The antiprion compound 6-Aminophenanthridine inhibits protein folding activity of the ribosome by direct competition

Yanhong Pang¹, Sriram Kurella¹, Cécile Voisset², Dibyendu Samanta³, Debapriya Banerjee¹, Ariane Schabe¹, Chanchal Das Gupta³, Hervé Galons⁴, Marc Blondel² and Suparna Sanyal¹*

¹Department of Cell and Molecular Biology, Uppsala University, Box-596, BMC, 75124, Uppsala, Sweden

²Institut National de la Santé et de la Recherche Médicale UMR1078, Université de Bretagne Occidentale, Faculté de Médecine et des Sciences de la Santé, Etablissement Français du Sang (EFS) Bretagne, CHRU Brest, Hôpital Morvan, Laboratoire de Génétique Moléculaire, Brest, F-29200, France

³Department of Biophysics, Molecular Biology and Genetics, Calcutta University, Kolkata-700009, India

⁴Laboratoire de Chimie Organique 2, CNRS UMR 8601, Université Paris Descartes, 4 avenue de l'Observatoire, 75270 Paris Cedex 6, France

* Correspondence
Telephone: +46 18 4714220
Fax: +46 18 4714262
E-mail: suparna.sanyal@icm.uu.se

Running title: 6AP inhibits PFAR by direct competition

Present Address:
[Dibyendu Samanta], Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY-10461
[Ariane Schabe], Max-Delbrück-Centrum for Molekular Medicine, Robert-Rössle-Str. 10, 13125 Berlin-Buch, Germany

Keywords: PFAR, 6AP, ribosome, prion, ribosomal RNA

Background: 6-Aminophenanthridine (6AP) is an inhibitor of the protein folding activity of the ribosome (PFAR).

Results: The protein substrates and 6AP bind at common sites on ribosomal RNA; mutations at those sites abolish binding and inhibit PFAR.

Conclusion: 6AP competitively obstructs the protein binding sites and thereby inhibits PFAR.

Significance: We have clarified the mechanism by which 6AP inhibits PFAR.

SUMMARY
The domain V of the 23S/25S/28S ribosomal RNA (rRNA) of the large ribosomal subunit constitutes the active center for the protein folding activity of the ribosome (PFAR). Using in vitro transcribed domain V rRNAs from Escherichia coli and Saccharomyces cerevisiae as the folding modulators and human carbonic anhydrase (HCA) as a model protein, we demonstrate that PFAR is conserved from prokaryotes to eukaryotes. It was shown earlier that 6-Aminophenanthridine (6AP), an antiprion compound, inhibits PFAR. Here, using UV cross-linking followed by primer extension, we show that the protein substrates and 6AP interact with a common set of nucleotides on the domain V of 23S rRNA. Mutations at the interaction sites decrease PFAR and result in loss or change of binding pattern for both the protein substrates and 6AP. Moreover, kinetic analysis of HCA refolding shows that 6AP decreases the yield of the refolded protein, but does not affect the rate of refolding. Thus, we conclude that 6AP competitively...
occludes the protein substrates from binding to rRNA and thereby inhibits PFAR. Finally, we propose a scheme clarifying the mechanism by which 6AP inhibits PFAR.

INTRODUCTION

It has been shown over the past two decades, that the ribosome is able to refold about twenty different proteins in vitro (1-5). The protein folding activity of the ribosome (referred as PFAR) is not restricted to any particular species or groups of organisms since ribosomes from various sources were shown to possess this activity (1,2,6). Also, the protein substrates of PFAR are not limited to a specific protein-family; proteins from diverse sources with various properties could be folded by ribosomes (4). The active site for PFAR lies in the large subunit of the ribosome (50S in bacteria and 60S in eukaryotes) and, similar to the peptidyl transferase activity, involves the ribosomal RNA (rRNA) (2,7). In fact, both of these crucial functions of the ribosome share the same active center, i.e. the domain V of the 23S rRNA in bacteria and 25S/28S in eukaryotes (7-10). The same domain from mitochondrial ribosome also displays activity in refolding proteins (6,11). This RNA domain (referred hereafter as ‘domain V rRNA’) is usually free from any ribosomal protein and lies in the subunit interface of the 70S/80S ribosome. However, upon splitting of the ribosomal subunits it gets exposed on the surface of the large subunit. Thus, in vitro, 50S/60S ribosomal subunits show a more pronounced protein folding activity than the fully assembled 70S/80S ribosomes (12,13).

Despite series of in vitro demonstrations of PFAR, a question still remains open in the field, i.e. is PFAR functional in the modern cells or is it an evolutionary relic representing function of an ancient protein-production machine? Although there are few reports of PFAR in living bacterial cells (14,15), the in vivo context of PFAR has not been fully established. One recent finding, however, has linked PFAR to the living cells and moreover, associated it with diseases of higher eukaryotes. It has been shown that two unrelated compounds 6-Aminophenanthridine (6AP) and Guanabenz acetate (GA), with demonstrated activity against yeast ([PSF] and [URE3]) and mammalian (PrPSc) prions bind to rRNA and inhibit PFAR (15-17). The correlation between the antiprion activity of these two drugs and their ability to specifically inhibit PFAR suggest that PFAR could be involved in the establishment or maintenance of the prion processes in cells. This notion was further reinforced by the discovery of a 6AP derivative called 6API, where the 6-amino group of 6AP is substituted with 2-butan-1-ol, that was inactive in both the reversion of the prion phenotype in vivo and inhibition of PFAR in vitro (15). In a different context, PFAR was suggested to be involved in another amyloid-based disease—oculopharyngeal muscular dystrophy (OPMD), which is an inherited myodegenerative disease caused by the aggregation of PABPN1 protein into amyloid fibers within the nucleus of muscle cells (18). Thus, even though PFAR’s involvement in prion processes is yet to be directly demonstrated, 6AP and GA constitute valuable tools for studying PFAR (19).

