Mode of action of the antiprion drugs 6AP and GA on ribosome assisted protein folding

ARTICLE *in* BIOCHIMIE · MARCH 2011

Impact Factor: 2.96 · DOI: 10.1016/j.biochi.2011.03.002 · Source: PubMed

7 AUTHORS, INCLUDING:

Yanhong Pang
Uppsala University
5 PUBLICATIONS **30** CITATIONS
SEE PROFILE

Cécile Voisset
French Institute of Health and Medical Rese…
52 PUBLICATIONS **2,599** CITATIONS
SEE PROFILE

Herve Galons
Université René Descartes - Paris 5
75 PUBLICATIONS **1,479** CITATIONS
SEE PROFILE

Marc Blondel
Université de Bretagne Occidentale
58 PUBLICATIONS **1,371** CITATIONS
SEE PROFILE

Biochimie xxx (2011) 1–8

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Mode of action of the antiprion drugs 6AP and GA on ribosome assisted protein folding

Suzana Dos Reis [a,1,2], Yanhong Pang [a,1], Neelanjan Vishnu [a,3], Cécile Voisset [b,c,d,e], Hervé Galons [f], Marc Blondel [b,c,d,e], Suparna Sanyal [a,*]

[a] Department of Cell and Molecular Biology, Uppsala University, Box-596, BMC, 75124 Uppsala, Sweden
[b] INSERM U613, Brest F-29200, France
[c] Univ Brest, Faculté de Médecine et des Sciences de la Santé, F-29200, France
[d] Etablissement Français du Sang (EFS) Bretagne, Brest F-29200, France
[e] CHRU Brest, Hop Morvan, Laboratoire de Génétique Moléculaire, Brest F-29200, France
[f] Laboratoire de Chimie Organique 2, CNRS UMR 8601, Université Paris Descartes, 4 avenue de l'Observatoire, 75270 Paris Cedex 6, France

ARTICLE INFO

Article history:
Received 9 February 2011
Accepted 1 March 2011
Available online xxx

Keywords:
Protein folding
Prion
Ribosome
Antiprion drugs
Carbonic anhydrase

ABSTRACT

The ribosome, the protein synthesis machinery of the cell, has also been implicated in protein folding. This activity resides within the domain V of the main RNA component of the large subunit of the ribosome. It has been shown that two antiprion drugs 6-aminophenanthridine (6AP) and Guanabenz (GA) bind to the ribosomal RNA and inhibit specifically the protein folding activity of the ribosome. Here, we have characterized with biochemical experiments, the mode of inhibition of these two drugs using ribosomes or ribosomal components active in protein folding (referred to as 'ribosomal folding modulators' or RFMs) from both bacteria *Escherichia coli* and yeast *Saccharomyces cerevisiae*, and human carbonic anhydrase (HCA) as a sample protein. Our results indicate that 6AP and GA inhibit the protein folding activity of the ribosome by competition with the unfolded protein for binding to the ribosome. As a result, the yield of the refolded protein decreases, but the rate of its refolding remains unaffected. Further, 6AP- and GA mediated inhibition of RFM mediated refolding can be reversed by the addition of RFMs in excess. We also demonstrate with delayed addition of the ribosome and the antiprion drugs that there is a short time-span in the range of seconds within which the ribosome interacts with the unfolded protein. Thus we conclude that the protein folding activity of the ribosome is conserved from bacteria to eukaryotes and most likely the substrate for RFMs is an early refolding state of the target protein.

1. Introduction

In addition to its central role in protein synthesis, the ribosome is also known to assist protein folding [1,2]. It was first demonstrated in 1992 that ribosomes isolated from *Escherichia coli* could act as a protein folding modulator for refolding fungal glucose 6-phosphate dehydrogenase and bacterial alkaline phosphatase *in vitro* [3]. Later, the folding activity of the ribosome was confirmed by refolding many other proteins such as lactate and malate dehydrogenase, RTEM-beta lactamase, lysozyme etc [1,4–10], with ribosomes from various sources such as eubacteria, archaea, eukaryotes and mitochondria [4,5,7–10]. Additional experiments were also performed *in vivo* to demonstrate that the ribosome possesses a general activity in protein folding even in the context of the living cell [11]. Since its discovery, a lot of attention has been paid to identify the active center on the ribosome responsible for its folding activity. This activity resides in the large subunit of the ribosome and can be traced to the domain V of 23S rRNA in bacteria or its equivalent parts in the eukaryotic ribosome, also known to be responsible for peptide bond formation [12,13]. This particular domain is located at the interface of the two subunits in the 70S ribosome and is not masked by any ribosomal protein in the 50S subunit. It has been proposed that a part of the domain V of 23S rRNA transiently traps the unfolded protein to protect it from

Abbreviations: RFM, Ribosomal folding modulator; 6AP, 6-aminophenanthridine; GA, Guanabenz; HCA, human carbonic anhydrase; rRNA, ribosomal RNA.

* Corresponding author. Tel.: +46 18 471 4220; fax: +46 18 471 4262.
E-mail address: suparna.sanyal@icm.uu.se (S. Sanyal).

[1] First authors with equal contribution.

[2] Present address: CNRS-UPS; IPBS Institut de Pharmacologie et Biologie Structurale, F-31077 Toulouse, France.

[3] Present address: Medizinische Universität Graz, Institute Für Molecular Biologie und Biochemie (Vorklinik) Harrechgasse 21/III, 8010 Graz, Austria.

0300-9084/$ – see front matter © 2011 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.biochi.2011.03.002

misfolding and then the other part releases the protein thereby driving it to the productive folding pathway [14,15]. It was unclear for a long time how the unfolded protein reaches the active folding site in the ribosomal subunit interface until it has been shown that unfolded proteins could split the ribosome into its subunits and thus gain full access to the active site on the 50S subunit for its refolding [16,17]. Thus in summary, the ribosome provides a unique opportunity to coordinate the protein synthesis activity of its peptidyl transferase center with the protein folding process [18]. Detailed understanding of how these two activities are coordinated needs further investigation. The ribosomal components active in protein folding *in vitro* (70S ribosome, 50S subunit, 23S rRNA from bacteria or equivalent components from eukaryotes) are referred generally as ribosomal folding modulators (RFMs) in this paper.

