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Biophysical Chemistry 50 (1994) 3-16

Biophysical Chemistry

Crystallography of halophilic ribosome: the isolation of an internal ribonucleoprotein complex

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(Received 5 January 1994)

Abstract

Crystals of 50S ribosomal subunits from *Haloarcula marismortui* diffracting to 2.9 Å resolution were grown. Because of their large unit cells and the extremely weak diffracting power, almost all X-ray crystallographic analysis of these crystals must be performed with intense synchrotron radiation. At ambient temperature, all ribosomal crystals decay upon the first instance of X-irradiation. To overcome this severe sensitivity, procedures for data collection at cryo temperature were developed. Under these conditions the crystals can be irradiated for periods sufficient for the collection of more than one data set from an individual crystal (days or weeks) with no observable damage. They also can be stored for months, to resume interrupted measurements. To assist the interpretation of the anticipated electron density map, a specific internal nucleoprotein complex of protein HmaL1 and a stretch of H23S rRNA was isolated from the halophilic ribosome. The fragments of the 23S rRNA protected by the protein from nuclease digestion were sequenced. Alignment of the sequences of some archaebacterial L1-specific RNA fragments to the corresponding parts of eubacterial and eukaryotic rDNAs, localized the sequence identities to two distinct regions. Chimeric complexes were reconstituted with the corresponding *E. coli* ribosomal components, indicating a rather high homology, despite the evolution distance. A feasible secondary structure of the rRNA stretch participating in this complex was found to be compatible with the one proposed for the corresponding part in the *E. coli* ribosomal RNA.

Key words: Haloarcula marismortui; Ribosomes; X-ray crystallography; Ribonucleoprotein complexes

1. Introduction

The ribosome is the universal cell organelle which facilitates the intricate and essential process of the enzymatic translation of the genetic

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Abbreviations: r-proteins stands for ribosomal proteins and rRNA for ribosomal RNA. E, B, T, and H in front of a name of a ribosomal protein shows its bacterial source (*Escherichia coli*; *Bacillus stearothermophilus*; *Thermus Thermophilus* and *Haloarcula marismortui*, respectively). L shows that the protein is of the large (50S) subunit, and S of the small (30S) one. The symbol: HmaL# indicates that this particular protein is homologous to protein # from *E. coli*. H50S is the large subunit of *H. marismortui*.

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SSDI 0301-4622(94)00004-4

information into proteins. It is a nucleoprotein assembly, built of two subunits of unequal size, which associate upon initiation of protein synthesis. A typical bacterial ribosome (called also 70S, according to its sedimentation coefficient) contains about a quarter of a million atoms and is of a molecular weight of around 2.3 million dalton (1.45 and 0.85 for the large, 50S, and the small, 30S, subunits, respectively). Three chains of rRNA, of over 4500 nucleotides, account for about two-thirds of the mass of the ribosomes, the rest are some 57–73 different proteins, depending on the source.

Intensive biochemical, biophysical and genetic studies shed light on several functional and evolutionary aspects of protein biosynthesis and provided some information on the spatial *in situ* proximities between several ribosomal components, the secondary structure of ribosomal RNA and the approximate locations of some functional centers (reviewed in refs. [1,2]). However, for illuminating the molecular mechanism of protein biosynthesis a reliable molecular model is essential. Therefore we undertook X-ray crystallography studies.

1.1. The superiority of the halophilic ribosomes

Numerous attempts to crystalize the traditionally studied hence well characterized *E. coli* ribosome, carried out in several laboratories, led to marginal success [3]. However, intact, modified and complexed ribosomal particles from thermophilic or halophilic bacteria yielded crystals suitable for X-ray and neutron crystallographic studies at various resolution limits (reviewed in refs. [4,5]. All crystals of ribosomal particles grown in our laboratories were obtained from biologically active particles and the crystalline material retains its integrity and activity for long periods, despite the natural tendency of the ribosomes to disintegrate.

It was found that the quality of the crystals is directly related to the level of extremeness in the natural environment of the bacterium. Thus, ribosomes from normal eubacteria or from moderate halophiles did not crystallize, and those from extreme thermophiles crystallize better than those from moderate ones. This finding may be related to the higher stability of the thermophilic and halophilic ribosomes.

The Dead Sea contains the highest salt concentrations of any natural bodies of water in the world. Despite this unusual salinity, it supports the growth of several species of micro-organisms, all of which exhibit the unusual property of withstanding high salt concentrations and elevated temperatures. One of these organisms is the archaebacterium *H. marismortui*. The ribosomes of this bacterium function at 3.5–4 M salinity at up to 60°C, conditions which usually cause the dissociation of nucleoprotein assemblies and the denaturation of isolated proteins.

1.2. Crystallographic studies

An extensive search for the suitable ionic composition for crystal growth and the development of a sophisticated seeding procedure, led to the growth of six crystal forms of the 50S and one of the 30S subunits from this bacterium [4–8]. A special procedure was designed which allows data collection from crystals immersed in solutions of compositions similar to those of the physiological environment within the cells, which were grown at the lowest concentrations of salts essential to avoid disintegration of the halophilic ribosomes [7,8].

The crystals of H50S diffract to the highest resolution obtained so far from crystals of ribosomal particles: 2.9 Å (Fig. 1). They are of reasonable mosaicity (0.2–0.3°) and adequate mechanical stability [8]. As mentioned above, a fairly halophilic environment is essential for crystals growth. Furthermore, in addition to KCl and NH_4Cl, a substantial amount of Mg^{2+} (50–100 mM) and minute amounts of Cd^{2+} (1–2 mM) are required for obtaining good crystals. The influence of Mg^{2+} on the quality of these crystals may be attributed to its involvement in maintaining the integrity of the ribosomal particles as well as to its probable participation in the crystal network [11]. However, the exceptional contribution of a few mM of Cd^{2+}, at concentrations found to be harmless to *H. marismortui* ribosomes, is still unexplained. Thus, crystals grown under the same