In the present work, we elucidate how 6AP inhibits PFAR. Using UV cross-linking followed by primer extension, we show that the protein substrates of PFAR and 6AP (but not the inactive analog 6API) interact with largely overlapping sites of the domain V of 23S rRNA. Mutations in the interaction sites not only abolish or change the interaction map of both the protein substrates and 6AP, but also decrease the protein folding activity of domain V of rRNA from both Escherichia coli (E. coli) and Saccharomyces cerevisiae (S. cerevisiae). Moreover, we show that 6AP does not affect the kinetics of PFAR but reduces the yield of the refolded protein. Our results lead to a simple model for PFAR inhibition by 6AP.

EXPERIMENTAL PROCEDURES

Proteins: Human carbonic anhydrase I (HCA) was expressed in E. coli and purified using column chromatography as described in (20). Proteins bovine carbonic anhydrase (BCA) and bacterial dihydrofolate reductase (DHFR) were purchased from Sigma. His-tagged T7-RNA polymerase was purified using IMAC after over-expression from the plasmid pET21a-T7 pol (lab strain).

In vitro transcription of domain V rRNA: Plasmids pGEM4Z and pAV164, containing
DNA sequences for domain V of 23S rRNA from *E. coli* and 25S rRNA from *S. cerevisiae*, respectively, were used as templates for transcription. Linear templates were prepared either by restriction digestion (e.g. pGEM4Z with EcoRI) or by PCR amplification of the target sequence. 1.5 µg of the linearized DNA template was mixed with transcription buffer (800 mM Hepes-NaOH (pH 7.5), 120 mM MgCl₂, 120 mM dithiothreitol and 8 mM spermidine). Next, 7 mM rNTP mix, 60 U RNase inhibitor (RiboLock, Fermentas) and 1.68 µM T7 RNA polymerase were added to it to start the transcription and incubated for 4 hours at 37°C. Then DNA templates were digested with RNase-free DNase I. RNA was precipitated with 3 M sodium acetate (pH 5.2) and ethanol after extraction with phenol and chloroform (1:1). Next, RNA was made free from nucleotides using nucleospin RNA clean-up kit (Macherey Nagel). RNA concentrations were measured in NanoDrop™ 1000 spectrophotometer (Thermo Scientific, V.3.6) and the quality was checked by running in a 4% denaturing urea-polyacrylamide gel.

**Mutagenesis of domain V rRNA:** Plasmids pGEM4Z and pAV164 were used for introducing base mutations on domain V of 23S rRNA from *E. coli* and 25S rRNA from *S. cerevisiae*, respectively. 19 variants of domain V of 23S rRNA and nine variants of domain V of 25S rRNA were created by using Quikchange mutagenesis (Stratagene). The mutations were confirmed by DNA sequencing and corresponding RNAs were transcribed and purified as described above.

**HCA refolding assay:** Human carbonic anhydrase (HCA) (30 µM) was denatured with 6 M guanidine hydrochloride (Gdn-HCl) by incubating overnight at 37°C. Refolding of HCA was done typically for 30 min at room temperature by 100 times dilution of the denatured mix in refolding buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 100 mM NaCl and 0.05 mM ZnSO₄) as described earlier (20), without (self-folding), or with domain V rRNA. For time-course experiments, samples were withdrawn from the refolding mix at different time points and assayed for HCA activity. Typical concentration of domain V rRNA in these experiments was 350 nM. The enzymatic activity of HCA was used as a measure of refolding considering the native enzyme activity (stored undiluted in ice) as 100%.

**UV cross-linking:** 6 M Gdn-HCl denatured proteins (HCA, BCA, and DHFR, all 30 µM) were diluted 100 times in the refolding buffer containing domain V of 23S rRNA (*E. coli*) (300 nM) and UV cross-linking was performed immediately in a GS GENE LINKER™, Bio-rad instrument, with 254 nm UV irradiation (600 mJ) (21). For cross-linking with 6AP, 300 nM domain V of 23S rRNA and 0.5 mM 6AP (or 6APi) were mixed and subjected to the same procedure as above. In both cases, the samples were kept on ice during irradiation to prevent heat damage of the RNA. The irradiated samples were precipitated by salt-ethanol and washed with 70% ethanol for primer-extension.

**Primer extension assay:** Primer (5′ACCCCGGATCCGCGCCCACGGCAGATGG3′) was labeled with [γ-32P] dATP at 37°C using T4 polynucleotide kinase (Fermentas) for 1 hour by the 5’-end labeling method (22). The labeled primer was incubated with the cross-linked RNA-protein or RNA-6AP/6APi complex (about 10 µg) at 65°C for 5 min. Primer extension was initiated by addition of Thermo Script Reverse Transcriptase (Invitrogen), at 55°C for about 1 hour and then incubated 15 min at 72°C for completion of the reaction. The products were precipitated and washed with 70% ethanol and run on a 6.5% polyacrylamide gel with 8 M urea, next to a sequencing ladder of domain V rDNA obtained using the same primer by Thermo Sequenase DNA Polymerase (Thermo Sequenase™ Sequencing Kit, USB; USA).

