Arsenite interferes with protein folding and triggers formation of protein aggregates in yeast

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Running title: Arsenite-induced protein aggregation
Summary
Several metals and metalloids profoundly affect biological systems, but their impact on the proteome and mechanisms of toxicity are not fully understood. Here, we demonstrate that arsenite causes protein aggregation in *Saccharomyces cerevisiae*. Various molecular chaperones were found to be associated with arsenite-induced aggregates indicating that this metalloid promotes protein misfolding. Using in vivo and in vitro assays, we show that proteins in the process of synthesis/folding are particularly sensitive to arsenite-induced aggregation, that arsenite interferes with protein folding by acting on unfolded polypeptides, and that arsenite directly inhibits chaperone activity. Thus, folding inhibition contributes to arsenite toxicity in two ways: by aggregate formation and by chaperone inhibition. Importantly, arsenite-induced protein aggregates can act as seeds committing other, labile proteins to misfold and aggregate. Our findings describe a novel mechanism of toxicity that may explain the suggested role of this metalloid in the etiology and pathogenesis of protein folding disorders associated with arsenic poisoning.

Keywords: arsenic toxicity/protein aggregation/protein degradation/protein folding/yeast
Introduction

Certain metals and metalloids are known to adversely impact living organisms. For example, arsenic (As), cadmium (Cd) and chromium (Cr) are clearly toxic to cells and several compounds containing these agents are classified as human carcinogens (Beyersmann and Hartwig, 2008). Metal/metalloid toxicity is often attributed to inhibition of protein function through binding to free thiols or other functional groups in native proteins, or through displacement of essential metal ions in metal-dependent proteins (reviewed in: (Beyersmann and Hartwig, 2008; Sharma et al., 2011; Wysocki and Tamás, 2010)). Recent in vitro studies indicated that certain metals and metalloids inhibit refolding of chemically denatured proteins (Ramadan et al., 2009; Sharma et al., 2008). Whether non-native or nascent proteins are targeted also in living cells is still unknown and the cellular basis of metal/metalloid toxicity is poorly understood.

Folding of most nascent proteins is co-translational and assisted by molecular chaperones (Frydman, 2001). However, the processes of protein folding and quality control are prone to errors and susceptible to environmental stress conditions resulting in aberrant protein conformers and aggregation, as in the case of several neurodegenerative diseases and age-related disorders (Buchberger et al., 2010; Goldberg, 2003). To prevent the accumulation of potentially toxic protein aggregates, cells utilize highly conserved defence mechanisms including molecular chaperones that facilitate disaggregation and reactivation of aggregated proteins, and the ubiquitin-proteasome pathway that contributes to aggregate removal through degradation (Buchberger et al., 2010; Goldberg, 2003; Liberek et al., 2008; Sharma et al., 2009).

Trivalent arsenic [arsenite, As(OH)₃ or As(III)] has been shown to interact with several native proteins in mammalian cells, such as actin, tubulin and thioredoxin reductase, and the classical view is that enzyme inhibition is due to binding (Aposhian and Aposhian, 2006; Menzel et al., 1999; Zhang et al., 2007). As(III) disrupts the actin and tubulin cytoskeleton in budding yeast Saccharomyces cerevisiae (Pan et al., 2010; Thorsen et al., 2009), but whether this is caused by direct binding to actin and tubulin is not known. S. cerevisiae responds to As(III) exposure by strongly enhancing expression of chaperone- and proteasome-encoding genes; up-regulation of proteasomal components is mediated by the transcription factor Rpn4p and cells lacking this factor are As(III)-sensitive (Haugen et al., 2004; Thorsen et al., 2007; Thorsen et al., 2009). Rpn4p is also required for Cd (Thorsen et al., 2009) and Cr tolerance (Holland et al., 2007). Together, these observations suggest that metal-treated cells may accumulate damaged proteins that need to be eliminated for optimal tolerance.
Although several lines of evidence indicate that protein homeostasis and quality control are affected by metals and metalloids, the current understanding of how these agents impact the proteome in living cells is limited. This study demonstrates that As(III) interferes with protein folding and triggers formation of protein aggregates in *S. cerevisiae*. We also show that As(III)-induced protein aggregates can act as seeds committing other, labile proteins to misfold and aggregate. This mechanism of toxic action may explain the suggested role of As(III) in the etiology and pathogenesis of certain protein folding disorders.
Results

Arsenite triggers protein aggregation in vivo. To investigate whether metals/metalloids cause protein aggregation in living yeast cells, we monitored the subcellular distribution of a green fluorescent protein (GFP)-tagged version of the chaperone Hsp104p that promotes protein disaggregation and refolding (Glover and Lindquist, 1998). Hsp104p-GFP was evenly distributed throughout the cytosol in unexposed cells, whereas As(III) (NaAsO₂) triggered Hsp104p-GFP redistribution to distinct foci (Fig. 1A), previously shown to represent sites of protein aggregation (Kawai et al., 1999; Lum et al., 2004). Cells exposed to 0.1 mM As(III) formed 1-3 foci/cell, while cells exposed to 0.5 mM As(III) formed larger and more foci/cell (Fig. 1A), indicating that As(III)-induced aggregate formation was concentration-dependent. Protein aggregation was triggered by intracellular As(III), since Hsp104p-GFP foci were absent in cells overexpressing the plasma membrane-localized As(III) efflux permease Acr3p (Wysocki et al., 1997) (Fig. 1B). To confirm biochemical association of Hsp104p with As(III)-induced protein aggregates, we exposed cells to 0.1 mM or 0.5 mM As(III), isolated aggregated proteins by sedimentation, and probed the localization of Hsp104p by Western blot analysis. For untreated cells, the majority of Hsp104p was in the soluble fraction, whilst As(III) exposure generated a concentration-dependent shift of Hsp104p from the soluble to the aggregate fraction (Fig. 1C). The total amount of Hsp104p increased in response to As(III), as reported earlier (Sanchez et al., 1992) (Fig 1C).