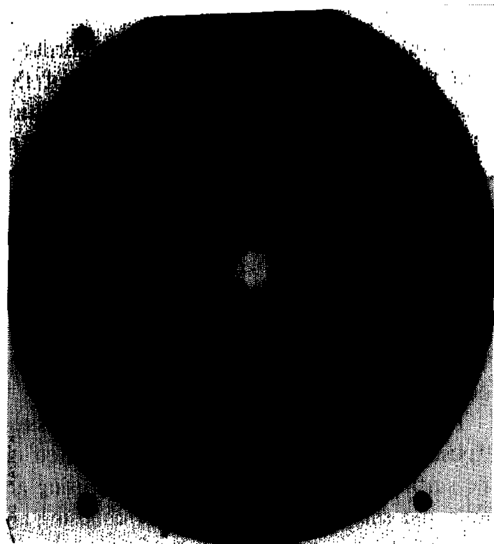

Fig. 1. A rotation photograph of a crystal of H50S, obtained at 90 K at Station F1/CHESS, operating at 5.3 Gev and 50 mA. Crystal to film distance = 220 mm, collimator = 0.1 mm^2; wavelength = 0.9091 Å.

conditions which yield 2.9 Å resolution, but without Cd^{2+}, diffract nominally to 4.5 Å but yield useful only to 7–9 Å resolution [4,8].

These crystals reach average size of 0.3 × 0.3 × 0.05 mm, have cell dimensions of 210 × 300 × 570 Å and $C222_1$ symmetry. A typical diffraction data set collected from these crystals contains over 150000 unique intensities for the resolution shell 3.5–50 Å. The evaluation of these data is still not a routine task, although some sophisticated computational tools have been developed specifically for this purpose. Nevertheless, the evaluated data are of quality comparable to that obtained from crystalline proteins of average size. Thus, for above 55% completeness, the typical values for R-merge (I) at 6 Å are in the range of 5–10% [12] and at 3 Å, 12–15% (A. Zaytzev-Bashan, H.A.S. Hansen, Z. Berkovitch-Yellin and A. Yonath, to be published).

Because of the weak diffracting power and the large unit cells of the ribosomal crystals, virtually all the crystallographic studies have to be performed with intense synchrotron radiation. At ambient temperature the diffraction pattern of these crystals decay within the first instances of irradiation. To eliminate the extreme radiation damage, data are collected at cryogenic temperatures (about −180°C) from shock frozen crystals. Under these conditions the crystals diffract with no observable decay for periods sufficient for the collection of a full set of data from individual crystals, and the irradiated crystals can be stored for months and still maintain their diffracting power. To facilitate cryogenic data collection, special experimental procedures had to be designed for accommodating the problematic features of the ribosomal crystals: fragility, sensitivity, thin edges, etc. [12,13].

The phasing of X-ray amplitudes remains the less predictable and most difficult part in structure determination. Phase information is crucial for constructing electron-density maps, and cannot be directly measured. The classical methods for phasing are MIR and SIR (multiple and single isomorphous replacement, respectively), both require specific and quantitative attachment of heavy atoms at a limited number of sites within the unit cell. Useful derivatives of proteins of average size consist of one or a few heavy-metal atoms (such as Pt, Hg, Au, Ag etc.). Much heavier compounds are needed for ribosomes. Therefore, to reach the required electron density we are using a stable and water-soluble form of an undecagold cluster (GC, m.w. 6200 Da), with a core of 11 gold atoms linked directly to each other [14,15]. Simulation studies showed that this cluster is of adequate phasing power at low and medium resolution, providing quantitative binding and reasonable isomorphism (N. Volkmann and H. Bartels, to be published).

Conventional derivatization of crystals of biological macromolecules is achieved by soaking the crystals in solutions of mM amount of heavy-atom salts. This is a rather chancy process, which, for structures of complexity similar to that of ribosomes, is likely to lead to multi-site binding. To facilitate quantitative and site specific derivatization, a monofunctional reagent was developed from the undecagoldcluster. A chemically reactive non-chiral handle of a limited flexibility and length, imitating the peptide bond, was attached to the undecagold cluster [5,14,15]. Preliminary derivatization experiments were performed on

50S subunits from *Bacillus stearothermophilus*. One ribosomal protein, BL11, was removed by mutagenesis [16] or by a stepwise addition of salts [17]. The cluster was bound quantitatively to the isolated ribosomal protein, exploiting its only –SH moiety. The modified protein was, in turn, reconstituted into the cores of mutated ribosomes, lacking it, obtained by cell growth on a special antibiotics, thiostrepton [16]. The crystals of the so obtained fully derivatized particles yielded data of reasonable quality, currently used in phasing attempts (H. Bartels, H.A.S. Hansen, Z. Berkovitch-Yellin, I. Agmon, W.S. Bennett, A. Zaytzev-Bashan, I. Levin, F. Schlünzen, N. Volkmann, A. Dribin and A. Yonath, to be published).

1.3. Derivatization, biochemical and genetics of halophilic ribosomes

Severe difficulties were encountered in attempts to adapt the procedure described above to the halophilic ribosomes. Because of the significant resistance of the halophilic ribosomes to mutations [18] no single-protein-depleted core particles could be designed. Also, such core could not be constructed by chemical methods similar to those used for eubacteria, namely the elevation of the concentrations of salts in the media, as the natural environment of the halophilic ribosome is almost salt-saturated. Furthermore, in contrast to incorporation of the gold-cluster bound protein BL11 into depleted cores of *B. stearothermophilus*, protein HmaL11, the presumed halophilic homolog of BL11, can be reconstituted into core particles only when its sulfhydryl group is free.

Procedures for selective detachment of a few halophilic r-proteins, exploiting dioxane, were developed, and cores of 50S particles, depleted of proteins HmaL1, HmaL6, HmaL10, HmaL11 and HmaL12 were prepared. All detached proteins could be fully reconstituted and the so obtained particles crystallize under the same conditions as native 50S subunits do. However, as mentioned above, blocking the –SH group of one of them, HmaL11, prevented its incorporation into the core particles. In this way cores of 50S subunits, depleted of protein HmaL11, were obtained. The ribosomal subunits lacking protein HmaL11 crystallize under the same conditions as the native H50S subunits and show apparent isomorphism with them, indicating that the removal of this protein caused neither major conformation changes in the ribosome, nor disturbances in the crystal's network. The crystals of the depleted particles reach average size of 0.2 × 0.2 × 0.05 mm, and diffract well to 10 Å resolution. In later stages, maps constructed from the differences between the diffraction of the native and depleted particle should facilitate the interpretation of the electron density maps, by showing the location of HmaL11.