**RESULTS**

**Refolding of HCA with domain V rRNAs:** HCA denatured in 6 M Gdn-HCl was subjected to refolding in the presence of *in vitro* transcribed domain V of 23S rRNA from *E. coli* and 25S rRNA from *S. cerevisiae*. In comparison to self-folding, which resulted in ~25% of native HCA activity, both domain V rRNAs increased the refolding to 38-40% (Figure 1A). Titration with domain V rRNAs showed an optimal RNA concentration approximately in one to one ratio with the
HCA concentration during refolding. Furthermore, HCA refolding with both domain V rRNAs could be fully inhibited by 6AP to the level of self-folding (Figure 1A inset). Consistent with earlier report, there was no effect of 6AP on self-folding (Figure 1B) (20).

6AP mediated inhibition of the protein folding activity of domain V of 23S rRNA:
Since domain V of rRNA constitutes the active center for PFAR (7), we have studied the mode of action of 6AP as an inhibitor, using domain V of 23S rRNA as a folding modulator. With increasing concentrations of 6AP, domain V assisted refolding of HCA decreased gradually from 38% to 25% (Figure 1B-C). The data were fitted with hyperbolic function using Origin 8.0 software. The half-maximal inhibitory concentration (IC₅₀) was estimated from the fitted curve as 90 µM, similar to earlier measurements with other ribosome-borne folding modulators e.g. 70S ribosome (20).

Next, refolding of HCA was monitored as a function of time without (self-folding) or with domain V of 23S rRNA and the rates were determined by fitting the resulting curves with single exponential function. The estimated rates in both reactions were similar (Figure 1C, Table 1), which is consistent with a previous report where 23S rRNA was used as the folding modulator (20). Addition of 6AP in gradually increasing concentrations did not alter the rate (Figure 1C, Table 1), although the extent of refolding was reduced as seen in the single time-point assays (Figure 1B).

Binding sites of three protein substrates on domain V of 23S rRNA:
The protein binding sites on the central loop and the lower part of domain V of 23S rRNA have been reported earlier for BCA, lysozyme, malate dehydrogenase and lactate dehydrogenase protein substrates using UV cross-linking followed by primer-extension (21,23). We have extended these observations by using HCA and DHFR as protein substrates. Our results, in good agreement with previously published results (21,23), showed a similar interaction map for all three proteins. Five major interaction sites spanning U2474-A2476, U2492-G2494, G2553-C2556, A2560-A2564 and U2585-G2588 residues were identified (Figure 2A). When zoomed into the tertiary structure of the ribosome, these sites lie in close proximity to each other creating a binding pocket (Figure 2B). Since the same sets of nucleotides were identified as the interaction sites for various proteins, these sites must be of general importance for PFAR.

Binding sites of 6AP on domain V of 23S rRNA:
We have mapped the interaction sites of 6AP on domain V of 23S rRNA in the same way as in the case of the protein substrates. As shown in Figure 3A, ten 6AP binding sites have been identified by UV cross-linking followed by primer extension assay. These are U2473-C2475, U2491-G2494, C2499-C2501, A2513-C2515, G2553-C2556, U2561-U2562, A2564-A2565, U2586-G2588, A2598-G2599 and C2601-A2602 (Figure 3A-B). Interestingly, six of these sites (those underlined) showed partial or complete overlap with the protein binding sites mentioned earlier (Figure 2A). This overlap of binding sites on the rRNA suggests that 6AP competes with the protein substrates for the binding sites on domain V of 23S rRNA.

When 6APi was reacted in the same way as 6AP, only full-length product was obtained in the primer extension experiment, similar to the control reaction with just domain V of 23S rRNA (Figure 3A). This result shows that 6APi, in contrast to 6AP, does not interact with domain V rRNA, which also explains its inactivity towards inhibition of PFAR. In another control experiment, primer extension was done without UV treatment. In this case too, only full-length products were seen with both 6AP and 6APi (Figure 3A). Thus, it confirmed that the bands seen after UV cross-linking with 6AP were indeed due to 6AP binding to domain V rRNA and not because of degradation of the labeled transcript. The possibility of RNase contamination was further eliminated by running the reactions in a standard urea-acrylamide gel, which showed only intact RNAs (data not shown).

Effect of mutations in the domain V of 23S/25S rRNA on HCA refolding:
The domain V rRNA, also the center for ribosomal peptidyl transferase activity, is a highly conserved region of the ribosome. When compared between distant species such as E. coli and S. cerevisiae, most of the protein and 6AP binding sites showed a high degree of
6AP inhibits PFAR by direct competition

Conservation (Figure 3C). In order to understand the importance of specific nucleotides in PFAR, we have mutated several bases of domain V rRNA from both E. coli (23S) and S. cerevisiae (25S), especially those forming 6AP and protein binding sites, and tested the resulting mutants in the HCA refolding assay.

