Early Assembly Proteins of the Large Ribosomal Subunit of the Thermophilic Archae bacterium Sulfolobus

IDENTIFICATION AND BINDING TO HETEROLOGOUS rRNA SPECIES*

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Studies of ribosome structure in thermophilic archae bacteria may provide valuable information on (i) the mechanisms involved in the stabilization of nucleic acid-protein complexes at high temperatures and (ii) the degree of evolutionary conservation of the ribosomal components in the primary kingdoms of cell descent. In this work we investigate certain aspects of RNA/protein interaction within the large ribosomal subunits of the extremely thermophilic archae bacterium Sulfolobus solfataricus. The ribosomal proteins involved in the early reactions leading to in vitro particle assembly have been identified; it is shown that they can interact with the RNA in a temperature-independent fashion, forming a thermally stable "core" particle that can subsequently be converted into complete 50 S ribosomes. Among the protein components of the core particle, those capable of independently binding to 23 S and 5 S RNA species have also been identified. Finally, we show that the early assembly proteins of Sulfolobus large ribosomal subunits are able to interact cooperatively with 23 S RNAs from other archae bacteria or from eubacteria, thereby suggesting that RNA/protein recognition sites are largely conserved within prokaryotic ribosomes. By contrast, no specific binding of the archae bacterial proteins to eukaryotic RNA could be demonstrated.

Our current model of ribosome structure and function is principally based on Escherichia coli particles. Ribosomes, however, while performing the same basic functions in all living organisms, exist in three lineage-specific versions: eubacterial, archaebacterial, and eukaryotic. So far, ribosome diversity has been scarcely explored, yet, considerably more information on both eukaryotic and archaebacterial organisms is needed for understanding the evolution of the translational machinery.

In the present study we analyze certain structural aspects of the large ribosomal subunit of Sulfolobus solfataricus, an extremely thermophilic archae bacterium that thrives optimally in sulfur hot springs at 85–87°C. Sulfolobus ribosomes are interesting in two respects: they are derived from a species belonging to the most primitive archae bacterial group, the Crenarchaeota (1); they are structurally stable and maintain translational efficiency and fidelity at temperatures close to the boiling point of water (2). The availability of an in vitro system for the total reconstitution of active large subunits of Sulfolobus ribosomes (3) enables one to study in detail the structure and function of the particle.

Here we report the identification of a subset of Sulfolobus large subunit proteins which are localized in the interior of the particle and participate in the early events of ribosome assembly. Among these, the most likely candidates for the role of primary RNA-binding proteins have also been identified. We also show that most of early assembly proteins of Sulfolobus ribosomes are able to bind cooperatively to heterologous 23 S-like RNA species from both other archae bacteria and eubacteria.

MATERIALS AND METHODS

Preparation of rRNA, Ribosomal Proteins, Ribosomes, and Ribosomal Subunits—S. solfataricus ribosomes and ribosomal subunits were prepared as described previously (2, 3). rRNA (total ribosomal or purified rRNA species, as required) and total large subunit proteins (TP50) were prepared as detailed elsewhere (2, 4). Escherichia coli and Halofex indigenous rRNAs were extracted from 70 S ribosomes kindly supplied by Dr. R. Amils (Madrid). Saccharomyces cerevisiae rRNA was extracted from 80 S ribosomes prepared according to the procedure described in Ref. 5.

Partial Assembly Experiments—To prepare reconstitution intermediates, 1–10 A260 units of Sulfolobus 23 S RNA were incubated for 1 h at 20°C with a 3-fold molar excess of TP50 in a buffer containing 300 mM KC1, 30 mM Tris-HCl, pH 6.8, 15 mM Mg(OAc)2, 5 mM β-mercaptoethanol.

