvents, organic polymers and a variety of chromatographic procedures. These methods have been widely used for the successful purification of a large number of proteins and enzymes. In the case of purification of ribosomal proteins extracted from ribosomes, no initial fractionation has been developed. Total ribosomal proteins were processed by several chromatographic methods. Split proteins which are removed by salt washing of ribosomes were also used as starting material for purification. *S. acidocaidanus* ribosomal proteins could not be stripped in a step-wise manner from ribosomes with increasing salt concentration. We have come across a report in the literature that novobiocin, a potent inhibitor of DNA gyrase, can precipitate purified histones in solutions (Cotten *et al.*, 1986). The precipitation of some purified proteins (histones, carbonic anhydrase, myoglobin, cytochrome-C and soybean trypsin inhibitor) with novobiocin indicated that the effectiveness of precipitation was dependent on the arginine content as well as the arginine clusters in proteins. Those proteins with high arginine content were precipitated with low concentration of novobiocin (500 μg/ml), while those with the low arginine content, inspite of the basic nature of the proteins, either required high concentration of novobiocin for precipitation or did not precipitate at all (Cotten *et al.*, 1986). These observations on novobiocin precipitation of proteins prompted us to try novobiocin for differential precipitation of ribosomal proteins. As shown in the results section, four groups of r-proteins were obtained by novobiocin precipitation. The relationship between the arginine content and clusters in *S. acidocaidanus* r-proteins and the precipitability by novobiocin could not be analysed as there is no information available on the amino acid composition/sequence of most of r-proteins from this organism. Therefore, *E. coli*
ribosomal proteins, whose amino acid composition and sequences are known were also fractionated by novobiocin.

In the case of *E. coli* ribosomes only three groups of proteins were obtained along with trace amounts of a few proteins precipitated at 200 fg/ml novobiocin. This may be due to the difference in the arginine content of the ribosomal proteins from the two organisms. *S. acidocaldarius* ribosomes apparently contain several ribosomal proteins with high arginine content such that they were precipitated using only 200 µg/ml novobiocin. It was reported (Qaw and Brewer, 1986) that there is a relationship between the arginine content of proteins and their thermal stability. Hence the precipitation of several ribosomal proteins of *S. acidocaldarius* by low concentrations of novobiocin (200-500 µg/ml) suggests that there may be a higher content of arginine in these proteins which in turn would imply an enhanced thermal stability of these proteins from this acidothermophilic organism.

Furthermore, a comparison of the fractionation patterns of the ribosomal proteins from the two organisms indicates that structurally homologous proteins identified between *S. acidocaldarius* and *E. coli* ribosomes are present in the same novobiocin fraction. Proteins denoted L1 and L10 in *S. acidocaldarius* have been shown to be homologous to *E. coli* proteins L10 and L7/L12. These acidic proteins in both cases are present in the novobiocin 1000 jig/ml supernatant fraction. The two-dimensional gel electropherograms show that there is no relationship between the basicity of a protein and the concentration of novobiocin required for its precipitation. L24 which was not precipitated even by 1000 fg/ml novobiocin is more basic than the protein LU which was precipitated with 200 µg/ml concentration of the antibiotic. Furthermore, acidic proteins such as L10 of
*S. acidocaldarius* as well as one of the most basic proteins, L33, were present in the same group (1000 μg/ml novobiocin supernatant fraction).

Most of the proteins in the 500 fig/ml and the 1000 μg/ml novobiocin fractions of *E. coli* ribosomal proteins have a high arginine content (greater than 7 mole percent) except proteins S2 (5.4%) and L1(4.1%). In the case of 1000 μg/ml soluble fraction, most of the proteins present had an arginine content lower than 7 mole percent except S6 (8.5%) and L23 (7.7%). These results therefore support the observation that precipitation by novobiocin is to a greater extent dependent on the arginine content of the proteins, the exceptions may be due to protein-protein interactions as well as the absence of arginine clusters in the proteins.

The individual groups of proteins fractionated by novobiocin may be used as starting material for the separation of individual proteins by ion exchange and gel filtration chromatography. We suggest that this method may also be useful for the fractionation of complex protein mixtures such as cell extracts.
3.2: Thermal stability of ribosomes and characterisation of 50S core particle

Adaptation of certain thermophilic organisms to natural hostile environments allows them to survive and grow even at temperatures close to (Brock et al, 1972; Brierly and Brierly, 1973; De Rosa et al, 1975), or higher than the boiling point of water (Morita, 1980). Thermally tolerant ribosomes that are capable of code translation with fidelity and efficiency are essential for bacterial growth and survival in extremely hot niches where, ribosomes from other organisms undergo denaturation. So the nucleic acid and protein interactions in these organisms may be stronger than in the conventional mesophilic organisms. In the present study, an attempt is made to understand the thermal stability of *S. acidocaldarius* ribosomes. The rRNA and r-protein interactions were compared between *S. acidocaldanus* (which grows optimally at 87 °C and pH of 1-3) and mesophilic bacterium, *E. coli*. Thermal melting studies on rRNA and ribosomes from both the organisms were carried out. Studies were also carried out on the ribosome core particles obtained by hydrophobic chromatography of ribosomes from *S. acidocaldarius* and *E. coli*.

**Sepharose 4B chromatography of *S. acidocaldarius* and *E. coli* ribosomes:**

*S. acidocaldarius* and *E. coli* ribosomes were also compared by hydrophobic chromatography using reverse salt gradient on sepharose 4B. Ribosomal particles containing defined set of r-proteins were isolated by different methods and their thermal stability was also studied. The ribosomes are dissociated at low Mg**⁺ concentrations and are brought to the verge of
precipitation at a high concentration of ammonium sulphate. Under these conditions they are strongly adsorbed onto sepharose 4B. The ribosomal subunits are eluted by a decreasing gradient of ammonium sulphate. The method is based on the difference in hydrophobic interaction of subunits with the agarose gel in the presence of very low concentrations of Mg²⁺.

Around 200 mg of 70S ribosomes in 10 ml buffer were applied to a column of sephadex G-50. Ammonium sulphate was added to a final concentration of 1.5 M to the ribosome fractions eluting from the column within the void volume. The ribosomes were applied to the column of sepharose 4B equilibrated with the buffer containing 0.02 M Tris-Cl (pH 7.5) containing 1.5 M (NH₄)₂SO₄. Elution was performed with three volumes of the same buffer but with a linear reverse salt gradient of (NH₄)₂SO₄(1.25 M-0.02 M) and 3 ml fractions were collected. The fractions were analysed by measuring absorbance at 260 nm.

The sepharose 4B chromatography was performed under three different buffer conditions viz., in the absence of Mg²⁺, in the presence of 1 mM Mg²⁺; and in the presence of 5 mM Mg²⁺.