We next asked whether other xenobiotic metals also trigger protein aggregation in vivo. For this, we exposed yeast to As(III), Cd (CdCl₂) or Cr (CrO₃) using concentrations that inhibited growth to similar degrees (Jin et al., 2008), and quantified protein aggregation by counting the fraction of cells with Hsp104p-GFP foci. Although all agents triggered protein aggregation, they did so to various degrees (Fig. 1D); As(III) was the most potent (~75% cells had aggregates), followed by Cd (~50%) and Cr (~15%). We conclude that As(III) is a potent trigger of protein aggregation in vivo.

As(III)-induced aggregates are largely distinct from P-bodies and stress granules. As(III) is known to induce formation of cytosolic structures in mammalian cells called processing bodies (P-bodies) and stress granules (SG) that consist of mRNAs and various RNA-binding proteins (Balagopal and Parker, 2009). We asked whether As(III) triggers P-body and/or SG formation also in budding yeast, and whether Hsp104p is part of those structures. For this, we simultaneously monitored the distribution of Hsp104p-GFP and red fluorescent protein (RFP)-tagged Dcp2p (P-body marker) or cyan fluorescent protein (CFP)-tagged Pab1p (SG...
marker) in As(III)-exposed cells. Similar to mammalian cells, P-bodies and SGs were formed in As(III)-treated yeast cells (Fig. 1E). However, the majority of the Hsp104p-containing foci were separate from Dcp2p- and Pab1p-containing foci. Even though we cannot exclude that Hsp104p is part of P-bodies and/or SGs, our data suggest that Hsp104p-containing aggregates represent largely distinct structures in As(III)-exposed S. cerevisiae cells.

**Clearance of aggregates involves the proteasomal system.** We next monitored Hsp104p-GFP distribution over time. Hsp104p-GFP was localized to foci after 1 hour of As(III) exposure, but after 3 hours, the majority of wild type cells had an even cytosolic Hsp104p-GFP distribution like in untreated cells (Fig. 2A). In contrast, Hsp104p-GFP foci were still present at the 3-hour time-point in rpn4Δ cells lacking the transcriptional regulator of proteasomal gene expression Rpn4p (Fig. 2A). Western blot analysis confirmed that Hsp104p was present in comparable amounts and associated with aggregates similarly in wild type and rpn4Δ cells (Fig. 1C). Together, these data suggest that wild type cells can clear the cytosol from protein aggregates, whilst rpn4Δ cells are impaired in aggregate clearance.

To explore the role of the proteasome in aggregate clearance, we measured the activity of the 26S proteasome in cell extracts prepared from untreated and As(III)-exposed cells. As(III)-treated wild type cells had ~7-8-fold greater 26S activity (Fig. 2B) and a small increase in the amount of proteasomal components (Fig. 2C) compared to untreated control cells. Addition of As(III) to cell extracts from unexposed control cells did not affect 26S activity (Fig. 2B), indicating that As(III) does not stimulate 26S activity in vitro. To test whether transcriptional activation of proteasomal genes is required for enhanced 26S activity, we also performed activity measurements using the rpn4Δ mutant. Although rpn4Δ cells were capable of increasing proteasomal activity, this mutant had clearly lower 26S activity (Fig. 2B) and reduced amounts of proteasomal components (Fig. 2C) compared to the wild type, both in the absence and presence of As(III).

Deletion of the RPN4 gene sensitized cells to As(III) (Fig. 2D) as previously reported (Haugen et al., 2004; Thorsen et al., 2009). The proteasome-defective mutants cim5-1/rpt1-1 (defective in one of the ATPases of the 19S regulatory particle (Ghislain et al., 1993)) and pre1-1 pre4-1 (defective in the β-type subunits of the catalytic 20S core (Hilt et al., 1993)) were also clearly more As(III) sensitive than the corresponding wild type (Fig. 2D). Taken together, aggregate clearance involves the proteasomal system and enhanced proteasomal activity is important for As(III) tolerance.
**As(III) does not induce mistranslation.** One way As(III) could promote protein aggregation is by causing errors during translation. To test this, we used a yeast mutant carrying an ade1-14 UGA stop codon and scored red pigmentation as a read-out for mRNA mistranslation (Liu and Liebman, 1996). The drug paromomycin, that is known to cause mistranslation, suppressed the red pigmentation associated with the ade1-14 allele (Fig. 3A). As(III) did not cause any colour change of this mutant, and addition of both As(III) and paromomycin did not produce a stronger suppression of the red pigmentation than paromomycin alone (Fig. 3A). Cells grown in liquid medium were more sensitive to As(III) and paromomycin together than to either agent on its own (Fig. 3B). In contrast to As(III), Cr suppressed red pigmentation of the ade1-14 strain (Holland et al., 2007) and caused a strong synergistic toxicity with paromomycin (Fig. 3C), consistent with the notion that Cr induces mistranslation (Holland et al., 2007). We conclude that As(III) is not an efficient inducer of protein mistranslation.

