Chapter-2
MATERIALS & METHODS
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Bacterial strains:

The organisms used in the present study viz., Sulfolobus *acidocaldarius* strain DSM 639 and *Escherichia coli* A19 were obtained respectively from Deutsche Sammlung Von Mikroorganismen, Göttingen, Germany, and from Dr. A. R. Subramanian, Max-Planck Institute for Molecular Genetics, Berlin, Germany.

Bacterial growth:

*S. acidocaldarius* was grown at 75 °C for 40-48 hours with vigorous aeration in a medium containing 0.1% yeast extract, 0.1% bactotryptone, 0.5% casaminoacids, 0.1% glucose, 0.02% sodium chloride, 0.13% ammonium sulphate, 0.03% potassium dihydrogen phosphate, 0.025% magnesium sulphate, 0.07% calcium chloride. The pH of the medium was adjusted to 3.0 with 1 M sulphuric acid (Kikuchi and Asai, 1984). The growth was followed by the measurement of absorbance at 650 nm after every 6 hours. The bacterial cells were harvested after neutralising the culture with 1 M Tris base (5 ml/ litre). The cell pellets were stored frozen at -80 °C.

*E. coli* was grown in Luria broth at 37 °C with vigorous aeration according to Minks *et al* (1978). The mid-logarithmic phase cultures were chilled and harvested by centrifugation and stored frozen at -80 °C.

Isolation of ribosomes and supernatant fraction (S-100):

This was carried out according to Minks *et al.* (1978). All operations were carried out at 0-4 °C. Cells were ground with double the weight of alumina until
soft and sticky and extracted with buffer (3 ml/g cells), containing 20 mM Tris-Cl (pH 7.6), 50 mM potassium chloride, 10 mM magnesium acetate and 7 mM 2-mercaptoethanol (TKM$_{10}$Me), in the presence of 2 μg/ml DNase (RNAse free). The suspension was centrifuged for 10 minutes at 10,000 X g to sediment alumina and cell debris. The supernatant so obtained was again centrifuged at 30,000 X g for 30 minutes to obtain S-30 extract. The S-30 supernatant was centrifuged at around 1,00,000 X g for 4 hours 30 minutes to pellet ribosomes. The upper two-thirds of the supernatant (S-100) was collected, dialysed against TKM$_{10}$Me buffer containing 10% glycerol for 6 hours and stored frozen in aliquots. The crude ribosomal pellet was suspended in TKM$_{10}$Me buffer containing 1 M ammonium chloride and left at 4 °C for 15 hours. The ribosomes in 1 M ammonium chloride buffer were pelleted by centrifugation at 1,00,000 X g for 4 hours and the supernatant (ammonium chloride wash) was collected. The ribosomal pellet was suspended in TKM$_{10}$Me buffer containing 15% glycerol and stored frozen at -80 °C.

Isolation of *S. acidocaldarius* ribosomes using gel filtration chromatography and ion-exchange chromatography:

In addition to the conventional method for the isolation of ribosomes using ultra-centrifugation, we have developed a method for the isolation of ribosomes from *S. acidocaldarius*, which involves gel-filtration and ion-exchange chromatography. The method which is described below is rapid in comparison to the ultra-centrifugation method and the ribosomes thus obtained are not contaminated with high molecular weight non-ribosomal proteins.

