Structural and functional exchangeability of 5 S RNA species from the eubacterium *E.coli* and the thermoacidophilic archaeobacterium *Sulfolobus solfataricus*

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ABSTRACT

The role of 5 S RNA within the large ribosomal subunit of the extremely thermophilic archaeobacterium *Sulfolobus solfataricus* has been analysed by means of *in vitro* reconstitution procedures. It is shown that *Sulfolobus* 50 S subunits reconstituted in the absence of 5 S RNA are inactive in protein synthesis and lack 2–3 ribosomal proteins. Furthermore, it has been determined that in the course of the *in vitro* assembly process *Sulfolobus* 5 S RNA can be replaced by the correspondent RNA species of *E.coli*; *Sulfolobus* reconstituted particles containing the eubacterial 5 S molecule are stable and active in polypeptide synthesis at high temperatures.

INTRODUCTION

On the basis of sequence data, 5 S ribosomal RNAs from the three kingdoms of cell descent have been classified into four main classes of secondary structures: the eubacterial one, the eukaryotic one and two distinct archaeobacterial types, one encompassing the 5 S RNAs from halophilic and methanogenic archaeobacteria, the other comprising the 5 S RNAs from sulfur-dependent archaeobacteria and *Thermoplasma acidophilum*. Although the different classes of 5 S molecules conform to a same general pattern of folding, they can be distinguished from one another on the basis of several unique structural features (1,2,3). It is still unclear whether kingdom-specific structural idiosyncracies play any role in 5 S function; ribosome reconstitution experiments have shown that 5 S RNAs from several eubacteria (4) and also from the archaeobacteria *H.cutirubrum* and *T. acidophilum* (2) can be incorporated into functionally active *Bacillus stearothermophilus* 50 S subunits. 50 S subunits with reduced biological activity are instead obtained when eukaryotic 5 S species are tested in *B.stearothermophilus* reconstitution (2).

The extent of functional exchangeability of 5 S RNAs of extremely thermophilic archaeobacteria has not yet been explored. Such 5 S species, notably those of archaeobacteria belonging to the genus *Sulfolobus*, could provide a case of extreme specialization of function, as they possess an unusually high degree of secondary structure which may be required to ensure their stability at temperatures close to the boiling point of water (5,6).

Recently, we have reported a procedure for the *in vitro* reconstitution of active *Sulfolobus solfataricus* 50 S subunits from total RNA and 50 S subunit proteins (7). In the present paper, we report a more refined protocol which allows the reconstitution of *Sulfolobus* subunits from the purified 23 S and 5 S rRNA species. The procedure has been exploited to investigate i) the effect of 5 S omission on the structure and the function of *Sulfolobus* ribosomes, and ii) whether *Sulfolobus* 5 S can be functionally replaced by 5 S RNAs from other sources. Indeed, we show that 5 S from a mesophilic eubacterium (*E.coli*) can be

incorporated into reconstituted *Sulfolobus* subunits, with no apparent loss of function in polypeptide synthesis.

MATERIALS AND METHODS

Preparation of ribosomes, rRNA and ribosomal proteins

Sulfolobus solfataricus cells were grown, and ribosomal subunits prepared, as described by Londei et al. (7). Whole-cell RNA was prepared by phenol extraction as detailed previously (7). 23 S and 5 S rRNA species were purified by zonal centrifugation and chromatography, as described by Amils et al. (8); the integrity and purity of the isolated rRNA species were checked by gel electrophoresis of heat-denatured samples (8).

Total ribosomal proteins from *Sulfolobus* purified 50 S subunits (TP 50) were prepared according to Londei et al. (7).

5' end labelling of 5 S RNA with 32P

100 pmoles (0.1 A260 units) of either *Sulfolobus* or *E. coli* 5 S RNA species in 100 mM Tris/HCl (pH 8) were supplemented with 24 units alkaline phosphatase (Boehringer). The mixture (100 μ l) was incubated at 37°C for 60 min, then extracted twice with phenol and once with chloroform:isoamylalcohol (24:1). The RNA was precipitated with ethanol after adjusting the final aqueous phase at 200 mM KCl. The pellet was resuspended in 50 mM Tris/HCl (pH 7), 10 mM MgCl₂, 5 mM DTT and supplemented with 3 units polynucleotide kinase (Boehringer) and 30 μ Ci 32P- γ -ATP (10 mCi/mmol). The mixture (50 μ l) was incubated at 37°C for 30 min, then filtered through a Sephadex G-50 column made up in a 1 ml-syringe. Aliquots of the radioactive RNAs were diluted with unlabelled 5 S to final specific activities of 3200 cpm/pmol (*Sulfolobus*) and 4000 cpm/pmol (*E. coli*).