**Chaperones are associated with As(III)-aggregated proteins.** To better understand how As(III) impacts the proteome, we exposed wild type cells to 1.5 mM As(III) and isolated aggregated proteins by sedimentation. The isolated proteins were separated by SDS-PAGE and identified by mass spectrometry. A total of 143 aggregation-sensitive proteins were unambiguously identified in As(III)-exposed cells (Supplemental material). To characterize these proteins, we searched for functional categories (according to FunCat, Munich Information Center for Protein Sequences (MIPS)) that were significantly enriched (false discovery rate (FDR) < 5%) in the aggregated protein-set compared to the *S. cerevisiae* genome (Fig. 1F; Supplemental material). Highly overrepresented functions in the As(III)-aggregated protein-set were related to protein synthesis (>3-fold enrichment; Fischer’s exact test, *p*<10^{-9}), metabolism (1.9-fold; *p*=5x10^{-9}), proteins with binding function or cofactor requirement (1.9-fold; *p*=6x10^{-6}), protein folding and stabilization (>4-fold; *p*<3x10^{-4}), and unfolded protein response (>7-fold; *p*<10^{-9}). The latter groups contained various chaperones including Hsp104p and its co-chaperones Ydj1p and Ssa1p, ribosome-associated chaperones (Ssb2p, Zuo1p, Ssz1p, Egd1p, Egd2p), components of the CCT (chaperonin-containing T) complex (Cct1p/Tcp1p, Cct3p, Cct4p, Cct8p), and the Hsp70-co-chaperones Sis1p and Sse1p (Supplemental material). These chaperones are probably associated and cosedimented with their misfolded and aggregated protein substrates, suggesting that As(III) promotes protein misfolding in vivo.
As(III) targets proteins in the process of synthesis/folding and interferes with chaperone activity in vivo. To explore whether As(III) causes misfolding and aggregation of proteins during synthesis/folding or of already folded native proteins, we monitored Hsp104p-GFP distribution in cells treated simultaneously with As(III) and the protein synthesis inhibitor cycloheximide (CHX). Interestingly, no Hsp104p-GFP foci were formed when cells were exposed to As(III) in the presence of CHX, neither at the lower (0.1 mM) nor at the higher (0.5 mM) As(III) concentration (Fig. 4A). We next exposed cells to 42°C for one hour, a condition that can lead to thermal unfolding and aggregation of native proteins (Singer and Lindquist, 1998). Importantly, Hsp104p-GFP was redistributed to foci in response to high temperature both in the absence and presence of CHX (Fig. 4A), indicating that the mechanisms by which As(III) and heat cause protein aggregation are distinct. These data suggest that proteins in the process of synthesis/folding are likely to be the prime targets of As(III)-induced aggregation. To substantiate this finding, exponentially growing yeast cells were pulsed for 5 minutes with 35S-methionine to label newly synthesized proteins, in the absence or presence of As(III) where As(III) was added 15 minutes prior to 35S-methionine labelling. Next, translation was stopped by adding CHX and aggregated proteins were isolated by sedimentation. While equal amounts of radioactivity was incorporated into newly synthesized proteins in untreated and As(III)-exposed cells (total lysate), a higher proportion of the radioactivity was present in the aggregate fraction of As(III)-treated cells compared to untreated cells (Fig 4B). Quantification of the 35S-containing material showed a 2.5-fold increase in aggregation of newly synthesized proteins during As(III)-treatment compared to unexposed cells (Fig 4B).

The presence of ribosome-associated chaperones in the As(III)-aggregated protein-set (Supplemental material; see above) was another indication that nascent proteins are vulnerable to this metalloid. Co-translational protein folding in yeast is assisted by two functionally interconnected ribosome-associated chaperone systems called SSB-RAC (stress 70 B-ribosome-associated complex) and NAC (nascent polypeptide-associated complex) while the yeast Hsp110 chaperone Sse1p functions as a nucleotide exchange factor for both ribosome-associated and cytosolic Hsp70s (Frydman, 2001; Koplin et al., 2010). Loss of SSB (ssb1Δ ssb2Δ) or Sse1p (sse1Δ) function leads to protein aggregation and reduced cell viability under conditions of protein folding stress, these effects being aggravated by additional deletion of NAC (egd1Δ egd2Δ btt1Δ) (Koplin et al., 2010). Cells lacking either NAC or SSE (sse1Δ) were clearly more As(III)-sensitive than wild type cells, whilst NAC
SSE cells showed an additive sensitivity (Fig. 4C), consistent with the notion that As(III) causes protein folding stress. Unexpectedly, growth of wild type and the mutants lacking SSB or NAC SSB was similarly affected by As(III) (Fig. 4C), even though these mutants accumulate aggregated proteins and experience protein folding stress (Koplin et al., 2010). Interestingly, SSB and NAC SSB deficient cells have strongly reduced translational activity (Koplin et al., 2010); hence, diminished translational activity may protect cells from As(III) toxicity.

To investigate whether As(III) has an inhibitory effect on the chaperone/protein folding systems, we first induced aggregate-/Hsp104p-GFP foci formation by heat (42°C), then lowered the temperature to 30°C and monitored aggregate clearance in the absence or presence of As(III). To prevent de novo formation of As(III)-induced aggregates when shifting the temperature back to 30°C, we added CHX as indicated (Fig. 4D). 3 hours after the shift to 30°C, cells had cleared the cytosol from heat-induced aggregates/Hsp104p-GFP foci. In contrast, cells were defective in aggregate clearance in the presence of As(III) (Fig. 4D). Since As(III) does not inhibit but rather enhances proteasomal activity (Fig. 2), these data indicate that As(III) may interfere with chaperone activity in vivo.

**As(III) inhibits protein folding in vitro.** To further explore the impact of As(III) on protein folding, we monitored folding of firefly luciferase using several in vitro assays. As(III) inactivated native luciferase in a slow time-dependent process (Fig. 5A), indicating that the folded protein is not particularly susceptible to As(III)-mediated inactivation. In contrast, As(III) efficiently inhibited the spontaneous refolding of chemically denatured luciferase (Fig. 5B). When the refolding assay was performed in the presence of the *Escherichia coli* DnaK/DnaJ/GrpE chaperone system and ATP, the rate and yield of refolding in the absence of As(III) increased about 4-fold (Fig. 5C). The inhibitory effect of As(III) was somewhat more pronounced in the presence of chaperones than for spontaneous refolding (Fig. 5E); nevertheless, the DnaK/DnaJ/GrpE chaperone system was still capable of increasing the yield of refolding in the presence of metalloid (Fig. 5C). Finally, As(III) interfered with chaperone-mediated disaggregation and refolding of stable aggregates of heat-denatured luciferase (Fig. 5D); in this case the inhibitory efficacy was slightly lower than in the case of chemically denatured luciferase (Fig. 5E). Collectively, these data demonstrate that As(III) is a potent inhibitor of protein folding in vitro, acting primarily on unfolded polypeptides.
**Protein aggregation contributes to As(III) toxicity.** Since accumulation of aggregated proteins is potentially toxic to cells (Goldberg, 2003), we asked whether treatments that prevent or exacerbate protein aggregation also affect As(III) tolerance. Indeed, treating yeast cells with CHX not only diminished protein aggregation (Fig. 4A) but also resulted in improved As(III) tolerance (Fig. 4E). The opposite correlation also exists; cells defective in aggregate clearance (rpn4Δ) are sensitized to As(III) (Fig 2A,D). Hence, accumulation of protein aggregates generated during As(III) exposure contribute to the toxicity of this metalloid.

