Chapter 12
Non-native Proteins as Newly-Identified Targets of Heavy Metals and Metalloids

Sandeep K. Sharma, Pierre Goloubinoff and Philipp Christen

Abstract Heavy metal ions such as Cd^{2+}, Hg^{2+} and Pb^{2+} as well as metalloid arsenic(III) species very efficiently inhibit the refolding of chemically denatured proteins (IC_{50} values in nanomolar range). In their presence, the proteins misfold and aggregate. Denatured proteins appear to be much more susceptible to form high-affinity pluridentate complexes with heavy metals and metalloids than native proteins. In a denatured protein, the potential ligands of metal ions, the most important ones being cysteine and histidine residues, are more easily accessible for the toxic agents; moreover, denatured proteins with more flexible and motile backbones are more likely than folded native proteins to tolerate the formation of pluridentate protein–metal complexes with their defined geometry. In cells, the interference of metals with nascent and other non-native forms of proteins might manifest itself both in a quantitative deficiency of the affected proteins and the formation of proteotoxic aggregates. Possibly, the toxic effects of heavy metals and metalloids arise not only from their interaction with specific, particularly susceptible native proteins but also from a general derailing of protein folding. The toxic scope of heavy metals and metalloids thus could be more pleiotropic and extensive than assumed so far.

Introduction

Certain heavy metal ions and metalloids, e.g. iron, copper, manganese or zinc, act as cofactors of many proteins, enzymes in particular, and are essential components of living matter. However, these essential components in overdose as well as xenobiotic, i.e. non-essential, heavy metal ions and metalloids have proven to cause acute and chronic toxicoses in all forms of life (Hu 2005; Kosnett 2007), including carcinogenic effects (Waisberg et al. 2003) and prenatal and developmental defects

P. Christen (✉)
Department of Biochemistry, University of Zurich,
Winterthurerstrasse 190, 8057 Zürich, Switzerland
Fax: +41-44-635-6805
e-mail: christen@bioc.uzh.ch

G. Bánfalvi (ed.), *Cellular Effects of Heavy Metals*,
DOI 10.1007/978-94-007-0428-2_12, © Springer Science+Business Media B.V. 2011

(Bolin et al. 2006; Monnet-Tschudi et al. 2006; Wu et al. 2008). Environmental and occupational exposure of humans, in particular to cadmium, mercury, lead and arsenic, may entail severe health hazards. The very existence of this volume on *Cellular effects of heavy metals* testifies to the importance of heavy metal toxicity as a topic in medicine and public health. The interaction of heavy metal ions with living matter has also been medically exploited: organoarsenicals and mercury compounds were once used against syphilis and trypanosomiasis; calomel (Hg_2Cl_2) served as diuretic, laxative and antiseptic; sublimate ($HgCl_2$) and organic mercury compounds as antiseptic in the treatment of wounds and as conserving agent in certain vaccines. While these applications are now considered obsolete, other metal-containing compounds are still in medical use: bismuth subgallate is employed as internal deodorant and in cosmetic formulations, and a platinum complex (cisplatin) serves as a well established cytostatic agent. Recently, arsenic trioxide has been approved as a cytostatic against acute promyelocytic leukemia (Wang and Chen 2008).

While the general toxicity of heavy metals and arsenic is undisputed and their remarkably pleiotropic toxic effects are known in detail, the underlying molecular mechanisms are mostly unclear. General consensus holds that proteins are the prime targets of heavy metal ions and arsenicals; only few metals such as chromium, nickel and platinum are known to interact directly with DNA. Proteins have as yet been considered to be affected by metal ions in two different ways: the toxic metal ions either bind to free thiol and other functional groups of certain native proteins, or replace essential metal ions in metal-dependent proteins (Gurd and Wilcox 1956; Vallee and Ulmer 1972; Kägi and Hapke 1984; Fraústo da Silva and Williams 1993). Here, we review a third mode of how heavy metals and metalloids may interact with and impair cellular proteins. Folding proteins have proven much more susceptible to heavy metal ions and arsenicals than proteins that have reached their native state (Sharma et al. 2008; Ramadan et al. 2009). Heavy metal ions (at nanomolar concentration) and arsenic(III) compounds (at micromolar concentrations) have been found to inhibit the refolding of chemically denatured proteins. Conceivably, nascent proteins and other forms of non-native proteins in cells are affected by heavy metals and metalloids in the same way.