Reconstitution procedure

50 S ribosomal subunits of *Sulfolobus solfataricus* were reconstituted from the separated rRNA and protein components following the two-step procedure described by Londei et al. (7). One A260 unit (50 pmol) of whole-cell RNA, or 0.5 A260 units (25 pmol) of purified 23 S RNA plus 0.02 A260 units (25 pmol) of purified 5 S RNA (from either *E. coli* or *Sulfolobus solfataricus*) were mixed with 2–3 A260 equivalents of TP 50 (1 A260 equivalent is the amount of proteins extracted from 1 A260 unit of 50 S subunits) in the presence of 20 mM Mg²⁺, 300 mM K⁺, and 10 mM spermine hydrochloride. After 45 min heating at 65°C the Mg concentration was raised to 40 mM and incubation was continued at 80°C for another 60 min.

Table 1. Poly-phenylalanine synthesizing activity of *Sulfolobus solfataricus* 50 S subunits reconstituted in the absence and in the presence of (homologous and heterologous) 5 S RNA.

		counts/min assay mix	pmol phe/ pmol 50 S	% activ.
Native 50 S		29,250	24.3	100
	total RNA	21,692	18.1	74
Reconst.	23 S alone	4,670	3.8	15.9
50 S	23 S + <i>Sulfolobus</i> 5 S	18,850	15.7	64.4
	23 S + <i>E. coli</i> 5 S	20,730	17.3	70.1

Each assay mixture (110 μ l) contained: KCl, 30 mM; Tris/HCl pH 7, 20 mM; Mg acetate, 18 mM; spermine, 3 mM; ATP, 3 mM; GTP, 1 mM; poly U 120 μ g; 20 μ M³H phenylalanine (specific activity 100 counts/min/pmol); native 30 S subunits, 10 pmol; native or reconstituted 50 S subunits, 12 pmol; S-100, 2–3 μ l. The mixtures were incubated at 75°C for 40 min, and hot trichloroacetic acid precipitable radioactivity was determined as in (7).

Fig.1. Sedimentation profiles of *Sulfolobus solfataricus* 50 S subunits: a) native subunits; b) subunits reconstituted from purified 23 S RNA and total large subunit proteins (TP 50), in the absence of 5 S RNA; c) subunits reconstituted from 23 S RNA, TP 50 and purified *Sulfolobus* 5 S; d) subunits reconstituted from 23 S RNA, TP 50 and purified *E.coli* 5 S.

Structural and functional analysis of the reconstituted subunits

The structural compactness and the sedimentation coefficient of the reconstituted particles were assessed by zone-velocity sedimentation in 10–30% (w/v) sucrose-density gradients containing 50 mM KCl, 20 mM Tris/HCl pH 7, 10 mM Mg acetate, and using rRNAs and native ribosomal subunits as the references. To determine their protein content, sucrose-gradient purified reconstituted particles were collected by centrifugation at 45,000 rpm for 20 h, and subjected to acetic acid treatment as described previously (7). The extracted proteins were analyzed by two-dimensional gel electrophoresis following the procedure described in (9). The synthetic capacity of the reconstituted particles was assayed using a poly(U)-directed cell-free system at 75°C after complementation with an amount of native *Sulfolobus solfataricus* 30 S subunits approximately equimolar to that of reconstituted 50 S (7; see also Legend to Table 1).

RESULTS

Reconstitution of Sulfolobus subunits with and without 5 S

Total reconstitution of *Sulfolobus* 50 S subunits was performed as described previously

Fig.2. Sedimentation profiles of reconstitution mixtures containing ^{32}P -labelled 5 S RNA species. 12 pmol *Sulfolobus* 23 S RNA were incubated with 20 pmol of either *E. coli* or *Sulfolobus* 5 S RNA, having specific activities of, respectively, 3200 cpm/pmol and 4000 cpm/pmol. The reconstitution mixtures were then centrifuged onto sucrose gradients and 0.3 ml fractions were collected. Upon measuring the absorbance at 260 nm and the radioactivity of each fraction, it was calculated that approx. 0.2 A260 units, or 7 pmol, of 50 S particles were formed following reconstitution, and that the radioactivity associated with the 50 S peak corresponded to 23,000 cpm, or 7.6 pmol, for *Sulfolobus* 5 S RNA, and to 26500 cpm, or 6.5 pmol, for *E. coli* 5 S RNA. a) Reconstitution with ^{32}P *Sulfolobus* 5 S; b) Reconstitution with ^{32}P *E. coli* 5 S; c) ^{32}P *Sulfolobus* 5 S (20 pmol) centrifuged on a same gradient with native 50 S subunits. Solid line: A260; broken line: radioactivity.