**As(III)-induced aggregates seed protein aggregation in the absence of As(III).** Protein aggregates can inhibit the native folding of other proteins, leading to inactivation and aggregation (Gidalevitz et al., 2006). We therefore addressed the possibility that stable As(III)-induced luciferase aggregates may inhibit native refolding of urea-unfolded luciferase in the absence of free As(III) ions. Following removal of free As(III) by gel filtration, we found that substoichiometric amounts of both As(III)-free aggregates (Fig. 6A) and As(III)-induced aggregates (Fig. 6B) inhibited native luciferase refolding. Remarkably, the IC₅₀ of As(III)-induced aggregates that was four times lower than that of As(III)-free aggregates (Fig. 6C). Thus, luciferase refolding was affected by substoichiometric concentrations of aggregated molecules, a phenomenon evocative of seed-induced proteinaceous aggregation, as previously observed in the case of many disease-causing protein aggregates (Aguzzi and Rajendran, 2009; Ben-Zvi and Goloubinoff, 2002). We conclude that As(III)-aggregated species have a strong inhibitory effect on de novo folding of proteins that have not encountered any metalloid during refolding. Hence, As(III)-aggregated species can act as seeds committing other, labile proteins to misfold and aggregate.
Discussion

Arsenic is a major environmental pollutant and chronic exposure is associated with neurotoxicity, cardiovascular abnormalities, nephrotoxicity, and with cancers of the skin, bladder and lung. This metalloid has also a long history of usage in medical treatment. Yet, the molecular details of its biological actions are not completely understood (Singh et al., 2011; Wysocki and Tamás, 2010). Here, we describe a novel mechanism of As(III) toxicity that involves accumulation of protein aggregates due to impaired protein folding in vivo.

**As(III) triggers protein aggregation.** We demonstrated that As(III) is a potent inducer of protein aggregation in *S. cerevisiae*; aggregate-formation is concentration-dependent and triggered by ‘free’ intracellular As(III) (Fig. 1). Moreover, our data indicated that As(III)-aggregated proteins can act as seeds committing other proteins to misfold and aggregate (Fig. 6). We identified 143 proteins that aggregated during As(III) exposure and various molecular chaperones were found to co-sediment with As(III)-induced protein aggregates (Fig. 1F, Supplemental material). These chaperones, including Hsp104p and its co-chaperones Ydj1p and Ssa1p, as well as the cytosolic chaperones Sis1p and Sse1p, are often found stalled on aggregates as a result of failed attempts to convert the aggregates into non-aggregated functional species (Shorter and Lindquist, 2008). Such a stalling effect adds to the well-established proteasome stalling, which is often observed in cases of ageing or misfolding diseases (Hinault et al., 2011; Kern et al., 2010; Macario and Conway de Macario, 2002).

Interestingly, heat and As(III) appear to affect the proteome in distinct ways; whilst heat may cause thermal unfolding and aggregation of native proteins, As(III) generates protein aggregation primarily by interfering with folding of nascent polypeptides (Fig 4). As(III) is a potent inducer of Hsp104p and other heat shock proteins; however, whilst cells lacking Hsp104p are 100- to 1000-fold more sensitive to heat than wild type cells, Hsp104p provides only a 2- to 3-fold survival advantage during exposure to lethal concentrations of As(III) (Sanchez et al., 1992). There are several ways to interpret these observations. From an aggregation point of view, aggregates produced by heat and As(III) may be fundamentally dissimilar. From as toxicity point of view, the main lesion produced by As(III) may not be accessible to Hsp104p (different compartment) or may not be a protein. From a chaperone point of view, As(III) may directly inhibit Hsp104p or other chaperones required for the restoration of folding homeostasis accounting for its poor performance in As(III) tolerance and its inability to dissolve aggregates when As(III) is present.
As(III) affects the folding of nascent proteins and inhibits the chaperone/protein folding systems. Several observations indicate that As(III) triggers protein aggregation primarily by targeting unfolded/nascent proteins. Firstly, the presence of ribosome-associated chaperones in the As(III)-aggregated protein-set suggested that nascent proteins are vulnerable to this metalloid. Indeed, cells defective in folding of nascent polypeptides were As(III)-sensitive probably due to enhanced protein folding stress (Fig. 4). Secondly, in vitro data demonstrated that native firefly luciferase was moderately affected by As(III), whilst spontaneous and chaperone-mediated luciferase refolding were strongly inhibited (Fig. 5). The notion that unfolded/nascent proteins are the main target for As(III)-induced aggregation in vivo was further supported by the translational shut-down experiment, in which CHX prevented As(III)-induced protein aggregation, and by the fact that As(III)-exposed cells showed a 2.5-fold increase in aggregation of 35S-labelled material (Fig 4A,B). Thirdly, in vitro data revealed that As(III) had a greater impact on the refolding protein than on the chaperone system; DnaK/DnaJ/GrpE increased the yield of luciferase refolding 3.3-fold in presence of 600 µM As(III) and 4.3-fold in the absence of metalloid. Hence, 600 µM As(III) resulted in ~24% lower chaperone activity while the spontaneous refolding luciferase had lost ~70% of its efficiency (compare final yields in Fig. 5B,C). Although As(III) acts primarily on nascent proteins, the in vitro experiment above showed that this metalloid also interferes directly with chaperone activity. This interference was confirmed in vivo since As(III) inhibited clearance of heat-induced protein aggregates (Fig. 4D). Thus, folding inhibition contributes to As(III) toxicity in two ways; by aggregate formation and by chaperone inhibition.