*S. acidocaldarius* cells (10 gms) were suspended in 25 ml of TKM$_{10}$Me containing 0.5 M ammonium chloride buffer. The suspended cells were lysed
using 0.5% NP-40 and 0.1% Triton-X-100. The suspension was incubated on ice for 30 minutes. Pancreatic DNAse (100 μg) was added to the suspension and again incubated for 20 minutes. The suspension was centrifuged at 30,000 X g for 30 minutes. The supernatant (S-30) obtained was loaded on sephacryl S-200 column (bed volume 250 ml), which was previously equilibrated with TKM\textsubscript{10},Me containing 0.5 M ammonium chloride buffer. The column was eluted with the same buffer and fractions (5 ml each) were collected. The absorbance of diluted fractions was measured at 260 nm and 280 nm. The ribosome peak fractions in the void volume were analysed by slab gel electrophoresis and were pooled. The pooled fraction was dialysed against TKM\textsubscript{10},Me containing 50 mM ammonium chloride buffer and passed through a DEAE-cellulose column equilibrated with the same buffer. Non-specifically bound material was eluted using TKM\textsubscript{10},Me containing 100 mM ammonium chloride buffer. The DEAE-cellulose column was later eluted with TKM\textsubscript{10},Me containing 0.7 M ammonium chloride buffer. Absorbance of the column fractions was measured at 260 nm and 280 nm. The peak fractions of this elution were pooled, dialysed against TKM\textsubscript{10},Me and stored frozen at -80 °C.

**Isolation of *S. acidocaldarius* ribosomes by ultra-centrifugation:**

*S. acidocaldarius* cells (10 gms) were suspended in 25 ml of TKM\textsubscript{10},Me containing 0.5 M ammonium chloride buffer at 4 °C. NP-40 and Triton-X-100 were added to final concentrations of 0.5% and 0.1% respectively. The suspension was incubated at 4 °C for 30 minutes. Pancreatic DNAse (around 100 μg) was added and incubated for another 20 minutes. After complete lysis of cells, the suspension was centrifuged at 30,000 X g for 30 minutes. The S-30 fraction was centrifuged at 1,10,000 X g for 4 hours 30 minutes. The pelleted
ribosomes were washed and suspended in $\text{TKM}_{10}\text{Me}$ containing 1 M ammonium chloride buffer and left at 4 °C for 15 hours and clarified at 10,000 X g for 10 minutes to remove the aggregated material. The supernatant was centrifuged at 1,10,000 X g for 4 hours 30 minutes to pellet the salt washed ribosomes.

**Slab gel electrophoresis in the presence of SDS:**

Protein samples were electrophoresed on 12.5% polyacrylamide slab gels containing SDS as described by Laemmli, (1970). The ratio of acrylamide to bis acrylamide was 30:0.8. Separating gel (12.5%) was in 0.375 M Tris-Cl (pH 8.8), 0.1% SDS. A stacking gel of 5% polyacrylamide in 0.125 M Tris-Cl (pH 6.8), 0.1% SDS was used. Protein samples were treated with 0.1% SDS and 1% 2-mercaptoethanol and heated at 65 °C for 15 minutes or boiled at 100 °C for 2 minutes. Electrophoresis was carried out at 120 Volts until the tracking dye reached the bottom of the gel. The electrode buffer was 0.05 M Tris, 0.38 M glycine, 0.1% SDS (pH 8.3). After electrophoresis the gels were washed in 7.5% acetic acid for 20 minutes and stained with 0.15% Coomassie brilliant blue R-250 in 50% methanol and 7.5% acetic acid for 2 hours. The gels were destained with a solution containing 5% methanol and 7.5% acetic acid.

**Extraction of ribosomal proteins from ribosomes:**

Ribosomal proteins (r-proteins) were extracted from *S. acidocaldarius* and *E. coli* ribosomes according to the method of Hardy *et al.* (1969). Two volumes of 100% acetic acid (glacial) containing 0.05% magnesium acetate were added to one volume of ribosomes (10 mg/ml) and left on ice for 45-60 minutes with constant stirring. After 1 hour, the suspension was centrifuged at 15,000 X g for 20 minutes. Supernatant was removed carefully and the pellet was re-extracted with
67% acetic acid containing 0.1 M magnesium acetate (0.5 volumes). Supernatant was collected after recentrifugation at 15,000 X g for 20 minutes to remove the precipitated RNA. Both the supernatants containing r-proteins were pooled and precipitated with 5 volumes of ice cold acetone. After 15 hours, the r-proteins were pelleted at 10,000 X g for 10 minutes in Sorvall SS-34 rotor. Ribosomal proteins were dissolved in 8 M urea and 7 mM 2-mercaptoethanol, dialysed against the same solution for 6 hours and stored frozen at -80 °C.

