



Cite this: *Chem. Commun.*, 2015, 51, 4544

Advances in the molecular understanding of biological zinc transport†

Claudia A. Blindauer

Received 19th December 2014,
Accepted 20th January 2015

DOI: 10.1039/c4cc10174j

www.rsc.org/chemcomm

Between 5 and 10% of all proteins of a given organism are estimated to require zinc for function, and hence zinc is essential for almost any given metabolic process. It is therefore of great interest to understand major players and mechanisms that ensure the tight and correct control of zinc distribution and speciation in organisms and their individual cells. Significant progress has been made in recent years regarding 3-dimensional structures and modes of action of zinc sensor proteins, membrane-bound zinc transporters for cellular and sub-cellular uptake and efflux, as well as intracellular binding proteins. This feature article highlights advances in structures, zinc-binding sites and thermodynamics of proteins that are involved in zinc homeostasis and trafficking, including developments in understanding the metal selectivity of proteins.

1. Introduction: the growing importance of zinc in biological systems

Since the discovery of zinc as an essential element for fungi,¹ animals² including humans,³ plants and bacteria, and of the first enzyme to be shown to require zinc for activity in 1939,⁴ much has been learned about zinc in biological systems, ranging from effects on the whole organismal level, over the identification of important zinc-binding proteins, down to structural, thermodynamic, and kinetic details of zinc–protein interactions.^{5,6}

Zinc deficiency affects up to two billion people worldwide.³ Its multiple systemic effects include growth retardation, weight loss, infertility, mental and emotional disorders, impaired immune function, skin lesions and hair loss.³ The most salient recognition of the importance of zinc in human health was delivered by the 2008 Copenhagen Consensus conference, which ranked supplying zinc and vitamin A to over 100 million malnourished children as their highest priority solution to advance global welfare.⁷ Besides the drastic consequences of severe zinc deficiency and their alleviation by zinc supplementation,⁸ the more subtle impacts of zinc homeostasis on ageing,^{9,10} neurodegenerative diseases,^{11–13} cancer,^{14–16} the immune system,¹⁷ and energy metabolism^{18,19} are active study areas.

Apart from its crucial importance for human health, zinc plays also vital roles in the physiology of all other organisms.

The impact on plants is illustrated by several-fold increases (up to 600%) in crop yields upon fertilisation of zinc-deficient soils in Anatolia with zinc.²⁰ Zinc-deficient soils are widespread, and may contribute to zinc deficiency in humans, especially where they subsist on cereal-based diets which are rich in zinc-chelating phytate.²¹ Furthermore, the growth of eukaryotic phytoplankton, in particular coccolithophores and diatoms, has been suggested to be limited by zinc availability in certain regions of the oceans, with consequences for global carbon balances.²² The significance of zinc for bacteria is less well understood, but from the facts that most bacteria have dedicated systems for high-affinity zinc uptake^{23–25} and respond to zinc starvation,^{26–28} it may be concluded that zinc is also an essential nutrient for most if not all bacteria. This is also borne out by observations that zinc availability is actively reduced during a host's acute phase response to bacterial infection,^{29,30} and that zinc is required for full virulence for at least some pathogens.^{31–33} Influencing zinc homeostasis at the host–pathogen interface³⁴ offers exciting new avenues for antimicrobial therapy, for example the inhibition of virulence factors such as anthrax lethal factor by zinc chelation.³⁵ Conversely, it has also been recognised that free zinc is an unexpectedly potent cellular toxin,³⁶ emphasising the importance of highly efficient homeostatic mechanisms.

For many of the above examples, one of the most exciting current fields in zinc biochemistry concerns understanding zinc “on the move” – in particular in the context of signalling.^{37,38} In terms of inter-cellular signalling, the “gluzineric” neurons of the mammalian forebrain,^{13,39} and the “zinc sparks” emitted by fertilised oocytes⁴⁰ are most notable. Along with the respective biological studies on the organismal and cellular level, progress in this area is being facilitated on the one hand by the

Department of Chemistry, University of Warwick, Coventry, CV4 7AL, UK.

E-mail: c.blindauer@warwick.ac.uk; Fax: +44 (0)24 76 524112;

Tel: +44 (0)24 76 528264

† Electronic supplementary information (ESI) available: Tables S1 and S2. See DOI: 10.1039/c4cc10174j



development of dedicated fluorescent probes,^{37,41–45} including genetically encoded FRET sensors,^{46,47} and the associated imaging studies, and on the other by the discovery and characterisation of proteins involved in zinc transport and homeostasis.