This work also adds new knowledge on how As(III) affects the actin and tubulin cytoskeleton. The CCT chaperonin complex is required for proper folding of actin and tubulin, as well as many other substrates (Frydman, 2001). As(III) was previously shown to disrupt the actin and tubulin cytoskeleton in vivo (Pan et al., 2010; Thorsen et al., 2009) and to inhibit actin folding by CCT in vitro (Pan et al., 2010). However, it was not clear how As(III) inhibited CCT since neither substrate binding nor ATPase activity of CCT was affected by this metalloid (Pan et al., 2010). Here, we found four out of eight CCT components as well as the CCT substrate β-tubulin (Tub2p) in the set of As(III)-aggregated proteins. Currently, we do not know whether As(III) causes aggregation of de novo synthesized CCT components or whether nascent chains of actin and tubulin aggregate during folding, thereby pulling CCT into the insoluble fraction. In any case, aggregation of CCT components will probably affect the folding process, increase the cellular load of unfolded actin and tubulin, and delay cytoskeleton recovery during As(III) exposure.
Collectively, our in vivo and in vitro data show that folding of unfolded/nascent proteins is affected by As(III), resulting in accumulation of aggregated proteins. The fact that cells defective in chaperones and protein folding systems are As(III)-sensitive underscores their importance for protection against metalloid toxicity.

**Protein aggregation and clearance contribute to As(III) toxicity and tolerance, respectively.**

We showed here that a failure in aggregate clearance correlates with As(III) sensitivity whilst diminished aggregation enhanced tolerance (Figs 2, 4). Accumulation of toxic aggregates puts a higher demand on the proteasomal system. Indeed, *S. cerevisiae* responded to As(III) by enhancing 26S proteasome activity and this increase in proteasomal activity was shown to be important for tolerance (Fig. 2). While the amount of proteasomal components was slightly elevated, the 26S activity rose about 7-fold. Moreover, *rpn4Δ* cells were capable of increasing 26S activity in response to As(III), despite being defective in transcriptional activation of proteasomal gene expression (Haugen et al., 2004). Apparently, proteasomal activity and/or assembly is regulated post-transcriptionally during As(III) stress. Mammalian cells respond to As(III) by inducing expression of the AIRAP protein which then associates with the proteasome. Although the exact role of AIRAP in vivo is not fully understood, AIRAP clearly improves proteasome stability and activity in vitro (Stanhill et al., 2006). Whether yeast has a protein with similar function as mammalian AIRAP, is currently unknown.

Our data also suggested that diminished translational activity can protect cells from As(III) toxicity (Fig. 4). Consistent with this view, recent genome-wide studies revealed that mutations in genes encoding ribosomal protein and biogenesis factors result in greater As(III) tolerance (Dilda et al., 2008; Pan et al., 2010). Given that cells treated with the translational inhibitor CHX accumulated fewer aggregates and displayed improved tolerance (Figs 4A,E), it is tempting to speculate that cells slow down protein synthesis during As(III) exposure to avoid protein aggregation and toxicity. The fact that As(III)-exposed cells strongly down-regulate expression of genes with functions related to protein synthesis with a concomitant up-regulation of chaperone and proteasomal gene expression (Haugen et al., 2004; Thorsen et al., 2007) supports this notion.

**Is protein aggregation a general mechanism of metal action?** We showed that several metals can trigger protein aggregation in vivo (Fig. 1). Although As and Cd as well as lead (Pb) and mercury (Hg) efficiently inhibit protein folding in vitro (Ramadan et al., 2009; Sharma et al., 2008; Sharma et al., 2011), Cd and Cr appeared less potent than As(III) in triggering protein aggregation in vivo. It is possible that Cd and Cr do not enter cells as efficiently as As(III). Alternatively, the observed difference in potency could be a
consequence of distinct modes of biological action; for example, Cr causes protein mistranslation (Holland et al., 2007) whilst As(III) does not (Fig. 3); As(III) interferes with CCT activity in vitro whilst Cd does not (Pan et al., 2010). The fact that Cd is very efficient in inhibiting protein folding in vitro but less potent in triggering protein aggregation in vivo, could potentially be explained by a lack of CCT inhibition given that CCT participates in the folding of as much as 10-15% of all cytosolic proteins in mammalian cells (Thulasiraman et al., 1999).

Conclusions. This study demonstrated that As(III) is a potent inhibitor of protein folding, and a powerful trigger of protein aggregation in vivo. This novel mechanism of As(III) toxicity does not contradict previously identified targets and modes of action such as induction of oxidative stress, impairment of DNA repair and disruption of enzyme function (Aposhian and Aposhian, 2006; Beyersmann and Hartwig, 2008; Singh et al., 2011; Wysocki and Tamás, 2010). Chronic arsenic exposure is associated with a variety of neurodegenerative disorders caused by aberrant protein folding including Parkinson’s and Alzheimer’s diseases (Gong and O’Bryant S, 2010; Singh et al., 2011), and our data suggest that the deleterious effects of As(III) may result both from short-term, direct interactions of relatively high concentrations of As(III) ions with folding proteins, alongside long-term indirect interactions of low concentrations of As(III)-aggregated seeds that in turn commit other, labile proteins to misfold and aggregate. In mammals, these effects may become amplified with age when the efficiency of chaperone- and protease-dependent proteostasis declines (Hinault et al., 2011). Hence, the mechanism of action described here could contribute to the effects of arsenic in such protein folding diseases or other human disorders.
Materials and methods

Yeast strains, plasmids and growth conditions. *S. cerevisiae* strains and plasmids used in this study are listed in Table 1. The HSP104-GFP *rpn4Δ* strain was generated by crossing BY4741 *HSP104-GFP-HIS3-MX6* with BY4742 *rpn4Δ::KanMX4* followed by sporulation, tetrad dissection and selection according to (Kaiser et al., 1994). Yeast strains were grown on rich YPD medium (1% yeast extract, 2% peptone, 2% glucose) or on minimal SC (synthetic complete) medium (0.67% yeast nitrogen base) supplemented with auxotrophic requirements and 2% glucose as a carbon source. Growth assays were carried out in liquid medium or on plates as previously described (Warringer and Blomberg, 2003; Wysocki et al., 2004). Sodium arsenite (NaAsO₂), chromium trioxide (CrO₃), cadmium chloride (CdCl₂), paromomycin sulfate, and cycloheximide (CHX) (all from Sigma-Aldrich) were added to the cultures at the indicated concentrations. Mistranslation was scored using a qualitative plate assay as previously described (Holland et al., 2007).