As shown in Figure 4A, most of the mutated variants of the domain V of 23S rRNA showed highly reduced refolding activity; less than 50% compared to the wild-type (Table 2). Highest defect (> 85%) was seen with individual or group mutations at positions UUG2492-94, UU2561-62, UAG2586-88 on 23S rRNA (Figure 4A, Table 2). In comparison, mutations at positions G2472, UC2474-75, UA2563-64, UU2554-55 and UC2500-01 showed lesser degree of defect (50 – 75%). When 25S rRNA mutants were tested in the same assay, mutations at positions UGUC2873-76 were most defective (Figure 4B, Table 2). UUG2861-63 mutants also showed significant defect (~75%); these nucleotides correspond to UUG2492-94 on 23S rRNA (Figure 3C), suggesting that these residues play crucial role in PFAR.

Control mutations CGG2486-88AUU on 23S rRNA, and A2820C and G2841A on 25S rRNA, which did not correspond to the protein or 6AP interaction sites, showed no reduction in HCA refolding (Figure 4A-B, Table 2). Thus, these results not only pinpointed the nucleotides important for PFAR but also confirmed that the cross-linking sites do represent actual interaction sites for the protein substrate on domain V rRNA. It is interesting to note that no preference for base alteration was seen in the HCA refolding assay. In case of functionally important residues (such as U2492-G2494 in E. coli), changes from purine to purine or purine to pyrimidine and vice versa caused similar deleterious effects on rRNA assistance for HCA refolding (Figure 4A, Table 2).

In order to ensure that the mutations did not introduce any global change in the structure of domain V rRNA, wild-type and three mutant variants of domain V rRNA (CGG2486-88AUU, UU2561-62AA, UAG2586-88CAA) were subjected to circular dichroism (CD) analysis. Furthermore, using CD at 270 nm, the temperature melting profiles of these RNAs were recorded. All the tested RNAs produced essentially identical CD spectra and temperature melting profiles (Figure 5) confirming that the base mutations did not alter the secondary structure of the RNA. The difference in CD spectra between 20°C and 85°C indicates loss of folding in all tested RNAs with increase in temperature. It also confirms that the in vitro transcribed domain V rRNAs are structured under our experimental condition (20°C).

Loss of both protein and drug interaction sites due to mutations in the domain V of 23S rRNA: The loss of protein folding activity due to mutations on domain V rRNA called for a crosscheck of the interaction sites using mutant rRNAs. Among the mutations tested in the HCA refolding assay, UUG2492-94CCA and UAG2586-88CCA showed major loss of PFAR (Figure 4A). When these mutant RNAs were subjected to UV cross-linking and primer extension, distinct changes in the interaction patterns were seen for both HCA and 6AP. For UUG2492-94CCA rRNA, the bands corresponding to these nucleotides were completely missing for both HCA and 6AP (Figure 6A-B, top panels), while other bands remained intact. Similarly, in the case of UAG2586-88CCA, major change in the banding pattern was observed at this site (Figure 6A-B, bottom panel). For HCA, the bands corresponding to U2586-G2588 nucleotides became weaker compared to the wild-type (Figure 6A, bottom panel), while for 6AP these bands were completely missing (Figure 6B, bottom panel). These results confirmed that UUG2492-94 and UAG2586-88 indeed interact with both the protein substrate and 6AP. Thus, a correlation can be envisaged between PFAR and the mode of action of 6AP.

**DISCUSSION**

Domain V rRNA is the universal active center for PFAR:

It was shown earlier that the active site of PFAR lies in the domain V of 23S rRNA in bacterial ribosome (7,8,24). It was also shown that PFAR is a universal activity borne by prokaryotic, eukaryotic as well as mitochondrial ribosomes (4,7,25). This is not surprising considering the high degree of sequence conservation of the domain V rRNA.
6AP inhibits PFAR by direct competition through evolution. However, it was not known whether or not an in vitro transcribed domain V of the eukaryotic rRNA retains protein folding activity. Our results put an affirmative end to that discussion as the domain V of 25S rRNA from *S. cerevisiae* could refold HCA as efficiently as the domain V of 23S rRNA from *E. coli* (Figure 1A). Furthermore, protein folding activity of both the domain V rRNAs was inhibited similarly when treated with 6AP (Figure 1A, inset). Thus, we conclude that similar to peptidyl transferase activity, domain V based PFAR is conserved from bacteria to eukaryotes.

6AP inhibits the protein folding by domain V rRNA by direct competition:

The inhibition of PFAR by 6AP has been reported earlier using various ribosomal components as protein folding modulators (70S, 50S and 23S rRNA from *E. coli* and 80S from *S. cerevisiae*) (15,20). Based on the refolding kinetics, it was suggested that 6AP competes with the protein substrates for the binding sites on rRNA, although direct proof in support of this claim was unavailable (20). Our present results show that the binding sites of 6AP on domain V of rRNA highly overlap with those of the protein substrates (Figure 2-3). Moreover, specific base mutations in the overlapping sites not only alter the interaction of both the protein substrate and 6AP with domain V rRNA (Figure 6A-B), but also decrease PFAR (Figure 4A-B). Thus, it can be concluded that 6AP competitively occludes the protein substrates from gaining access to the functionally important interaction sites on the domain V rRNA and thereby inhibits PFAR.

How 6AP preferentially inhibits PFAR is an open question. Earlier results have shown that there is no effect of 6AP on global protein synthesis (15). When tested on di- or tri-peptide formation no decrease in the level of ML di- or MLL tri-peptide was seen in 60 sec even with high concentrations of 6AP, which would completely inhibit PFAR (data not shown). However, detailed understanding of the action of 6AP in protein synthesis requires fast kinetics or single-molecule based analysis, which are foreseeable steps in the future investigation of this topic. Also, it is important to characterize the structure of the ribosome in complex with 6AP, which will certainly add to the understanding of how 6AP selectively targets PFAR.