Principles of Protein Folding

The amino acid sequence of a protein determines its unique three-dimensional structure, which corresponds to the energetically most favorable spatial arrangement of the polypeptide chain (Anfinsen 1973). The refolding of chemically denatured proteins is initiated by abolishing the denaturing conditions, e.g. by dilution of the denaturing agent; thus, the total polypeptide chain simultaneously takes part in the refolding process. The intracellular folding of nascent proteins, however, appears to be co-translational, i.e. to start already in the first synthesized, amino-terminal segment before the synthesis of the polypeptide chain has been completed. Moreover, because of molecular crowding, the folding of many proteins

in cells is assisted by molecular chaperones, specialized proteins that improve the yield of folding and, in many cases, driven by ATP hydrolysis, rescue proteins, which are misfolded because of heat and other types of cellular stress. All major classes of molecular chaperones comprise heat-inducible members, their expression being markedly enhanced at elevated temperature and other conditions of cellular stress (for reviews, see Georgopoulos and Welch 1993; Sharma et al. 2009). Despite the apparently more complex mechanisms of intracellular protein folding as compared to *in vitro* protein folding, the general principle that a folding polypeptide chain spontaneously seeks to attain the conformation of lowest free energy still holds true.

Interaction of Heavy Metals with Functional Groups of Proteins

Heavy metal ions form monodentate and pluridentate complexes with S, N, O atoms in proteins. The most important ligands are the thiol groups of cysteine residues and the imidazole groups of histidine residues because they produce the most stable complexes (Table 12.1). The values of the dissociation equilibrium constants K'_d given in Table 12.1 are those for monodentate complexes; pluridentate (multidentate) complexes, in which the metal ion coordinates with more than one ligand in the same protein, are much more stable, their K'_d values roughly equating to the product of the K'_d values of the monodentate complexes of the individual ligands. Metalloproteins form such highly stable pluridentate complexes with their essential metal ions, most complexes having tetrahedral (with four ligands) or octahedral (six ligands) geometry (Gurd and Wilcox 1956; Vallee and Ulmer 1972; Kägi and Hapke 1984). Engagement of a folding protein in highly stable pluridentate protein–metal complexes has recently been found to interfere gravely with the formation of the native protein structure (Sharma et al. 2008; Ramadan et al. 2009). The reasons for folding proteins being more susceptible to heavy metals than native proteins seem obvious: the side chains in unfolded proteins are not only more exposed to the solvent but also more flexible and motile than in native folded proteins and thus more prone to be incorporated as ligands in pluridentate metal complexes.

Table 12.1 Monodentate complexes of functional groups in proteins with heavy metal ions: dissociation equilibrium constants and pK_a values

	K'_d at pH 7			Approximate pK_a in proteins
	Cd^{2+}	Hg^{2+}	Pb^{2+}	
Thiol group	2.5 μM	0.063 nM	13 μM	9.4
Imidazole group	2.0 mM	200 μM	6.3 mM	6.5
Carboxyl group	16 mM	2.5 μM	13 mM	4.6

K'_d is the apparent dissociation equilibrium constant at pH 7 of the reaction $ML \leftrightarrows M+L$, where ML is the 1:1 complex of the metal ion M and ligand L (Kägi and Hapke 1984)

Interference of Heavy Metals with the Refolding of Chemically Denatured Proteins

Cd^{2+}, Hg^{2+} and Pb^{2+} at nanomolar concentrations have been found to inhibit the spontaneous refolding of chemically denatured luciferase (Fig. 12.1). The metal ions affect the refolding of the protein without apparent delay. In contrast, the native protein is much less affected by the metal ions under the same conditions, being inactivated only to a limited degree in a slow time-dependent process (Fig. 12.2).

The dose–response curves for refolding inhibition (Fig. 12.3a) reveal IC_{50} values in the two-digit nanomolar range (Table 12.2), whereas the dose–response curves for inactivation of native luciferase show less than 50% inhibition even at the highest concentration (500 nM) of Cd^{2+} and Pb^{2+} (Fig. 12.3b).

It is important to note that the concentration of luciferase was 350 nM (20 μg/ml) in all experiments; at lower concentrations, the reproducibility of the measurements had proven unsatisfactory. Therefore, the IC_{50} values had to be determined at metal ion concentrations considerably lower than the 350 nM concentration of the target protein and thus cannot serve for quantitatively estimating the stability of the protein–metal complexes underlying the folding inhibition. The IC_{50} values measured under these conditions (Table 12.2) perforce underestimate the folding-inhibitory effect of the metal ions, particularly that of Hg^{2+}.