1.1 Zinc homeostasis: general premises

Cells of all organisms accumulate total concentrations of zinc in the high micromolar range.^{48,49} It can be estimated that at least around 9% of all human proteins require zinc for correct activity,⁵⁰ and up to 15% of all human proteins (8681 out of 56 376 structurally modelled target gene products) have been predicted to contain at least one “real” zinc-binding site.⁵¹ The respective proteins comprise zinc-dependent enzymes, thousands of zinc-finger proteins, proteins that are regulated by zinc, and proteins involved in zinc homeostasis. Several elegant studies^{52,53} have led to the conclusion that dissociation constants for zinc-binding proteins should be in the range of the prevailing “free” zinc concentrations in the compartments where these proteins fold or operate.^{48,49,54} Estimates for free Zn^{2+} range from picomolar⁵⁵ to femtomolar⁴⁸ in *E. coli*, and reliable estimates for eukaryotic cells are in the single-digit nanomolar to picomolar range.^{49,56} Although cells have considerable zinc-buffering capacity at their disposal,^{48,56} substantial deviations from these steady-state concentrations may occur on various time scales, be it as a consequence of changes in zinc levels in the environment of the cell, or in the course of physiological zinc signalling events.

Cells are dynamic systems; there is hence a permanent influx and efflux of compounds including metal ions such as zinc. The total zinc content of a cell is thus primarily controlled by the action of more or less specific membrane-bound transporters (Fig. 1).

Fluxes of zinc depend in the first instance on the abundance of the transporters in the appropriate location and on the concentration of zinc available for transport.⁵⁷ The abundance of the transporters can be regulated on the transcriptional level; this is generally mediated by zinc-dependent transcription factors, also termed zinc sensor proteins, some of which are mentioned in Fig. 1 and Table S1 (ESI†). In eukaryotic systems, the activity of a transport protein may also be regulated post-transcriptionally⁵⁸ and post-translationally, e.g. through metal-regulated protein trafficking.⁵⁹ Zn^{2+} may also allosterically regulate the activity of the transporters (see Section 2.2). Furthermore, the importance of metal-modulated protein turnover is also increasingly recognised, especially for proteins involved in zinc sensing.^{60,61}

For the regulation of free cytosolic Zn^{2+} concentrations, most eukaryotes and some bacteria synthesise metallothioneins, small cysteine-rich proteins with metal-binding properties ideally suited for intracellular zinc buffering (Section 2.3.1). A recent addition to the portfolio of intracellular zinc trafficking proteins is the COG0523 family (Section 2.3.2).

Besides the total cellular and free cytosolic Zn^{2+} concentrations, those of various cellular compartments also need to be maintained within desired levels, because they may contain Zn-dependent proteins. The granules for insulin storage and

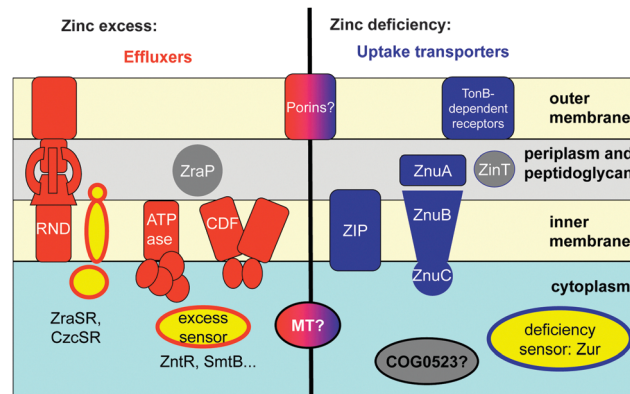


Fig. 1 Major players in zinc homeostasis, exemplified for Gram-negative bacteria. Proteins involved in efflux are shown in red, those involved in uptake in blue. Chaperones are shown in grey, and sensor proteins in yellow. The two-component systems that regulate expression of RND systems or ZraP are also illustrated, with a membrane-spanning sensor histidine kinase and a cytosolic DNA-binding protein. ZIP and CDF transporters are also ubiquitously used for zinc uptake and efflux from the cytosol of eukaryotes. Zn-transporting ATPases are also known for plants, but not for mammals. ABC transporters, TonB-dependent receptors, and RND systems for zinc are only known for bacteria. The ExbB/ExbD proteins providing energy to the TonB receptor are not shown. Intracellular zinc-binding metallothioneins (MTs) may be of benefit in both excess and deficiency conditions, and outer-membrane porins may permit both uptake and efflux.

secretion by pancreatic β -cells are a prominent example, but a less well-known yet rather momentous fact is the finding that failure to maintain an adequate zinc level in the endoplasmatic reticulum triggers the unfolded protein response.⁶² Similarly, even zinc levels in the periplasm of Gram-negative bacteria are regulated by sensor proteins and (metallo-)chaperone proteins (see Section 2.3.3),^{28,63,64} perhaps to ensure that periplasmic zinc-requiring enzymes, e.g. alkaline phosphatase, can acquire their cofactor, whilst excluding Zn^{2+} from adventitious sites in other proteins.