When S. acidocaldarius 70S ribosomes were loaded and eluted on sepharose 4B in the absence of Mg²⁺ using reverse salt gradient, ribosomes were fractionated into five groups as five different peaks (fig. 10). Each peak fraction was separately pooled and extracted with acetic acid for r-proteins and analysed by two-dimensional electrophoresis of Geyl et al. (1982), which revealed that each group contained predominantly a distinct set of proteins. The first peak contained stripped ribosomal proteins from both the subunits. These proteins were called as the split protein fraction-I. The second peak contained L7/L12 and some other ribosomal proteins from both the subunits. These proteins were called as split protein fraction-II. The third peak contained 30S particle containing some r-
Fig. 10: Chromatography of *S. acidocaldarius* ribosomes on Sepharose 4B in the absence of Mg$^{++}$ ions: Elution was carried out with 3 volumes of a linear reverse salt gradient \{1.5 M—0.02 M (NH$_4$)$_2$SO$_4$\}. 
Figure 10
proteins in diminished amount. Peak 4 corresponds to the undissociated 70S particle which contains all the proteins from 30S and 50S subunits (albeit some proteins in reduced quantity). Peak 5 corresponds to 50S core particle and contains around 15-18 ribosomal proteins from the 50S subunit. The proteins present in each peak fraction were identified by comparing with the gel patterns of control 30S and 50S ribosomal subunit proteins (fig. 11A; B; C; D; E; F) and the results are presented in Table-5.

When S. acidocaldarius ribosomes were loaded and eluted from sepharose 4B in the presence of 1 mM Mg$^{2+}$ using reverse salt gradient, ribosomes were fractionated into three distinct peaks (fig. 12). In this case, the first peak represents 30S particle. The second peak represents the undissociated 70S particle containing all the ribosomal proteins from both the 30S and 50S subunits with some r-proteins in diminished amounts. The third peak contains 50S core particle proteins. The number of proteins in this group were from 15-18. The proteins present in each group were extracted with acetic acid. These extracted ribosomal proteins were subjected to two-dimensional gel electrophoresis and the proteins were identified as before (fig. 13A; B; C; D; Table-6).

S. acidocaldarius ribosomes were chromatographed on sepharose 4B in the presence of 5 mM Mg$^{2+}$ using reverse salt gradient. The profile (fig. 14) was similar to that obtained when chromatography was performed in the presence of 1 mM Mg$^{2+}$. Ribosomal proteins present in each peak were identified after two-dimensional gel electrophoresis (fig. 15A; B; C; D; Table-7). Although, the elution profiles (1 mM and 5 mM Mg$^{2+}$) did not show any peaks corresponding to split proteins. The fractions eluting ahead of 30S peak contained some r-proteins (as in fig. 10).
Fig. 11: Two-dimensional gel electrophoretic patterns of different peak fractions of sepharose 4B chromatography (no Mg^{2+}); (A) Total ribosomal protein, (B) Peak-1 ribosomal proteins, (C) Peak-2 ribosomal proteins, (D) Peak-3 ribosomal proteins, (E) Peak-4 ribosomal proteins, (F) Peak-5 ribosomal proteins.
Table - 5
Sepharose 4B chromatography of *S. acidocaldarius* ribosomes in the absence of Mg$^{++}$ ions.

<table>
<thead>
<tr>
<th>Sepharose 4B Peaks</th>
<th>Proteins present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak-1</td>
<td>S13, S15, S24, L1, L10 (faint), L13, L14 (faint), L15 (faint), L24 (faint), L25, L26, L27, L28.</td>
</tr>
<tr>
<td>Split proteins</td>
<td></td>
</tr>
<tr>
<td>Peak-2</td>
<td>S13, S15 (faint), S24, L10 (faint), L13, L14 (faint), L15, L24, L25, L26, L27.</td>
</tr>
<tr>
<td>Split proteins</td>
<td></td>
</tr>
<tr>
<td>Peak-3</td>
<td>S4, S6, S7, S8, S10, S11, S12, S13, S14, S15, S16, S17, S18, S19, S20, S23, S24, S25, S27, L10 (faint), a.</td>
</tr>
<tr>
<td>30S particle</td>
<td></td>
</tr>
<tr>
<td>Peak-4</td>
<td>All 70S r-proteins are present</td>
</tr>
<tr>
<td>70S particle</td>
<td></td>
</tr>
<tr>
<td>50S core particle</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 12: Chromatography of *S. acidocaldarius* ribosomes on Sepharose 4B in the presence of 1 mM Mg$^{++}$ ions: Elution of ribosomes was affected by 3 volumes of a linear reverse salt gradient.
Figure 12
Fig. 13: Two-dimensional gel electrophoretic patterns of different peak fractions of sepharose 4B chromatography (1 mM Mg^{++}):(A) Total ribosomal protein, (B) Peak-1 ribosomal proteins, (C) Peak-2 ribosomal proteins, (D) Peak-3 ribosomal proteins.
Table - 6
Sepharose 4B chromatography of *S. acidocaldarius* ribosomes in the presence of 1 mM Mg$^{2+}$ ions.

<table>
<thead>
<tr>
<th>Sepharose 4B Peaks</th>
<th>Proteins present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak-1 30S particle</td>
<td>S3, S4, S6, S7, S8, S10, S12, S13, S14, S15, S16, S17, S18, S19, S20, S23, S24, S25, S27, L10 (faint), a.</td>
</tr>
<tr>
<td>Peak-2 70S Particle</td>
<td>All 70S r-proteins are present</td>
</tr>
</tbody>
</table>
Fig. 14: Chromatography of *S. acidocaldarius* ribosomes on Sepharose 4B in the presence of 5 mM Mg$^{++}$ ions: Elution of ribosomes was affected by 3 volumes of a linear reverse salt gradient.
Figure 14
Fig. 15: Two-dimensional gel electrophoretic patterns of different peak fractions of sepharose 4B chromatography (5 mM Mg"⁺"):(A) Total ribosomal protein, (B) Peak-1 ribosomal proteins, (C) Peak-2 ribosomal proteins, (D) Peak-3 ribosomal proteins.
**Table - 7**

*Sepharose 4B* chromatography of *5. acidocaldarius* ribosomes in the presence of 5 mM Mg\(^{++}\) ions.