The incubation mixtures were centrifuged on 10–30% (w/v) sucrose density gradients made in 30 mM KC1, 30 mM Tris-HCl, pH 7, 5 mM Mg(OAc)2 to isolate the reconstituted particles, the appropriate fractions were collected from the gradients and subjected to overnight high-speed centrifugation. The particles were resuspended in 40 mM NH4Cl, 20 mM Tris-HCl, pH 7, 10 mM Mg(OAc)2, 5 mM β-mercaptoethanol (ribosome buffer) containing 50% (v/v) glycerol; they were then treated with acetic acid to extract the proteins. These were separated by two-dimensional electrophoresis and stained with Coomassie Blue as described previously (6).

Routinely, the poly(U)-dependent poly(Phe) synthesizing activity of the particles was assayed on samples directly taken from the incubation mixture as described in Ref. 3. When specified, one-half of the mixture was adjusted to 12 mM with respect to spermine and to 20 mM with respect to Mg2+, and incubation was continued for another 60 min at 75°C.

Preparation of "Core" Particles—To prepare core particles, about 10 A260 units of purified Sulfolobus 50 S subunits were allowed to stand overnight at 0°C in 10 mM Tris-HCl, pH 7, 5 mM Mg(OAc)2 containing either 1 M LiCl, 2 M urea; 2 M LiCl, 4 M urea; 3 M LiCl, 6 M urea; or 4 M LiCl, 8 M urea. The core particles were separated from the split proteins by high speed centrifugation over a "cushion" of 0.5 M sucrose in 500 mM NaH4Cl, 20 mM Tris-HCl, pH 7, 10 mM Mg(OAc)2 acetate, 5 mM β-mercaptoethanol. The particles were analyzed by
Sucrose gradient centrifugation and subjected to activity measurements and protein extraction and analysis.

Preparation of Hybrid Particles—About 5 A260 units of large ribosomal subunit RNA from *E. coli*, *H. mediterranei*, or *S. cerevisiae* were incubated for 1 h at 40 °C with a 3-fold molar excess of Sulfolobus TP50 in the presence of 300 mM KCl, 30 mM Tris-HCl, pH 6.8, 20 mM Mg(OAc)₂, and 5 mM β-mercaptoethanol. The particles were isolated by sucrose gradient centrifugation, collected, and analyzed for activity in poly(Phe) synthesis and protein composition. In this case, the protein gels were stained by the silver nitrate method (7).

For thermal denaturation experiments, the purified hybrid particles were dissolved in 10 mM Tris-HCl, pH 7, 0.1 mM Mg(OAc)₂ at A260 = 0.5. Hyperchromicity/temperature curves were recorded as described in Ref. 3.

Protein Blotting and rRNA Hybridization—About 200 μg of Sulfolobus TP50 were separated by two-dimensional electrophoresis following the method of Geyp et al. (8). After electrophoresis, the gels were washed with 30 min in 0.7% acetic acid, and the proteins were transferred to a nitrocellulose filter by electrophoresis (3 h at 280 mA at 4 °C in 0.7% acetic acid). The filter was then washed for 60 min with several changes of binding buffer (500 mM KCl, 20 mM Tris-HCl, pH 7, 20 mM Mg(OAc)₂) and then incubated for 1 h at room temperature in binding buffer containing 32P-labeled Sulfolobus large subunit RNA (23 S or 5 S) or purified 5 S RNA (about 2 × 10⁶ cpm of each, corresponding to 5 pmol). After incubation, the filter was extensively washed with binding buffer, stained with fast green to reveal protein spots, and then dried and exposed.

RESULTS

Reconstitution Intermediates of Sulfolobus Large Ribosomal Subunits—As reported elsewhere (3), the large subunits of Sulfolobus ribosomes can be reassembled in *vitro* from the separate RNA and protein components by a two-step procedure in which high temperature and a suitable concentration of polyamines (thermine or spermine) represent the critical parameters. The assembly mechanism, however, was not analyzed in detail; specifically, it was left undecided whether high temperature is obligatorily required for the primary interactions between the RNA and protein components of the thermophilic ribosome.

