The chaperonin of the archaeon *Sulfolobus solfataricus* is an RNA-binding protein that participates in ribosomal RNA processing

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The 60 kDa molecular chaperones (chaperonins) are high molecular weight protein complexes having a characteristic double-ring toroidal shape; they are thought to aid the folding of denatured or newly synthesized polypeptides. These proteins exist as two functionally similar, but distantly related families, one comprising the bacterial and organellar chaperonins and another (the so-called CCT-TRiC family) including the chaperonins of the archaea and the eukaryotes. Although some evidence exists that the archaeal chaperonins are implicated in protein folding, much remains to be learned about their precise cellular function. In this work, we report that the chaperonin of the thermophilic archaeon *Sulfolobus solfataricus* is an RNA-binding protein that interacts specifically *in vivo* with the 16S rRNA and participates in the maturation of its 5' extremity *in vitro*. We further show that the chaperonin binds RNA as the native heterooligomeric complex and that RNA binding and processing are inhibited by ATP. These results agree with previous reports indicating a role for the bacterial/organellar chaperonins in RNA protection or processing and suggest that all known chaperonin families share specific and evolutionarily ancient functions in RNA metabolism.

Keywords: archaea/chaperonin/RNA binding/rRNA processing/*Sulfolobus*

Introduction

The chaperonins are essential cellular proteins that form barrel-shaped high molecular weight complexes. They are thought to assist the folding of newly synthesized polypeptides and to promote the refolding of denatured ones (Hartl, 1996). The chaperonins exist in two distinct evolutionary versions, similar in structure but only distantly related in amino acid sequence (Hartl *et al.*, 1994; Hartl, 1996). The first family includes the 60 kDa heat-shock proteins of the bacteria and the mitochondria (Hsp60), and the chloroplast chaperonins, which are not heat-shock proteins. The typical representative of this group is *Escherichia coli* GroEL, a high molecular weight

complex of 14 identical subunits, arranged as two seven-subunit rings stacked on top of one another.

The second group, called the CCT-TRiC family, includes the chaperonins of the archaea and the eukaryotes. Like their bacterial counterparts, they form toroidal multimeric complexes which, however, contain several different subunits. Unlike the archaeal chaperonins, those of eukaryotes are not heat-shock proteins.

The chaperonin of the thermophilic archaeon *Sulfolobus shibatae* (growing optimally at ~70°C) formerly was described as a homomultimeric complex containing 18 copies of a 55 kDa polypeptide, termed TF55, arranged as two stacked nine-subunit rings (Trent *et al.*, 1991). Later, the 18mer complex was found actually to be composed of two different proteins, TF55 (re-termed the β -subunit) and another polypeptide of similar molecular weight and related primary structure (termed the α -subunit) (Kagawa *et al.*, 1995). The chaperonin complex is very abundant in *S.shibatae* cells already under normal growth conditions, and is increased further upon heat shock, namely exposure of the cells to a temperature of 86–88°C (Trent *et al.*, 1990). Similar two-subunit heterooligomeric chaperonin complexes have also been described in other archaea (Phipps *et al.*, 1991; Knapp *et al.*, 1994; Waldmann *et al.*, 1995).

Although the participation of the chaperonins (especially *E.coli* GroEL) in protein folding seems to be well established, there are several reports in the literature suggesting that these large protein complexes may also be involved in other functions. For instance, *E.coli* GroEL itself has been implicated in the processing of the 5S rRNA (Sohlberg *et al.*, 1993), and in the degradation of certain mRNAs (Georgellis *et al.*, 1995), while the mitochondrial Hsp60 have been suggested to participate in mRNA processing (Sanyal *et al.*, 1995). As to the TRiC family chaperonins, their role in protein folding remains to be fully elucidated, and in any case seems to be less general than that of the bacterial chaperonins (Frydman *et al.*, 1992; Hartl, 1996).