To create potential heavy-atom binding sites, exposed reactive moieties are being inserted on the surface of the halophilic ribosome, using genetic and chemical procedures. For choosing appropriate locations for insertions of exposed reactive groups we benefit from the advanced stage of the sequencing of the halophilic r-proteins [19] and from studies on surface mapping, performed either by limited proteolysis [20] or by direct chemical determination of exposed amino and sulfhydryl groups under different salt concentrations (S. Weinstein, C. Paulke, I. Sagi, C. Glotz, I. Levin, V. Weinrich and F. Franceschi, to be published). As the genes of these particular proteins have been cloned [21], the insertion of chemically reactive side chains into the surface of the ribosome became feasible.

Several mutants were produced by site directed *in vitro* mutagenesis. Concentrating on the provision of exposed sulfhydryls, a cys codon was inserted into protein HmaL1. The mutated genes were cloned into the vector, pET11d, and the mutated proteins were overexpressed in *E. coli*. A similar procedure was applied for protein HmaL11. The natural –SH group of this protein, which, upon any type of modification, prevents its incorporation into core particles, was exchanged by ser. Similar to the native proteins, the mutated and over-expressed ones could be reconstituted into ribosomal cores lacking them. Screening for the suitability of the mutated proteins for binding heavy atom clusters is in progress, and indications for the incorporation of protein HmaL11 in which amino acid 33 is a cysteine into core 50S particles, have been obtained.

2. An internal halophilic ribonucleoprotein complex

Assuming that the intrinsic conformations of those ribosomal components which crystallize are maintained even when they are detached from the ribosomes, the atomic structures of ribosomal components should assist the interpretation of the electron density map of the entire particle. For the last three decades extensive efforts have been made in several laboratories to crystallize isolated ribosomal components from eubacteria. These include proteins, internal clusters of them, internal RNA/protein complexes and whole or fragmented RNA chains (summarized in ref. [22]). So far experience shows that in general assembled ribosomal particles crystallize more readily than their isolated components. In fact, the majority of the r-proteins could not be crystallized, and the structures of most of the crystallized components have still not been solved, mainly due to the poor internal order of these crystals. This rather poor yield indicates that the conformations of many ribosomal components are dictated by their environment, namely by the network of internal contacts which construct the skeleton of the ribosome. Thus, those proteins that do not possess an independent intrinsic conformation may loose their *in situ* conformation when detached from the scaffolding ribosome.

Protein BL11 is an example of a ribosomal protein which undergoes significant, albeit reversible, conformational changes upon isolation from the 50S subunit. On the ribosome its –SH group is exposed and reactive, whereas in solution this group is burried and can become reactive only under denaturating conditions (6 M urea). However, the lost native conformation can be regained upon reconstitution into core particles, even when large chemical moieties are bound to its –SH group [14,15].

Although it remains to be seen whether the structures of the crystallized isolated r-proteins do reflect their *in situ* conformations, the mere ability to crystallize may indicate the existence of intrinsic fold. The superiority of the thermophilic isolated r-proteins for crystallographic studies is evident. This shows that under proper conditions these components can be trapped in a unique conformation. Encouraged by these findings, we focused our efforts on the purification and the crystallization of isolated components from halophilic ribosomes. Internal small and defined ribonucleoprotein complexes, may be more suitable for crystallographic studies than isolated proteins or rRNA fragments, as they are more likely to maintain the native conformation, since in such complexes the micro environment may be kept.

The isolation of an internal riboprotein complex benefitted from investigations on the integrity of the halophilic ribosomes. Illuminating is the dependence of the number of exposed sulfhydryls in the 50S subunits on the relative concentrations of the potassium and the magnesium ions. Thus, up to 15 sulfhydryls become exposed upon lowering the KCl concentration from 3 to 1.5 M, in the presence of about 10 mM Mg^{2+}. However, even at low KCl (1.2–1.5 M), the presence of 20–25 mM Mg^{2+} was sufficient to keep the number of exposed sulfhydryls close to its minimum, namely 2–3 (S. Weinstein, I. Sagi and V. Weinrich, to be published). Since the increase of exposed sulfhydryls may indicate partial "opening" (or unfolding) of the structure of the ribosome, it seems that a few mM of Mg^{2+} have the same influence on the compactness of the halophilic ribosomes as 1–1.5 M KCl do.

A ribonucleoprotein complex of protein HmaL1 and a stretch of H23S rRNA was isolated by decreasing the salt concentration below the minimum which is needed for preserving the structure of the entire 50S subunit. This 23S rRNA-protein complex was separated from the dissociated r-proteins and the 5S rRNA on a linear sucrose gradient. Fractions containing the 23S rRNA were pooled and the proteins were extracted from the complex and analyzed on a SDS gel (Fig. 2). For demonstrating quantitative separation, the gels were loaded with equal amounts of equivalent units of r-proteins. Essentially only one protein, HmaLl, was consistently detected, in a few preparations trace amounts of two other proteins, HmaL12 and HmaL18, were also observed. In addition, it was found that protein HmaL1 was missing in the fractions of

the free proteins and the 5S rRNA (Fig. 3). This indicates that among the 23S RNA binding proteins, protein HmaL1 has the highest specific affinity, so that it remains bound in quantitative amounts to the 23S rRNA throughout the extraction procedure.