Whether 6AP binds to other domains of the rRNA is yet another open question. As 6AP has planar structure, which presumably makes it prone to layered stacking between the RNA bases, detailed understanding of the chemistry of 6AP-rRNA interaction needs further investigation beyond the scope of the present work (D. Banerjee, manuscript in preparation).

Model of domain V rRNA assisted refolding and 6AP inhibition:

We propose a simple model to explain the mechanism of protein folding with domain V rRNA and its inhibition by 6AP (Figure 7). According to this model, the unfolded protein substrate (U) collapses to an early folding intermediate (I) immediately after dilution of the denaturant. In the case of self-folding, a fraction of the intermediate I (~25%) rapidly primes to a productive folding intermediate (I*), which slowly folds to the active, folded state (F). The remaining ~75% is trapped in a misfolded state (mF), possibly through a different intermediate (I-). The rate limiting step in this folding pathway is I* → F, with an average time (t1/2 = 1/k) of 6 - 7 min. It is clear from our results that the rate of refolding of HCA does not change in the presence of domain V rRNA (Figure 1B, Table 1). Hence, the role of the domain V rRNA as protein folding modulator is probably to increase the fraction of the productive intermediate I* (~40%) through transient trapping of I rather than to modulate the actual folding step (I* → F). 6AP inhibits the ‘trapping’ reaction by competing with I for the binding sites on domain V rRNA. As a result, the protein follows the self-folding pathway. That is why at high concentration of 6AP, which presumably blocks all protein interaction sites on domain V rRNA, refolding only to the extent of self-folding could be achieved. This model can be extrapolated to elucidate PFAR in general as the other ribosomal folding modulators (e.g. 70S ribosome, 50S subunit and 23S rRNA), similar to domain V rRNA, modulate only the extent of refolding but the rate of refolding remains unaffected (20).

Our current knowledge about the nature of the interaction between protein substrates and the domain V rRNA is quite limited. The common interaction map of various protein
6AP inhibits PFAR by direct competition

substrates on domain V of 23S rRNA suggests a general mechanism of PFAR for all protein substrates. The multiple interaction sites on domain V rRNA indicate that the transition I → I* might involve multiple steps of interaction between the protein substrate and the domain V rRNA, the sequence of which remains open for future investigations.

Correlation between PFAR and prion:
As discussed above, the binding of 6AP and PFAR both involve common sets of nucleotides on the domain V rRNA (Figures 2 and 3), and both are inhibited when these nucleotides are altered by mutation (Figures 4 and 6). Since 6AP possesses well documented antiprion activity, but do not interact directly with the prion protein (16,26), our results, in line with earlier reports (15,20), suggest indirectly that PFAR might be involved in prion formation. However, the possibility of alternative mechanisms by which 6AP inhibits prion phenotype cannot be completely overruled.

The involvement of nucleic acids in formation of prions is not a new concept. Independent studies have shown that nucleic acid interactions facilitate prion fibril formation (27-29), although the mechanism of such processes is not known. Detailed knowledge about the in vivo substrates of PFAR as well as direct kinetic analysis will be required to corroborate our hypothesis about the involvement of PFAR in prion mechanisms.

ACKNOWLEDGEMENTS

We acknowledge Lars Hellman, Uppsala University for allowing us to use the UV cross-linker. We also thank Susan Liebman, University of Illinois, Chicago, USA for providing us construct for 25S rRNA. Xueliang Ge and Petar Kovachev are acknowledged for help in manuscript preparation.

FOOTNOTES

This work was supported by Swedish Research Council (individual grants from M and NT sections, VR-SIDA (Swedish Research Link) and Linnaeus grant to Uppsala RNA Research Center; Carl Tryggers Stiftelse; Wenner Gren Stiftelse (postdoc stipendium for DB); Knut and Wallice Wallenberg Foundation (to RiboCORE), Vinnova / DBT (India), and especially SSF-Dalen (Sweden-France bilateral collaboration) program to SS. YP is partially supported by a scholarship from the Chinese Scholarship Council. MB and CV acknowledge funding support from Inserm, from CRITT Santé Bretagne from the Région Bretagne and the ANR “Blanche” from the French government.

FIGURE LEGENDS

Figure 1. Refolding of HCA with domain V rRNA and the effect of 6AP.
(A) Refolding of denatured HCA (final concentration 300 nM) with various concentrations of domain V (Dom V) of 23S rRNA (E. coli-●-) and 25S rRNA (S. cerevisiae-▲-). The extent of refolding (30 min at room temperature) is estimated as the percentage of native HCA activity (stored undiluted in ice). The inset shows the effect of 6AP (300 µM) on HCA refolding without and with domain V rRNAs from E. coli and S. cerevisiae (labeled as S. cere). The results are average of minimum three individual measurements and error bars represent standard deviation.
(B) Refolding of HCA (300 nM, 30 min at room temperature) in the presence of increasing concentrations of 6AP (A), without (self-refolding) (-■-) or with (-○-) the domain V of E. coli 23S rRNA (300-400 nM). The IC₅₀ (half maximal inhibitory concentration) is determined from the x-intercept drawn at half maximal refolding considering the difference between the self and domain V of 23S rRNA assisted refolding as 100%. (C) Time course of HCA refolding without (self-refolding) (-■-) or with domain V of 23S rRNA at different concentrations of 6AP (-○-). The curves are fitted with a single exponential equation using Origin 8.0 and the rates (mean of minimum three experiments) are estimated from the respective fits.
**Figure 2.** Interaction sites of the protein substrates on the domain V of 23S rRNA.
(A) Primer extension analysis on domain V of 23S rRNA after UV cross-linking without any protein (1) or with HCA (2), DHFR (3) and BCA (4). The first 4 lanes show sequencing ladders as indicated on top of the lanes.
(B) Illustration of the common interaction sites of the protein substrates on the three-dimensional structure of the 50S subunit from *E. coli* in bronze (PDB identifier 3UOS). The stem loop part of domain V of 23S rRNA (A2009-A2434) is in green and the circular central loop (A2435-G2668) is in blue with the nucleotides corresponding to the protein interaction sites (identified in A) in yellow. The interaction sites are labelled in the zoomed panel above.