In this work, we show that the chaperonin of *Sulfolobus solfataricus*, an extremely thermophilic archaeon closely related to *S.shibatae*, is an RNA-binding protein that interacts specifically *in vivo* with the 16S rRNA. We further show that *in vitro* *S.solfataricus* chaperonin introduces a specific processing cut within the 5'-external transcribed spacer (5' ETS) of the pre-rRNA. The cleavage site shows sequence homology with early processing sites in the 5' ETS of yeast and mammalian pre-rRNAs. Both RNA binding and cleavage are inhibited by ATP, a well-known modulator of chaperonin function. On the whole, the results support the notion that the chaperonins of all known families have specific and evolutionarily ancient functions in RNA metabolism.

Fig. 1. *Sulfolobus solfataricus* chaperonin interacts with the 16S rRNA. (A) Purification of *S.solfataricus* chaperonin. SDS-PAGE of F1 = whole-cell extract; F2 = 20S fraction recovered from sucrose gradients; and F3 = chaperonin purified from F2 by ammonium sulfate precipitation and gel filtration. Ch = chaperonin. (B) Hybridization analysis with a 16S-specific probe of the RNA immunoprecipitated with the anti-chaperonin antibodies. IP = immunoprecipitate; PI = precipitate with the pre-immune serum; tot. RNA = control rRNA. (C) Hybridization analysis of non-denatured chaperonin-RNA complexes: a, with a 16S-specific probe; b, with a 23S-specific probe; c, Western blot with the anti-chaperonin antibodies. Ch = chaperonin; 16S, 30S, 50S = control RNA and ribosomal subunits.

Results

Sulfolobus solfataricus chaperonin interacts with the 16S rRNA

In the thermophilic archaeon *S.solfataricus*, as is the case in its close relative *S.shibatae* and in other archaea, the 60 kDa chaperonins are represented by two related polypeptides of 55–60 kDa, termed the α - and β -subunits. These proteins form high molecular weight complexes that sediment at ~20S and constitute a sizeable fraction (3–5%) of the total cellular proteins under normal growth condition (Trent *et al.*, 1991). From cell lysates of *S.solfataricus* (strain MT4) grown at physiological temperature (80°C) we purified the chaperonin complex to near homogeneity (Figure 1A). In the course of the purification procedure, we noticed that the chaperonin consistently carried over a nucleic acid component, which seemed to be physically associated with the protein complex. To determine whether this was the case, the chaperonin was immunoprecipitated using specific polyclonal antibodies raised against the gel-purified 60 kDa band, and the immunocomplexes were treated with proteinase K and phenol to extract the putative nucleic acid fraction. Preliminarily, we verified that the antibodies recognized exclusively the chaperonin subunits among the proteins of a *S.solfataricus* cell lysate (data not shown).

A nucleic acid fraction, identified as RNA by differential RNase and DNase treatment (data not shown), did co-immunoprecipitate with the chaperonin. Most of it consisted of a high molecular weight band which by Northern hybridization was shown to be the 16S rRNA (Figure 1B). No hybridization signal appeared using a probe specific for the 23S rRNA.

Further independent proof of a specific association between the chaperonin and the 16S rRNA was obtained by electrophoresing a purified chaperonin preparation under non-denaturing conditions and analysing it by Northern hybridization (after *in situ* denaturation) using probes specific for the 16S and 23S rRNAs. The 16S-specific probe revealed a high molecular weight band which, by parallel Western blotting, was indeed shown to contain the chaperonin (Figure 1C). As before, no hybridization signal was obtained with the 23S-specific probe, confirming that the large ribosomal subunit RNA was not associated with the chaperonin to any significant extent.

Both mature and immature 16S rRNA molecules associate with the chaperonin

The association between an rRNA and the chaperonin was of unclear significance. To start unravelling this problem,

Fig. 2. Primer extension mapping of the 5' end of the chaperonin-bound 16S rRNA. The RNA was extracted from the immunocomplexes precipitated with the anti-chaperonin antibodies. The top arrow and sequence indicate the transcription start site; the bottom arrow and sequence indicate the mature 5' end of the 16S rRNA.

we determined the maturation and assembly state of the chaperonin-associated 16S rRNA.