The isolation of the HmaL1-specific rRNA fragments was performed by subjecting the complex to a limited RNase-A digestion. Since the ribonucleoprotein complex was found to be stable in solutions of rather high salt concentrations, the resulting complex of protein HmaL1 and the specifically bound RNA fragments could not be fractionated from the digested RNA and the ribonuclease on a polyacrylamide gel by the procedure that was established for RNA-protein complexes from non halophilic sources [23]. Instead, the digest was separated on a Sephacryl S-200 column. The elution profile of the column indicated that HmaL1 was bound to specific RNA fragments (Fig. 4). The substantial co-migration of protein HmaL1 and the RNA-fragment on the column is most probably due to tight protein-RNA interactions. Thus, protein HmaL1 was detected in aliquotes of the rRNA containing fraction which were loaded on linear sucrose gradients and subjected to extensive centrifugation.

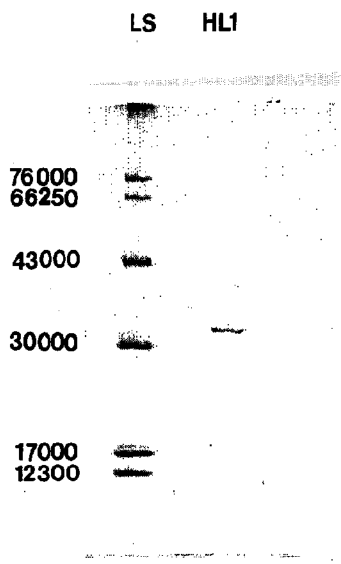

Fig. 2. SDS-gel analysis of the proteins present in the 23S rRNA ribonucleoprotein complex of *H. marismortui*. The proteins were extracted from the isolated 23S rRNA ribonucleoprotein complex and directly loaded 'on a 10–22% polyacrylamide gel. A lengths standard was included as a marker.

Evolutionary aspects. The sequences of the fragments of the 23S rRNA which were protected by protein HmaL1 from RNase-A digestion were directly determined by enzymatic RNA sequencing of the 3′-termini and 5′-termini. Three discrete fragments were detected (157, 164 and 178 bases in lengths) and isolated, all share an identical internal sequence of 154 nucleotides (Fig. 5) and two of them have a common 5′-terminus. The rRNA component of this complex shows two regions of a high homology to its corresponding stretch in *E. coli*, despite the significant evolutionary distance. Thus, it was found that stable heterologous complexes can be formed between the halophilic 23S RNA and protein L1 from *E. coli*, and between the *E. coli* 23S rRNA fragment and HmaL1 [5]. It remains to be seen whether the preservation displayed by this complex indicates that the structural elements of the ribosome have been rather conserved throughout evolution.

To assess the uniqueness of this complex, we investigated heterologous complexes. Increasing amounts of either protein HmaL1 or E1I were incubated with a constant quantity of RNAs previously extracted from 70S ribosomes of either *H. marismortui* or *E. coli*. In each reconstitution experiment the salt concentrations were adjusted to the physiological salt conditions of the proteins in the halophilic ribosomes. The reconstitution mixtures were fractionated on a sucrose gradients. The fractions of 5S RNA, 16S RNA and 23S RNA were analyzed for bound proteins. HmaL1 and EL1 were exclusively found in the fractions containing the 23S RNA. For a quantitative analysis of the bound proteins in particular ribonucleoprotein complexes, the ribosomal proteins were analyzed on SDS gels and scanned spectrophotometrically. Limited digestion of the ribonucleoprotein complexes was carried out with RNase A.

The association of protein EL1 with 23S RNAs and 26S RNAs from organisms remote in evolution from *E. coli* was reported earlier [24]. In these studies the specificity of the binding was based on the following criteria: (a) the protein