In the present work, we investigated whether the assembly of Sulfolobus ribosomes could be initiated at low temperature by adjusting the ionic conditions of the first step of the reconstitution process. For that purpose, RNA from purified large subunits (containing 23 and 5 S species) was incubated with TP50 (1:3 molar ratio) at various temperatures between 0 and 20 °C in the presence of 300 mM KCl, 20 mM Tris-HCl, pH 6.8, and 15 mM Mg(OAc)₂. At all of the temperatures assayed, albeit most efficiently at 20 °C, the reaction resulted in the production of a discrete particle of about 40 S (Fig. 1). Although the 40 S particles were completely inactive in poly(Phe) synthesis (not shown); however, they retained a high degree of thermal stability, as shown by their unaltered sedimentation behavior following prolonged incubation at 85 °C (Fig. 3c).

To this end, reconstitution intermediates were prepared using purified 23 S RNA and a 5-fold molar excess of 32P-end-labeled 5 S RNA. Fractionation of the reconstitution mixtures on sucrose gradients showed that the radioactive 5 S RNA was not incorporated within the incomplete particles but was present in the finally reconstituted subunits (results not shown). Further and more direct evidence that protein L5 participates in the binding of 5 S RNA is reported below.

Partial Disassembly of Sulfolobus Large Subunits—That most protein components of the low temperature reconstitution intermediate are indeed located in the interior of the subunit was confirmed by partial disassembly of native 50 S ribosomes. These were subjected to unfolding treatments in order to generate protein-deficient core particles. To this end, the 50 S subunits were allowed to stand overnight at 4 °C in either: 1 M LiCl, 2 M urea; 2 M LiCl, 4 M urea; 3 M LiCl, 6 M urea; or 4 M LiCl, 8 M urea. The core particles were then separated from the dissociated proteins by sedimentation through a cushion of 0.5 M sucrose.

Surprisingly, the 50 S ribosome was found to be converted into a discrete core particle of about 40 S regardless of the LiCl/urea concentration used (Fig. 3). The cores were inactive in poly(Phe) synthesis (not shown); however, they retained a high degree of thermal stability, as shown by their unaltered sedimentation behavior following prolonged incubation at 85 °C (Fig. 3c).

Additional proof that identical core particles are produced over a 4-fold range of LiCl/urea concentrations was obtained from determinations of the particles' protein composition. All of the cores were found to contain the same set of 20 proteins, a representative pattern of which is shown in Fig. 2C (treatment with 3 M LiCl, 6 M urea).

A comparison of the patterns in Fig. 2, B and C, shows that the core proteins largely correspond to those occurring in the low temperature reconstitution intermediate. The cores contain five additional proteins not found in the intermediate, namely L2, L5, L25, L27, and L30; only one of the "intermediate" proteins, L17, is not detected in the cores. Also, electrophoresis of the RNA extracted from the cores (not shown) evidenced that they contained 5 S RNA, in accordance with the presence of protein L5 (see also below).

RNA Binding Proteins—On the whole, the partial reconstitution and partial disassembly experiments define a set of 15 proteins (L1, L4, L6, L7, L9, L11, L13, L14, L15, L16, L18, L19, L21, L22, L24) that appear to be involved in early
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assembly events and to be located in the interior of the 50 S subunit. To determine which of these were primary RNA binding proteins (i.e., proteins capable of interacting strongly and individually with rRNA) we performed "Western blotting" assays.

*Sulfolobus* large ribosomal subunit proteins were separated by two-dimensional gel electrophoresis following the method of Geyl *et al.* (8). This procedure differs from that used previously to resolve and number *Sulfolobus* TP50 (4, 6) in that the second dimension gel contains 4 M urea and no sodium dodecyl sulfate; this was necessary to avoid irreversible denaturation of the proteins. Indeed, preliminary experiments showed that proteins blotted onto nitrocellulose after separation on sodium dodecyl sulfate-containing gels were no longer able to hybridize in a specific fashion to RNA probes, even if subjected to various "renaturing" procedures prior to blotting (not shown).