Proteasomal activity measurements. Yeast growing exponentially in SC medium were either untreated or exposed to 0.5 mM As(III). After 3 hours of exposure, cells were harvested by centrifugation, washed twice in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) and resuspended in 700 µl cold extraction buffer (10% glycerol, 10 mM Tris-HCl pH 7.5, 1mM EDTA, 0.5mM DTT, 5 mM MgCl₂, pH 7.4). Cells were disrupted with glass beads and vortex (3 x 20 seconds with 2 minutes on ice in between) followed by centrifugation. The protein concentration of the resulting supernatant (whole-cell extract) was quantified according to (Bradford, 1976). To measure proteasomal activity, whole-cell extracts (20 µg of total protein) were incubated with 200 µM of the fluorogenic proteasome substrate suc-LLVY-AMC (Biomol International) for one hour at 30°C, and fluorescence intensity of the free AMC formed was monitored in a Synergy2 Multi-Mode Microplate Reader (BioTek). Proteasomal activity was also measured in the presence of 20 µM MG132 proteasome inhibitor (Biomol International), and 7-amino-4-methylcoumarin was used for obtaining the standard curve (Biomol International).

Microscopy. Yeast cells expressing GFP-, CFP- and RFP-fusion proteins of interest were grown to mid-log phase in SC medium containing appropriate amino acid requirements. Cells were washed twice with water or PBS and the GFP, CFP or RFP signals were observed in living cells using a Leica DM RXA (Leica Microsystems) or Zeiss Axiovert 200M (Carl Zeiss MicroImaging) fluorescence microscope. The microscopes were equipped with 100x HCX PL
Fluotar 1.30 (Leica) and Plan-Apochromat 1.40 (Zeiss) objectives and appropriate fluorescence light filter sets. Images were captured with digital camera (Hamamatsu C4742-95 (Hamamatsu Photonics) or AxioCamMR3(Zeiss)) and QFluoro (Leica) or AxioVision (Zeiss) software, and processed with Photoshop CS (Adobe Systems). Where indicated, cells were treated with As(III) or heat (42°C) in the absence or presence of 0.1 mg/ml cycloheximide. To compare protein aggregation by different metals, cells were exposed to 0.4 mM As(III), 0.4 mM Cr, or 5 µM Cd for 1 hour and Hsp104p-GFP localization monitored as above. To quantify protein aggregation, the fraction of cells with Hsp104p-GFP foci was determined by visual inspection of 350-900 cells.

**Protein detection and identification.** Wild type (BY4742) and rpn4Δ strains were grown to exponential phase (A_{600} ~ 0.6) in YPD medium and equivalent cell numbers (10 A_{600} units) were used to analyse protein aggregation as described previously (Rand and Grant, 2006). Briefly, cells were disrupted in lysis buffer (50 mM potassium phosphate buffer, pH 7, 1 mM EDTA, 5% glycerol, 1 mM phenylmethylsulfonyl fluoride and Complete Mini protease inhibitor cocktail (Roche)), and the membrane and aggregated proteins were isolated by centrifugation at 15,000 g for 20 minutes. Membrane proteins were removed by washing twice with 320 µl lysis buffer and 80 µl of 10% Igepal CA 630 (NP-40) (Sigma-Aldrich), centrifuging at 15,000 g for 20 minutes each time, and the final aggregated protein extract was resuspended in 100 µl of lysis buffer. Soluble and aggregated protein extracts were analysed by 12% reducing SDS-PAGE and Western blotting using an anti-Hsp104p antibody (kindly provided by Mick Tuite, University of Kent). Bound antibody was visualized by chemiluminescence (Pierce) after incubation of the blot in donkey anti-rabbit immunoglobulin-horseradish peroxidase conjugate (Amersham Pharmacia Biotech). To detect proteasomal components by Western blot, primary antibodies targeting Rpt1p and Rpt3p (diluted 1:5,000; Abcam) were used whilst an antibody targeting the kinase Hog1p (diluted 1:2000; Santa Cruz Biotechnology) was used as loading control. Secondary donkey anti-goat and donkey anti-rabbit antibodies (diluted 1:15,000; LI-COR Biosciences) and the Odyssey® Infrared Imaging System (LI-COR Biosciences) were used for visualization.

For protein identification, yeast cells were exposed to 1.5 mM As(III) for one hour, aggregated proteins were isolated as above, and the SDS-PAGE gels were stained using colloidal Coomassie blue (Sigma-Aldrich). Proteins were excised, trypsin-digested, and identified using liquid chromatography-mass spectrometry (LC-MS) in the Biomolecular Analysis Facility (Faculty of Life Sciences, University of Manchester). Proteins were identified using the Mascot
mass fingerprinting programme (www.matrixscience.com) to search the NCBInr and Swissprot databases. As a control, we also isolated and identified proteins that aggregated in the absence of As(III). Proteins found to aggregate both under control conditions and As(III) exposure were considered as generally aggregation-prone and were not included in the analyses below.

Newly synthesized proteins were labelled with $^{35}$S-methionine as previously described (Koplin et al., 2010). Cells were first starved for 1 hour in methionine-free SC medium, then labelled with 20 µCi/ml $^{35}$S-methionine for 5 min, quickly chilled and treated with 300 µg/ml CHX to stop protein translation. Cells were disrupted with glass beads and vortex (3 x 1 minute) in lysis buffer (20mM Na-phosphate, pH 6.8, 10mM DTT, 1mM EDTA, 0.1% Tween, 1mM PMSF, protease inhibitor cocktail and 3 mg/ml zymolyase). Supernatants were adjusted to equal protein concentrations and aggregated proteins were pelleted at 16,000 g for 20 minutes at 4°C. Proteins were separated by SDS-PAGE (10%) and visualized by autoradiography (Molecular Imager FX, Bio-Rad).

Bioinformatics and statistical analyses. Enriched functional categories were set with FDR<5%. Enrichments were calculated by taking the ratio of the relative protein content in the As(III)-aggregated set to the relative content in the genome for each category. $p$-values were calculated by Fisher’s exact test.