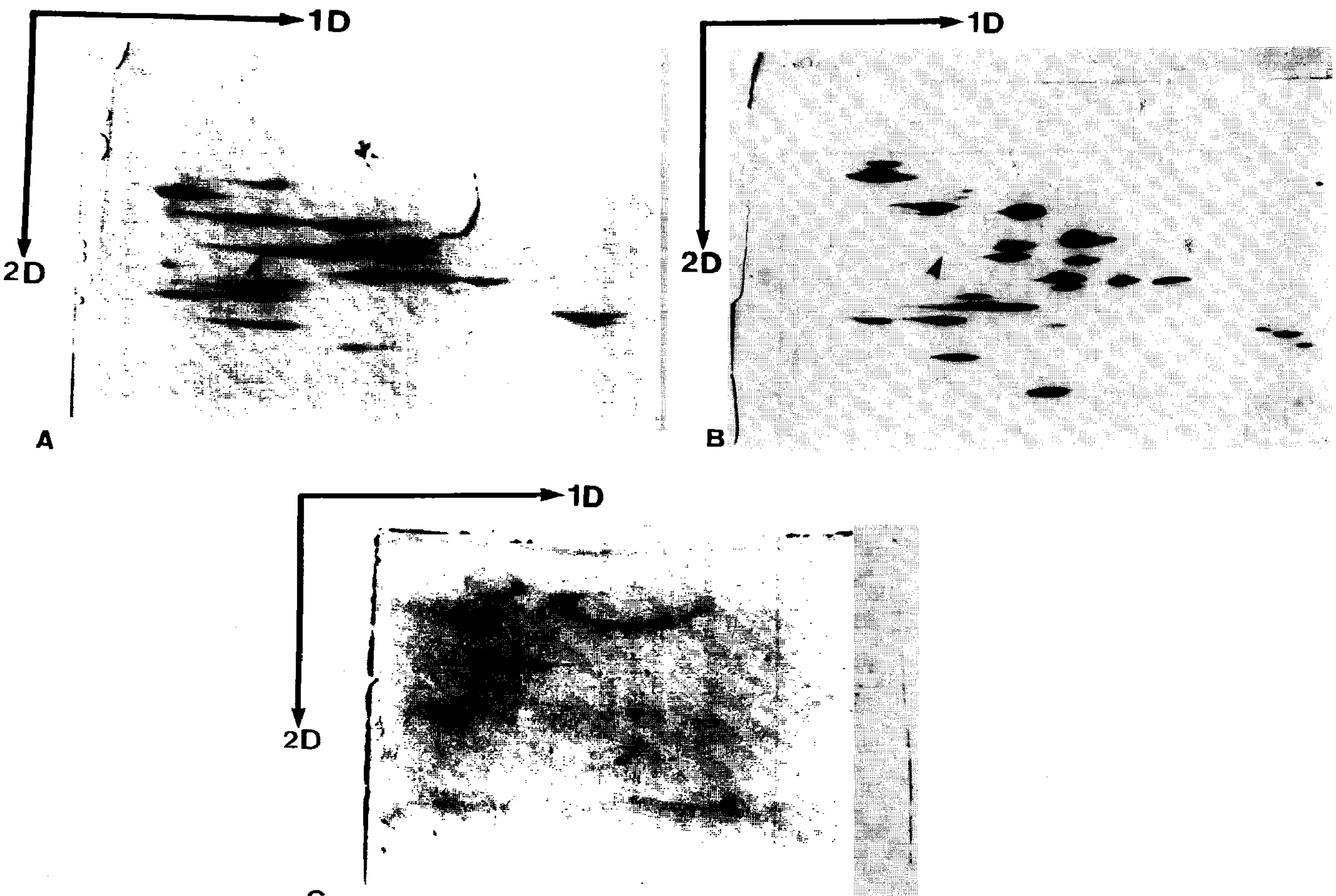

Fig. 3. Identification of proteins in the 235 rRNA ribonucleoprotein complex of *H. marismortui* using two dimensional gel electrophoresis. After the 50S subunits were extracted with EB-buffer, the released ribosomal components (r-proteins, 5S rRNA and the 23S rRNA ribonucleoprotein complex) were separated on a linear sucrose gradient. (A) A total protein extract of the 50S subunit of *H. marismortui* for comparison, protein HmaL1 is marked with an arrow. (B) shows the two dimensional gel electrophoresis of the fractions containing the dissociated r-proteins. The arrow indicates the empty position of HmaL1. Protein HmaL1, exclusively found in the fractions of the ribonucleoprotein complex, is shown in (C).

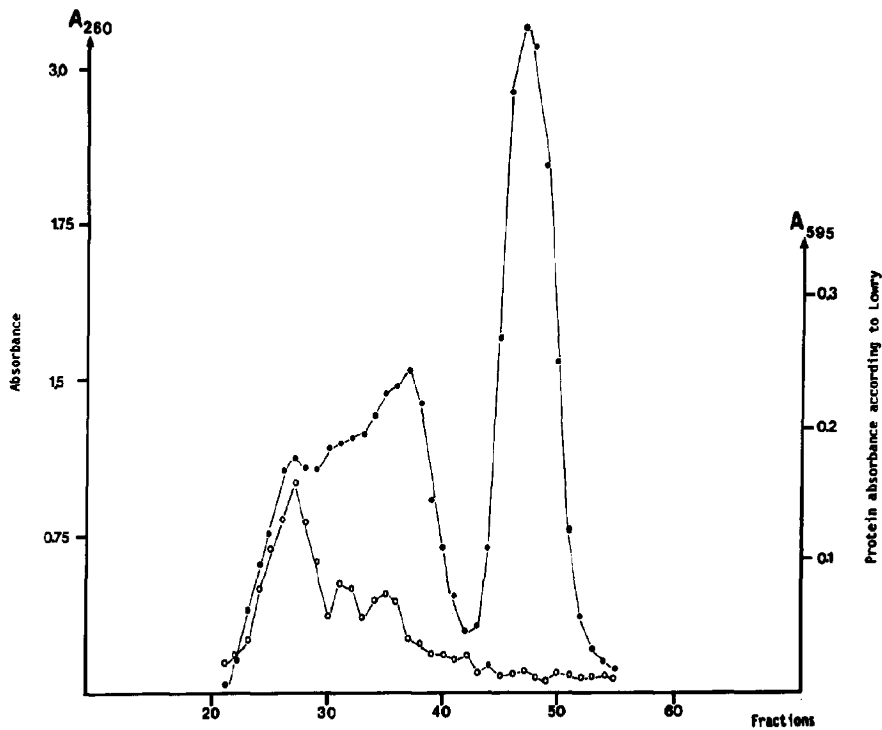

Fig. 4. Isolation of the HmaL1-specific rRNA fragments. The 23S RNA complex was digested with RNaseA, and the resulting products were separated on a Sephacryl S-200 column. The elution of the nucleic acids was monitored at A260. The quantity of the protein containing fractions were estimated according to Lowry. The highest concentration of HmaL1 was found in fraction 27, where the peak of the RNA fragments was found. RNaseA was found in fractions 34–36, showing that it was clearly separated from the HmaL1-protected fragments. The last nucleic acids peak consisted of the fractions including the RNA digestion products.

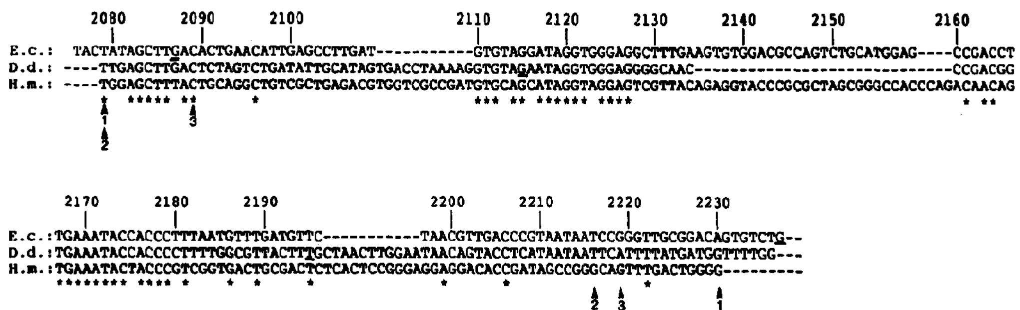

Fig. 5. Sequences from *H. marismortui* 23S rDNA comprising the 178 nucleotide fragment of the HmaL1-binding site aligned to the 23S rDNA region that encodes for the homologous protein binding region in RNA from *E. coli* and the corresponding segment of *D. discoideum* 26S rDNA. Sequence identities are marked with asterisks. The first and the last nucleotide of the rRNA fragment masked by protein L1 in the homologous [32] and in the heterologous complex [23] are underlined in the 23S rDNA and 26S rDNA sequences. Arrows (1: 178 nucleotides, 2: 164 nucleotides, 3: 157 nucleotides) define the sequence of the three 23S rRNA segments from *H. marismortui*, which were protected by protein HmaL1 during the partial hydrolysis of the HmaL1-23S rRNA complex with RNaseA.