To map the 5' end of the complex-bound 16S rRNA, we preliminarily sequenced a tract of the 5' region of the *S. solfataricus* (strain MT4) rDNA operon, including the entire 5' ETS, which turned out to be 139 nucleotides in length (Figure 2), equal to that of *S. shibatae* (Reiter *et al.*, 1987) and slightly shorter than that of *Sulfolobus acidocaldarius* (145 nucleotides; Durovic and Dennis, 1994). The 5' end of the 16S rRNA extracted from the immunocomplexes precipitated with the anti-chaperonin antibodies was mapped by primer extension. Most molecules ended at the mature 5' terminus A₁₃₉ (Figure 2); however, there was also a minority of immature 16S rRNA species, as indicated by the presence of extension products containing 5' ETS sequences. The longest of these terminated at the putative transcription initiation site G₁ (top arrow in Figure 2) and therefore derived from entirely unprocessed molecules. A few other termination sites were apparent, the strongest falling at C₁₃₁, a few nucleotides upstream from the mature 5' end. These may correspond to partially processed molecules or result from RNA fragmentation or pausing in reverse transcription.

The 3' end of the chaperonin-associated 16S rRNA was determined by RT-PCR and found to be the fully mature form in the entire population of molecules (data not shown).

Finally, we investigated whether the chaperonin-bound 16S rRNA was assembled with any small ribosomal

subunit proteins. To this end, the immunocomplexes obtained by precipitation with the anti-chaperonin antibodies were probed with polyclonal antibodies that reacted specifically against all the proteins of *S. solfataricus* 30S subunits (Figure 3). The results, shown in Figure 3, revealed that a few (no more than three or four) small ribosomal subunit proteins, having molecular weights of between 20 and 30 kDa, were indeed associated with the chaperonin-RNA complex. However, most proteins, especially the bulk of the smaller ones (10–20 kDa), were apparently missing. Notably, *S. solfataricus* 30S subunits contain only six polypeptides comprised in the range 20–30 kDa (Schmid and Böck, 1982; Londei *et al.*, 1983), most of which are primary RNA-binding proteins that interact with the 16S rRNA in the initial stages of ribosome assembly (P. Londei, unpublished results). Thus, the ribosomal proteins associated with the chaperonin-RNA complex may correspond to those establishing the earliest interactions with the 16S rRNA.

The chaperonin is involved in the processing of the 5' ETS of the 16S rRNA

The results reported in the above paragraph hinted at a possible function of the chaperonin in rRNA maturation and/or in the initial stages of ribosome assembly. To investigate these possibilities, we first asked whether the chaperonin intervened in the processing of the 5' ETS of the pre-rRNA.

As described previously (Durovic and Dennis, 1994),

Fig. 3. Western blot revealing the 30S subunit proteins associated with the rRNA-chaperonin complex. The indicated samples, resolved by SDS-PAGE, were subjected to Western blot analysis using polyclonal antibodies obtained by immunizing rabbits with *S.solfataricus* purified 30S subunits. TP30 = total proteins extracted from *S.solfataricus* purified 30S subunits; TP50 = total proteins extracted from *S.solfataricus* purified 50S subunits; IP = proteins immunoprecipitated with the specific anti-chaperonin antibodies; PI = proteins precipitated with the corresponding pre-immune serum. The arrows on the right indicate the main ribosomal protein bands consistently appearing in the immunocomplexes precipitated with the anti-chaperonin antibodies. The large bands close to the top of the gel correspond to the antibody heavy chain. The positions of the molecular weight markers are indicated on the left.

the maturation of the small ribosomal subunit RNA in *Sulfolobus* resembles in some aspects the corresponding eukaryotic process. Particularly, the removal of the 5' ETS is performed via sequential endonucleolytic reactions that do not require the formation of a stem between the inverted repeats flanking the 16S rRNA gene, as occurs in bacteria. The reaction can be assayed conveniently using a model pre-rRNA substrate, consisting of an *in vitro* transcript spanning the 5' ETS and a short tract of the 16S coding sequence (Durovic and Dennis, 1994). Accordingly, we investigated whether a radiolabelled synthetic RNA obtained by *in vitro* transcription of a truncated *S.solfataricus* 16S rRNA gene, including the 5' ETS of 139 nucleotides (preceded by ~15 nucleotides of plasmid sequence) and ~100 nucleotides of 16S coding sequence (Figure 4, top), could be cleaved specifically by the chaperonin.