Reduced glutathione and the chelating agent EDTA attenuate the inhibitory effect of Cd^{2+}. However, neither agent rescues protein that has become misfolded in the presence of cadmium ions (Sharma et al. 2008). The ATP-dependent Hsp70 molecular chaperone system (DnaK/DnaJ/GrpE of *Escherichia coli*) significantly reduces the refolding inhibitory effect of Cd^{2+} (Table 12.2). The cyclic action of this chaperone system includes the following steps: ATP-DnaK with fast binding and release kinetics binds the substrate, i.e. the non-native protein; DnaJ stimulates the hydrolysis of DnaK-bound ATP, thus converting ATP-DnaK to ADP-DnaK with slow kinetics and high affinity for the substrate (Palleros et al. 1993; Schmid et al. 1994); pulling action of tightly bound DnaK disentangles the misfolded substrate (De Los Rios et al. 2006; Sharma et al. 2009); GrpE exchanges DnaK-bound ADP with ATP, thus triggering the release of the substrate or its re-entry into the chaperone cycle (Siegenthaler and Christen 2006). Per cycle, one DnaK molecule hydrolyzes one ATP molecule, the rate of ATP hydrolysis thus corresponds to the rate of the chaperone cycle.

Measurement of ATP consumption clearly demonstrates an acceleration of the chaperone cycle, i.e. an increased engagement of the chaperone system, due to the metal-induced misfolding of luciferase (Fig. 12.4). The steady-state ATPase activity of DnaK/DnaJ/GrpE in the absence of denatured luciferase is relatively slow and not affected by Cd^{2+}. Denatured luciferase increases the ATPase activity through *cis*-activation of the DnaK-ATPase by DnaJ in ternary (ATP-DnaK)-luciferase-DnaJ complexes (Han and Christen 2003). The additional presence of Cd^{2+} increases the ATPase activity even more. Apparently, the perturbation of luciferase refolding by the metal ion almost doubles the chaperone load (Fig. 12.4).