Prions are non-conventional infectious pathogens responsible for a group of fatal neurodegenerative diseases like Creutzfeldt–Jacob disease in human, Bovine Spongiform Encephalopathy (BSE) in bovines or scrapie in ovines and caprines. The "protein only" hypothesis states that the prion protein replicates without the use of any specific nucleic acid [19] and can spread by contamination with the prion infected food or tissue [20]. Prion proteins can exist mainly in two isoforms; (i) the cellular form or PrP^{C}, found in all cell types in the membrane (GPI-anchored protein) composed mostly of α-helices, and (ii) the pathology-associated form PrP^{Sc}, found in prion infected cells, rich in β-sheet structures, resistant to protease K [21] that can aggregate easily into amyloid fibers [22]. According to the most commonly accepted prion-hypothesis, PrP^{Sc} is able to convert normal PrP^{C} proteins into the infectious isoform PrP^{Sc} by changing their conformation [23].

The prion phenomenon is not restricted to the mammals. Indeed prions also exist in lower eukaryotes, *e.g.* [URE3] and [*PSI*+] in the budding yeast *Saccharomyces cerevisiae* [24–26] or [Het-s] in filamentous fungus *Podospora anserina* [27]. As these fungal prions are safe and nonhazardous to work with, they provide good model systems to study mammalian prion propagation and also provide a platform for high-throughput screening of the compounds effective against prion diseases. A yeast-based simple and economic method for safe and rapid screening of antiprion drugs has been recently set-up [28,29]. Indeed, most of the compounds isolated using this yeast-based method turned out to be active against mammalian prion *ex vivo* in various mammalian cell-based assays [28–30] and also *in vivo* in a murine model for prion-based diseases [31]. This finding not only validated the yeast-based approach for high-throughput screening but also proved that the yeast prions can be used as relevant models for studying mammalian prion diseases. Conversely, some of the antiprion drugs isolated using mammalian cell-based assays, like Quinacrine (QC) or Chlorpromazine (CPZ) were also found to be active against [URE3] and [*PSI*+] yeast prions in the yeast-based assay [28].

Two of the antiprion drugs identified with the yeast-based assay are 6-aminophenanthridine (6AP) and Guanabenz (GA). Although chemically different, both of the drugs are highly effective in reverting the prion phenotype in yeast and murine model cells [28,31]. In principle, at least two general mechanisms of action could be envisioned for antiprion drugs. They can either act in *cis* by targeting prion fibers or, alternatively, they can interfere with the cellular mechanism(s) involved in prion propagation (action in *trans*). 6AP and GA do not interact with prion proteins (from either mammals or fungi), instead the 'ribosome' is their main cellular target [32]. We have shown recently that 6AP and GA bind to the ribosome in a rRNA dependent manner and selectively inhibit the protein folding activity of the ribosome without affecting its function in protein synthesis [32]. Since 6AP and GA are the first identified specific inhibitors of the protein folding activity of the ribosome, these two drugs constitute unique tools to investigate the mechanism of this novel function.

In this work, using biochemical assays, we have characterized the mode of action of 6AP and GA in inhibiting the protein folding activity of the RFMs from both bacteria *E. coli* and yeast *S. cerevisiae* using human carbonic anhydrase (HCA) as a sample protein. Our data demonstrate that rRNA is the specific target for both drugs irrespective of the source of the ribosome and that these drugs inhibit ribosome assisted refolding of HCA in a competitive manner. Furthermore, using 6AP as a tool, we have estimated the time-span required for the interaction between the ribosome and the substrate protein (HCA) while refolding.

2. Material and methods

2.1. Enzymes and reagents

Human carbonic anhydrase I (EC 4.2.1.1) (HCA) (molecular weight 29,800 Da) cloned in vector pET3A (kind gift from Prof. C. K. Dasgupta, Calcutta, India) was over-expressed in *E. coli* BL21(DE3). Since HCA contains a Zn atom in its active center, 0.5 mM $ZnSO_4$ was added in the culture media LB containing 50 μg/ml Ampicilin. The enzyme was purified using DEAE-cellulose treatment followed by gel filtration chromatography (Superdex 75) using TN buffer (50 mM Tris–HCl, pH − 7.5, 100 mM NaCl) following the published protocol with minor modifications [33–35]. The single-band pure protein was stored in TN buffer in −80 °C. All the laboratory reagents were of analytical grade.

2.2. Drugs

6AP was synthesized as previously described [36,37]. GA was purchased from Sigma–Aldrich.

2.3. Ribosome

70S ribosome and its subunits were purified from *E. coli* MRE600 grown to mid log phase. Cells were suspended in two fold volume of ice cold opening buffer (20 mM Tris–HCl, pH − 7.5, 10.5 mM MgAc, 3 mM β-mercaptoethanol, 100 mM NH_4Cl, 0.5 mM EDTA) and disrupted by French press. First, cell debris was removed by centrifugation at 20,000 × *g* for 40 min and then the supernatant was cleared by a short centrifugation of 30 min at 81000 × *g*. Next, the cleared supernatant was overlaid onto an equal volume of sucrose cushion (20 mM Tris–HCl, pH 7.5, 10.5 mM MgAc, 3 mM β-mercaptoethanol, 500 mM NH_4Cl, 0.5 mM EDTA, 37.5% sucrose) and ultra-centrifuged at 71000 × *g* for 17–19 h in Ti 50.2 rotor at 4 °C. The pellet containing ribosomes was dissolved in high salt solution (20 mM Tris–HCl, pH 7.5, 800 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 7 mM β-mercaptoethanol) for removal of the ribosome associated proteins and chaperons, then pelleted through another 1.5 volume sucrose cushion (20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 800 mM KCl, 25% sucrose) by ultra- centrifugation at 71000 × *g* for 17 h at 4 °C. Resulting ribosome pellet was dissolved in low salt solution (20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$) and stored at −80 °C for later use. 80S ribosomes were purified from *S. cerevisiae* MN272 3Fα strain, following essentially the same procedure as for 70S ribosomes from *E. coli*.

For subunit preparation 70S ribosomes were dissolved in low Mg buffer (20 mM Tris–HCl, pH 7.5, 3 mM MgAc, 3 mM β-mercaptoethanol, 300 mM NH_4Cl, 0.5 mM EDTA). Then 50S and 30S subunits were isolated by ultracentrifugation at 75000 × *g* for 17 h at 4 °C on 10%–35% sucrose gradient. The 50S and 30S ribosomal subunits were collected by ultracentrifugation at 75000 × *g* for ~20 h at 4 °C and stored on −80 °C.

23S rRNA from *E. coli* 50S subunits and total RNA from *S. cerevisiae* 80S ribosomes were isolated by repeated (8–10 times)

Please cite this article in press as: S.D. Reis, et al., Mode of action of the antiprion drugs 6AP and GA on ribosome assisted protein folding, Biochimie (2011), doi:10.1016/j.biochi.2011.03.002

phenol-chloroform extraction following the procedure described in [14]. Both RNA samples free from all ribosomal proteins were stored in DEPC treated water at −20 °C.