Indeed, when incubated in the presence of the purified chaperonin, the model pre-rRNA substrate was cut neatly at a single position, yielding two unequal fragments of ~60 and ~180 nucleotides. The reaction required a high temperature (75°C) and was essentially complete after 20 min (Figure 4). Importantly, the cleavage activity was specific for the model pre-rRNA, since it did not attack a control RNA transcript of similar length and composition but unrelated to the rRNA (Figure 3E and F).

Confirmation that the endonuclease activity truly coincided with the chaperonin and was not attributable to some trace contaminant was obtained by immunoprecipitating the chaperonin with the specific antibodies and by showing that the recovered immunocomplexes were still capable of cleaving specifically the model pre-rRNA when incubated at 75°C under the appropriate conditions (Figure 4B and C).

Fig. 4. *In vitro* processing of the pre-rRNA 5' ETS. The model pre-rRNA substrate depicted on the top was treated as follows. First four lanes (0–20 min): time course of the processing reaction at 75°C in the presence of the purified chaperonin; A = 20 min incubation at 37°C with the purified chaperonin; B = incubation for 15 min at 75°C with the immunoprecipitated chaperonin; C = same as B but with the precipitate with the pre-immune serum; D = incubation at 75°C with the purified chaperonin in the presence of 1 mM ATP; E = control non-ribosomal RNA (see Materials and methods), unincubated; F = control RNA incubated for 20 min at 75°C with the purified chaperonin. The cleavage site at –94 (site 0) is indicated in the top diagram. The bars on the right indicate the positions of the two fragments resulting from cleavage.

By primer extension, the cleavage site on the model pre-rRNA was found to lie at C₄₆ (Figure 5), corresponding to an authentic processing site, the first of the three previously identified *in vivo* and *in vitro* in the 5' ETS of *S.acidocaldarius* pre-rRNA (Durovic and Dennis, 1994), and also to a putative processing site identified in the pre-rRNA of *S.solfataricus* (Reiter *et al.*, 1987). We concluded that *S.solfataricus* chaperonin was involved directly in initiating pre-rRNA processing, by promoting a specific early cleavage within the 5' ETS at a site we call 'site 0'.

Interestingly, the *S.solfataricus* site 0 lies in a position similar to that of the early processing site termed A0 in the 5' ETS of yeast pre-rRNA (Venema and Tollervey, 1995), namely ~90 nucleotides upstream from the mature 5' end of the 16S rRNA. Furthermore, the sequence surrounding the *S.solfataricus* site (CA↓CUUAU), which is largely conserved in other *Sulfolobus* species, has homology with the sequence at the yeast A0 site (AUC↓UUCUA) (Yeh Lee-Chuan and Lee, 1992) and also with the sequence in the mammalian 5' ETS wherein the pre-rRNA is first cleaved (A↓CUCUUA↓G in the mouse) (Kass *et al.*, 1987; Craig *et al.*, 1991). Particularly, a consensus CUU motif seems to be shared by all these processing sites.

Fig. 5. Primer extension mapping of the cleavage site on the model pre-rRNA. Top: a scheme illustrating the structure of the model pre-rRNA and of its expected extension product. Bottom: primer extension assay performed on the model pre-rRNA unincubated or after 20 min incubation at 75°C under processing conditions. The sequence at the cleavage site is shown on the left: the asterisk indicates the 5' terminus of the cleaved pre-rRNA.