<table>
<thead>
<tr>
<th>Sepharose 4B Peaks</th>
<th>Proteins present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak-1</td>
<td>S4, S6, S7, S8, S10, S12, S13, S14, S15, S16, S17, S18, S19, S20, S23, S24, S25, S27, L10, a, b.</td>
</tr>
<tr>
<td>30S particle</td>
<td></td>
</tr>
<tr>
<td>Peak-2</td>
<td>All 70S r-proteins are present</td>
</tr>
<tr>
<td>70S particle</td>
<td></td>
</tr>
<tr>
<td>50S core particle</td>
<td></td>
</tr>
</tbody>
</table>
We have performed sepharose 4B chromatography of *S. acidocaldarius* cell lysates. *S. acidocaldarius* S-30 was obtained by lysing the cells with Triton X-100 and NP-40 as described in the materials and methods section. The S-30 was dialysed against T 1.5 M (NH₄)_2SO₄ buffer for a period of 72 hours with four changes of buffer at 4 °C. After 72 hours, the precipitated material was removed by centrifugation of suspension at 15,000 RPM for 15 minutes. The clarified supernatant was loaded on to sepharose 4B and eluted with a reverse salt gradient. Four distinct peaks were obtained after elution (fig. 16), each peak was separately pooled and extracted with acetic acid for r-proteins and analysed by two-dimensional gel electrophoresis (fig. 17A; B; C). The first and second peaks contain S-100 proteins and stripped ribosomal proteins. Whereas the third peak corresponds to undissociated 70S particles which contains all the ribosomal proteins from both the subunits. The fourth peak corresponds to the 50S core particle. The proteins present in both of these particles were identified (Table-8). The last peak obtained under all the conditions (of chromatography on sepharose 4B) is a 50S core particle which contains an identical set of proteins.

**Sepharose 4B chromatography of *E. coli* 70S ribosomes in the absence of Mg^{++} ions:**

For comparison, *E. coli* 70S ribosomes were loaded on sepharose 4B in the absence of Mg^{++} and eluted with a reverse salt gradient as described in the materials and methods section. As described previously by Kirillov *et al.* (1978), *E. coli* ribosomes were fractionated into three distinct peaks, the first peak contained pure 30S subunits. The second and third peaks contained two partially unfolded forms of 50S subunit showing a good separation on sepharose 4B (fig. 18). Two-dimensional gel electrophoresis of proteins of peak 1 shows that it
Fig. 16: Chromatography of *S. acidocaldarius* S-30 extract on Sepharose 4B in the absence of Mg$^{++}$ ions: S-30 obtained from 20 grams of *S. acidocaldarius* cells was dialysed against buffer containing T$_{20}$, 1.5 M (NH$_4$)$_2$SO$_4$ for a period of 72 hours. The dialysed S-30 was chromatographed on sepharose 4B column as in fig. 1.
Figure 16
Fig. 17: Two-dimensional gel electrophoretic patterns of different peak fractions of sepharose 4B chromatography (S-30): (A) Total ribosomal protein, (B) Peak-3 ribosomal proteins, (C) Peak-4 ribosomal proteins.
Table -8

Sepharose 4B chromatography of *V. acidocaldarius* S-30 in the absence of Mg²⁺.

<table>
<thead>
<tr>
<th>Sepharose 4B Peaks</th>
<th>Proteins present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak-3</td>
<td>All 70S r-proteins are present</td>
</tr>
<tr>
<td>70S particle</td>
<td></td>
</tr>
<tr>
<td>Peak-4</td>
<td>L2, L3, L4, L6, L7, L8, L11, L12,</td>
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<tr>
<td></td>
<td>L14, L15, L19, L20, L23, L27,</td>
</tr>
<tr>
<td></td>
<td>L30, L33, a</td>
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</tbody>
</table>
Fig. 18: Chromatography of *E. coli* ribosomes on Sepharose 4B in the absence of Mg$^{++}$ ions: Elution of ribosomes was affected by 3 volumes of a linear reverse salt gradient.
contains ribosomal proteins of 30S subunit whereas peak 2 and peak 3 contain some ribosomal proteins of *E. coli* 50S subunit as reported by Kirillov et al. (1978).

**Thermal melting analysis of ribosomes and rRNA from *S. acidocaldarius* and *E. coli***

The thermal stability of *S. acidocaldarius* and *E. coli* ribosomes were monitored by following the increase in $A_{260}$ with rise in temperature. The hyperchromicities vs temperature curves in fig (19A) show that the melting transitions of *S. acidocaldarius* ribosomes increase steeply after a threshold value. For instance, the melting range for *S. acidocaldarius* ribosomes was only 4 °C compared to 9 °C for the corresponding *E. coli* counterparts.

The tendency of ribosomes to aggregate on heating is avoided by keeping the Mg$^{++}$ concentration low (0.08 mM). *E. coli* ribosomes appear to be stable upto 53-54 °C, after this temperature there was gradual increase in $A_{260}$. The melting curve gave a $T_m$ of 71 °C, *S. acidocaldarius* ribosomes do not show any increase in $A_{260}$ until 73 °C and the melting curve gave a $T_m$ of about 87 °C.

*E. coli* rRNA appeared to be stable upto 40 °C and displayed a $T_m$ of 68 °C, whereas *S. acidocaldarius* rRNA which is found to be stable upto 50 °C, displayed a $T_m$ of 80 °C (fig. 19B). In both cases, rRNA begins melting at temperatures much lower than the temperature at which ribosome denaturation is initiated, the melting transition being generally broader for rRNA than for ribosomes. These results indicate the large stabilisation of rRNA secondary structure by the binding of ribosomal proteins. However there are important quantitative differences between *E. coli* and *S. acidocaldarius* with respect to stability of rRNA in ribosomes.
Fig. 19: Thermal melting profiles of ribosomes and rRNA from *S. acidocaldarius* and *E. coli*

(A) (o) *S. acidocaldarius* ribosomes.
   (•) *E. coli* ribosomes.

(B) (o) *S. acidocaldarius* rRNA.
   (•) *E. coli* rRNA.
Figure 19
The significant result in fig (19A & B) is that the increment in ribosome stability with increasing thermophily is greater than the increment in stability for the isolated rRNA. This is evident by comparing the differences in \( \delta T_{m} \) between ribosome and rRNA. The difference is small, only 3 °C for the *E. coli* rRNA and ribosomes, but is 8 °C for *S. acidocaldarius* rRNA and ribosomes. Accordingly, the ratio of ribosome \( T_{m} \) to that of rRNA \( T_{m} \) increases with increasing thermal tolerance of the organisms.

**Thermal melting analysis of ribosomal subunits and ribosomal subunit rRNA from *S. acidocaldarius* and *E. coli*:**

Intact salt washed ribosomal subunits were subjected to thermal melting analysis and compared to the free rRNA species melted in the same solvent (0.08 mM Mg\(^{++}\)). *S. acidocaldarius* 50S ribosomal subunit melts with a \( T_{m} \) of 87 °C (fig. 20A), which is equivalent to the \( T_{m} \) of *S. acidocaldarius* 70S ribosomes. In comparison to *S. acidocaldarius* 50S subunits, 30S subunit melts with a \( T_{m} \) of 85 °C (fig. 20B) which is less by 2 °C. The free rRNA species from these subunits melt with a \( T_{m} \) of 80 °C (fig. 21A) and 79 °C (fig. 21B) respectively.