2.4. Denaturation and refolding of HCA

HCA was denatured at a concentration of 30 μM with 6 M guanidine hydrochloride, and 30 μM EDTA (pH 8.0) at 37 °C overnight. For refolding, denatured HCA was diluted 100 fold (final concentration 300 nM) and was incubated at 25 °C for 30 min in refolding buffer (20 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl and 0.05 mM $ZnSO_4$) without (self) or with RFMs (300–400 nM) pre-incubated in the refolding mix. For inhibition studies, the drugs 6AP and GA were also incubated in the refolding mix for about 10 min before the denatured HCA was added for refolding. The activity of the refolded enzyme was assayed by adding 8 mM para-nitro-phenyl acetate (PNPA) to the refolding mixture in the room temperature and measuring the increase in A_{400} with 1 min scan in a Hitachi U-2900 spectrophotometer. The extent of HCA refolding was estimated by the ratio of the activity of the refolded enzyme to that of the same concentration of the native enzyme stored undiluted in ice.

For time course experiments fixed volume aliquots were removed from the refolding mix at different time points and directly assayed for HCA activity. For delay experiments 70S ribosome and 6AP drugs were added in the refolding mix at different time points from the start of refolding.

3. Results

3.1. Refolding of HCA with ribosomal folding modulators (RFMs) from E. coli and S. cerevisiae

We have studied the effect of the ribosomal folding modulators (RFMs) from bacterial and eukaryotic sources on the refolding of HCA. The enzyme when refolded in the absence any modulator (self-folding) regained only 25 ± 3% of native HCA activity. In comparison, significant to moderate recovery of its activity was observed when it was refolded in the presence of the 70S ribosome (60 ± 3%), 50S subunit (70 ± 3%) and 23S rRNA (45 ± 4%) isolated from *E. coli*. Similar refolding was also seen when 80S ribosomes from *S. cerevisiae* were used as RFM (60 ± 3%). These results are summarized in Fig. 1, where the refolding of HCA expressed as the percentage of native HCA activity is plotted against the relative concentration of the RFMs. In almost all cases maximum refolding was achieved when HCA to RFM ratio was roughly 1:1; in line with earlier observations [3,5]. The extent of refolding of HCA varied to some extent with the activity of the RFMs, which is probably dependent on the correct conformation and folding of the rRNA in the respective RFM. The highest refolding was seen with the 50S subunits, which can be attributed to the fact that the protein folding center of the ribosome i.e. the domain V of 23S rRNA is practically exposed in the 50S subunit and thus readily available to the protein substrate whereas it is partly masked by the 30S subunit in the 70S ribosome. In contrast, the lowest refolding was seen with 23S rRNA as RFM, which is probably due to partial loss of its conformation during the removal of the ribosomal proteins. In line with previous reports [5,9], the 30S subunits and the 16S rRNA did not show any protein folding activity (data not shown).

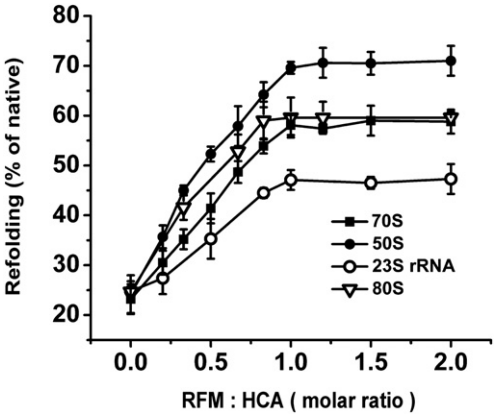

Fig. 1. Refolding of denatured HCA with bacterial and eukaryotic RFMs. HCA denatured with 6M Gdn-HCl was refolded in the presence of 70S ribosome (-■-), 50S subunit (-●-) and 23S rRNA (-○-) from *E. coli* MRE600 and 80S ribosome (-▽-) from yeast *S. cerevisiae*. The *X*-axis represents the molar ratio of the RFMs to HCA in the refolding reaction. On the *Y*-axis refolding is plotted as a percentage of the activity of the native HCA enzyme in identical concentration stored on ice. The first point in all refolding curves is without RFMs, indicating the percentage of self-folding.

3.2. 6AP and GA inhibit protein folding activity of bacterial RFMs

We have studied the effect of the drugs 6AP and GA on the folding activity of the RFMs from *E. coli*. As shown in the Fig. 2A and B, an increase in the concentration of 6AP and GA in the refolding reactions resulted in the decreased refolding of HCA in the presence of 70S/50S/23S rRNA as RFMs. These two drugs, however, showed no inhibitory effect on the self-folding of HCA. Also, no effect of the drugs was seen on the activity of native HCA even when present in high concentration (data not shown). From 6AP and GA titration curves, the half maximal inhibitory concentrations (IC_{50}) for these two drugs were estimated. For all three bacterial RFMs tested in these assays, the IC_{50} value for both 6AP and GA was around 90 μM. These values are quite close to the *in vivo* dosage of the drugs in yeast [32] and therefore of high physiological relevance.

Further, we have tested whether or not the drugs 6AP and GA affect the rate of 23S rRNA assisted refolding of HCA. For that, we have conducted time course experiments for HCA refolding in the presence of various concentrations of 6AP (Fig. 2C) and GA (Fig. 2D). The rate of 23S rRNA assisted refolding of HCA ($0.14 \pm 0.08\ s^{-1}$) was quite similar to the rate of its self-folding ($0.17 \pm 0.03\ s^{-1}$) suggesting that the interaction between the RFM and HCA is not the rate limiting step. Furthermore, the refolding rates did not alter in the presence of 6AP and GA in various concentrations although the amount of the refolded HCA was highly compromised. These results suggest that most likely 6AP and GA compete with HCA for the binding on the active site of the RFMs and just like the classical competitive inhibitors these drugs do not alter V_{max} of the reaction but increases K_m for the substrate. If this model is true then it should be possible to rescue the refolding of HCA from 6AP and GA inhibition by adding RFMs in excess.