Sulfolobus chaperonin is an RNA-binding protein

To substantiate further the notion that *Sulfolobus* chaperonin is a component of the rRNA processing machinery, we sought to obtain direct evidence that the protein complex bound RNA. To this end, the purified chaperonin and the radiolabelled model pre-rRNA substrate were incubated at low temperature (37°C) to allow RNA–protein binding but to prevent processing from occurring. After 15–20 min, the samples were UV-irradiated and finally subjected to extensive digestion with various ribonucleases.

Denaturing PAGE revealed a single protein band carrying cross-linked RNA, which migrated at ~60 kDa and superimposed on the stained chaperonin. Western blot assays confirmed that this band indeed contained the chaperonin (Figure 6A). The labelled band disappeared upon digestion with proteinase K, thus proving that it corresponded to an RNA–protein complex.

To eliminate formally the residual possibility that the RNA-binding 60 kDa band was a different protein that co-migrated with the chaperonin, the cross-linked RNA–protein complexes were precipitated with the specific anti-chaperonin antibodies prior to denaturing PAGE analysis. As expected, the immunoprecipitated chaperonin carried the radioactive RNA fragments (Figure 6B), thus confirming that it was indeed the RNA-binding protein. The pre-rRNA–chaperonin interaction appeared to be specific, since it was not prevented by the addition to the incubation

Fig. 6. Detection of the RNA–chaperonin complexes by UV cross-linking. The pre-rRNA substrate was incubated for 15 min at 37°C with the purified chaperonin, then treated as described in Materials and methods. (A) Cross-linked RNA–protein complexes (± proteinase K) after fractionation by SDS–PAGE and autoradiography (A) or Western blot with the anti-chaperonin antibodies (WB). (B) SDS–PAGE of the cross-linked complexes after immunoprecipitation with the anti-chaperonin antibodies (IP-αCh) or the pre-immune serum (PI); the complexes are visualized on the left by Western blot (WB) or on the right by autoradiography (A). Prot K = treatment with proteinase K. (C) Cross-linked complexes fractionated by non-denaturing electrophoresis, ± proteinase K or ± 1 mM ATP. The complexes are visualized by autoradiography (A) on the left, and by Coomassie Blue staining (C) on the right. The arrows on the right indicate the positions of the native heterooligomeric chaperonin in its two possible conformations (top) and of the free chaperonin subunits (bottom).

mixture of up to a 50-fold excess of the same control RNA transcript used in the processing assay (data not shown).

The cross-linked RNA–protein complexes were also analysed by non-denaturing PAGE to determine which of the different conformational states of the chaperonin was competent for RNA binding. In fact, *Sulfolobus* chaperonin has been reported to cycle through different aggregation states (i.e. bitoroid, single-ring, free subunits) which seem to have different capacities to bind polypeptide substrates, the free subunits having the highest affinity for denatured proteins (Quaite-Randall *et al.*, 1995). Most of the RNA was attached to the native bitoroidal chaperonin complex (Figure 6C) which, under the electrophoresis conditions used, yields a typical double band pattern. The two components are believed to correspond to an ‘open’ (upper) and a ‘closed’ (lower) conformer of the 18meric chaperonin (Quaite-Randall *et al.*, 1995), both of which seem to be equally able to interact with the model pre-rRNA. Only negligible amounts of radioactivity were found associated with the free subunits.

Finally, since the interaction of the chaperonins with

their protein substrates is known to be modulated by the binding and hydrolysis of ATP, we asked whether ATP influenced the RNA-binding capacity of *S.solfataricus* chaperonin. When the cross-linking experiments were performed in the presence of 1 mM ATP, the chaperonin-bound RNA was reduced to barely detectable amounts (Figure 6C). By analogy with the protein model, we presume that ATP promotes a fast dissociation of the RNA-chaperonin complex, although other mechanisms of action are possible. What is important to emphasize here, however, is that the addition of ATP also resulted in an almost complete inhibition of the cleavage at site 0 (Figure 4, lane D), thus demonstrating that the chaperonin-RNA interaction and RNA processing were indeed causally linked.