**Estimation of protein:**

The concentration of protein in different samples were determined according to LowTy *et al.* (1951) using bovine serum albumin as the standard.

**Two dimensional gel electrophoresis:**

Two dimensional gel electrophoresis (2-D electrophoresis) was performed according to the method of Geyl *et al.* (1981). Protein samples were in 6 M urea, 10 mM DTT and 10 mM bis tris-acetic acid pH 4.0. Basic fuchsin was used as a marker dye. Ribosomal proteins (100 µg in 100 µl) were placed on first dimensional (0.3 X 10 cm) 4% polyacrylamide gels in 0.0568 M bis tris-acetic acid (pH 5.0), 6 M urea, 6.5 mM EDTA and 0.1% bis acrylamide. The upper electrode buffer was 0.01 M bis tris-acetic acid (pH 4.0) and the lower electrode buffer was 0.18 M potassium acetate (pH 5.0). The first dimensional gel electrophoresis was performed at 110 Volts at 4 °C for 5-6 hours. After the run the gels were removed and placed on the top of two dimensional slab gels (18.6% polyacrylamide and 0.48% methylene bis acrylamide, 1% of 5 N KOH and 6 M urea at pH 4.5).
Electrophoresis in the second dimension was performed at 15 °C and 110 Volts until the tracking dye (which had been used in the first dimension) was migrated to the bottom of gel. Electrode buffer in second dimension was glycine-acetic acid (0.28 M glycine at a pH of 4.5 adjusted with acetic acid). After the electrophoretic run, the gels were removed and stained in 0.15% Coomassie brilliant blue R-250 in 50% methanol and 7.5% acetic acid. The gels were then destained with a solution containing 5% methanol and 7.5% acetic acid.

**Fractionation of r-proteins using novobiocin:**

A fractionation method involving precipitation of r-proteins with novobiocin has been developed to fractionate ribosomal proteins of the archaeon, *Sulfolobus acidocaldarius* and bacterium, *Escherichia coli*. Ribosomal protein solutions at a concentration of 0.2 mg/ml were dialysed against 20 mM Tris-Cl (pH 8.0), 1 M ammonium chloride and 7 mM 2-mercaptoethanol. Novobiocin stock solution (10 mg/ml) in water was added to the dialysed ribosomal protein solution to a concentration of 200 µg/ml. The solution was mixed, incubated on ice for 30 minutes and centrifuged at 20,000 X g at 4 °C for 15 minutes. The supernatant was collected and the precipitated protein was dissolved in 8 M urea and 7 mM 2-mercaptoethanol. The concentration of novobiocin in the above supernatant was raised to 500 µg/ml and after incubation on ice for 30 minutes, it was again centrifuged to obtain proteins precipitated at this novobiocin concentration. The supernatant fraction was collected again and treated with 1000 µg/ml novobiocin. Both supernatant and precipitated protein fractions were obtained as above. The final supernatant fraction was treated with five volumes of acetone and the precipitated protein was dissolved in 8 M urea, 7 mM 2-mercaptoethanol. The precipitated protein at each novobiocin concentration was
estimated by Lowry's assay. Proteins in each novobiocin fraction were identified by two dimensional electrophoresis of Geyl et al. (1981). The numbering of the ribosomal proteins S. acidocaldarius was according to (Schmid and Bock, 1982) and those of E. coli according to Geyl et al. (1981).

Isolation of ribosomal core particles by hydrophobic chromatography:

Hydrophobic chromatography of E. coli ribosomes was developed by Kirillov et al. (1978) to dissociate E. coli ribosomes into subunits. Chromatography of S. acidocaldarius ribosomes yielded 50S subunit core particles and 30S subunits lacking some proteins. Ribosomes in low concentrations of magnesium acetate and high concentrations of ammonium sulphate are strongly adsorbed onto sepharose 4B. This method is based on the difference in the hydrophobic interaction of subunits with the agarose gel at very low concentration (or in the absence) of Mg++ ions.