**Figure 3.** Interaction sites of 6AP on the domain V of 23S rRNA.
(A) Primer extension analysis on domain V of 23S rRNA after UV cross-linking in the absence (lane 1) or in the presence of 6AP (2) and 6APi (3). Lanes 4 – 6 are control experiments for lanes 1 – 3 without UV treatment. The first 4 lanes show sequencing ladders as indicated on top of the lanes.
(B) Mapping of the 6AP interaction sites (in green) on the secondary structure of the domain V of 23S rRNA. The nucleotides interacting with protein substrates (shown in Figure 3) are indicated by black boxes.
(C) The alignment of domain V rRNA sequences from *E. coli* (NC000913) and *S. cerevisiae* (*S. cere*) (U53879) showing strong overlap and high conservation of the sequences involved in the interaction with protein substrates (black boxes) and antiprion drugs (green boxes). The yellow highlights indicate the sequences subjected to mutagenesis, in red font are the ones which showed loss of protein folding activity (see Figure 4).

**Figure 4.** Effect of mutations in domain V rRNA on the domain V rRNA assisted refolding of HCA. Refolding of HCA without (self) or with domain V of *E. coli* 23S rRNA (A) and *S. cerevisiae* 25S rRNA (B), wild-type or with mutations in various base positions as indicated below the bars. The grid lines indicate the level of self-folding (bottom grid), wild-type domain V rRNA assisted folding (top grid) and midpoint between the top and the bottom grid (middle grid), for easy inspection of the degree of defect with the individual mutants.

**Figure 5.** Circular Dichroism (CD) analysis of the wild-type and the mutant domain V rRNAs. The wild-type and three variants of domain V of 23S rRNA carrying mutations CCG2486–88AUU, UU2561–62AA, UAG2586–88CCA are subjected to CD analysis to judge the secondary structure. The measurements were done in water using quartz cuvette of path length 1 mm in a Jasco-815 CD spectrometer with RNA concentration 0.5 µM. (A) CD spectra at 20°C and (B) at 85°C. The broken line in (B) indicates the spectrum of the wild type domain V at 20°C as a reference. (C) CD profiles of temperature dependent melting of wild type and mutant RNAs where the change in CD signal was monitored at a fixed wavelength (270 nm) with a temperature gradient of 1 degree/min.

**Figure 6.** Effect of mutations in domain V rRNA on the interaction map of HCA and 6AP. The interaction sites of HCA (A) and 6AP (B) on the domain V of 23S rRNA (*E. coli*) wild-type (1), mutants UUG2492-94CCA (2), and UAG2586-88CCA (3). Lane 4 is control with only domain V rRNA without HCA (A) or 6AP (B). The bands corresponding to the mutation sites are marked with boxes.

**Figure 7.** A simple model for PFAR and 6AP action. The unfolded state (U) collapses to an intermediate (I) upon dilution of the denaturant. For self-folding, I transforms either to a folding competent intermediate (I*) which folds slowly to the native state (F), or to another intermediate (I-) which leads to the misfolded state mF. The domain V rRNA assisted refolding proceeds through a fast trapping reaction which facilitates conversion of I to I*, thereby driving more I molecules to the productive folding pathway (I* → F). 6AP inhibits the trapping reaction by binding to the overlapping sites on domain V rRNA. As a result domain V rRNA assisted pathway gets blocked and the proteins fold via self-folding pathway.
### TABLES

| Domain Assisted Folding | $K_{obs}$ (min$^{-1}$) | Refolding (%) |
|-------------------------|------------------------|---------------|
| Dom V RNA assisted fold | 0.18 ± 0.03            | 38.5 ± 1.5    |
| Dom V + 6AP (10 µM)     | 0.18 ± 0.03            | 36 ± 1        |
| Dom V + 6AP (100 µM)    | 0.17 ± 0.02            | 30 ± 1.5      |
| Dom V + 6AP (300 µM)    | 0.17 ± 0.04            | 22 ± 2        |
| Self-folding            | 0.15 ± 0.02            | 23 ± 2        |

**Table 1.** The rate and % refolding (native = 100%) of HCA without (Self-folding) or with domain V of 23S rRNA in the absence and in the presence of increasing concentrations of 6AP.