bound exclusively to its cognate RNA when incubated with a mixture of different RNA molecules, (b) the molar protein: RNA ratio in the complex reached plateau values between 0.2:1 and 1.3:1 in the presence of an excess of protein and (c) bound protein remained attached to a specific RNA fragment in a mixture of digestion products resulting from limited RNase hydrolysis of a complex containing intact ribosomal RNA. Fig. 6 shows all possible variations of the homologous and heterologous reconstitution experiments (EL1 binding to the 23S rRNA of *E. coli*, EL1 binding to the 23S rRNA of *H. marismortui*, HmaL1 binding to the 23S RNA of *H. marismortui* and HmaL1 binding to the 23S rRNA of *E. coli*). In all cases the binding of the protein component to the 23S RNA reached saturation, although in the heterologous complexes the stoichiometry was rather low. Interestingly, it was shown that the protein recognizes specifically the 23S rRNA in the mixture of different RNAs, although the yield of the heterologous complexes is not always quantitative. Hence, we concluded that the archaebacterial protein HmaL1 and the eubacterial protein EL1 can recognize their cognate binding sites on both archaebacterial and eubacterial 23S RNA chains, despite the evolutionary distance and the substantial differences in the physiological environment and standard binding conditions for the ribosomes of these two bacteria.

Ribosomes were found to be highly conserved throughout evolution. Numerous phylogenetic studies were based on the comparative analysis of individual ribosomal components. Thus, it was found that protein EL1 recognizes defined binding sites on 23S rRNA and 26S RNA chains from various organisms of the three kingdoms (eubacteria, archaebacteria and eucaryotes) and forms specific heterologous ribonucleoprotein complexes with those chains [24]. Little is known about the function of protein L1 in the ribosome. However, it was suggested that the capability of protein L1 to bind to its mRNA transcript may facilitate a feedback translational regulation for its own synthesis [24–29].

For a better insight into some evolutional aspects of the rRNA features recognized by protein HmaL1, these archaebacterial rRNA fragments were compared to the sequence of *E. coli* 23S rDNA [30] and to the 26S rDNA sequence from the eucaryote *Dictiostelium discoideum* [31] Fig. 5. The protein L1 binding site was determined by limited nuclease digestion of a heterologous complex of *D. discoideum* 26S rRNA and protein L1 from *E. coli* [23], whereas the *E. coli* specific rRNA segment was obtained from nuclease digestion of the homologous complex [32]. The L1 binding sites on the rRNAs of these three different organisms exhibit two regions of highly conserved sequences (nucleotides 2110–2128 and

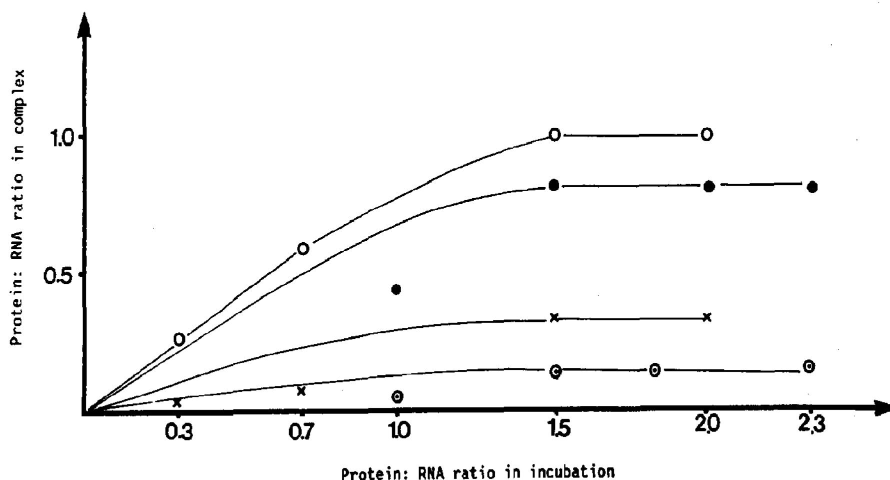

Fig. 6. Saturation curves of the binding of L1 to 23S RNA.

2161–2179, *E. coli* numbering), which are assumed to be part of the conserved L1 binding site.

Although the rRNA fragments share highly conserved sequences in their central part, they have extended ends of different lengths. Among these, the archaebacterial sequences are the longest. Recent attempts to characterize the L1 binding sites of defined RNA fragments, obtained by bacteriophage RNA polymerase transcription of cloned DNA sequences, showed that the minimal size of the 23S rRNA fragment from *E. coli* that recognizes protein EL1 (nucleotides 2120–2178) lies within this conserved region (P. Dong, P.B.F. Cahill and R.A. Zimmermann, private communication). The variations in the lengths of the fragments and the fact that they extend the minimum recognition site of L1 may indicate that parts of the longer nuclease resistant fragments possess inherent RNA folds which cannot be hydrolyzed by nucleases.

The compatibility of several potential secondary structures with the sequences that encompass the L1-binding site in *H. marismortui* 23S rRNA was assessed. Fig. 7 shows that an extended stem and loop can be formed, analogous to the proposed secondary structure of the L1-binding site in *E. coli* [29]. The primary sequences of these loops revealed two highly conserved consensus regions consisting of a 5′GGAG and a 5′UGAAAUAC. Within the 5′GGAG sequence A2126 is considered to make direct contact with protein L1 in *E. coli*, whereas the homologous purine rich region seems to be important for maintaining the conformation of the L1 binding site (P. Dong, P.B.F. Cahill and R.A. Zimmermann, private communication).