In vitro folding assays. *Escherichia coli* DnaK was expressed and purified as described previously (Feifel et al., 1996; Sharma et al., 2008), whilst DnaJ and GrpE (Schönfeld et al., 1995a; Schönfeld et al., 1995b) were kindly provided by Dr. H. J. Schönfeld (F. Hoffmann-La Roche, Basel, Switzerland). *Photinus pyralis* luciferase was obtained from Sigma-Aldrich. Protein concentrations were determined and the proteins stored as described previously (Sharma et al., 2008).

To prepare chemically denatured luciferase, 1 mg lyophilized luciferase was dissolved in 1 ml of 50 mM Tris acetate, 50 mM potassium perchlorate, 15 mM magnesium acetate, pH 7.5, and precipitated by adding five volumes of acetone (−20°C, 30 min). After centrifugation (10 min, 10,000 g, 4°C) the pellet was re-dissolved in 1 ml of denaturing buffer (6 M guanidine HCl, 100 mM Tris acetate, 5 mM Tris[2-carboxyethyl]phosphine (TCEP, a non-thiol reducing agent), 50 mM potassium perchlorate, 15 mM magnesium acetate, pH 7.5).

To prepare stable aggregates of heat-denatured luciferase, 700 nM luciferase was incubated for 5 min at 45°C in 50 mM Tris acetate, 50 mM potassium perchlorate, 15 mM magnesium acetate, pH 7.5 (Ben-Zvi et al., 2004; De Los Rios et al., 2006). The residual activity after
heat exposure was ~2% of the initial. Luciferase activity was measured as described previously (Bischofberger et al., 2003) using a Victor Light 1420 Luminescence Counter (Perkin-Elmer).

To study the effect of pre-formed aggregates on luciferase refolding, chemically denatured luciferase was allowed to refold spontaneously at 25°C (final concentration of luciferase 1 μM) in refolding buffer, without or with 1 mM As(III) (NaAsO₂). About 20% and 5% of luciferase was refolded at 25°C in 2 hours, without and with 1 mM As(III), respectively. These samples containing 80% and 95% aggregated luciferase were separated from free As(III) using PD SpinTrap G-25 columns (GE Healthcare Life Sciences), and thereafter used to study their effect on the refolding of luciferase.

**Supplemental material.** Supplemental Table 1 contains a full list of As(III)-aggregated proteins identified in this study and the functional categories (according to FunCat, MIPS) that were significantly enriched in the aggregated protein-set compared to the *S. cerevisiae* genome.
Acknowledgments

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References


Figure legends

**Fig. 1. As(III) causes protein aggregation.**

(A) As(III) triggers protein aggregation/Hsp104p redistribution. Hsp104p-GFP localization was monitored by fluorescence microscope in living wild type cells before and after 1 hour of exposure to 0.1 or 0.5 mM As(III).

(B) Intracellular As(III) causes protein aggregation. Hsp104p-GFP localization was monitored in wild type cells without or with overexpression of the arsenite efflux permease Acr3p after 1 hour of exposure to 0.5 mM As(III).

(C) Hsp104p is associated with protein aggregates in As(III) exposed cells. Wild type and rpn4Δ cells were exposed to As(III) for 1 hour and aggregated and non-aggregated (supernatant) protein fractions were isolated and separated on SDS-PAGE. The membranes were probed by Western blot analysis using an antibody against Hsp104p. Hsp104p levels were also detected in total cell lysates (0.5 mM As(III), 1 hour).

(D) Quantification of protein aggregation in response to metals. Hsp104p-GFP distribution was scored in cells exposed to 0.4 mM As(III), 0.4 mM Cr, or 5 µM Cd for 1 hour (these concentrations inhibited growth to the same extent). The fraction of cells with at least one aggregate/Hsp104p-GFP foci was determined by visual inspection of 700-900 cells per condition.

(E) Hsp104p-containing protein aggregates are largely distinct from P-bodies and stress granules. Hsp104p-GFP localization was monitored simultaneously with Dcp2p-RFP (P-body marker) or Pab1p-CFP (SG marker). Wild type cells with chromosomally tagged Hsp104p were transformed with the indicated plasmids and treated with 1 mM As(III) for 1 hour before visualization using fluorescence microscope. Dcp2p-RFP, Pab1p-CFP and Hsp104p-GFP were evenly distributed throughout the cytosol in unexposed cells.

(F) Functional categories that are significantly enriched (false discovery rate (FDR) < 5%) in the As(III)-aggregated protein-set compared to the *S. cerevisiae* genome.

**Fig. 2. Clearance of aggregates requires the proteasomal system.**

(A) Aggregate clearance is defective in rpn4Δ cells. Hsp104p-GFP localization was monitored by fluorescence microscope in living wild type and rpn4Δ cells before and during exposure to 0.1 mM As(III).

(B) Proteasomal activity is stimulated by As(III). Exponentially growing wild type and rpn4Δ cells were either untreated (control; black) or exposed to 0.5 mM As(III) for 3 hours (in vivo;
light grey). 26S proteasomal activity was measured in wild type and \textit{rpn4Δ} cell extracts by monitoring the rate of cleavage of the fluorogenic proteasomal substrate suc-LLVY-AMC. As(III) was also added to cell extracts prepared from unexposed cells (in vitro; dark grey) to investigate whether proteasomal activity is regulated in vitro. The data shown were obtained from four independent experiments and activity is represented in arbitrary units (AU). The size of each box is determined by the 25th and 75th percentiles, the whiskers represent the maximum and minimum values, and the horizontal lines show the median, respectively.

(C) Amount of proteasomal components. Western blot analysis of proteasomal components in wild type and \textit{rpn4Δ} cells before and after As(III) exposure. Antibodies targeting the proteasomal subunits Rpt1p and Rpt3p were used whilst an antibody targeting the kinase Hog1p was used as loading control.

(D) Mutants defective in proteasomal activity are As(III) sensitive. Cells were grown in liquid medium and 10-fold serial dilutions of the cultures were spotted onto agar plates with or without As(III). Growth was monitored after 2-3 days at 30°C.