To test this hypothesis we conducted HCA refolding experiments by adding increasing amount of 23S rRNA in the refolding reaction in the presence of the inhibitors 6AP and GA at high concentration. As shown in the Fig. 2E and F, the addition of an excess of 23S rRNA resulted in gradual recovery of the HCA refolding even in the presence of the drugs. With HCA to 23S rRNA ratio 1: 13 for 6AP and 1: 8 for GA, the effect of the drugs could be completely overcome and maximum refolding could be achieved. This result fits very well with the classical model of competitive inhibition where K_m for the enzyme–substrate interaction increases in the presence of the inhibitor and the inhibition on the substrate can be rescued by the addition of the enzyme in excess. Although the data collected here are not sufficient for the estimation of the exact K_m value for HCA and 23S rRNA interaction, we

Please cite this article in press as: S.D. Reis, et al., Mode of action of the antiprion drugs 6AP and GA on ribosome assisted protein folding, Biochimie (2011), doi:10.1016/j.biochi.2011.03.002

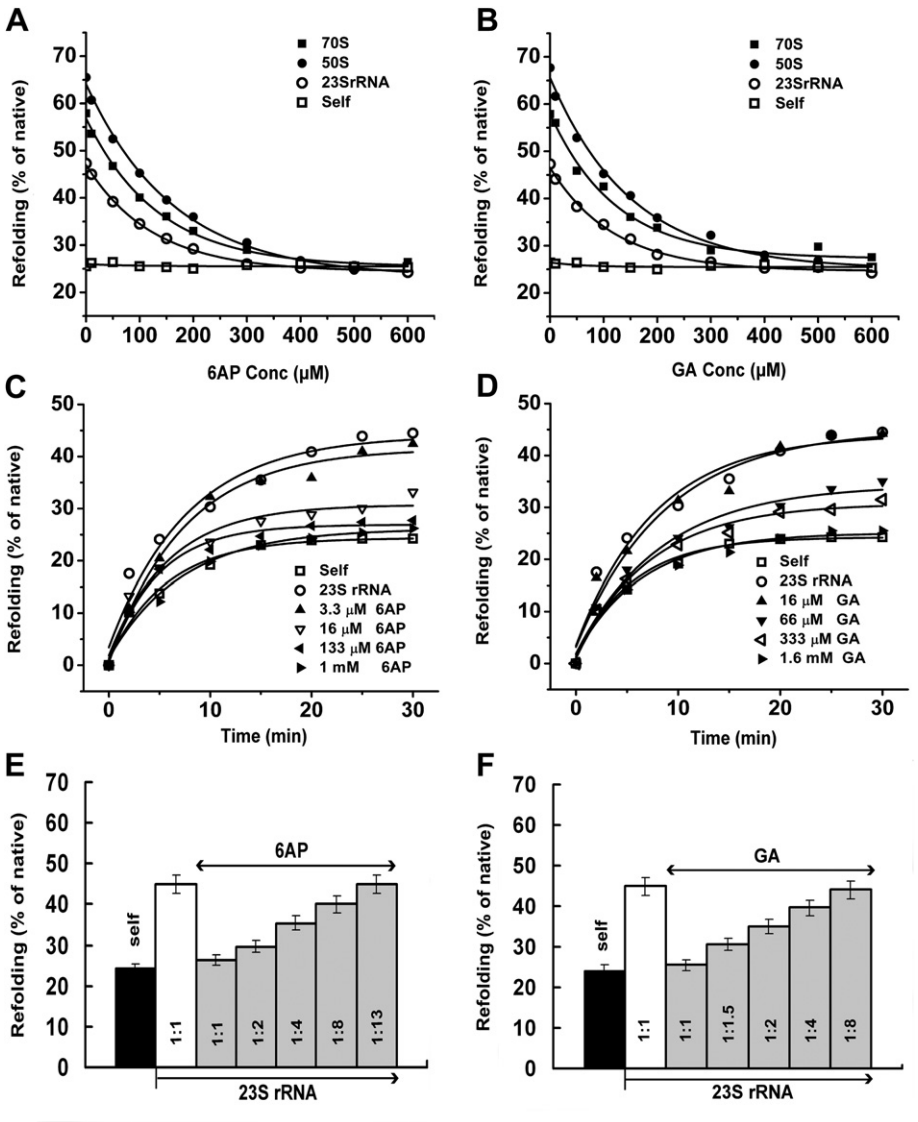

Fig. 2. Antiprion drugs 6AP (left panels) and GA (right panels) inhibit the protein folding activity of the bacterial RFMs. The effect of increasing concentration of 6AP (A) and GA (B) on HCA refolding (percentage of native HCA activity), without (-□-) and with RFMs 70S ribosome (-■-), 50S subunit (-●-) and 23S rRNA (-○-) (all 300–400 nM) from bacteria *E. coli* MRE600. The IC_{50} (half maximal inhibitory concentration) is determined from the *x*-intercept drawn at half maximal refolding considering the difference between the self and RFM assisted folding as 100%. Panels (C) and (D) present time courses of HCA refolding with 400 nM 23S rRNA as RFM and different concentration of 6AP and GA respectively. The curves are fitted with a single exponential equation using Origin 8.0 and the rates are estimated from the respective fits. Panels (E) and (F) show that 6AP and GA (1 mM each) mediated inhibition of HCA refolding can be rescued by the addition of 23S rRNA in excess. The labels on the bars indicate the molar ratio between HCA and 23S rRNA. The double headed arrow above the bars indicates that those reactions were done with 6AP (E) and GA (F). The first bar in black indicates self folding of HCA.

can certainly conclude that 6AP and GA competitively inhibit the RFM-assisted folding of HCA.

3.3. 6AP and GA also inhibit refolding activity of eukaryotic RFMs in a similar fashion

We have reported earlier the protein folding activity of the 80S ribosome from yeast. As 80S ribosome and particularly its RNA are highly conserved in the eukaryotes, we choose to use 80S ribosome from yeast to test the effect of the drugs on their folding activity.

When HCA was refolded in the presence of 80S ribosome isolated from *S. cerevisiae*, about 60 ± 3% refolding was observed, which is comparable with *E. coli* 70S assisted refolding and significantly higher than its self-folding (~25%) (Fig. 3A and B). Total RNA extracted from 80S ribosomes also showed 45 ± 3% reactivation (Fig. 3C and D). Titration of 6AP and GA in the refolding mix strongly reduced the yield of active HCA refolded with 80S ribosomes (Fig. 3A and B); or total RNA extracted from it (Fig. 3C and D). Also, as seen in the case of 23S rRNA assisted refolding of HCA, no effect on the rate of HCA refolding was observed when 80S ribosomes or total RNA from 80S was used as the folding modulator (data not shown). Furthermore, HCA refolding could be rescued despite the presence of 6AP and GA (0.5 mM each) by increasing the concentration of 80S ribosomes in the refolding reaction (Fig. 3E and F). These data suggested that the working principle of the RFMs is rRNA dependent and conserved from the bacteria to eukaryotes.