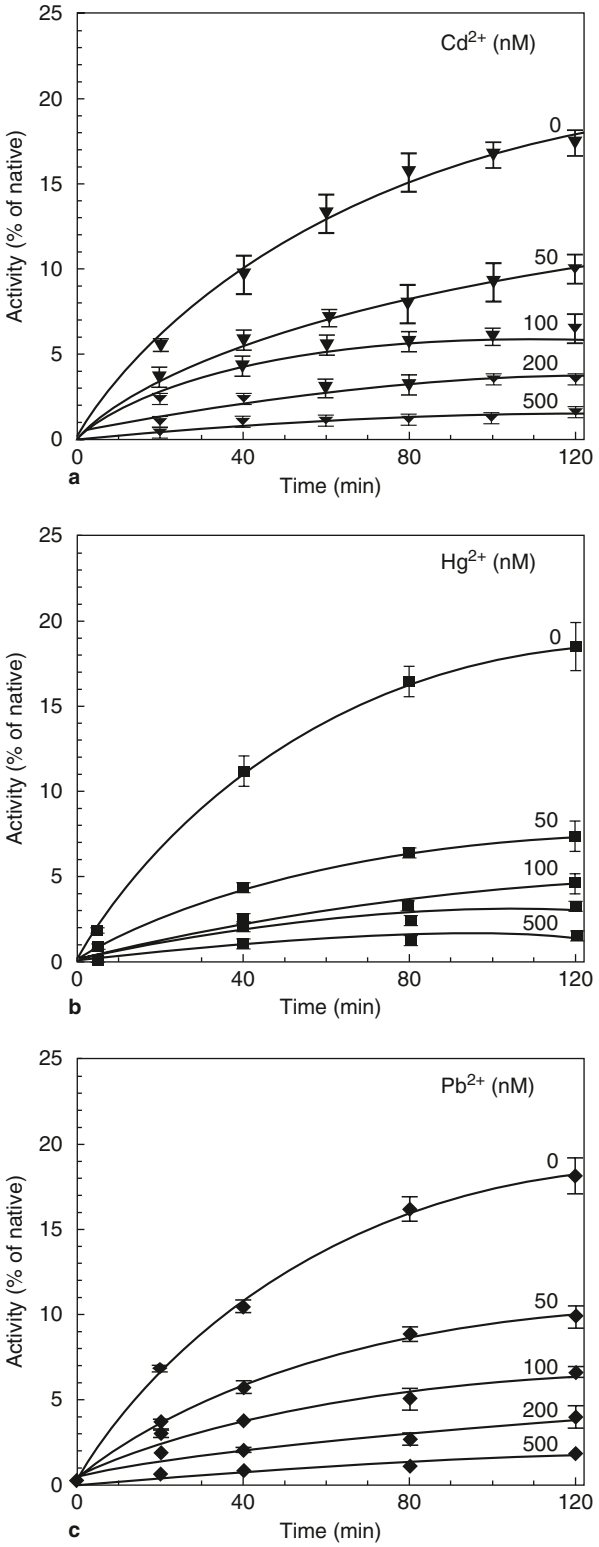

Fig. 12.1 Inhibition of luciferase refolding by Cd^{2+}, Hg^{2+} and Pb^{2+}. Luciferase (17.5 μM) was chemically denatured in 6 M guanidine hydrochloride, 50 mM Tris acetate, 5 mM TCEP (Tris[2-carboxyethyl]phosphine, a non-thiol reducing agent), pH 7.5, for 30 min at 25°C. Spontaneous, unassisted refolding 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 Cd^{2+} **a**, Hg^{2+} **b** and Pb^{2+} **c**. Luciferase activity was measured in samples of the refolding solution at the indicated times (for details, see Sharma et al. 2008). Error bars represent the SEM from three independent experiments

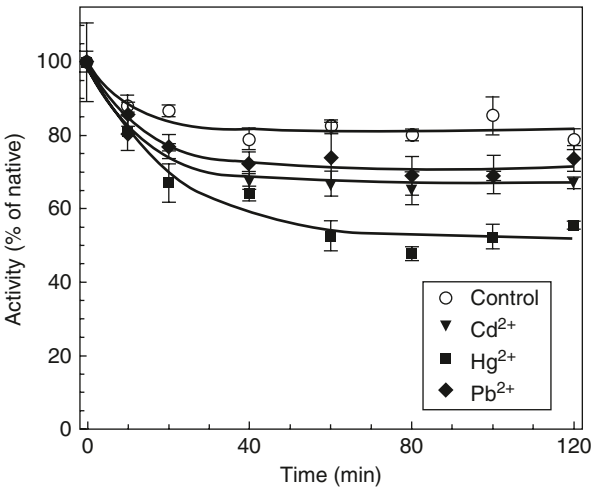

Fig. 12.2 Effect of metal ions on the enzymic activity of native luciferase. The effect of Cd^{2+}, Hg^{2+} and Pb^{2+} (100 nM) on the enzymic activity of native luciferase (350 nM) was tested in refolding buffer at 25°C under the same conditions as used for the refolding of chemically denatured luciferase (Fig. 12.1)