It was proposed that in *E. coli* the competition between protein L1 binding sites on the rRNA and mRNA regulates the translation of the L11-L1 polycistronic m-RNA. The homologous ribosomal protein genes from halophilic bacteria are clustered in the same order as in *E. coli*. However, the transcription was found to be different; in halobacteria protein L1 is translated from a L1-L10-L12 tricistronic mRNA [21,33,34]. Recent results suggest that in the archaebacterium *Methanococcus vannielii*, the L1 binding region

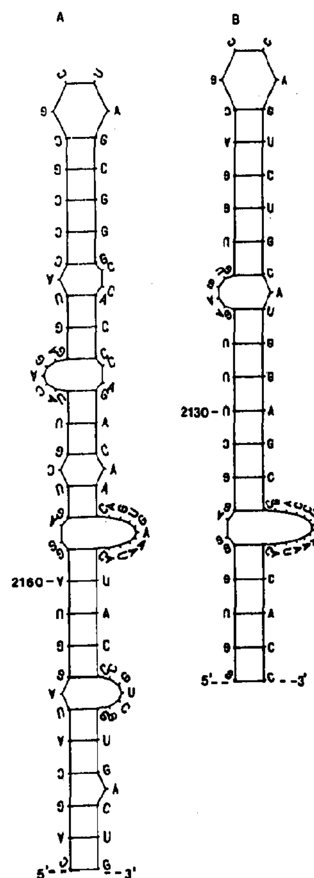

Fig. 7. (A) Possible secondary structures of the L1-binding site on the *H. marismortui* 23S rRNA. (B) The model of the secondary structure of the corresponding sequence from *E. coli* [29].

seems to be located within the structural gene [35]. Thus, protein L1 can act as a feedback repressor, by abolishing the translation of the L1-mRNA even when the leader sequence was deleted (Hanner, Mayer, Stoeffler and Piendl, private communication), in contrast to *E. coli*, where the homologous binding site is located in the untranslated leader region.

A thorough search for primary and secondary sequences that may generate the L1 binding site on the L1 mRNA of *H. marismortui*, did not reveal a definite homologous structure. The fact that the motif of 5′GGAG occurs five times within the L1 transcript renders a reliable prediction to be more difficult. Comparison of the homologous

L1 mRNA sequences from *H. marismortui* with the recently predicted L1 binding site on the leader sequence of the related organism *Halobacterium cutirubrum* [34] showed that within this region only the conserved stretch is the Shine–Dalgarno sequence. Since it is assumed that the regulatory function of the EL1 binding sequence on its own mRNA is expressed only when the amount of the newly synthesized protein EL1 exceeds the stoichiometry needed for the assembly of the ribosome, the low level of conservation in the halophilic bacteria may reflect a lower regulating potential of the halophilic homolog to EL1.

3. Concluding remarks

Bearing in mind the potential contribution of the detailed three-dimensional structures of isolated ribosomal components to the crystallographic studies of assembled ribosomal particles, we developed procedures for the preparation of *in situ* complexes with defined compositions. We concentrated on the particles which yield the best crystals, the 50S ribosomal subunits from *H. marismortui*. We report here the isolation and the characterization of a highly stable ribonucleoprotein complex containing protein HmaL1, the halophilic homolog to L1 from *E. coli* and a stretch of the 23S rRNA, masked by it.

This ribonucleoprotein complex displays unusual integrity at extreme salt concentrations, as does the ribosome from which it was extracted. High resolution structures of such complexes should also be instrumental for the analysis of the forces holding together proteins and nucleic acids at high salt concentrations and shed light on the intriguing questions of stability and integrity of multi-components cellular assemblies.

Furthermore, the object of our studies, ribosomes from halophilic bacteria, may be exploited for evolution investigations. Indeed, the formation of heterologous complexes with the counterparts of *E. coli* ribosomes and the comparisons of the sequence of the stretch of the rRNA composing this complex, yielded some interesting information.

4. The procedures

Ribosomes were prepared according to ref. [6].

Crystals of 50S subunits from *H. marismortui* were grown by vapor diffusion in Linbro dishes at 19°C from 6–8 μl of 80–120 OD_{260} H50S subunits in solution of 1.6 M KCl, 0.5 M NH_4Cl, 0.01 M $MgCl_2$, containing traces of non-physiological additives: 5–7% polyethylene glycol (m.w. 6000) and 1–2 mM $CdCl_2$. The crystals were stored in solutions of 3 M KCl, 0.5 M NH_4Cl and 0.01 M $MgCl_2$, 10% polyethylene glycol (m.w. 6000) and 1–2 mM $CdCl_2$. For data collection 18% ethylene glycol were added, serving as a cryogen. The procedures for crystallographic data collection and for the evaluation of these data are described in refs. [4,8,13].

The monofunctional undecagold cluster was prepared and bound to ribosomal particles according to refs. [5,14,15].

Exposed sulfhydryls were determined as described in refs. [14, 20]. Preliminary attempts to label and identify exposed amino groups are currently being developed.

Two-dimensional gel electrophoresis of isolated ribosomal proteins was performed according to [36].

The detachment of selected ribosomal proteins from the 50S and 30S subunits of *H. marismortui* by dioxane is described in refs. [5,8]. The detached proteins were incorporated into the depleted core particles by incubating both components at 55°C for 40 min. A few proteins could also be detached from the 50S subunits by lowering the salt concentrations to 0.5 M KCL, 0.05 M $MgCl_2$ and 0.10 Tris–HCl pH = 7.0.

The sequencing of the halophilic ribosomal proteins is described in refs. [5,37]. For overexpression of selected r-proteins, their genes were cloned from a genomic-library or by the polymerase chain reaction [38]. The genes were cloned in pET11d and overexpressed in *E. coli* BL21 (DE3 pLysS) [39]. The overexpressed proteins were purified by ion-exchange chromatography [40].

Extraction of the complex of protein HmaL1 and the 23S rRNA: the ribonucleoprotein complex was released from 50S-ribosomal subunits by

dialysis against buffers containing low Mg concentration. The 50S subunits were diluted to 200 A260/ml in extraction buffer EB (1.2 M KCl, 10 mM Tris–HCl pH 7.8, 0.3 mM MgCl2) and dialyzed extensively at 4°C over night against the same buffer. The resulting ribonucleoprotein complex, containing 23SrRNA, was separated on a linear sucrose gradient (5–25% sucrose in extraction buffer). Centrifugation was carried out using a TST414-rotor for 3.5 h at 39000 rpm and 10°C. The rRNA peaks were located by measuring the absorption at 260 nm.