**Fig. 2.** Gel electrophoretic protein analysis of *Sulfolobus* native 50 S subunits (A), "low temperature reconstitution intermediates" (B), and "core particles" prepared with 3 M LiCl, 6 M urea (C). The proteins were extracted from gradient-purified particles by acetic acid treatment; electrophoresis was as specified under "Materials and Methods."

**Fig. 3.** Sedimentation analysis of "core particles." a, sucrose density gradient profile of 50 S subunits treated with 2 M LiCl, 4 M urea; b, 50 S subunits treated with 4 M LiCl, 8 M urea; c, same as in b but treated at 85 °C for 30 min. The arrows indicate the position of native 50 S subunits.

32P-Labeled RNA extracted from purified *Sulfolobus* large ribosomal subunits (containing 23 and 5 S RNA in equimolar amounts) was used as the probe. Hybridizations were performed at room temperature in the presence of 300 mM K+ and 20 mM Mg2+; under these conditions about 10 proteins were consistently found to interact with the RNA (Fig. 4A). Although the electrophoretic system used in this case differed from that used for the previous experiments, the radioactive spots could be unambiguously identified because the first-dimension run was identical in the two systems. The labeled proteins were L1, L4, L5, L6, L9, L11, L12, L13, L15, and L21. All of them, with the exception of L5 and L12, belong to the previously identified subset of early assembly proteins.

As explained above, there was evidence indicating that protein L5 was involved in the binding of 5 S RNA. To assess this point, hybridization experiments were performed using purified *Sulfolobus* 5 S RNA as the probe; the result was the exclusive labeling of protein L5 (Fig. 4B). Thus, L5 most probably acts as a primary 5 S RNA binding protein in *Sulfolobus* ribosomes; importantly, the experiment also indicates that RNA/protein binding is indeed specific under the conditions employed.

The binding specificity was further checked by adding to the hybridization mixtures (containing either 23 + 5 S RNA or only 5 S RNA) a 100-fold excess of unlabeled 16 S RNA; under these conditions the hybridization patterns were superimposable to those of Fig. 4, thus indicating that 16 S RNA does not compete with the large subunit RNA species for protein binding. Furthermore, no signals were detected when labeled 16 S RNA was directly hybridized to blotted TP50 (not shown).

**Binding of Sulfolobus TP50 to Heterologous rRNA Species—** To obtain information about the degree of phylogenetic conservation of the ribosomal components involved in initiating particle assembly, we tested the ability of *Sulfolobus* TP50 to
recognize rRNA molecules from distantly related archaeabacteria, and from eubacteria and eukaryotes.

Binding of Sulfolobus TP50 to RNA extracted from purified large ribosomal subunits of the halophilic archaeabacterium H. mediterranei, the eubacterium E. coli, and the eukaryote S. cerevisiae was assayed under a variety of experimental conditions. The ionic environment ultimately adopted contained 300 mM KCl, 20 mM Tris-HCl, pH 7.0, and 20 mM Mg(OAc)$_2$. Incubations were carried out at 40 °C for 60 min.

As shown in Fig. 5, sedimentation analysis of reconstitution mixtures containing either E. coli or H. mediterranei rRNA revealed the presence of a discrete particle of about 40 S. The formation of such a particle was strictly dependent upon the presence of at least 15 mM concentrations of Mg$^{2+}$ ions; by contrast, polyamines such as spermine or spermidine were not required (data not shown). Although the hybrid particles could also be observed at relatively low temperatures (15–20 °C), temperatures between 37 and 44 °C strongly stimulated their formation (not shown). By contrast, in developing conditions allowing the formation of a homogeneous particle from yeast rRNA and Sulfolobus proteins. Such attempts invariably resulted in the appearance of polydisperse arrays of absorbance peaks, indicating the presence of heterogeneous ribonucleoprotein complexes and also extensive RNA degradation (Fig. 5c).