3.4. Determination of the time-span required for the interaction of RFM with the unfolded HCA and the drugs 6AP and GA

In order to determine the time-span of the interaction between the unfolded protein (HCA) and RFM we have added 70S ribosomes

Please cite this article in press as: S.D. Reis, et al., Mode of action of the antiprion drugs 6AP and GA on ribosome assisted protein folding, Biochimie (2011), doi:10.1016/j.biochi.2011.03.002

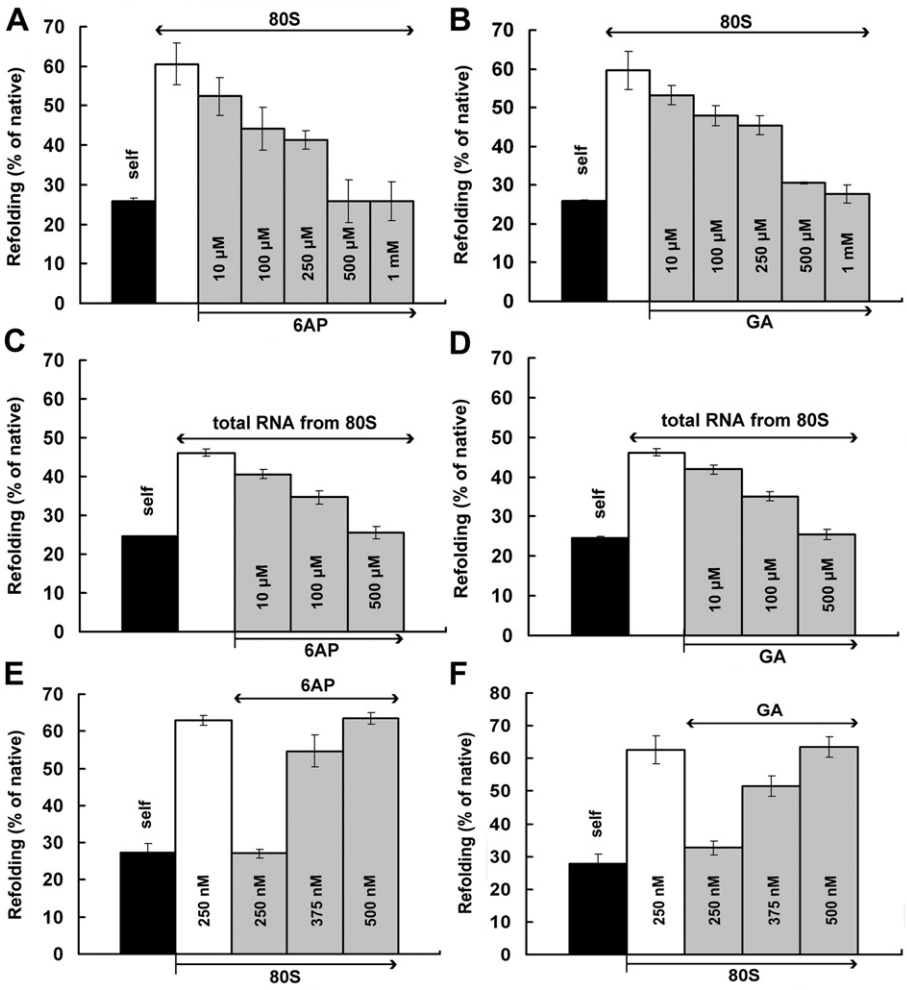

Fig. 3. Effect of 6AP (left panels) and GA (right panels) on refolding of HCA with eukaryotic RFMs from *S. cerevisiae*. 80S ribosomes in (A) and (B) and total RNA extracted from 80S in (C) and (D) increase the extent of HCA refolding compared to its self-folding (first bar in black). The refolding is estimated as a percentage of native HCA activity stored undiluted in ice. 6AP (A) and (C) and GA (B) and (D) added in increasing concentration inhibit RFMs and thereby reduce the extent of HCA refolding as seen for bacterial RFMs. (E) and (F) shows gradual recovery from 6AP and GA (0.5 mM each) mediated inhibition by the addition of 80S ribosome in excess (concentrations indicated in the respective bars). In all these experiments HCA concentration during refolding was 300 nM.

in the refolding reaction for HCA at various time points (Fig. 4A). Maximum refolding of HCA was obtained only when 70S ribosomes were either pre-incubated in the refolding reaction prior to the addition of denatured HCA or added simultaneously with it. Even a small delay of 5 s in the addition of 70S ribosomes resulted in a significant reduction of HCA refolding. No assistance from 70S ribosomes in the refolding of HCA was seen if it was added after ~30 s from the start of the refolding reaction. This result suggests that there is a very short time window in the early phase of protein folding during which the RFMs interact with the unfolded protein.

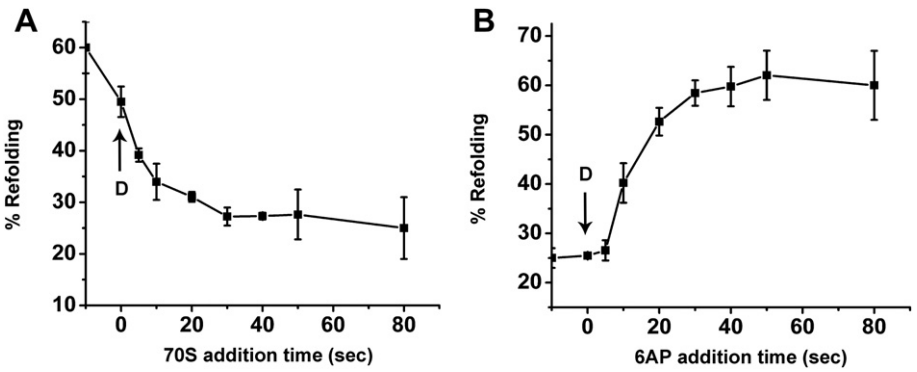

Fig. 4. Estimation of the time-span for unfolded protein interaction with 70S RFM. (A) The effect of delay in addition of 70S in HCA refolding reaction. (B) The effect of delay in the addition of 6AP in 70S assisted HCA refolding. In both panels the arrow labelled with D indicates the start point of refolding by addition of the denatured HCA in the refolding mix.

Please cite this article in press as: S.D. Reis, et al., Mode of action of the antiprion drugs 6AP and GA on ribosome assisted protein folding, Biochimie (2011), doi:10.1016/j.biochi.2011.03.002

This observation is further validated using 6AP-mediated inhibition of 70S assisted refolding of HCA. As shown in Fig. 4B, 6AP was added at different time points in HCA refolding reaction with 70S pre-incubated in it. Again, complete inhibition of 70S assisted refolding with 6AP was seen only when the drug was present in the reaction prior to the dilution of the unfolded HCA or was added simultaneously with it. Further delay in 6AP addition resulted in lesser inhibition of HCA refolding; no inhibition was seen if 6AP was added later than ~30 s. This is in very good correlation with 70S delay experiments (Fig. 4A) and supports the model of competitive inhibition. Thus, these results suggest that the RFMs act very early on the protein refolding process, thereby preventing the unfolded proteins to interact with each other and blocking aggregation.