To detect the presence of proteins in fractions containing dissociated r-proteins after low salt treatment, the upper fractions of the sucrose gradient which contain both r-proteins and the 5S rRNA, were analyzed on two dimensional polyacrylamide gels. For a quantitative comparison, each gel was loaded with equal amounts of equivalent units (one equivalent unit corresponds to 1 A(260) 50S subunits) of r-proteins as shown in Fig. 2.

For large scale production of this complex, HmaL1 was cloned, overexpressed and isolated by DEAE-sepharose CL6BP chromatography. The DNA coding for the fragment of rRNA protected by HmaL1 was obtained from chromosomal DNA using PCR. One of the oligo-nucleotides used for this reaction contained at the 5′ end sequence a T7 RNA polymerase promoter. The fragment, obtained by the PCR reaction, was subsequently transcribed *in vitro* with T7 RNA polymerase using the procedure of ref. [41].

Preparation of HmaL1-specific rRNA fragments. To isolate the HmaL1-specific rRNA fragments, the complex was subjected to a limited RNase-A digestion. Pancreatic RNaseA (United States Biochemical Corporation) was added to the HmaL1-RNA complex at an enzyme/substrate concentration of 9 units/200 e.u. (1 e.u. corresponded to 1 A (260) 50S subunit) in digestion buffer (1.2 M KCl, 0.5 M NH_4Cl, 20 mM Tris–HCl pH 7.8, 30 mM $MgCl_2$). Incubation was continued for 3 h at 20°C. The digest was loaded directly on a Sephacryl S-200 column of 200 ml bed volume equilibrated in the digestion buffer. Fractions of 3.7 ml were collected at a flow rate of 0.3 ml/min. Aliquotes from the effluent were precipitated in trichloroacetic acid (10% wt/v final concentration) and analyzed on SDS gels [42].

Sequence analysis of protein HmaL1-specific rRNA fragments. The rRNA fragments were extracted from the ribonucleoprotein complex with phenol, dephosphorylated with alkaline phosphatase (calf intestinal mucosa, Pharmacia) at a ratio of 15 pmol RNA: 0.1 unit enzyme and separated on a 10% polyacrylamide gel made in 8.3% urea. The methylene blue-stained RNA bands were eluted from the minced gel according to ref. [43]. Methylene blue was removed by extracting the elution-buffer three times with *n*-butanol and the RNA-fragments were recovered by precipitation with 2.2 volume of absolute ethanol at −80°C. 5′-end-labelling of the RNA with T4 polynucleotid kinase (Pharmacia) and gamma-^{32}PATP (New England Nuclear) was based on the procedure of [44]. 3′-end-labelling of the RNA with T4 RNA ligase (Pharmacia) and 5′^{32}PpCp (New England Nuclear) was carried out according to ref. [45]. The labelled fragments were then purified by electrophoresis on 20% polyacrylamide gels in 50 mM Tris-borate, pH 8.3, 1 mM EDTA, 7 M urea, located by autoradiography, and eluted as described above in the presence of yeast carrier tRNA. Individual fragments were subjected to enzymatic sequence analysis as described [46–48], alkaline hydrolysis of the RNA fragments were carried out according to ref. [49].

Formation of homologous and heterologous protein–RNA complexes protein HmaL1 was extracted from the complex with 23SrRNA using dioxane according to [40]. The RNAs were extracted with phenol from 70S subunits of *E. coli* and *H. marismortui*. Protein EL1 together with either 23S rRNA from *E. coli H. marismortui* were incubated in Rec4 buffer (20 mM Tris–HCl pH 7.8, 4 mM $MgCl_2$, 400 mM NH_4Cl, 4 mM 2-mercaptoethanol) at 44°C for 20 min [50]. Both HmaL1-H23S rRNA and HmaL1-E23S rRNA complexes were reconstituted in 3 M KCl, 0.5 M NH_4Cl, 20 mM $MgCl_2$, 20 mM Tris–HCl pH 7.8, 4 mM 2-mercaptoethanol and incubated for 60 min at 55°C. The ribonucleoprotein complexes were fractionate by sucrose-gradient sedimenta-

tion (5–25% sucrose made in the appropriate reconstitution buffers). Protein HmaL1 and EL1 were extracted from the corresponding complexes with acetic acid (2.2 V/V glacial acetic acid and 10 mM $MgCl_2$ final concentration) and loaded on SDS gels. The gels were scanned spectrophotometrically and the amounts of the proteins were determined from standards relating staining intensity of known quantities of protein HmaL1 and protein EL1.

Acknowledgement

These studies were initiated under the inspiration and guidance of the late Professor H.G. Wittmann. Purified protein EL1 was a gift from Dr. K.H. Nierhaus (MPI for Mol. Genetics, Berlin) and we thank him for this. We also appreciate the advice and the possibility to use the equipment of E. Mandelkow and J. Biernat. We thank our colleagues, P. Dennis, S. Weinstein, V. Weinrich, I. Sagi, B. Donzelman, Z. Berkovitch-Yellin, W.S. Bennett, H.A.S. Hansen, K. von Böhlen, A. Zaytzev-Bashan, J. Thygesen, I. Agmon, M. Eisenstein, N. Volkmann, H. Bartels, F. Schlünzen, R. Sharon, I. Levin, A. Dribin and J. Harms for their interest and H.S. Gewitz, T. Arad, I. Dunkel, C. Glotz, C. Paulke, S. Meyer, B. Romberg, J. Muessig, J. Piefke and R. Albrecht for excellent technical assistance. Data were collected at CHESS, SSRL, KEK/PF and the EMBL and the MPG SR stations at DESY. This work was supported by research grants from BMFT (MPBO 180), NIH (GM34360), DFG (Yo-11/1-2), DARA (50QV 86061) and the Kimmelman Center for Biomolecular Structure and Assembly. A.Y. holds the Martin S. Kimmel Professorial chair.

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