The specificity of RNA/protein interactions in the heterologous particles was demonstrated by the experiments illustrated in Fig. 6. Increasing amounts of Sulfolobus TP50 were incubated with a fixed amount of E. coli total ribosomal RNA; sedimentation analysis of the incubation mixture and electrophoresis of acetic acid extracts of the purified absorbance peaks showed that Sulfolobus proteins interact exclusively with E. coli 23 S RNA, leaving 16 S RNA unmodified. In addition, incubation of E. coli 23 S RNA with Sulfolobus small subunit proteins did not result in the formation of a particle but only in extensive degradation of the rRNA (not shown).

Hybrid particles containing either halobacterial or eubacterial RNA species were inactive in poly(U)-directed polyphenylalanine synthesis. Electrophoretic analysis of the protein moieties of gradient-purified particles showed that both

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**Fig. 4.** Hybridization patterns of large subunit RNAs to nitrocellulose-blotted TP50. A, hybridization with 23 + 5 S RNA as the probe; B, hybridization with purified 5 S RNA. The experiments were carried out as described under “Materials and Methods.”

**Fig. 5.** In vitro recognition between Sulfolobus TP50 and noncognate RNA species. a, sucrose density gradient profile of incubation mixtures (1 h at 40 °C under the conditions specified under “Materials and Methods”) containing Sulfolobus TP50 and E. coli large subunit RNA; b, the same with H. mediterranei large subunit RNA; c, the same but with S. cerevisiae large subunit RNA. The arrows, from left to right, indicate the position of 23 S RNA and native 50 S subunits, respectively.

**Fig. 6.** Specificity of RNA/protein interactions in the hybrid particles. Left panel: a, sucrose density gradient profile of E. coli total ribosomal RNA (1 A$_{260}$ unit); b, the same amount of E. coli RNA after incubation with approximately 40 μg of Sulfolobus TP50; c, same as in b but with approximately 80 μg of Sulfolobus TP50. Right panel: the two absorbance peaks detected in experiment c were purified from the gradients and subjected to acetic acid treatment in order to extract any proteins complexed with the RNAs; the extracts were electrophoresed on sodium dodecyl sulfate-containing 12% acrylamide gels. Lane 1, protein extract of the faster moving peak; lane 2, protein extract of the slower moving peak; lane 3, control TP50.
contained the same set of about 20 proteins, a representative pattern of which is shown in Fig. 7. Interestingly, these proteins essentially coincide with those found in the Sulfolobus core particles; this indicates that the early-assembly proteins of Sulfolobus ribosomes are also capable of interacting with other prokaryotic RNAs.

The degree of structural compactness of the hybrid particles was analyzed by thermal melting experiments. The hyperchromicity/temperature profiles (Fig. 8) show that the hybrid particles have about the same $T_m$ value as $E. coli$ native large ribosomal subunits, although melting over a somewhat broader temperature range than the latter. This indicates that (i) the hybrid particles are somewhat less compact than complete eubacterial subunits and that (ii) the thermophilic proteins do not possess any intrinsic capacity of conferring a high thermal stability to the mesophilic rRNA.

**DISCUSSION**

*Architecture of Sulfolobus Ribosomes*—The results in the present report allow several conclusions. A first series of considerations regards the structure of Sulfolobus ribosomes and the mechanisms whereby the organelles withstand thermal denaturation.

It had been established previously that the in vitro assembly of Sulfolobus subunits demands temperatures close to that required for optimal cell growth (87 °C). Although the precise role of heat in promoting particle assembly had not been defined, there was reason to believe that temperatures below 60 °C hindered any effective interaction among the ribosomal components (3). Instead, the results in the present report show that the initial assembly reactions for the thermophilic ribosome, including RNA/protein recognition, are basically independent of temperature; heat, however, is required to convert low temperature reconstitution intermediates into active 50 S subunits, probably by promoting a conformational change that enables the incomplete particle to bind the late assembly proteins. The above findings are in agreement with the known assembly pattern of $E. coli$ large ribosomal subunits (9), in which the first reconstitution intermediate, containing about 20 proteins, can be formed at 0 °C while heat activation is subsequently required to complete the assembly process.