4. Discussion

Studies on the ribosome as protein synthesis machinery of the cell have been in focus for the last 50 years. However, in the past ten years, the field has undergone dramatic development during which several high resolution crystallographic structures and cryo-EM reconstructions of the ribosome and its subunits, alone or bound with tRNA, mRNA and the translation factors became available [38]. Simultaneously, biochemical assays on protein synthesis in combination with molecular dynamics simulation have also progressed in such a way that it is now possible to understand the mechanism of individual steps of protein synthesis in near-atomic detail [39,40]. The main chemical step in protein synthesis, peptide bond formation, is catalysed by ribosomal RNA from the large subunit (50S in bacteria and 60S in eukaryotes); the catalytic site (also called the peptidyl transferase center) being the domain V of 23S rRNA in bacteria and 25S/28S in eukaryotes. Interestingly, the RNA sequence of this domain is highly conserved in all kingdoms of life and the central part of this domain is present even in the mitochondrial ribosome where several segments of rRNA have been substituted by the addition of ribosomal proteins. Thus, it is clear that the domain V of 23S rRNA or its equivalent RNA in eukaryotic ribosomes has been playing a central role in protein synthesis since the evolution of this function in the ribosome.

Folding nascent protein chains to their active form is a novel function of the ribosome in addition to its central role in protein synthesis [1,2]. Although the mechanism of protein folding by the ribosome has not been characterized yet at the molecular level, it has been documented for about 25 different proteins from various sources, with ribosomes from all three kingdoms of life [2]. Interestingly, the protein folding activity of the ribosome is a rRNA dependent function and the active site for this function has been mapped to the domain V of 23S rRNA of the large subunit of the bacterial ribosome (or its equivalent parts of the eukaryotic ribosomes) (Fig. 1), which, as discussed above is also responsible for the ribosome's protein synthesis function [16,41]. The finding that ribosomes can fold proteins opened many interesting questions such as whether protein folding is a co-translational or post-translational function of the ribosome, or whether it is a cis- or trans- activity of the ribosome with respect to its protein synthesis activity. Another important question in this context is whether the protein folding function of the ribosome coevolved with the protein synthesis function or not, and if not then which of the two functions appeared first. Although this is a highly debatable topic it is interesting to note that the domain V of 23S rRNA transcribed *in vitro* is capable of refolding proteins [13,15,41], whereas several attempts to form peptide bonds with similar *in vitro* transcripts have not been successful.

Antibiotics, which block protein synthesis in bacteria, have also been effective in blocking protein folding activity of the ribosome [11,12]. Thus, the discovery that the antiprion drugs 6AP and GA inhibit only the folding activity of the ribosome without affecting its protein synthesis activity [32] opened a great possibility for separating these two fundamental functions of the ribosome. The protein folding activity of the ribosome was often viewed as an evolutionary relic, *i.e.* the function of the ribosome in a primitive cell way back in evolution. The specific inhibition of the protein folding activity of the ribosome by 6AP and GA, which cure the prion phenotype in yeast and other eukaryotic cells, brought this novel function into the context of the modern cell. This is also evident as RFMs from two different kingdoms could refold HCA with similar efficiency.

Enzyme inhibitors are usually classified into three major groups based on their mode of operation. These are i) competitive, ii) noncompetitive and iii) uncompetitive inhibitors. Among these, the competitive inhibitors have been characterized very well with steady-state enzyme kinetics. In competitive inhibition, the inhibitor competes with the substrate for its binding site on the enzyme and thereby prevents the substrate from getting access to the active catalytic site. As a result, K_m between the enzyme and the substrate increases in the presence of the inhibitor, but V_{max} remains unaltered. As shown in the results section, 6AP and GA mediated inhibition of the protein folding activity of the ribosome matched very closely with this model. Most significantly, addition of 6AP and GA in HCA refolding experiments with RFMs greatly reduced the amount of refolded HCA molecules although the rate of the refolding was unchanged (Figs. 2A–D, 3A–D). It should be noted that the rate of RFM assisted refolding of HCA was essentially similar to that of its self refolding irrespective of the type and the source of the RFMs and also, independent of the presence of the antiprion drugs 6AP and GA (Fig. 2C and D). This observation has two important implications. First, it suggests that the interaction of the RFMs with the protein substrate is transient in nature, which drives the unfolded protein molecules into the productive folding pathway but does not influence their rate of folding. Second, most probably 6AP and GA compete with the unfolded protein for the same binding sites on the rRNA and thus occlude RFMs from interacting with the unfolded protein substrates. As a result, the number of the protein molecules directed to the productive folding pathway by the RFMs becomes less eventually resulting into lower percentage of refolding. Additional support to this analysis comes from the experiments where 6AP and GA mediated inhibition of HCA refolding with RFMs could be overcome by supplying RFMs in excess (Figs. 2E–F, 3E–F).

It should be noted that even with high concentrations of the drugs 6AP and GA (0.5–1 mM) the percentage of RFM-assisted HCA refolding was never less than its self-folding (Figs. 2A–D, 3A–D). Also, self-folding of HCA was completely uninfluenced by these two drugs. This result indicates primarily that 6AP and GA do not interact directly with HCA or in general terms with the protein substrate. This is also evident from the fact that 6AP and GA impose no inhibition on activity assay of HCA. It also demonstrates that the self-folded protein molecules belong to a special conformational group which can transform from the unfolded to the active folded state without any assistance and since 6AP and GA target RFMs, the self-folded molecules escape the inhibition by 6AP and GA.

The indication that the RFMs interact transiently with the substrate proteins prompted us to design experiments for measuring the time required for the interaction of the RFM with the protein substrate. For this, 70S was added to the HCA refolding reaction with gradual delay and the extent of refolding was measured in each case. The plot in Fig. 4A clearly indicates that the time needed for the 70S and unfolded HCA interaction is between 0 and 30 s, after which 70S addition could not assist HCA refolding. Consistent with this result, an effective inhibition of 70S assisted folding of HCA with 6AP was only obtained when the drug was added within the first 30 s from the beginning of HCA refolding. These results suggest that most probably the RFMs recognize and interact with the unfolded protein substrate at a very early stage of its folding and therefore could not

Please cite this article in press as: S.D. Reis, et al., Mode of action of the antiprion drugs 6AP and GA on ribosome assisted protein folding, Biochimie (2011), doi:10.1016/j.biochi.2011.03.002

be effective if added later when the substrate protein has essentially lost the particular conformation crucial for recognition by RFMs due to self- or misfolding. In fact, this is a remarkable difference with classical enzyme reactions, where the substrate remains essentially unchanged throughout the process and the enzyme molecules are recycled several times between the substrates. However, in an RFM catalysed refolding reaction the RFM cannot be recycled as the substrate changes its conformation with time. It also explains why RFMs are needed in a nearly 1:1 ratio with the protein substrate.