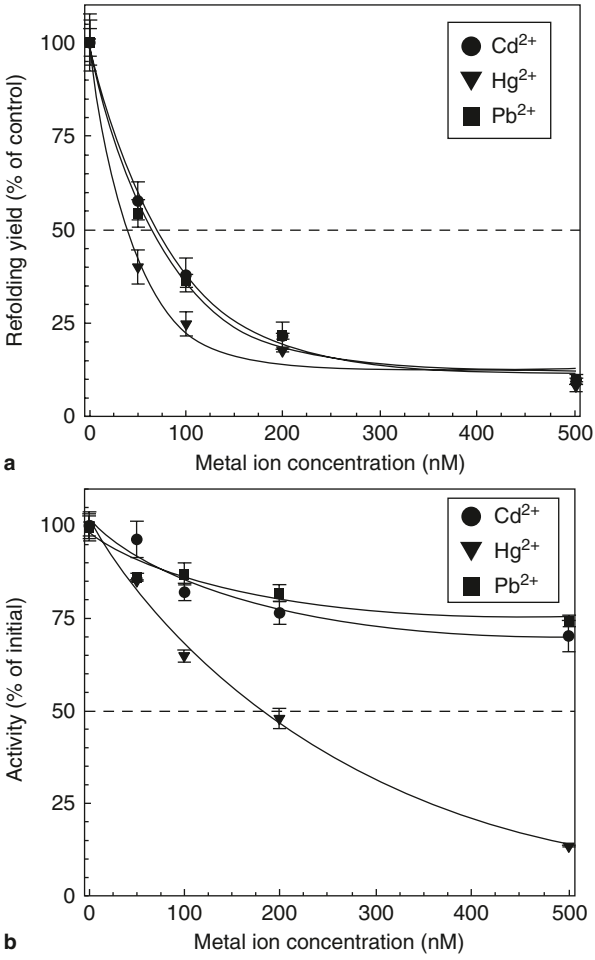

Fig. 12.3 Dose–response curves of Cd^{2+}, Hg^{2+} and Pb^{2+} for inhibition of refolding and inactivation of native enzyme. **a** Inhibition of spontaneous refolding of luciferase by Cd^{2+}, Hg^{2+} and Pb^{2+}. Chemically denatured luciferase (350 nM) was refolded at 25°C in the presence of increasing concentrations of metal ions. Luciferase activity was measured as a function of time, and the final yield of activity after 120 min (expressed as percentage of the yield in the absence of metal ion) plotted *vs* metal concentration. **b** Inactivation of native luciferase by Cd^{2+}, Hg^{2+} and Pb^{2+}. Luciferase (350 nM) was incubated for 120 min at 25°C with the indicated concentrations of metal ions. The residual activity after 120 min is plotted

Table 12.2 IC_{50} values of Cd^{2+}, Hg^{2+} and Pb^{2+} for inhibition of protein refolding

	IC_{50} value (nM)			Cysteine residues (Number per protomer)
	Cd^{2+}	Hg^{2+}	Pb^{2+}	
Luciferase				4
spontaneous refolding	66±11	40±3	63±6	
chaperone-assisted refolding	100±5	53±2	140±11	
Lactate dehydrogenase				5
spontaneous refolding	68±2	58±6	74±9	
Malate dehydrogenase				8
spontaneous refolding	300±45	290±16	520±44	
Glucose-6-phosphate dehydrogenase				0
spontaneous refolding	340±15	230±18	>600	

For chaperone-assisted refolding of luciferase, the refolding solution additionally contained 3.5 μM DnaK, 0.7 μM DnaJ, 1.4 μM GrpE and 5 mM ATP. The IC_{50} values with SEM were calculated from three independent experimental data sets

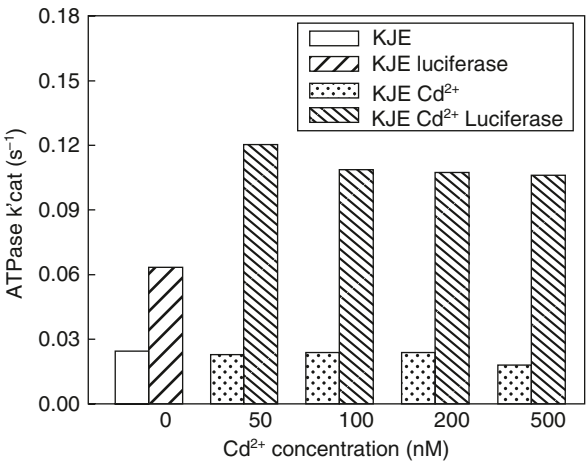

Fig. 12.4 Cd^{2+} increases the chaperone load due to luciferase refolding. The steady-state ATPase activity of the of DnaK/DnaJ/GrpE (KJE) molecular chaperone system in the presence of the indicated concentrations of Cd^{2+} was measured in the absence and presence of 350 nM refolding luciferase

The increased chaperone load is to be attributed to a higher incidence of misfolded polypeptide chains. An enhanced expression of cellular heat shock proteins, in particular of Hsp70, is indeed observed in cells exposed to heavy metal ions (Wagner et al. 1999; Han et al. 2007; Kusakabe et al. 2008; for reviews, see Hall 2002; Ahsan et al. 2009).

In addition to luciferase, we have tested with three other proteins whether their refolding was inhibited by metal ions (Table 12.2). Cysteine-containing lactate dehydrogenase proved as susceptible as cysteine-containing luciferase, while cysteine-containing malate dehydrogenase and cysteine-less glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* were somewhat less affected.