*E. coli* large subunit rRNA melts with a \( T_{m} \) of 66 °C (fig. 21A) and the small subunit rRNA melts with a \( T_{m} \) of 68 °C (fig. 21B) in comparison to ribosomal subunits which melt with a \( T_{m} \) of 72 °C (50S subunit fig. 20A) and 70 °C (30S subunit fig. 20B).

Thermal melting studies on the ribosomal particles obtained by the sepharose 4B chromatography were carried out. The 30S particles, 70S particles and the 50S core particles obtained under different chromatographic conditions gave melting profiles with a \( T_{m} \) of 85.4 °C, 86.8 °C and 87 °C respectively (fig. 22, 23, 24, 25).
Fig. 20: Thermal melting profiles of ribosomal subunits from *S. acidocaldarius* and *E. coli*

(A) (○) *S. acidocaldarius* SOS ribosomal subunit.
     (●) *E. coli* SOS ribosomal subunit.

(B) (○) *S. acidocaldarius* 30S ribosomal subunit.
     (●) *E. coli* 30S ribosomal subunit.
Figure 20
Fig. 21: Thermal melting profiles of 23S and 16S rRNA from
*S. acidocaldarius* and *E. coli*
(A) (o) *S. acidocaldarius* 23S rRNA, (•) *E. coli* 23S rRNA.
(B) (o) *S. acidocaldarius* 16S rRNA, (•) *E. coli* 16S rRNA.
Figure 21
Fig. 22: Thermal melting profiles of ribosomal particles obtained by sepharose 4B chromatography of *S. acidocaldarius* ribosomes (no Mg$^{2+}$).

(A) 30S particle (peak-3), (B) 70S particle (peak-4),
(C) 50S core particle (peak-5).
Figure 22

[Graph showing A260 against Temperature in °C]
Figure 22
Fig. 23: Thermal melting profiles of ribosomal particles obtained by sepharose 4B chromatography (1 mM Mg^{++}).
(A) 30S particle (peak-1), (B) 70S particle (peak-2),
(C) 50S core particle (peak-3).
Figure 23
Figure 23
Fig. 24: Thermal melting profiles of ribosomal particles obtained by sepharose 4B chromatography (5 mM Mg$^{++}$).
(A) 30S particle (peak-1), (B) 70S particle (peak-2),
(C) 50S core particle (peak-3).
Figure 24

A

B

Temperature in °C

A_{260}

A_{260}
Figure 24
Fig. 25: Thermal melting profiles of ribosomal particles obtained by sepharose 4B chromatography (S-30).
(A) 70S particle (peak-3), (B) 50S core particle (peak-4).
Figure 25
Association and dissociation of ribosomal subunits:

The ability of SOS core particles obtained by sepharose 4B chromatography to associate with 30S subunits to form 70S ribosomes was tested. As a control, \textit{S. acidocaldarius} 50S and 30S subunits which were purified by gradient centrifugation were tested for reassociation in buffer containing 20 mM Tris-Cl(pH 7.6), 40 mM Mg$^{++}$, and 3 mM spermine. Control 50S and 30S subunits reassociated with the formation of 70S particle (fig. 26). When \textit{S. acidocaldarius} 50S core particle was incubated with the 30S subunit under associating conditions it failed to reassociate to form 70S particle. The reason for this may be that the contact sites which are necessary for association are either absent or unavailable in the 50S core particle.

Discussion:

Results presented in this section show that the large and small ribosomal subunits of the extreme thermoacidophilic archaeon \textit{S. acidocaldarius} are substantially more heat stable and exhibit a greater degree of co-operative interactions than the mesophilic organism, \textit{E. coli}.

The remarkable heat resistance of \textit{S. acidocaldarius} SOS and 30S ribosomal subunits in comparison to \textit{E. coli} subunits can be accounted by the presence of increased G+C content of rRNA as well as efficient stabilisation of rRNA by ribosomal proteins (our results and Cammarano et al.. 1982; 1983). Tm of \textit{S. acidocaldarius} free rRNA is only 12 °C higher than the Tm of \textit{E. coli} rRNA, whereas the Tm \textit{S. acidocaldarius} ribosomes is 19 °C higher than \textit{E. coli} ribosomes. These results suggest that ribosomes are more heat stable than isolated rRNA indicating contribution of r-proteins for the thermal stability of ribosomes. Furthermore, the greater stability of the bihelical domains of rRNA within the
Fig. 26: *S. acidocaldarius* 30S ribosomal subunits were incubated with 50S subunits or 50S core particle for reassociation and analysed by sucrose density gradient centrifugation.

(o) 30S and 50S ribosomal subunits, (●) 30S subunit and 50S core particles, (v) Control 30S and 50S subunits under dissociation conditions.
Figure 26
thermophilic ribosomes reflects more extensive interactions between functional groups of ribosomal proteins and rRNA domains. In ribosomes from *S. acidocaldanus*, bonds other than those maintained by Mg$^{++}$ bridges are probably extensive to prevent the intra-particle packing from collapsing to an unfolded form under conditions that promote extensive unfolding, and presumably, loss of some ribosomal proteins from the mesophile ribosomes (Altamura *et al.*, 1991).

The chemical composition of *C. acidophila* (genus *S. acidocaldanus*) ribosomes revealed that *C. acidophila* 30S subunits are about 280,000 Da heavier than bacterial 30S subunits, owing to both a larger number of proteins and a greater average molecular weight of the protein components. In comparison to 30S subunits, *C. acidophila* 50S subunits are about 150,000 Da heavier than their *E. coli* counterparts. The increased protein to RNA ratio may possibly be related to the elevated thermal stability of *C. acidophila* ribosomes (Londei *et al.*, 1983). Our melting studies also revealed a correlation between the high temperature of growth of *S. acidocaldanus* and the high temperature (87 °C) required to start the melting of the thermophilic ribosomes.

In addition to ultra-centrifugation, the other method used for the isolation of *E. coli* ribosomal subunits is sepharose 4B chromatography using reverse salt gradients (Kirimlov *et al.*, 1978). Chromatography using reverse salt gradients was applied earlier for the separation of proteins (Sargent and Graham, 1964) and transfer RNA as well (Holmes *et al.*, 1975).