Do RFMs fall in the category of the protein chaperones? The answer is 'yes', if we compare the kinetics of protein folding as in both cases the yield of the refolded protein molecules increases but the rate of the refolding remains essentially unaltered. On the other hand, if we compare the mechanism of action of chaperones and RFMs the answer is 'no', as the RFMs unlike chaperones lack the ability to unfold the misfolded proteins. Also, most likely the RFMs recognize only a specific conformation of the substrate compared to a wide range of the unfolded and misfolded substrates recognized by chaperones, as evident from our delay experiments. Further experiments will be needed to clarify these issues. Our kinetic results add more evidences for a putative involvement of ribosome assisted protein folding in prion propagation. Starting from the first demonstration, a nearly perfect correlation exists between the antiprion activity of the two drugs and their ability to inhibit the protein folding activity of the ribosome [29,32]. This correlation is further strengthened as two close chemical derivatives of 6AP and GA (namely, 6APi and GAi) inactive in reverting the prion phenotype turn out to be also inactive in inhibiting this activity [32]. It has been recently shown that the protein folding activity of the ribosome could also be involved in another amyloid-based disease, OPMD (oculopharyngeal muscular dystrophy), which is an inherited myodegenerative disease due to the aggregation of PABPN1 protein within the nuclei of muscle cells [42]. Thus in summary, the protein folding activity of the ribosome could play an important and general role in various diseases linked to protein misfolding.

5. Conclusion

The antiprion drugs 6AP and GA inhibit the protein folding activity of the ribosome by competition with the protein substrate for binding to the ribosome. This result suggests a direct link between the ribosomal protein folding activity and various misfolding diseases.

Acknowledgement

SS acknowledges the funding support from the Swedish Research Council (*Vetenskapsrådet – NT, M, Linnéstöd, equipment)*, VR-SIDA (SRL), Göran Gustafsson Stiftelse, Carl Tryggers Stiftelse and Wenner Gren Stiftelse. SDS was a long-term research fellow funded by EMBO. Part of the funding for YP is provided by the Chinese Scholarship Council. MB and CV acknowledge funding support from Inserm and the ANR "Blanche" from the French government.

References

[1] W. Kudlicki, A. Coffman, G. Kramer, B. Hardesty, Ribosomes and ribosomal RNA as chaperones for folding of proteins, Fold Des 2 (1997) 101–108.
[2] D. Das, A. Das, D. Samanta, J. Ghosh, S. Dasgupta, A. Bhattacharya, A. Basu, S. Sanyal, C. Das Gupta, Role of the ribosome in protein folding, Biotechnol. J. 3 (2008) 999–1009.
[3] B. Das, S. Chattopadhyay, C. Das Gupta, Reactivation of denatured fungal glucose 6-phosphate dehydrogenase and E. coli alkaline phosphatase with *E. coli* ribosome, Biochem. Biophys. Res. Commun. 183 (1992) 774–780.
[4] A.K. Bera, B. Das, S. Chattopadhyay, C. Dasgupta, Refolding of denatured restriction endonucleases with ribosomal preparations from *Methanosarcina barkeri*, Biochem. Mol. Biol. Int. 32 (1994) 315–323.
[5] S. Chattopadhyay, B. Das, A.K. Bera, D. Dasgupta, C. Dasgupta, Refolding of denatured lactate dehydrogenase by *Escherichia coli* ribosomes, Biochem J. 300 (Pt 3) (1994) 717–721.
[6] S.C. Sanyal, D. Bhattacharyya, C. Das Gupta, The folding of dimeric cytoplasmic malate dehydrogenase. Equilibrium and kinetic studies, Eur. J. Biochem. 269 (2002) 3856–3866.
[7] B. Das, C.D. Gupta, Reconstitution of denatured *E. coli* alkaline phosphatase with *E. coli* ribosome, Indian J. Biochem. Biophys. 29 (1992) 512–515.
[8] R.H. Argent, A.M. Parrott, P.J. Day, L.M. Roberts, P.G. Stockley, J.M. Lord, S.E. Radford, Ribosome-mediated folding of partially unfolded ricin A-chain, J. Biol. Chem. 275 (2000) 9263–9269.
[9] B. Das, S. Chattopadhyay, A.K. Bera, C. Dasgupta, 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 (1996) 613–621.
[10] J. Ghosh, A. Basu, S. Pal, S. Chowdhuri, A. Bhattacharya, D. Pal, D.K. Chattoraj, C. DasGupta, Ribosome-DnaK interactions in relation to protein folding, Mol. Microbiol. 48 (2003) 1679–1692.
[11] S. Chattopadhyay, S. Pal, D. Pal, D. Sarkar, S. Chandra, C. Das Gupta, Protein folding in *Escherichia coli*: role of 23S ribosomal RNA, Biochim. Biophys. Acta. 1429 (1999) 293–298.
[12] S. Chattopadhyay, B. Das, C. Dasgupta, Reactivation of denatured proteins by 23S ribosomal RNA: role of domain V, Proc. Natl. Acad. Sci. U S A 93 (1996) 8284–8287.
[13] D. Pal, S. Chattopadhyay, S. Chandra, D. Sarkar, A. Chakraborty, C. Das Gupta, Reactivation of denatured proteins by domain V of bacterial 23S rRNA, Nucleic Acids Res. 25 (1997) 5047–5051.
[14] S. Pal, S. Chandra, S. Chowdhury, D. Sarkar, A.N. Ghosh, C.D. Gupta, Complementary role of two fragments of domain V of 23 S ribosomal RNA in protein folding, J. Biol. Chem. 274 (1999) 32771–32777.
[15] S.C. Sanyal, S. Pal, S. Chowdhury, C. DasGupta, 23S rRNA assisted folding of cytoplasmic malate dehydrogenase is distinctly different from its self-folding, Nucleic Acids Res. 30 (2002) 2390–2397.
[16] D. Samanta, D. Mukhopadhyay, S. Chowdhury, J. Ghosh, S. Pal, A. Basu, A. Bhattacharya, A. Das, D. Das, C. DasGupta, Protein folding by domain V of *Escherichia coli* 23S rRNA: specificity of RNA-protein interactions, J. Bacteriol. 190 (2008) 3344–3352.
[17] A. Basu, D. Samanta, D. Das, S. Chowdhury, A. Bhattacharya, J. Ghosh, A. Das, C. Dasgupta, In vitro protein folding by *E. coli* ribosome: unfolded protein splitting 70S to interact with 50S subunit, Biochem. Biophys. Res. Commun. 366 (2008) 598–603.
[18] G. Kramer, D. Boehringer, N. Ban, B. Bukau, The ribosome as a platform for co-translational processing, folding and targeting of newly synthesized proteins, Nat. Struct. Mol. Biol. 16 (2009) 589–597.
[19] S.B. Prusiner, Novel proteinaceous infectious particles cause scrapie, Science 216 (1982) 136–144.
[20] S.B. Prusiner, Prions, Proc. Natl. Acad. Sci. U S A 95 (1998) 13363–13383.
[21] C. Govaerts, H. Wille, S.B. Prusiner, F.E. Cohen, Evidence for assembly of prions with left-handed beta-helices into trimers, Proc. Natl. Acad. Sci. U S A 101 (2004) 8342–8347.
[22] A.S. Cohen, Clinical aspects of amyloidosis, including related proteins and central nervous system amyloid, Curr. Opin. Rheumatol. 6 (1994) 68–77.
[23] J. Stockel, J. Safar, A.C. Wallace, F.E. Cohen, S.B. Prusiner, Prion protein selectively binds copper(II) ions, Biochemistry 37 (1998) 7185–7193.
[24] J.M. Parry, B.S. Cox, Photoreactivation of ultraviolet induced reciprocal recombination, gene conversion and mutation to prototrophy in *Saccharomyces cerevisiae*, J. Gen. Microbiol. 40 (1965) 235–241.
[25] R.B. Wickner, [URE3] as an altered URE2 p.otein: evidence for a prion analog in *Saccharomyces cerevisiae*, Science 264 (1994) 566–569.
[26] M. Denis-Duphil, F. Lacroute, Fine structure of the ura 2 locus in *Saccharomyces cervisiae*. I. In vivo complementation studies, Mol. Gen. Genet. 112 (1971) 354–364.
[27] V. Coustou, C. Deleu, S. Saupe, J. Begueret, The protein product of the het-s heterokaryon incompatibility gene of the fungus *Podospora anserina* behaves as a prion analog, Proc. Natl. Acad. Sci. U S A 94 (1997) 9773–9778.
[28] S. Bach, N. Talarek, T. Andrieu, J.M. Vierfond, Y. Mettey, H. Galons, D. Dormont, L. Meijer, C. Cullin, M. Blondel, Isolation of drugs active against mammalian prions using a yeast-based screening assay, Nat. Biotechnol. 21 (2003) 1075–1081.
[29] S. Bach, D. Tribouillard, N. Talarek, N. Desban, F. Gug, H. Galons, M. Blondel, A yeast-based assay to isolate drugs active against mammalian prions, Methods 39 (2006) 72–77.
[30] D. Tribouillard, S. Bach, F. Gug, N. Desban, V. Beringue, T. Andrieu, D. Dormont, H. Galons, H. Laude, D. Vilette, M. Blondel, Using budding yeast to screen for anti-prion drugs, Biotechnol. J. 1 (2006) 58–67.
[31] D. Tribouillard-Tanvier, V. Beringue, N. Desban, F. Gug, S. Bach, C. Voisset, H. Galons, H. Laude, D. Vilette, M. Blondel, Antihypertensive drug guanabenz is active in vivo against both yeast and mammalian prions, PLoS One 3 (2008) e1981.
[32] D. Tribouillard-Tanvier, S. Dos Reis, F. Gug, C. Voisset, V. Beringue, R. Sabate, E. Kikovska, N. Talarek, S. Bach, C. Huang, N. Desban, S.J. Saupe, S. Supattapone, J.Y. Thuret, S. Chedin, D. Vilette, H. Galons, S. Sanyal, M. Blondel, Protein folding activity of ribosomal RNA is a selective target of two unrelated antiprion drugs, PLoS One 3 (2008) e2174.
[33] J.M. Armstrong, D.V. Myers, J.A. Verpoorte, J.T. Edsall, Purification and properties of human erythrocyte carbonic anhydrases, J. Biol. Chem. 241 (1966) 5137–5149.