Mechanism of Folding Inhibition by Heavy Metal Ions

The efficiency of folding inhibition as expressed by the reciprocal of the IC_{50} values was $Hg^{2+} > Cd^{2+} > Pb^{2+}$ with all four proteins that have as yet been were tested (Table 12.2). This order correlates with the relative stability of the monodentate complexes of these metal ions with thiol, imidazole and carboxylate groups in proteins (Table 12.1). However, the IC_{50} values of Cd^{2+} and Pb^{2+} (very tight binding Hg^{2+}, probably due to gross underestimating of its IC_{50} value as mentioned above, is an exception) are much lower than the dissociation equilibrium constants of the monodentate complexes. We infer from this discrepancy that the refolding-inhibitory protein–metal complexes are pluridentate rather than monodentate complexes, the metal ions being bound to several appropriately positioned liganding side chains of the denatured protein molecule. The possibility of metal ions interacting with two to six ligands and forming pluridentate complexes with their metal-specific geometry (Gurd and Wilcox 1956; Vallee and Ulmer 1972; Kägi and Hapke 1984; Fraústo da Silva and Williams 1993) is of course much higher in a denatured protein with its more flexible and motile polypeptide chain. The example of glucose-6-phosphate dehydrogenase (Table 12.2) shows that such chelate-like structures are even formed in proteins that are devoid of cysteine residues and apparently form stable pluridentate complexes exclusively with the more weakly binding imidazole and carboxylate ligands.

Xenobiotic heavy metals other than cadmium, mercury or lead as well as overdosed essential heavy metals are of course also to be expected to perturb protein folding. Depending on the affected protein and the type of metal ion or metalloid, differential effects on the kinetics and thermodynamics of the folding trajectory of the protein will ensue.

Interference of As(III) Species with Oxidative Refolding of Disulfide Bond-Containing Proteins

Results very similar to those obtained with heavy metal ions have been reported by Ramadan et al. (2009) with three different arsenic(III)compounds, such as arsenite (arsenous acid, $As(OH)_3$) and monomethylarsenous acid ($CH_3As(OH)_2$) as inhibitors of oxidative protein refolding. Three different disulfide-bonded extracellular proteins were tested: lysozyme and ribonuclease A, each with four disulfide bridges, and riboflavin-binding protein with nine disulfide bridges. Low micromolar concentrations of the arsenicals efficiently inhibited the oxidative refolding of the chemically denatured proteins. The arsenicals bind rapidly and tightly to the cysteine residues of the reduced denatured proteins, three and two thiol groups coordinating with one molecule of arsenite and monomethylarsenous acid, respectively. Reduced glutathione (5 mM) weakens the inhibitory effect, which, however, still prevails. The interactions of the arsenic(III) compounds

with the reduced proteins are complex and not amenable to quantitative analysis, a tentative estimate by the authors of this review suggests IC_{50} values in the one-digit micromolar range.

In comparison with heavy metal ions, arsenicals thus seem somewhat less efficient in disturbing protein folding and their mode of inhibition, i.e. preventing the oxidative formation of structurally indispensable disulfide bonds, is different from that of heavy metal ions, which form pluridentate complexes comprising also protein side chains other than thiols. Despite these differences, the consequences of heavy metal ions and arsenicals interfering with protein refolding are in fact very similar: in both cases, aggregates of inactive misfolded proteins are produced with an increased propensity for binding thioflavin-T (Sharma et al. 2008; Ramadan et al. 2009), indicative of β-structured protein aggregates (LeVine 1999). Similar to heavy metals, arsenic induces the expression of heat-shock genes (Johnston et al. 1980; Levinson et al. 1980) and causes an accumulation of ubiquitinated cellular proteins (Kirkpatrick et al. 2003; Stanhill et al. 2006).

Possible Sequels of Protein Folding Inhibition in Cells

The results reviewed here indicate that the toxic scope of heavy metals and metalloids like arsenic might be greater than assumed as yet. Both groups of toxic agents might interact not only with specific native proteins that are particularly susceptible, but also, at least in principle, with any protein in non-native state. All nascent polypeptide chains are at least transitorily potential targets, and any proteins in other non-native states, e.g. proteins under heat or other cellular stress as well as natively unfolded (intrinsically unstructured) proteins (for a review, see Fink 2005), might also be affected. Denatured and other non-native proteins are indeed well known to be much more susceptible to proteolytic attack and to chemical modification, because the cleavable bonds and functional groups that are buried in the native protein become exposed upon denaturation (Means and Feeney 1971).