| Variants of *E. coli* 23S domain V rRNA | Refolding Efficiency % of wild-type | Degree of defect |
|-----------------------------------------|-------------------------------------|------------------|
| Wild-type                               | 100                                 | 0 ± 10 %         |
| CGG2486-88AUU                           | 104.4                               |                  |
| UC2474-75AU                             | 56.2                                | 10 - 50 %        |
| UC2500-01AU                             | 44.3                                |                  |
| UA2563-64AU                             | 41.1                                | 50 - 75 %        |
| UC2554-55AA                             | 31.6                                |                  |
| G2472U                                  | 30.7                                |                  |
| UAG2586-88CCA                           | 22.2                                | 75 - 85 %        |
| A2587C                                  | 19.7                                |                  |
| U2493C                                  | 14.1                                |                  |
| G2494C                                  | 13.4                                | 85 - 90 %        |
| G2588A                                  | 13.1                                |                  |
| U2586C                                  | 11.2                                |                  |
| G2494A                                  | 7.8                                 | 90 - 95 %        |
| UUG2492-94CCA                           | 7.2                                 |                  |
| U2493G                                  | 3.5                                 |                  |
| U2492C                                  | 1.6                                 |                  |
| UU2561-62AA                             | 1.5                                 | > 95 %           |
| UUG2492-94GCC                           | 1.1                                 |                  |
| U2493G                                  | -5.5                                |                  |

**Table 2.** The domain V rRNA mutants analysed and sorted according to their refolding efficiency estimated as (refolding<sub>domain V mutant</sub> - self-folding) / (refolding<sub>wild-type domain V</sub> - self-folding) from the results presented in Figures 4A-B, and expressed in percentage relative to the wild-type domain V rRNA (100%). The third column represents degree of defect (100% - refolding efficiency).
References

1. Bera, A. K., Das, B., Chattopadhyay, S., and Das Gupta, C. (1994) Protein folding by ribosome and its RNA. Curr. Sci. 66, 230-232
2. Das, B., Chattopadhyay, S., Bera, A. K., and Das Gupta, C. (1996) In vitro protein folding by ribosomes from Escherichia coli, wheat germ and rat liver - The role of the 50S particle and its 23S rRNA. Eur. J. Biochem. 235, 613-621
3. Argent, R. H., Parrott, A. M., Day, P. J., Roberts, L. M., Stockley, P. G., Lord, J. M., and Radford, S. E. (2000) Ribosome-mediated folding of partially unfolded ricin A-chain. J. Biol. Chem. 275, 9263-9269
4. Das, D., Das, A., Samanta, D., Ghosh, J., Das Gupta, S., Bhattacharya, A., Basu, A., Sanyal, S., and Das Gupta, C. (2008) Role of the ribosome in protein folding. Biotech. J. 3, 999-1009
5. Kudlicki, W., Coffman, A., Kramer, G., and Hardesty, B. (1997) Ribosomes and ribosomal RNA as chaperones for folding of proteins. Fold Des. 2, 101-108
6. Suljoadikusumo, I., Horikoshi, N., and Usheva, A. (2001) Another function for the mitochondrial ribosomal RNA: Protein folding. Biochemistry 40, 11559-11564
7. Chattopadhyay, S., Das, B., and Das Gupta, C. (1996) Reactivation of denatured proteins by 23S ribosomal RNA: Role of domain V. Proc Natl Acad Sci U S A. 93, 8284-8287
8. Sanyal, S. C., Pal, S., Chowdhury, S., and Das Gupta, C. (2002) 23S rRNA assisted folding of cytoplasmic malate dehydrogenase is distinctly different from its self-folding. Nucleic Acids Res. 30, 2390-2397
9. Pal, D., Chattopadhyay, S., Chandra, S., Sarkar, D., Chakraborty, A., and Das Gupta, C. (1997) Reactivation of denatured proteins by domain V of bacterial 23S rRNA. Nucleic Acids Res. 25, 5047-5051
10. Pal, S., Chandra, S., Chowdhury, S., Sarkar, D., Ghosh, A. N., and Gupta, C. D. (1999) Complementary role of two fragments of domain V of 23S ribosomal RNA in protein folding. J. Biol. Chem. 274, 32771-32777
11. Das, A., Ghosh, J., Bhattacharya, A., Samanta, D., Das, D., and Das Gupta, C. (2011) Involvement of mitochondrial ribosomal proteins in ribosomal RNA-mediated protein folding. J. Biol. Chem. 286, 43771-43781
12. Basu, A., Samanta, D., Bhattacharya, A., Das, A., Das, D., and Das Gupta, C. (2008) Protein folding following synthesis in vitro and in vivo: Association of newly synthesized protein with 50S subunit of E. coli ribosome. Biochem. Biophys. Res. Commun. 366, 592-597
13. Basu, A., Samanta, D., Das, D., Chowdlury, S., Bhattacharya, A., Ghosh, J., Das, A., and Das Gupta, C. (2008) In vitro protein folding by E-coli ribosome: Unfolded protein splitting 70S to interact with 50S subunit. Biochem. Biophys. Res. Commun. 366, 598-603
14. Chattopadhyay, S., Pal, S., Pal, D., Sarkar, D., Chandra, S., and Das Gupta, C. (1999) Protein folding in Escherichia coli: role of 23S ribosomal RNA. Biochim. Biophys. Acta. 1429, 293-298
15. Tribouillard-Tanvier, D., Dos Reis, S., Gug, F., Voisset, C., Beringue, V., Sabate, R., Kikovska, E., Talarek, N., Bach, S., Huang, C. H., Desban, N., Sape, S. J., Supattapone, S., Thuret, J. Y., Chedin, S., Vilette, D., Galons, H., Sanyal, S., and Blondel, M. (2008) Protein folding activity of ribosomal RNA is a selective target of two unrelated antiprion drugs. PloS One. 3, e2174
16. Bach, S., Talarek, N., Andrieu, T., Vierfond, J. M., Mettey, Y., Galons, H., Dormont, D., Meijer, L., Cullin, C., and Blondel, M. (2003) Isolation of drugs active against mammalian prions using a yeast-based screening assay. Nat Biotechnol. 21, 1075-1081
17. Tribouillard-Tanvier, D., Beringue, V., Desban, N., Gug, F., Bach, S., Voisset, C., Galons, H., Laude, H., Vilette, D., and Blondel, M. (2008) Anthypertensive drug guanabenz is active in vivo against both yeast and mammalian prions. PLoS One. 3, e1981
18. Barbezier, N., Chartier, A., Bidet, Y., Buttsstedt, A., Voisset, C., Galons, H., Blondel, M., Schwarz, E., and Simonelig, M. (2011) Antiprion drugs 6-aminophenanthridine and guanabenz reduce PABPN1 toxicity and aggregation in oculopharyngeal muscular dystrophy. Embo Mol Med. 3, 35-49
6AP inhibits PFAR by direct competition