All the experiments were performed in two stages at 4°C. In the first stage S. acidocaldarius ribosomes were transferred by gel-filtration through a column of sephadex G-50 into a buffer containing 0.02 M Tris-Cl (pH 7.6) with desired concentrations of magnesium acetate and 0.6 M ammonium sulphate. Ammonium sulphate was added to a final concentration of 1.5 M to the pooled ribosome fractions eluting in the void volume of G-50 column.

Ribosomes were applied to a column of sepharose 4B (100 ml bed volume) equilibrated with the buffer containing 0.02 M Tris-Cl (pH 7.5), containing 1.5 M ammonium sulphate and desired concentration of magnesium acetate. The column was eluted with three volumes of reverse salt gradient of ammonium sulphate (1.5
M to 0.02 M) in the same buffer. Fractions were collected and analysed by measuring absorbance at 260 nm, after appropriate dilution.

**Hydrophobic chromatography for the isolation of 50S core particles directly from S-30:**

In this method, S-30 was prepared by lysing 15 gms of cells with Triton-X-100 and NP-40 as described previously. The S-30 obtained was dialysed against buffer containing 0.02 M Tris-Cl (pH 7.5), 15 M ammonium sulphate buffer for 72 hours. The precipitated material was removed by centrifugation at 15,000 X g for 15 minutes. The clear supernatant was loaded on sepharose 4B equilibrated with 0.02 M Tris-Cl (pH 7.5), 15 M ammonium sulphate buffer. Elution of the column was carried out by linear reverse salt gradient of 1.5-0.02 M ammonium sulphate in buffer containing 0.02 M Tris-Cl (pH 7.5). Absorbance of the fractions was measured at 260 nm after appropriate dilution and the fractions containing 50S core particle and 70S particle were pooled, dialysed against TKM$_{10}$Me buffer and stored frozen at -80 °C.

**Isolation of ribosomal subunits by density gradient centrifugation:**

*S. acidocaldarius* and *E. coli* 70S ribosomes were dialysed against buffer containing high salt and low magnesium acetate (0.02 M Tris-Cl pH 7.6, 1 mM magnesium acetate, 0.5 M ammonium chloride). The dialysed ribosomal solution was clarified by low speed centrifugation, carefully layered on the top of 8-38% linear sucrose gradients and centrifuged for 2 hours 40 minutes at 40,000 RPM in VTi-50 Beckman rotor. Fractions were collected from the bottom of the gradient tube. The absorbance of the fractions was measured at 260 nm after appropriate dilutions. Fractions corresponding to 30S and 50S subunits were pooled.

The
subunits were immediately brought to 10 mM magnesium acetate by the addition of 1 M magnesium acetate and concentrated by DEAE-cellulose chromatography and were stored in aliquots at -80 °C.

**Association and dissociation of ribosomal subunits and core particles:**

The buffers used for association and dissociation were 20 mM Tris-Cl (pH 7.6), 50 mM ammonium chloride, 40 mM magnesium acetate, 3 mM spermine and 7 mM 2-mercaptoethanol (Buffer A). 20 mM Tris-Cl (pH 7.6), 1 mM magnesium acetate, 500 mM ammonium chloride, 7 mM 2-mercaptoethanol (Buffer B) respectively.

50S subunits (12 Å) and 30S subunits (8 Å) in 20 mM Tris-Cl (pH 7.6), 50 mM ammonium chloride, 10 mM magnesium acetate and 7 mM 2-mercaptoethanol were mixed and magnesium acetate and spermine were added to the concentrations of the buffer A. The mixture was incubated at 45 °C for 40 minutes and subsequently loaded onto a 8-38% linear sucrose gradient in buffer A. The sample was centrifuged at 40,000 rpm for 2 hours 20 minutes in a VTi-50 rotor. Fractions were collected from the bottom of the gradient tube. The absorbance of the fractions was measured at 260 nm after appropriate dilutions and the values plotted on a graph.