Unlike the case of *E. coli* 70S ribosome which gets completely dissociated into two subunits on sepharose 4B, a significant amount of 70S particle is present in the form of undissociated state in the case of *S. acidocaldanus* ribosomes. This could be due to the stabilisation of interaction between 30S and 50S subunits in
the presence of high salt concentration in the case of thermophilic ribosomes. The
30S particle and 50S core particle prepared by chromatography showed that they
contain undegraded 16S and 23S rRNA molecules.

The *E. coli* 50S subunits were eluted from sepharose 4B in two different
states, viz., (i) in a slightly unfolded 47S form and (ii) highly unfolded 33S form
(Kirillov *et al.*, 1978). Similar results (unfolding of 50S subunits into 46S and 28S
forms) were observed earlier by Gavrilova *et al.*, (1966). In case of
*S. acidocaldarius* there is only one form of 50S subunit core particle which elutes
as the last peak. A significant amount of 50S core particle was obtained when the
ribosomal sample was freshly prepared and the chromatography performed in the
absence of Mg++. A large amount of ribosomal proteins associated with the 50S
subunit were stripped off and the 50S core particle that was tightly bound to the
sepharose 4B through ionic interactions was eluted with a lower concentration of
salt. Londei *et al.* (1986b), reported that *in vitro* reconstitution of 50S subunits
require a two step procedure, viz., one at a temperature of 45 °C and the other at a
temperature of 65 °C which bring about the conformational change in the core
particle and allow the remaining proteins to be incorporated into 50S subunit. The
protein composition of the core particle formed in the first step of reconstitution is
very similar to the protein composition of the 50S core particle obtained by us.
The protein composition of 50S core particle was also similar to that of 50S core
particle obtained by treatment with 2 M LiCl/4 M urea or 3 M LiCl/6 M urea or 4

Unlike *E. coli* ribosomes, which undergo stepwise disassembly following
treatments with increasing concentrations of LiCl (*Nierhaus* and Montejo, 1973),
*S. acidocaldarius* large ribosomal subunits are relatively resistant to uncompacting
treatments (*Cammarano et al.*, 1983). LiCl and urea treatment results in the
removal of some 12 external proteins, leaving behind a compact and thermally stable core particle. Attempts to remove additional proteins from the core resulted in the complete disaggregation of the particle, thus indicating that the component RNA and protein molecules are held together by very strong co-operative interactions. Hydrophobic bonds in protein/protein contact surfaces probably play an important role in conferring a high degree of stability to the thermophilic ribosome.

Preliminary studies of 50S core particles showed that they are inactive in reassociation with 30S subunits. Hence, the chromatographically isolated 50S core particles serve only as a source for the isolation of primary rRNA binding proteins. This method has no limitations with respect to the quantity of subunits to be isolated in one run. By increasing the volume of sepharose 4B column to several hundred milli-litres and increasing the content of amount of ribosomes loaded, we can prepare large quantities of ribosomal subunit core particles.

Ramakrishnan et al. (1986) reported that *E. coli* 30S subunits isolated by the method of Kirillov et al. (1978) were active in protein synthesis *in vitro* and comigrated on sucrose gradients with subunits obtained by zonal sucrose gradient centrifugation. Their work shows that the subunits obtained by this technique were deficient in S3 and S14. These two proteins are removed from 30S subunits during chromatography and are eluted in a protein peak that elutes before 30S peak. Both S3 and S14 have been located on the periphery of the subunit by neutron triangulation technique (Moore et al., 1986) These proteins are known to be on the periphery of the subunit and close to each other The assembly map of Mizushima and Nomura (1970) indicates that they are among the last proteins to bind to the 30S in the assembly of the subunit *in vitro*. In a similar fashion the two split fraction proteins of *S. acidocaldarius* which were obtained from
*S. acidocaldarius* on sepharose 4B chromatography, could be the last proteins to be assembled on both the subunits. *S. acidocaldarius* 30S subunit particle obtained under different conditions contained most of the ribosomal proteins indicating strong interaction of the 30S proteins with 16S rRNA under these conditions. Attempts were made to obtain 30S core particles by other methods (see chapter 3.3).

The stability of subunit particles was analysed by thermal melting experiments. The 30S particle and the 50S core particles which are obtained under different conditions when subjected to thermal melting analysis, melt with a Tm which is identical to that of 30S and 50S subunits. In other words, the 50S core particle contains all the minimum i-proteins which are necessary for protecting the rRNA against thermal denaturation, and which may be directly interacting with the rRNA molecule. These proteins are the most likely candidates for early assembly proteins.
3.3: Characterisation of 30S core particle

Extensive work has been carried out on the protein-RNA interactions in *E. coli* ribosome (Held *et al.*, 1974; Zimmerman *et al.*, 1979, Wirrmann, 1982; Brimacombe *et al.*, 1983; Wittmann, 1983). Concerning the 30S ribosomal subunit, it has been shown by Nomura's group and is generally accepted that seven ribosomal proteins (S4, S7, S8, S13, S15, S17, S20), independently and specifically bind to the 16S RNA (Held *et al.*, 1974). Co-operative interactions between ribosomal proteins and 16S RNA have also been identified (Brimacombe *et al.*, 1978). The results provide the basis for the assembly of the 30S subunits in *E. coli*.

30S subunit particle obtained by hydrophobic chromatography contained most of the 30S subunit proteins. In order to know the primary 16S rRNA binding proteins, 30S core particle was isolated by salt/urea extraction of 30S subunits. In this chapter, we present the results obtained on treatment of *S. acidocaldarius* 30S subunits with different concentrations of LiCl and/or urea to isolate 30S core particles and identification of the primary 16S rRNA binding proteins.

**Isolation of 30S core particles:**

**Treatment with LiCl:**

*S. acidocaldarius* 30S ribosomal subunits (obtained by density gradient) were treated with four different concentrations of LiCl (1 M, 2 M, 3 M, 4 M) as the only chaotropic agent in 20 mM Tris-Cl (pH 7.6), 2 mM Mg++, 150 mM KCl, 7 mM 2-mercaptoethanol buffer. Core particles were separated from extracted proteins by ultra-centrifugation. The proteins present in the core particles were extracted and analysed by two-dimensional gel electrophoresis (fig. 27A, B, C, D,
Fig. 27: Two-dimensional gel electrophoretic patterns of *S. acidocaldarius* 30S ribosomal core particle proteins obtained at different concentrations of LiCl:
(A) 30S ribosomal proteins, (B) 1 M LiCl core particle, (C) 2 M LiCl core particle, (D) 3 M LiCl core particle, (E) 4 M LiCl core particle, Sample loaded-125 μg.
**Table - 9**

*V. acidocaldarius* 30S ribosomal proteins **present in**
different LiCl core particles

<table>
<thead>
<tr>
<th>LiCl Concentration</th>
<th>Proteins present</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M LiCl 30S core particle</td>
<td>S4, S6, S7, S10, S11, S12, S14, S15, S16, S17, S18, S19, S20, S27, a, b, c.</td>
</tr>
<tr>
<td>2 M LiCl 30S core particle</td>
<td>S4, S6, S7, S10, S11, S12, S14, S15, S16, S18, S19, S20, S27, a, b, c.</td>
</tr>
<tr>
<td>3 M LiCl 30S core particle</td>
<td>S4, S6, S7, S10, S11, S12, S14, S15, S16, S18, S19, S20, S27, a, c.</td>
</tr>
<tr>
<td>4 M LiCl 30S core particle</td>
<td>S6, S10, S11, S16, S27, a.</td>
</tr>
</tbody>
</table>
E). The proteins present in 1 M; 2 M, 3 M and 4 M LiCl core particles were identified (Table-9) When the concentration of LiCl was increased from 2 M to 4 M, only six proteins were associated with the 16S rRNA and these could be binding to rRNA by very strong ionic or hydrophobic interactions.