As demonstrated by the partial disassembly experiments, the interactions enabling Sulfolobus ribosomes to withstand both thermal and “chemical” stress occur at the level of the tridimensional packing of the core components. Unlike $E. coli$ ribosomes, which can undergo stepwise disassembly following treatment with increasing concentrations of LiCl (10), Sulfolobus large ribosomal subunits are extremely resistant to uncompacting treatments (11). By using combinations of LiCl and urea they can be stripped 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 collapse of the particle, thus indicating that the component RNA and protein molecules are held together by strongly cooperative interactions. Probably, hydrophobic bonds in protein/protein contact surfaces play an essential role in conferring a high degree of stability to the thermophilic ribosome.

The analysis of partially assembled and disassembled particles has allowed us to delineate a preliminary pattern of the topographical hierarchy of Sulfolobus large subunit proteins. A further search has led to the identification of a set of eight to nine proteins capable of binding strongly and individually to the 23 S RNA. A similar number of RNA binding proteins has been found in $E. coli$ large ribosomal subunits (12); at present, however, lack of sequence information about Sulfolobus proteins prevents any identification of possible homologies between the archaeabacterial and the eubacterial 23 S RNA binding proteins.

A final remark concerns the 5 S RNA binding proteins. A previous study (4) had led to the identification of three proteins (L5, L8, and L19) as involved in 5 S RNA binding within Sulfolobus large ribosomal subunits. According to the present results, only one of them (L5) is capable of interacting strongly with the isolated 5 S RNA. This situation appears to

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1. P. Londei, unpublished results.
be very similar to that found in E. coli ribosomes, which contain three 5 S RNA binding proteins, only one of which (L18), however, can form stable complexes in vitro with purified 5 S RNA (12, 13).

Heterologous Interactions—As discussed above, no special or “extreme” conditions are required to correctly initiate the in vitro assembly of Sulfolobus ribosomes. This finding enabled us to test whether, and to which extent, cross-recognition could occur between archaeabacterial proteins and non-cognate RNA species.

We found that discrete hybrid particles, containing about two-thirds of the proteins of Sulfolobus large ribosomal sub-units, are formed following incubation of Sulfolobus TP50 with 23 S rRNAs from distantly related archaeabacteria or from eu-bacteria. The results appear significant for two reasons. First, most of the proteins capable of recognizing the non-cognate RNA species coincide with those composing the Sulfolobus core particles and include all of the RNA binding proteins. Second, the heterologous ribosomal components appear to be capable of cooperative interaction, as the reaction consistently yields a discrete and homogeneous particle rather than a heterogeneous array of ribonucleoprotein complexes. These results indicate that the RNA molecules under study, regardless of evolutionary distances, all possess the conformational information required to interact with the non-cognate proteins and to successfully proceed through the early and intermediate stages of the ribosome assembly process. On the other hand, the inability of the hybrid particles to complete the reconstitution process may be explained by surmising that either (i) the late assembly reactions include the participation of the less conserved rRNA domains, which are unable to recognize the heterologous proteins; or (ii) the hybrid particles are endowed with structural faults that hinder a conformational rearrangement critical for promoting the binding of late assembly proteins. Although the two hypotheses are not necessarily alternative, the second one seems more likely in view of the well documented importance of conformational changes during ribosome assembly (9, 14).

A final comment regards the (apparent) lack of compatibility between archaeabacterial proteins and eukaryotic (yeast) large ribosomal subunit RNA. This result seems to indicate the existence of marked structural differences between the eukaryotic and the archaeabacterial/eubacterial RNA molecules; if so, such differences may reside in the presence in the eukaryotic rRNAs of insertions (the so-called “expansion segments”) (15) which could form unique structural motifs. However, it should be pointed out that suitable conditions for the in vitro reassembly of eukaryotic ribosomes have not been described yet. It is possible, therefore, that the lack of interaction between Sulfolobus proteins and eukaryotic rRNA is to be ascribed to some inadequacy of the experimental protocol rather than to an inherent incompatibility of the RNA/protein recognition sites.

REFERENCES