The susceptibility of proteins to folding inhibition by heavy metal ions or arsenicals may be assumed to depend on various structural features: first, on the number of cysteine and histidine residues and the distribution of such residues along the polypeptide chain, which determines their accessibility and the steric feasibility of forming pluridentate complexes; and second, the relative rates of complex formation and of attaining the native structure of the protein. In the completely folded protein most potentially liganding groups will be buried; moreover, the formation of pluridentate complexes with their specific geometry would require at least partial unfolding of the protein. Importantly, formation of protein–metal complexes, in contrast to proteolysis or chemical modification, is extremely fast, the rate constants of divalent metal ions for substitution of inner-sphere water of aquo ions being in the range of 10^7–10^9 s^{-1} (Fraústo da Silva and Williams 1993). The fast rate of complex formation may explain that even the folding of a fast-folder protein like ribonucleaseA is impaired by arsenic(III) species (Ramadan et al. 2009).

In the case of heavy metal and metalloid poisoning, the derailing of protein folding in general might not only lead to a loss of function, i.e. to a quantitative shortage of the affected proteins, but might manifest itself in the formation of toxic protein aggregates. Under these circumstances, cellular protein homeostasis might become imbalanced as observed in folding diseases (Chiti and Dobson 2006; Gidalevitz et al. 2006) and possibly in aging (Cohen et al. 2006).

Conclusions

We have clear-cut experimental evidence that heavy metals and metalloids very efficiently interfere with *in-vitro* protein refolding, and there is irrefutable evidence that these agents are highly toxic to all forms of life. However, there is a missing link between the *in-vitro* observation on the molecular level and the vast toxicological data stock. The missing link is experimental evidence that heavy metals and metalloids interfere with protein folding and induce formation of toxic protein aggregates not only *in vitro* but also in cells. There is correlative, but not cogent, evidence for this cause-and-effect relationship. Cells exposed to heavy metals and arsenicals invariably respond with an induction of heat shock proteins and an accumulation of ubiquitinated proteins (Johnston et al. 1980; Levinson et al. 1980; Wagner et al. 1999; Kirkpatrick et al. 2003; Othumpangat et al. 2005; Stanhill et al. 2006; Han et al. 2007; Kusakabe et al. 2008; for reviews, see Hall 2002; Ahsan et al. 2009).

The biological defense mechanisms against the sequels of heavy metal poisoning indeed are, in the order of their employment, reduced glutathione, the intracellular concentration of which being 5 mM or higher (Bánhegyi et al. 2007); ubiquitous metal-binding metallothioneins (Kägi and Schäffer 1988; Klaassen et al. 2009) and, additionally, in plants the enzymically synthesized phytochelatins (Freisinger 2008); the cellular chaperone network, in particular Hsp70 and Hsp60; and finally the gated proteases. If all these lines of defense fail, the deposition and compaction by aggresomes in less toxic inclusions, which may be degraded by lysosomal autophagy, provide a last resort (for reviews, see Hinault et al. 2006; Sharma et al. 2009). The *in-vivo Unfolded Protein Response* to heavy metal or metalloid poisoning might thus relate to the *in-vitro* observations that the refolding of proteins in the presence of a heavy metal ion results in an increased chaperone load (Fig. 12.4) and that folding inhibition by both heavy metals and arsenicals results in an accumulation of thioflavinT-binding aggregates (Sharma et al. 2008; Ramadan et al. 2009).

Future experimental efforts should focus on *in-vivo* experiments aimed at assessing the extent of the interference of heavy metals and metalloids with intracellular non-native proteins. The perturbation of the folding of cellular proteins in general, if existing, could contribute to explaining the pleiotropic, yet metal-specific, symptomatology of heavy metal poisoning (Waisberg et al. 2003; Hu 2005; Kosnett 2007). This mode of toxic action might not only be important in the pathogenesis of classic heavy metal poisoning, but also underlie so far unknown or inexplicable

consequences of exposure of living organisms to heavy metals, including certain protein folding diseases (Barnham et al. 2004; Chiti and Dobson 2006; Wu et al. 2008), autoimmune responses (Rowley and Monestier 2005), and subtle chronic impairments of health that are still undefined (Hu 2005; Cohen et al. 2006).

Acknowledgements This work was supported in part by a grant from the Swiss National Science Foundation (3100A0-109290) to PG. We thank J.H.R. Kägi for valuable discussions.

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