**Treatment with urea:**

Urea was used in moderate ionic strength buffers (20 mM Tris-Cl pH 7.6, 2 mM Mg++, 150 mM KCl, 7 mM 2-mercaptoethanol), as the only denaturant, to detect proteins that bind to RNA strongly. Core particles were isolated from extracted proteins by ultra-centrifugation The proteins present in the core particles were extracted and analysed by two-dimensional gel electrophoresis (figs. 28A, B, C, D). The core particles obtained by 1 M, 2 M and 4 M urea contained similar set of proteins. The protein composition of these core particles were similar to the 3 M LiCl core particles. The r-proteins present in urea core particles were identified (Table-10).

**Treatment with LiCl and urea:**

*S. acidocaldarius* ribosomal subunits were treated with four different concentrations of LiCl and urea to see the combined effect of chaotropic agent and a denaturant. The four concentrations used were 1 M LiCl/2 M urea, 2 M LiCl/4 M urea, 3 M LiCl/6 M urea and 4 M LiCl/8 M urea in 20 mM Tris-Cl (pH 7.6), 2 mM Mg++, 150 mM KCl, 7 mM 2-mercaptoethanol or 20 mM Tris-Cl (pH 7.6), 20 mM Mg++, 150 mM KCl, 7 mM 2-mercaptoethanol buffer. Core particles were isolated by ultra-centrifugation. The proteins present in the core particles were extracted and analysed by two-dimensional gel electrophoresis (figs. 29A, B).
Fig. 28: Two-dimensional gel electrophoretic patterns of *S. acidocaldarius* 30S ribosomal core particle proteins obtained at different concentrations of urea:
(A) 30S ribosomal proteins, (B) 1 M urea core particle,
(C) 2 M urea core particle, (D) 4 M urea core particle,
Sample loaded-125 µg.
### Table - 10

*S. acidocaldarius* 30S ribosomal proteins present in different Urea core particles

<table>
<thead>
<tr>
<th>Urea Concentration</th>
<th>Proteins present</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1 M Urea</strong></td>
<td>S3, S4, S6, S7, S8, S10, S11, S12, S14, S15, S16, S17, S22, S23, S25, S27. a.</td>
</tr>
<tr>
<td>30S core particle</td>
<td></td>
</tr>
<tr>
<td><strong>2 M Urea</strong></td>
<td>J S3, S4, S6, S7, S8, S10, S11, S15, S16, S17, S27. a</td>
</tr>
<tr>
<td>30S core particle</td>
<td>J</td>
</tr>
<tr>
<td><strong>4 M Urea</strong></td>
<td>S3, S4, S6, S7, S10, S11, S15, S16, S17, S27. a</td>
</tr>
<tr>
<td>30S core particle</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 29: Two-dimensional gel electrophoretic patterns of *S. acidocaldarius* 30S ribosomal core particle proteins obtained at different concentrations of LiCl and urea:
(A) 30S ribosomal proteins, (B) 1 M LiCl/2 M urea core particle, Sample loaded-125 μg.
Large number of the ribosomal proteins in the 30S subunit were stripped off at a concentration of 1 M LiCl/2 M urea. The proteins associated with the 16S rRNA in the 1 M LiCl/2 M urea core particles were identified (Table-11). The protein composition of the 1 M LiCl/2 M urea core particle was similar to 3 M LiCl core particles. At higher concentrations of LiCl/urea (2 M LiCl/4 M urea, 3 M LiCl/6 M urea) only protein-a was present in substantial amount, with trace amounts of S4, S6, S8 and S10. At 4 M LiCl/8 M urea concentration protein-a was also absent. Treatment with LiCl/urea at two different concentrations of Mg^{++} (2 mM and 20 mM) did not show any notable difference in the core particle protein composition.

Thermal melting studies were carried out on the core particles obtained by the three different treatments. Core particles obtained by treatment with different concentrations of LiCl (fig. 30) or urea (fig. 31) melted with a Tm of about 84.5 °C. Melting analysis of the core particles obtained by 1 M LiCl/2 M urea gave a Tm of 85.7 °C (which is close to Tm of intact 30S subunit as in fig. 32).

Discussion:

Structural and functional studies on prokaryotic ribosomes have benefited enormously from the ability to separate ribosomal proteins and rRNA apart and to reassemble the dissociated ribosomal subunit components again into biologically active particles. One of the widely used techniques for controlled dissociation of ribosomal constituents involves treatment of ribosomal subunits with high concentrations of monovalent cations, notably LiCl. Such a treatment results in the removal of a portion of the ribosomal proteins, leaving discrete core particles with a well-defined subset of the ribosomal proteins (Homann and Nierhaus, 1971; Nierhaus and Montejo, 1973; Nierhaus and Dohme, 1974). Study of core particles
**Table- 11**

*N. acidocaldarius* 30S ribosomal proteins present in different LiCl/urea core particles

<table>
<thead>
<tr>
<th>LiCl/Urea Concentration</th>
<th>Proteins present</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M LiCl/2 M Urea</td>
<td>S4, S5, S6, S8, S10, S11, S12.</td>
</tr>
<tr>
<td>30S core particle</td>
<td>S14, S15, S16, S17, S27, a, b, c.</td>
</tr>
</tbody>
</table>
Fig. 30: Thermal melting profiles of LiCl 30S core particles from *S. acidocaldarius*;

(o) 1 M LiCl core particle,

(•) 2 M LiCl core particle,

(v) 4 M LiCl core particle.
Figure 30

Temperature in °C

A_{260}
Fig. 31: Thermal melting profiles of urea 30S core particles from *S. acidocaldarius*:
(o) 1 M urea core particle,
(•) 2 M urea core particle,
(v) 4 M urea core particle.
Figure 31
Fig. 32: Thermal melting profiles of LiCl/urea 30S core particles from *S. acidocaldarius*:

(o) 1 M LiCl/2 M urea core particles obtained in the presence of 2 mM Mg$^{++}$

(*) 1 M LiCl/2 M urea core particles obtained in the presence of 20 mM Mg$^{++}$
Figure 32
have been useful in establishing structure-function relationships for various prokaryotic ribosomal components (Nierhaus, 1980) and continue to be used for this purpose (Nowotny and Nierhaus, 1982; Miamets et al., 1983). The identification of primary rRNA binding proteins in these core particles is particularly important because some of these proteins are surely expected to play a key role in providing the framework required for the correct assembly of ribosomes (Nowotny and Nierhaus, 1982; Bielka, 1979).