Discussion

In this work, we show that the 60 kDa chaperonin of the thermophilic archaeon *S.solfataricus* is an RNA-binding protein that interacts *in vivo* with the 16S rRNA and carries an endonuclease activity capable of cleaving *in vitro* at a specific site the 5' ETS of the pre-rRNA. The precise identity of the endonuclease is still uncertain; it may be an activity of the chaperonin itself or of a different polypeptide tightly associated with the 18meric complex. However, the findings that the model pre-rRNA used as the processing substrate interacts directly and specifically with the chaperonin, and that ATP inhibits both RNA binding and cleavage, provide compelling evidence in favour of the idea that the chaperonin is an active component of the rRNA processing machinery in *Sulfolobus*.

Although the present results demonstrate a direct involvement of *Sulfolobus* chaperonin in a single specific step of pre-rRNA processing, several features of the data suggest that the protein complex probably also plays a role in a number of later events. For instance, the fact that most of the chaperonin-associated 16S rRNA has a mature 5' extremity indicates that other nucleases, distinct from the activity cutting at site 0, must intervene to terminate 5' end processing while the rRNA remains in a chaperonin-bound state. A probable explanation for this finding is that after cleavage at site 0 the protein complex is still needed to maintain the RNA in the right conformation for the efficient completion of the processing pathway leading to the removal of the 5' ETS.

Moreover, the relative stability of the 16S rRNA-chaperonin complex *in vivo* and the fact that it also includes a small subset of ribosomal proteins (probably RNA-binding proteins) suggest that an adequate 'chaperoning' of rRNA conformation may also be required to assist the initial steps of 30S subunit assembly. The synthesis of the particle would then be completed after a correctly assembled early precursor has been released from the chaperonin, probably following ATP hydrolysis. These hypotheses, that currently are being tested in our laboratory, are in keeping with the expected functions of a molecular chaperone, which, however, would in this case act principally on RNA conformation.

The relationship between the RNA processing activity of *Sulfolobus* chaperonin and its postulated role in protein folding remains unclear. *Sulfolobus* chaperonin has been

reported to bind denatured proteins and to possess anti-aggregant activities (Trent *et al.*, 1991; Guagliardi *et al.*, 1995); however, it is still unknown whether these functions underlie a generalized role in protein folding. Also, the mechanism for protein binding and release is probably different from that of *E.coli* GroEL (Quaite-Randall *et al.*, 1995). Thus, more information on both the RNA- and protein-binding activities of the *Sulfolobus* chaperonin is needed before a meaningful comprehensive model of its functions can be delineated.

On the other hand, the finding that *Sulfolobus* chaperonin has a role in rRNA processing, however unexpected, fits with a number of previous findings implicating the bacterial/organellar chaperonins in the processing or protection of various RNAs (Sohlberg *et al.*, 1993; Georgellis *et al.*, 1995; Sanyal *et al.*, 1995; see Introduction). Here we demonstrate for the first time a participation in RNA metabolism of a chaperonin of the CCT-TRiC (archaeal/eukaryotic) family. This suggests that the handling of RNA substrates is a widespread and evolutionarily ancient function of the 60 kDa chaperonins, whose features and biological significance are still largely unexplored.

Materials and methods

Chaperonin extraction and purification

Sulfolobus solfataricus (strain MT4) cells were grown at 85°C, and crude cell lysates were prepared as previously described (Londei *et al.*, 1986). The high molecular weight components were sedimented by centrifugation at 100 000 g for 2 h. The sediment was resuspended in a buffer containing 500 mM NH₄Cl, 20 mM Tris-HCl pH 7.4, 10 mM Mg acetate, 5 mM β -mercaptoethanol, and was fractionated by zonal centrifugation on a 7–38% sucrose gradient in the same buffer. The fractions sedimenting at ~20S, enriched in chaperonin, were purified further by precipitation with 40% ammonium sulfate followed by gel filtration through a Sephacryl S-200 column.