70S ribosomes (20 Å) in 20 mM Tris-Cl (pH 7.6), 50 mM ammonium chloride, 10 mM magnesium acetate and 7 mM 2-mercaptoethanol were dialysed against buffer B (dissociating conditions), layered on a 8-38% linear sucrose gradient in buffer B and centrifuged at 40,000 rpm for 2 hours 20 minutes in a VTi-50 rotor. The fractions were collected from the bottom of the gradient tube and their absorbance analysed as before.
**S. acidocaldanus** 50S core particle (12 $A_{260}$) obtained by sepharose 4B chromatography of the 70S ribosomes (in the absence of Magnesium acetate) and (8 $A_{260}$) 30S subunits obtained by gradient centrifugation were mixed and incubated at 45 °C for 40 minutes in buffer A, by making appropriate additions. The sample was analysed as before on a 8-38% linear sucrose gradients.

**Isolation of ribosomal RNA:**

Total ribosomal RNA, 16 S RNA and 23 S RNA from *S. acidocaldanus* and *E. coli* were isolated using the following procedure. Equal volume of TKM$_{10}$Me buffer saturated phenol was added to ribosomes or ribosomal subunits (10 mg/ml concentration) and vigorously shaken for 30 minutes at 4 °C. The mixture was separated into two layers by centrifugation at 12,000 X g in a Sorvall RC5C centrifuge. The aqueous layer was carefully taken without disturbing the interface. The phenolization was repeated six times. All the 7 phenol phases were combined and extracted once with one volume of TKM$_{10}$Me buffer. RNA present in aqueous phases was precipitated with 2 volumes of ethanol (pre-cooled at -20 °C) overnight. The precipitate was collected by centrifugation at 10,000 X g for 15 minutes in a SS-34 rotor in the Sorvall RC5C centrifuge. The pellet was dissolved in TKM$_{10}$Me buffer, re-precipitated with 2 volumes of cold ethanol and dissolved in the appropriate buffer chosen for thermal analysis.

**Thermal melting analysis:**

Ribosomes, ribosomal subunits, core particles and rRNA (1 $A_{260}$ each) in 1 mM Tris-Cl (pH 7.6) and 0.08 mM magnesium acetate was heated at a rate of 1 °C rise per minute and the increase in $A_{260}$ was continuously recorded in Philips PU 8700 UV/Vis spectrophotometer equipped with a thermoprogrammer (model
Prior to heating, the contents were gently mixed and incubated for 5 minutes at the starting temperature. Low magnesium concentration was chosen for preventing aberrant Tm values.

**Isolation of ribosomal subunit core particles:**

The 30S ribosomal subunit of *S. acidocaldarius* is protein rich in comparison to *E. coli* subunit. Treatment of *S. acidocaldarius* with increasing concentrations of LiCl and urea produces a core particle which contains a well defined subset of ribosomal proteins.

LiCl and urea were added to a final concentration of 1 M LiCl/2 M urea or 2 M LiCl/4 M urea or 3 M LiCl/6 M urea or 4 M LiCl/8 M urea to 10 mg/ml of 30S subunit in 20 mM Tris-Cl (pH 7.6), 50 mM potassium chloride, 2 mM magnesium acetate and 7 mM 2-mercaptoethanol or 20 mM Tris-Cl (pH 7.6), 50 mM potassium chloride, 20 mM magnesium acetate and 7 mM 2-mercaptoethanol and incubated overnight at 4 °C with constant stirring. The precipitated material was removed by centrifugation at 15,000 X g for 15 minutes. The core particles were separated from split proteins by centrifugation at 1,90,000 X g for 5 to 8 hours. The proteins present in the core particle were extracted with acetic acid and analysed by two dimensional gel electrophoresis.