The dissection of *S. acidocaldarius* ribosome structure and function is still in its infancy compared to the extensive body of knowledge collected on prokaryotic ribosomes. *S. acidocaldanus* 30S ribosomal subunits are peculiar in that they are richer in protein content than the prokaryotic 30S ribosomal subunit (Londei et al., 1983).

Here we report the identification of *S. acidocaldarius* small subunit proteins that are the primary 16S rRNA binding proteins and probably are the early assembly proteins. The analysis of partially disassembled particles obtained by different conditions (LiCl, urea and LiCl/urea) has allowed us to delineate a preliminary pattern of the topographical hierarchy of *N. acidocaldanus* 30S ribosomal subunit proteins into primary and secondary rRNA binding proteins.

Proteins of the small subunit of *N. acidocaldanus* were extracted by washing subunits with a series of buffers containing increasing concentrations of LiCl as the only chaotropic agent and separately with increasing concentrations of urea as denaturant. The proteins were also extracted by increasing concentrations of LiCl and urea to see the combined effect of denaturant and chaotropic agent. Under all conditions *S. acidocaldanus* 30S ribosomal subunits are disassembled into specific groups of soluble proteins and a core particle containing proteins and rRNA.
S. acidocaldarius 30S ribosomal subunits when treated with increasing concentration of LiCl, majority of 30S proteins were removed between 2 M LiCl and 4 M LiCl concentrations. In 4 M LiCl, rRNA is associated with 6 ribosomal proteins. The proteins which are stripped off from 30S subunit at 1 M and 2 M LiCl probably contain those proteins that had been predominantly held by ionic interactions. Proteins in the 4 M LiCl core particle may correspond to the primary 16S rRNA binding proteins. Extra proteins in 1 M LiCl/2 M urea; 4 M urea and 3 M LiCl core particles, other than the proteins in 4 M LiCl core particle, may represent those proteins that bind to the 16S rRNA subsequent to the binding of the primary rRNA binding proteins.

The degree of structural compactness of the core particles was analysed by thermal melting experiments. The 4 M LiCl core particle probably contains all the required r-proteins that directly interact with the rRNA molecule and protect it against thermal denaturation because the melting profiles of the core particles show Tm values close to that of the native 30S subunit.

E. coli 30S subunits release S1, S2, S3, S14 and S21 at 1 M LiCl concentration. Incubation in 2 M LiCl leads to the splitting of the next portion of proteins S5, S9, S10, S12, S13 and S20. Between 3 to 3.5 M LiCl, S6, S18, S11, S19 and then S16 and S17 are released. The residual 23S particles contain just four ribosomal RNA binding proteins S4, S7, S8, and S15 (Dijk and Littlechild, 1979; Littlechild and Malcolm, 1978). In contrast, yeast 40S subunits were found to be extremely sensitive to the salt as indicated by complete dissociation of the subunits at 0.5 M LiCl (El-Baradi et al., 1984).

Unlike LiCl, urea treatment of 30S subunit up to 4 M concentration did not result in dissociation of majority of the r-proteins. Composition of the core
particles obtained by urea treatment suggest that 30S subunits are relatively resistant to urea treatment.

The proteins which are present in 4 M LiCl core panicles are identified to be primary rRNA binding proteins as they are associated with rRNA under highly stringent conditions. The resistance of these proteins to LiCl extraction compares well with that exhibited by the strong RNA binding proteins from other classes of ribosomes (Rozier and Mache, 1984; El-Baradi et al., 1984; Nierhaus, 1980).

In *S. acidocaldarius* the 30S core particle is less stable than the 50S core particle. While the 30S core panicle is stable upto 1 M LiCl/2 M urea, the 50S core particle maintains its integrity even upto 4 M LiCl/8 M urea concentration. The results suggest that the *S. acidocaldarius* 50S subunit can withstand both thermal and chemical stresses better than 30S subunits.

Comparison of prokaryotic and eukaryotic core particles would help define both conserved and non-conserved features of ribosome structure. Furthermore, studies of ribosomal proteins and rRNA in thermophilic archaea may provide valuable information on the mechanisms involved in the stabilisation of nucleic acid and protein complexes at high temperatures and the degree of evolutionary conservation of ribosomal components in the primary kingdoms of the cell descent.
3.4: Homologous r-proteins between archaea and bacteria

Cross-reactions between ribosomal proteins from archaea, bacteria and eukaryotes have been analysed by means of immunodiffusion, immunoprecipitation, and one-dimensional immuno electrophoresis (Schmid and Bock, 1981). It was found that the immunological relatedness was group specific and therefore closely paralleled the relationships delineated by the 16S rRNA oligonucleotide data (Balch et al., 1979). The number of cross-reacting proteins was higher and the strength of their cross-reaction more intense when the organisms under comparison belonged to the same primary kingdom (Schmid and Bock, 1984). Only few cross-reacting proteins were detected when organisms from different kingdoms were compared. In the present work, immunologically homologous proteins between archaea (S. acidocaldarius) and bacteria (E. coli) were identified by two-dimensional gel electrophoresis of ribosomal proteins (from the two organisms) and immunoblotting.

Immunological homology between archaeal and bacterial ribosomal proteins:

Ribosomal subunits were isolated from S. acidocaldanus and E. coh by using vertical gradient centrifugation. The purity of the ribosomal subunits was checked by two-dimensional gel electrophoresis. Antiserum was raised against 50S and 30S ribosomal subunits of S. acidocaldanus and E. coli. IgG was isolated from the antisera as described previously.