19. Voisset, C., Thuret, J. Y., Tribouillard-Tanvier, D., Saupe, S. J., and Blondel, M. (2008) Tools for the study of ribosome-borne protein folding activity. *Biotech. J.* 3, 1033-1040
20. Dos Reis, S., Pang, Y., Vishnu, N., Voisset, C., Galons, H., Blondel, M., and Sanyal, S. (2011) Mode of action of the antiprion drugs 6AP and GA on ribosome assisted protein folding. *Biochimie.* 93, 1047-1054
21. Samanta, D., Mukhopadhyay, D., Chowdhury, S., Ghosh, J., Pal, S., Basu, A., Bhattacharya, A., Das, A., Das, D., and Das Gupta, C. (2008) Protein folding by domain v of Escherichia coli 23S rRNA: Specificity of RNA-protein interactions. *J Bacteriol.* 190, 3344-3352
22. Lewis, M. E., Sherman, T. G., Burke, S., Akil, H., Davis, L. G., Arentzen, R., and Watson, S. J. (1986) Detection of proopiomelanocortin mRNA by in situ hybridization with an oligonucleotide probe. *Proc Natl Acad Sci U S A.* 83, 5419-5423
23. Das, D., Samanta, D., Hasan, S., Das, A., Bhattacharya, A., Das Gupta, S., Chakrabarti, A., Ghorai, P., and Das Gupta, C. (2012) Identical RNA-protein interactions in vivo and in vitro and a scheme of folding the newly synthesized proteins by ribosomes. *J. Biol. Chem.* 287, 37508-37521
24. Chowdhury, S., Pal, S., Ghosh, J., and Das Gupta, C. (2002) Mutations in domain V of the 23S ribosomal RNA of *Bacillus subtilis* that inactivate its protein folding property in vitro. *Nucleic Acids Res.* 30, 1278-1285
25. Voisset, C., Saupe, S. J., and Blondel, M. (2011) The various facets of the protein-folding activity of the ribosome. *Biotech. J.* 6, 668-673
26. Gug, F., Oumata, N., Tribouillard-Tanvier, D., Voisset, C., Desban, N., Bach, S., Blondel, M., and Galons, H. (2010) Synthesis of conjugates of 6-aminophenanthridine and guanabenz, two structurally unrelated prion inhibitors, for the determination of their cellular targets by affinity chromatography. *Bioconjug Chem.* 21, 279-288
27. Gomes, M. P., Vieira, T. C., Cordeiro, Y., and Silva, J. L. (2012) The role of RNA in mammalian prion protein conversion. *Wiley interdisciplinary reviews. RNA.* 3, 415-428
28. Macedo, B., Millen, T. A., Braga, C. A., Gomes, M. P., Ferreira, P. S., Kraineva, J., Winter, R., Silva, J. L., and Cordeiro, Y. (2012) Nonspecific prion protein-nucleic acid interactions lead to different aggregates and cytotoxic species. *Biochemistry.* 51, 5402-5413
29. Silva, J. L., Vieira, T. C., Gomes, M. P., Rangel, L. P., Scapin, S. M., and Cordeiro, Y. (2011) Experimental approaches to the interaction of the prion protein with nucleic acids and glycosaminoglycans: Modulators of the pathogenic conversion. *Methods.* 53, 306-317
Figure 2

A

|   | G | A | U | C | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|---|---|---|
|   |   |   |   |   | U2474-A2476 |   |   |   |
|   |   |   |   |   | U2492-G2494 |   |   |   |
|   |   |   |   |   | G2553-C2556 |   |   |   |
|   |   |   |   |   | A2560-A2564 |   |   |   |
|   |   |   |   |   | U2585-G2588 |   |   |   |

B

Labels: U2474-A2476, U2492-G2494, G2553-C2556, A2560-A2564, U2585-G2588
Figure 7

Dom V RNA-assisted folding

U → I ~ Fast

Fast trapping

6AP

Dom V RNA: I

I* → I~

t_{1/2} ~ 6 min

~6 min

Dom V RNA

I* → I~ → F (~40%)

mF (~60%)

Self-folding

F (~25%)

mF (~75%)