(7), except that equimolar amounts of pure 23 S and 5 S rRNA species were used in place of whole-cell RNA. As the results in Table 1 show, the poly(phe) synthesizing activity of particles reconstituted from purified 23 S and 5 S RNAs is comparable to that of subunits reconstituted using whole-cell RNA (60–70% that of native 50 S subunits). The sucrose gradient profiles in Fig.1 also show that omission of 5 S RNA from the reconstitution mixtures has no major effect on the reassembly process, as the particles formed in the absence of the small RNA species display a sedimentation coefficient comparable to that of native 50 S particles. The experiments reported in Table 1, however, show that the synthetic capacity of the 5 S-deficient subunits is only approx. 15% that of particles reconstituted in the presence of 5 S RNA.

The uptake of 5 S RNA within the reconstituted subunits was also directly monitored and quantitated by means of the experiment illustrated in Fig.2a. Reconstitution mixtures containing ^{32}P -labelled 5 S RNA were incubated as usual and then subjected to sucrose gradient analysis. As the sedimentation profiles show, the bulk of radioactive molecules

Fig.3. Gel-electrophoretic patterns of proteins extracted from *Sulfolobus* 50 S subunits reconstituted in the absence of 5 S RNA (top panel) and in the presence of either *Sulfolobus* or *E.coli* 5 RNA species (bottom panel).

comigrates with the peak of 50 S subunits; by evaluating spectrophotometrically the amount of particles formed, and from the known specific activity of 5 S RNA, it was calculated that, as expected, about one picomole of the small RNA species is incorporated per picomole of reconstituted subunits (see also legend to Fig.2).

Fig.3 compares the gel-electrophoretic patterns of the proteins extracted from subunits reconstituted in the absence (top) or in the presence (bottom) of 5 S RNA. The latter is identical to that of proteins deriving from native *Sulfolobus* large subunits (not shown; see also ref.7); the former evidences that two proteins (numbered 8 and 19) are absent from 5 S deficient particles, while a third protein (n.5) appears to be present in much reduced amounts.

Reconstitution with heterologous (*E.coli*) 5 S RNA

To determine whether heterologous 5 S RNAs could be incorporated into *Sulfolobus* ribosomes, equimolar amounts (25 pmoles of each) of electrophoretically pure *Sulfolobus* 23 S RNA and *E.coli* 5 S RNA were mixed with 3 A260 equivalents of *Sulfolobus* TP 50 and the mixture was incubated under the standard conditions for the total reconstitution

of *Sulfolobus* 50 S subunits. Sucrose-gradient analysis of the reconstitution mixtures (Fig. 1) revealed the formation of a homogeneous particle sedimenting as the native 50 S *Sulfolobus* subunits. The effective incorporation of stoichiometric amounts of the heterologous small RNA species into the reassembled particles was directly assessed by performing reconstitution in the presence of radiolabelled *E. coli* 5 S RNA (Fig. 2b).

The synthetic capacity of the reconstituted particles containing the heterologous (*E. coli*) 5 S species was measured under the optimized ionic and temperature conditions for the *Sulfolobus* poly(U) assay system (30 mM KCl, 18 mM Mg acetate and 3 mM spermine at 75–80°C) in the presence of *Sulfolobus* 30 S subunits and *Sulfolobus* S-100. As the results in Table 1 show, reconstituted subunits containing the heterologous 5 S RNA exhibit the same synthetic activity (actually a somewhat higher one) as that of particles containing the homologous RNA species.

The protein gel-electrophoretic pattern of the reconstituted heterologous subunits shows the occurrence of a normal complement of *Sulfolobus* 50 S proteins (Fig. 3, bottom). Namely, the proteins that are lacking from the particles reassembled in the absence of 5 S RNA are again incorporated into functionally active subunits when the archaebacterial 5 S species is substituted for by the eubacterial one.

DISCUSSION

Properties of 5 S-deficient Sulfolobus 50 S subunits

The results of the reconstitution experiments in the present report show that, as it is the case for eubacterial ribosomes (10), 5 S RNA is dispensable for both the assembly and the stability of *Sulfolobus solfataricus* 50 S subunits. In fact, reconstitution mixtures lacking 5 S, when incubated under standard conditions (namely, high Mg and high temperature), yield compact, thermally stable particles having the same S value as that of the native 50 S subunits. Instead, as expected, 5 S RNA and its associated proteins are essential for ribosome function; it should be pointed out, however, that 5 S-deficient particles consistently exhibit significant amounts of residual poly(phe) synthesizing activity (10–20% that of native 50 S subunits). As similar results have been obtained with *E. coli* ribosomes (10), we think it unlikely that the observed activity is due to the presence in the ribosome-free supernatant of contaminating 5 S–protein complexes that are incorporated into the incomplete subunits during the poly(U) assay.