S. acidocaldanus 30S and 50S r-proteins were separated by two-dimensional gel electrophoresis (fig. 33) and transferred to nitrocellulose membranes. The membranes were immunoblotted with anti E. coli r-subunit...
Fig. 33: Two-dimensional gel electrophoretic pattern of ribosomal proteins from *S. acidocaldarius*.
(A) 30S proteins (100 μg), (B) 50S proteins (150 μg).
antibodies. Control experiments were also performed to determine optimum concentration of antibody by electrophoresis of *E. coli* subunit proteins, which showed cross-reaction of all the r-proteins with homologous antibodies. The *S. acidocaldarius* 30S subunit proteins cross-reacting with anti *E. coli* 30S were found to be S5, S6 and S11 proteins (fig. 34). The *S. acidocaldarius* 50S proteins cross-reacting with anti *E. coli* 50S were identified to be L8, LU and L16 (fig. 35). We have also performed immunoblotting experiments to identify the *E. coli r*-proteins (fig. 36) cross-reacting with anti *S. acidocaldarius* 50S and 30S antibodies. Proteins S5 and S18 (fig. 37) of *E. coli* 30S subunit strongly cross-reacted with anti *S. acidocaldahus* 30S and proteins L1, L3, L6 and L9 (fig. 38) of *E. coli* 50S subunit cross-reacted with anti *S. acidocaldarius* 50S.

**Discussion:**

It is now evident from the studies by Cammarano *et al* (1986) that there are two distinct classes of ribosomes within the archaeal kingdom. The ribosomes from the extreme halophiles and most of the methanogens are similar in size and composition to those of the bacteria while those from the thermophilic archaea and several methanogens (Schmid and Bock, 1982) are larger in mass and contain significantly more proteins than do the bacterial ribosomes. Antisera raised in rabbits against r-proteins of *Methanobacterium bryantii* cross-reacted with the ribosomes within the methanogens and with a member of the extreme halophiles. With the methods and the anti-total protein sera employed, there was no detectable cross-reaction with ribosomal proteins or ribosomes from *Sulfolobus* species, eubacteria or yeast (Schmid and Bock, 1984)

Out of all archaeal organisms, *S. acidocaldanus* is least characterised in terms of immunological homology to bacteria and eukaryotes. The
Fig. 34: Immunoblot of *S. acidocaldarius* 30S r-proteins with anti *E. coli* 30S.

Fig. 35: Immunoblot of *S. acidocaldarius* 50S r-proteins with anti *E. coli* 50S.
Fig. 36: Two-dimensional gel electrophoretic pattern of ribosomal proteins from *E. coli*.

(A) 30S proteins (100 μg), (B) 50S proteins (150 μg).
Fig. 37: Immunoblot of *E. coli* 30S r-proteins with anti *S. acidocaldarius* 30S.

Fig. 38: Immunoblot of *E. coli* SOS r-proteins with anti *S. acidocaldarius* 50S.
*S. acidocaldarius* proteins S5, S6 and S11 of small subunit and L8, L11 and L16 of large subunit were found to be immunologically homologous to *E. coli* r-proteins. Interestingly these proteins were present in 30S core particle obtained by 1 M LiCl and 2 M urea and the 50S core particle obtained by sepharose-4B chromatography. In *S. acidocaldarius*, except S5, all the proteins which are cross-reacting are identified to be primary rRNA binding proteins.

We could not detect cross-reaction of *S. acidocaldarius* L7/L12 with *E. coli* 50S antiserum and vice-versa, even though *E. coli* L7/L12 and *S. acidocaldarius* L7/L12 share several properties similar to each other such as low molecular weight; relative acidity; selective release from ribosome by high salt/ethanol and dimeric structure (Casiano *et al.*. 1990). A probable explanation is that in this particular case either the proteins are not immunologically homologous or the immunological epitopes derived from the tertiary and quartemary structures of L7/L12 are not available for cross-reaction on the nitro-cellulose membrane.

We are unable to detect cross-reaction of *S. acidocaldarius* L2 with total 50S antiserum of *E. coli* and vice versa. It has been reported that the antiserum raised against *E. coli* L2 protein did not react or else reacted only very weakly with ribosomal proteins from *S. solfataricus, T. tenax, D. mobilis* and *T. celer*. When ribosomal proteins of cytoplasmic ribosomes from eukaryotes were tested, the antibodies reacted with one ribosomal protein each from *S. cerevisiae* and *P. anserina*, but showed no reaction with r-proteins of the rat liver or chicken liver (Schmid *et al.*, 1984).

One of the 30S protein of *S. acidocaldarius* and *E. coli* which showed cross-reaction with *E. coli* and *V. acidocaldarius* 30S antiserum is protein S5 and is conserved in all life forms (*Wittmann-Liebold* and Greuer, 1978, Kimura, 1984, Scholzen and Arndt, 1991, *All-Robyn* *et al.*, 1990). S5 sequences are now known
from 13 prokaryotic, eukaryotic and archaeal organisms, although somewhat larger in later group of organisms. The structurally important residues that are crucial to its stuctural integrity are highly conserved, which reflects the invariance of the S5 structure. The molecule contains two distinct a/p domains that have structural similarities to several other proteins that are components of ribonucleoprotein complexes (Ramakrishnan and White, 1992). The *B. stearothermophilus* protein S5, is 55% homologous to its *E. coli* counterpart (Wittmann-Liebold and Greuer, 1978; Kimura, 1984) and its function is almost certainly identical in the two organisms.

The *E. coli* large subunit proteins which are cross-reacting with *S. acidocaldarius* 50S antiserum are L1, L3, L6 and L9. The L6 molecule contain many structurally important residues that are highly conserved and which therefore show that its overall fold is invariant between life forms (Kimura *et al*, 1981; Ceretti *et al*, 1983; Suzuki *et al*, 1990; Scholzen and Arndt, 1991; Golden *et al*, 1993). Immune electron microscopy localizes L6 to the same site in the *B. stearothermophilus* and *E. coli* (Hackl and Stoffler-Meilicke, 1988). A related and probably homologous eukaryotic protein labelled L9 has been identified in rat liver ribosomes (Suzuki *et al*, 1990) The other *E. coli* ribosomal protein whose amino acid sequence is highly conserved in five organisms (three bacterial and two Chloroplast) is L9. L9 has an important role in ribosome function, since it is present in all viable strains of *E. coli* (Dabbs *et al*, 1986) and anti L9 antibodies reduce protein synthesis by some 75% (Nag *et al*, 1991). Its general role may be best demonstrated by the 50S assembly map (Herold and Nierhaus, 1987) which show that L9 and L1 are the only proteins that interact directly with the 3' end of 23S *r*-RNA without the cooperation of other large subunit ribosomal proteins. L9
may be the essential scaffold molecule that maintains correct folding of this region of 23S r-RNA (Hoffman et al., 1993).

At present we do not know the correspondence between the proteins showing cross-reaction with heterologous antisera in S. acidocaldarius and E. coli. Further work is needed to determine the counterparts in S. acidocaldarius and E. coli among the cross-reactive proteins. Our studies also support the earlier observation that archaea contain far fewer proteins that are homologous to bacterial r-proteins.