Immunoprecipitations

Anti-chaperonin polyclonal antibodies were raised in rabbits using as the antigen the 60 kDa band excised and eluted from SDS-acrylamide gels. Antibodies to small ribosomal subunit proteins were obtained using as antigen the 30S subunits of *S.solfataricus* ribosomes purified by zonal centrifugation and high-salt washing (Londei *et al.*, 1986). Immunoprecipitations were preceded by a pre-clearing step during which 50 μ l of protein A-Sepharose CL-4B (Pharmacia) were incubated with the purified chaperonin for 1 h at 4°C in 500 μ l of 50 mM NH₄Cl, 20 mM Tris-HCl pH 7.4, 5 mM Mg acetate. The supernatant was supplemented with the antiserum (or the pre-immune serum in the controls), brought to 0.1% (v/v) with NP-40 and incubated for 1 h at 4°C. This step was repeated after bringing NP-40 to 0.5%. The immunocomplexes were washed three times with cold NET buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 mM EDTA containing 0.5% NP-40), and either heated at 95°C in reducing Laemmli buffer for SDS-PAGE and immunoblot analyses or assayed in the processing reaction.

Extraction and identification of the chaperonin-associated RNA

The chaperonin was immunoprecipitated with the specific antibodies as described above and the immunocomplexes were treated with a buffer containing 50 mM Tris-HCl pH 7.4, 5 mM EDTA, 300 mM NaCl, 1.5% SDS and 1.5 mg/ml proteinase K for 30 min at 37°C. The samples were extracted three times with phenol, once with chloroform/isoamylalcohol 99:1, and finally precipitated with ethanol. For hybridization analysis, the extracted RNA was run on denaturing agarose-formaldehyde gels. The 16S-specific probe was a *HindIII*-*HpaI* fragment of the *S.solfataricus* rDNA operon containing the entire 16S rRNA sequence. The 23S-specific probe was a *BamHI*-*XbaI* fragment of the same operon containing most of the 23S rRNA sequence.

For hybridization analysis of the native chaperonin-RNA complexes (Figure 1C), the chaperonin and the control rRNAs and ribosomal

subunits were run on non-denaturing 1% agarose gels and were blotted on Hybond N+ filters using 0.05 M NaOH as the blotting solution.

Primer extension experiments

Primer extension determination of the 5' terminus of the chaperonin-associated 16S rRNA and of the larger fragment obtained after *in vitro* processing was performed according to described techniques (Stern *et al.*, 1988). The primer was in both cases a 17mer oligonucleotide (5'-ACTCCATGGCTTAACC-3') complementary to the region 42–58 of *S.solfataricus* 16S rRNA.

In vitro processing

The radiolabelled pre-rRNA substrate (240 nucleotides) was obtained by *in vitro* transcription of a *S.solfataricus* 16S rRNA gene cloned in Bluescript SK and linearized with *Afl*III. The control RNA (290 nucleotides) was obtained by transcription of a synthetic minigene similar in base composition to the pre-rRNA substrate. An aliquot (100 ng) of each transcript (200 000–300 000 c.p.m.) was incubated for 20 min at 75°C (or as specified in Figure 3) with ~1 µg of purified or immunoprecipitated chaperonin. The reaction buffer contained 50 mM Tris pH 8, 10 mM MgCl₂ (final volume 30 µl). The reaction was stopped with 30 µl of 50 mM EDTA pH 8, 0.5% SDS. The products were resolved by electrophoresis on 8% acrylamide gels containing 8 M urea in TBE.

UV cross-linking

The radioactive pre-rRNA substrate (100 ng) was incubated with the purified chaperonin (1 µg) for 15 min at 37°C in 20 µl of 40 mM Tris pH 7.5, 50 mM KCl, 5 mM MgCl₂. The samples were exposed to radiation of 600 mJ/cm² under 254 nm UV light in an UV Stratalinker 1800 (Stratagene). After irradiation, the samples were digested with 1 µg of RNase A, 10 U of RNase T1, 5 U of RNase T2 and 5 U of RNase VI (per sample) for 15 min at 37°C and 15 min at room temperature. Some control samples were treated with proteinase K prior to RNase digestion. The products were resolved by conventional SDS–acrylamide gels or by non-denaturing gradient (4–12%) acrylamide gels made in 1.875 M Tris pH 8.9.

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