**Fig. 3. As(III) does not induce mistranslation.**

(A) Pigmentation assay. The \textit{ade1-14} mutant was grown in liquid medium and 10-fold serial dilutions of the cultures were spotted onto agar plates with/without As(III) and paromomycin as indicated. Pigmentation was scored after 4 days of growth at 30°C.

(B) Growth assay. Growth of wild type cells was monitored by measuring the optical density (OD) at 600 nm using a micro-cultivation approach. As(III) and paromomycin were added separately or together as indicated.

(C) Growth was monitored as above in the presence of Cr and/or paromomycin.

**Fig. 4. As(III) targets proteins in the process of synthesis/folding for aggregation and interferes with chaperone activity in vivo.**

(A) Inhibition of translation prevents As(III)-induced aggregate formation. Hsp104p-GFP localization was monitored in wild type cells exposed to As(III) or heat in the absence or presence of 0.1 mg/ml cycloheximide (CHX).

(B) As(III) enhances aggregation of newly synthesized proteins. Untreated or As(III)-exposed (0.5 mM) cells were pulsed for 5 minutes with \textsuperscript{35}S-methionine and aggregated proteins were isolated by sedimentation. 20 μg of total lysate and the isolated aggregate fractions were separated by SDS-Page for subsequent autoradiography (top). Incorporation of \textsuperscript{35}S-labelled
material into the various fractions was quantified by densitometric analysis. \(^{35}\)S incorporation in the aggregates is shown as a percentage of the total \(^{35}\)S-label incorporation (bottom).

(C) Growth assay. Growth of wild type and the indicated chaperone mutants in the presence of 1 mM As(III) was monitored by measuring the optical density (OD) at 600 nm using a micro-cultivation approach. Mutants used are SSB (ssb1\(\Delta\) ssb2\(\Delta\)), SSE (sse1\(\Delta\)) and NAC (egd1\(\Delta\) egd2\(\Delta\) btt1\(\Delta\)).

(D) As(III) inhibits chaperone activity. Cells were exposed to heat (42°C) for 1 hour to induce protein aggregation and then placed at 30°C. Aggregate clearance was monitored in the presence/absence of As(III) and CHX as indicated.

(E) Cycloheximide treatment improves As(III) tolerance. Wild type cells were treated with 0.1 mg/ml CHX for 1 hour before plating 10-fold serial dilutions of the cultures onto agar plates with or without As(III). Growth was monitored after 2-3 days at 30°C.

**Fig. 5. As(III) inhibits protein folding in vitro.**

(A) Native luciferase. Effect of As(III) on enzymatic activity of native luciferase (350 nM) in refolding buffer (50 mM Tris acetate, 100 mM potassium perchlorate, 15 mM magnesium acetate, pH 7.5) at 25°C.

(B) Spontaneous refolding. Luciferase (17.5 \(\mu\)M) was chemically denatured in 6 M guanidine hydrochloride, 50 mM Tris acetate, 5 mM TCEP (Tris[2-carboxyethyl]phosphine), pH 7.5, for 30 min at 25°C. Spontaneous refolding at 25°C was initiated through 1:50 dilution (final concentration of luciferase 350 nM) with refolding buffer (50 mM Tris acetate, 100 mM potassium perchlorate, 15 mM magnesium acetate, pH 7.5), containing the indicated concentrations of As(III). Luciferase activity was measured in samples of the refolding solution at the indicated times. Error bars represent the SEM from three independent experiments.

(C) Chaperone-assisted refolding of chemically denatured luciferase in the presence of 3.5 \(\mu\)M DnaK, 0.7 \(\mu\)M DnaJ, 1.4 \(\mu\)M GrpE and 5 mM ATP under otherwise identical conditions as in experiment 5B.

(D) As(III) inhibits of chaperone-mediated disaggregation and refolding of stable aggregates of heat-denatured luciferase. The aggregates (residual activity \(~2\%\) of native) were prepared by exposing 700 nM luciferase to 45°C for 5 min. On 1:2 dilution with refolding buffer, the aggregates were incubated with 3.5 \(\mu\)M DnaK, 0.7 \(\mu\)M DnaJ, 1.4 \(\mu\)M GrpE, 5 mM ATP and the indicated concentrations of NaAsO\(_2\) for 120 min at 25°C. In the control experiment, conducted in the absence of chaperones, no reactivation was observed.
(E) As(III) affects refolding of luciferase in a dose-dependent manner. Dose–response curves of arsenite for inactivation of native luciferase (see Fig. 5A; luciferase (350 nM) was incubated for 120 min at 25°C with the indicated concentrations of As(III); the residual activity after 120 min is plotted), inhibition of spontaneous refolding (Fig. 5B), chaperone-assisted refolding (Fig. 5C) and chaperone-mediated disaggregation and refolding of heat-denatured luciferase (Fig. 5D).

Fig 6. As(III)-aggregates act as seeds for further protein aggregation.
Spontaneous refolding of chemically denatured luciferase was followed as in Fig. 5B in the presence of the indicated concentrations of luciferase aggregates prepared, as described in Material and Methods, (A) in the absence, and (B) in the presence of 1 mM NaAsO₂. Luciferase activity was measured in samples of the refolding solution at the indicated times. Error bars represent the SEM from three independent experiments. (C) Dose response curves: Luciferase aggregates prepared in the presence of NaAsO₂ more strongly inhibit the refolding of luciferase (data points at 60 min from Fig. 6B) as compared to the luciferase aggregates prepared in the absence of NaAsO₂ (data points at 60 min from Fig. 6A).
Table 1. *S. cerevisiae* strains and plasmids used in this study.

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3A

Control

2.5 mM As(III)

50 μg/ml paromomycin

50 μg/ml paromomycin + 2.5 mM As(III)

3B

Growth (OD$_{600}$)

Time (h)

Control

2.5 mM As(III)

100 μg/ml paromomycin

2.5 mM As(III) + 100 μg/ml paromomycin

3C

Growth (OD$_{600}$)

Time (h)

Control

0.1 mM CrO$_3$

100 μg/ml paromomycin

0.1 mM CrO$_3$ + 100 μg/ml paromomycin
IC₅₀ values derived from Figure 5E

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*IC₅₀ values with SEM from three independent experimental data sets.