Electrophoretic analysis of the protein moiety of the 50 S particles reconstituted in the absence and in the presence of 5 S shows that *Sulfolobus* large subunits are similar to the corresponding eubacterial particles in containing two, perhaps three, 5 S-associated proteins. The likely candidates for the latter role are the proteins numbered 8, 19 and 5, having apparent molecular weights of about 20,000, 15,000 and 30,000 dal respectively; however, evidence that all three of them interact directly with 5 S RNA is still lacking.

The numbering used for the ribosomal proteins requires some further comment. For unclear reasons, the protein patterns obtained in the present work for *Sulfolobus* large subunits (either native or reconstituted) did not exactly match previously published ones (9), in spite of the fact that the same electrophoretic procedure was employed. As this made it difficult to use the same numbering system as in (9), to avoid confusion we preferred, for the purposes of the present paper, to renumber reproducible protein spots in order of decreasing electrophoretic mobility in the second dimension. With respect to the 5 S-associated proteins, however, we note that protein 5 of Fig. 3 most likely corresponds to

protein 4 of ref.9, while proteins 8 and 19 are, with less certainty, to be identified respectively as spots 10 and 25 of ref.9.

Properties of Sulfolobus subunits containing eubacterial 5 S RNA

A number of features distinguish the *Sulfolobus* 5 S base-pairing pattern from that of nearly all other 5 S species; the most conspicuous of these is the joining of two short helical segments, normally separated by an internal loop, in a long continuous helix containing only one mismatched base-pair (1,3,5). Overall, the unique features of *Sulfolobus* 5 S RNA concur in making the secondary structure of the molecule exceptionally stable; the calculated ΔG for thermal denaturation of *Sulfolobus* 5 S (-73 kCal/mol) is almost twofold larger than that of *E.coli* 5 S (approx. -40 kCal/mol) (6). This is normally interpreted as being necessary to prevent melting of the RNA under the extreme temperature conditions required for *Sulfolobus* growth (5).

However, we show in the present study that *E.coli* 5 S RNA can replace the endogenous RNA species of *Sulfolobus solfataricus* ribosomes with no loss of function in polypeptide synthesis at high temperatures. Remarkably, no modification of the standard reconstitution protocol for *Sulfolobus* large subunits (7) is required to build *E.coli* 5 S into the archaeobacterial particle, in spite of the fact that both steps of the *Sulfolobus* reassembly procedure are performed at temperatures much higher than those required for the *in vitro* reconstitution of *E.coli* large subunits (11). The functional equivalence between the eubacterial and the archaeobacterial 5 S RNAs is further stressed by the fact that both molecules are able to recognize the same set of *Sulfolobus* ribosomal proteins.

Altogether, the results in the present paper suggest that different classes of 5 S RNA molecules possess a highly conserved pattern of tertiary folding, which has undergone little variation throughout evolution and whose functional versatility has not been restricted by the ensuing primary and secondary structural variations.

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REFERENCES

1. Erdmann, V.A. and Wolters, J. (1986) Nucl. Ac. Res. 14 (suppl) r1-r58
2. Hartmann, R.K., Vogel, D.W., Walker, R.T. and Erdmann, V.A. (1988) Nucl. Ac. Res. 16, 3511-3524
3. Willekens, P., Huysmans, E., Vandenberghe, A., and De Wachter, R. (1986) System. Appl. Microbiol. 7, 151-159
4. Vogel, D.W., Hartmann, R.K., Bartsch, M., Subramanian, A.R., Kleinow, W., O'Brien, T.W., Pieler, T. and Erdmann, V.A. (1984) FEBS Lett. 169, 67-72
5. Stahl, D.A., Luehrsen, K.R., Woese, C.R. and Pace, N.R. (1981) Nucl. Ac. Res. 9, 6129-6137
6. Dams, E., Londei, P., Cammarano, P., Vandenberghe, A. and De Wachter, R. (1983) Nucl. Ac. Res. 11, 4667-4676
7. Londei, P., Teixidó, J., Acca, M., Cammarano, P., and Amils, R. (1986) Nucl. Ac. Res. 14, 2269-2285
8. Amils, R., Matthews, E.A., and Cantor, C.R. (1979) Methods in Enzymol. LIX, 449-461
9. Londei, P., Teichner, A., Cammarano, P., De Rosa M. and Gambacorta, A. (1983) Biochem. J. 209, 461-470
10. Dohme, F. and Nierhaus, K.H. (1976) Proc. Natl. Acad. Sci. USA 73, 2221-2225
11. Nierhaus, K.H. and Dohme, F. (1974) Proc. Natl. Acad. Sci. USA 71, 4713-4717