Please cite this article in press as: S.D. Reis, et al., Mode of action of the antiprion drugs 6AP and GA on ribosome assisted protein folding, Biochimie (2011), doi:10.1016/j.biochi.2011.03.002

[34] M.J. Carter, D.S. Parsons, The purification and properties of carbonic anhydrases from guinea-pig erythrocytes and mucosae of the gastrointestinal tract, Biochem. J. 120 (1970) 797–808.
[35] M.D. Lloyd, R.L. Pederick, R. Natesh, L.W. Woo, A. Purohit, M.J. Reed, K.R. Acharya, B.V. Potter, Crystal structure of human carbonic anhydrase II at 1.95 A resolution in complex with 667-coumate, A. novel anti-cancer agent, Biochem. J. 385 (2005) 715–720.
[36] F. Gug, S. Bach, M. Blondel, J.M. Vierfond, A.S. Martin, H. Galons, A single step synthesis of 6-aminophenanthridines from anilines and 2-chlorobenzonitriles, Tetrahedron 60 (2004) 4705–4708.
[37] F. Gug, M. Blondel, N. Desban, S. Bouaziz, J.M. Vierfond, H. Galons, An expeditious synthesis of 6-aminophenanthridines, Tetrahedron Lett. 46 (2005) 3725–3727.
[38] T.M. Schmeing, V. Ramakrishnan, What recent ribosome structures have revealed about the mechanism of translation, Nature 461 (2009) 1234–1242.
[39] S. Trobro, J. Aqvist, Mechanism of peptide bond synthesis on the ribosome, Proc. Natl. Acad. Sci. U S A 102 (2005) 12395–12400.
[40] C. Huang, C.S. Mandava, S. Sanyal, The ribosomal stalk plays a key role in IF2-mediated association of the ribosomal subunits, J. Mol. Biol. 399 (2010) 145–153.
[41] S. Chowdhury, S. Pal, J. Ghosh, C. DasGupta, Mutations in domain V of the 23S ribosomal RNA of *Bacillus subtilis* that inactivate its protein folding property in vitro, Nucleic Acids Res. 30 (2002) 1278–1285.
[42] N. Barbezier, A. Chartier, Y. Bidet, A. Buttstedt, C. Voisset, H. Galons, M. Blondel, E. Schwarz, M. Simonelig, Antiprion drugs 6-aminophenanthridine and guanabenz reduce PABPN1 toxicity and aggregation in oculopharyngeal muscular dystrophy, EMBO Mol. Med. 3 (2011) 35–49.

Please cite this article in press as: S.D. Reis, et al., Mode of action of the antiprion drugs 6AP and GA on ribosome assisted protein folding, Biochimie (2011), doi:10.1016/j.biochi.2011.03.002