Core particle of 30S subunits (10 mg/ml) was also isolated by treatment with only LiCl. Lithium chloride was added to a final concentration of 1 M LiCl, 2 M LiCl, 3 M LiCl, 4 M LiCl to 30S subunits obtained by ultra-centrifugation, and incubated overnight at 4 °C with constant stirring. The core particles were separated from split proteins by high speed centrifugation as previously described. Core particles were also isolated by treating 30S subunits with different concentrations of urea (1 M, 2 M, and 4 M urea).
Antisera to subunits and isolation of antibodies:

Antibodies to 30S and 50S subunits of *S. acidocaldarius* and *E. coli* were raised in rabbits as described by Stoffler and Wittman (1971). Subunits (each 500 µg) was emulsified with Freund's complete adjuvant and injected subcutaneously into rabbits at multiple sites. After four weeks, a booster injection of 200 µg protein in Freund's incomplete adjuvant was given through subcutaneous route. Rabbit was bled after one week of booster injection through the pinna vein. Blood was allowed to clot at 30 °C for 2 hours and at 4 °C for 15 hours. Antiserum was collected after centrifugation of clotted blood. The antisera were heated at 55 °C for 30 minutes to inactivate the complement proteins and fractionated with ammonium sulphate. Serum (10 ml) was mixed with 6 ml of saturated ammonium sulphate in 0.02 M Tris-Cl (pH 8.0), 1 mM EDTA and stirred gently at 30 °C for 30 minutes. The precipitate was spun down at 4,000 X g for 45 minutes. IgG pellet was dissolved in small volumes of 20 mM Tns-Cl (pH 8.0) and 1 mM EDTA. IgG was re-extracted with one-third volume of saturated ammonium sulphate solution. IgG precipitate was collected by centrifugation at 4,000 X g for 45 minutes, dissolved in small volumes of 70 mM sodium phosphate buffer (pH 6.3) and dialysed against the same buffer.

The crude IgG fraction was further purified by ion-exchange chromatography on DEAE-cellulose. The ion-exchanger was packed into a column and equilibrated with phosphate buffer at room temperature. Dialysed ammonium sulphate fraction was applied on the column and eluted with the same buffer. Fractions of 1 ml volume were collected and absorbance at 280 nm was measured. IgG peak fractions were pooled and concentrated by adding equal volumes of saturated ammonium sulphate (precipitated at 50% saturation). The IgG precipitate was collected by centrifugation at 15,000 X g for 15 minutes, dissolved in
phosphate buffered saline and dialysed against the same buffer. After dialysis, the concentrated antibody was distributed into aliquots and stored at -80 °C. Ouchterlony immunodiffusion was performed as per the standard protocol.

**Immunoblotting of two dimensional gels:**

This method was according to Towbin *et al.* (1979) with certain modifications. Ribosomal subunit proteins were extracted with acetic acid and subjected to two-dimensional gel electrophoresis of Geyl *et al.* (1981). Proteins were transferred from two-dimensional gels to nitro-cellulose membrane using the electrode buffer (25 mM Tris, 192 mM glycine, 20% methanol), for a period of 8 hours at 7 Volts. After the transfer, the blots were removed from the blotting apparatus and air-dried. Later the unbound sites were saturated by incubating the blots in blocking buffer containing 10 mM Tris-Cl (pH 8.1), 150 mM NaCl (T<sub>10</sub>NaCl<sub>150</sub>) and 3% BSA for 3 hours. The nitro-cellulose sheets were incubated with diluted antiserum (8:100) in blocking buffer for 4 hours and washed (6 X 5 minutes) with blocking buffer containing 0.05% NP-40. The blots were then incubated with peroxidase conjugated anti-rabbit IgG (1:1000) in blocking buffer for 2 hours. The membranes were again washed 6 times (6 X 5 minutes) with T<sub>10</sub>NaCl<sub>150</sub> buffer. Finally, the blots were developed in the substrate solution. The substrate solution was prepared freshly by adding 1 ml stock solution of 4-chloro-1-naphthol (3 mg/ml) in methanol and 10 μl of 30% H₂O₂ to 10 ml Tris-buffered saline (T<sub>10</sub>NaCl<sub>150</sub>). The reaction was terminated after 15 minutes by washing with distilled water and the membrane was air-dried.