While kinetic control is important in metal homeostasis, and some outcomes are achieved by the inclusion of irreversible steps,⁶⁵ thermodynamics of metal binding are at the heart of metal homeostasis. Therefore, the accurate determination of affinity constants is exceptionally important. However, it has been observed previously^{66–68} that this endeavour is substantially less straightforward than might be expected. As a rule of thumb, dissociation constants for cytoplasmic Zn^{2+} -binding proteins that are not at least nanomolar should be approached with scepticism, as such low affinities are not likely to be physiologically relevant under basal conditions. In contrast, micro- to nanomolar dissociation constants may be encountered in extracellular zinc-binding proteins as well as in membrane-bound transporters.

These rules pertain to both enzymes and homeostatic proteins, and pose an intriguing puzzle as to the interrelationship between thermodynamic and kinetic parameters of metal-ligand interactions, as enzymes and transporters need to achieve fundamentally different goals: firstly, a metal cofactor in an enzyme should not normally dissociate from the enzyme, whilst this is absolutely



necessary in a metal-homeostatic protein, ideally on a fairly rapid time scale – in essence, fast kinetics of metal binding and release are a necessity for homeostatic proteins. An example that this is achievable with similar metal affinities has been given by comparing the enzyme carbonic anhydrase (half-life of Zn in its binding site on the order of years) with metallothioneins (see Section 2.3.1), in which half-lives are on the order of seconds.⁶⁹ Few if any concrete data are available regarding on and off rates for Zn²⁺ binding by homeostatic proteins, but it has been argued that some dissociation reactions require the action of competing ligands to proceed at rates compatible with biological observations.⁶¹ The mobilisation of Zn²⁺ from a metallothionein by the glutathione/glutathione disulfide redox couple was one of the first illustrations of this principle.⁷⁰ Secondly, Zn²⁺ in an enzyme needs to exert catalytic activity, but this is clearly to be avoided during zinc transport (as well as in structural sites). It is hence not surprising that the structures and properties of the binding sites of zinc sensors⁷¹ and zinc transporters⁶⁵ are often significantly different from those in enzymes, although this is not always the case as will be seen in Section 2.

2. Structures and mechanisms of proteins involved in zinc homeostasis

The major components of cellular zinc homeostasis are summarised in Fig. 1. Broadly, zinc homeostatic proteins can be divided into sensors, membrane-bound transporters (Section 2.2), and intracellular binding proteins (Section 2.3). A thorough review on biological zinc sensing has recently appeared,⁷² structures and thermodynamics of several bacterial zinc sensor proteins have been discussed in detail in another excellent article,⁷³ and a dedicated review on the eukaryotic zinc sensor MTF-1 is also available.⁷⁴ Therefore, zinc sensor proteins will not be discussed in detail here, although some fundamental insights will be summarised in Section 2.1.

2.1 Zinc metalloregulation: zinc-responsive sensor proteins and DNA sequences

The combined efforts of studying the *in vivo* function of metal-responsive proteins and detailed biophysical studies of purified proteins have led to major advances in the understanding of metalloregulation. Broadly, there are two types of zinc-responsive transcriptional regulators, those that counteract zinc deficiency, and those that counteract zinc excess/toxicity (Fig. 1). Sensors for zinc excess trigger the expression of proteins involved in efflux (e.g. ATPases, CDF proteins, RND proteins; Section 2.2; Table S1, ESI[†]) or sequestration (metallothioneins, ZraP, Section 2.3; Table S2, ESI[†]), whereas sensors for deficiency mediate enhanced expression of uptake transporters (ABC systems, porins, ZIP proteins; Section 2.2; Table S1, ESI[†]), putative chaperones (Section 2.3; Table S2, ESI[†]), and proteins that reduce cellular zinc requirements. For instance, many bacterial zinc regulons comprise genes involved in zinc uptake, but also genes for alternative ribosomal proteins devoid of zinc binding sites.^{75,76} This may suggest that the ribosome is a

major zinc storage site in bacteria, and that this zinc can be mobilised in conditions of zinc starvation.

The best-understood zinc sensors are those found in bacteria.⁷⁷ Two general mechanisms are at work; sensors can either function as repressors or activators of gene transcription, in dependence on whether or not Zn²⁺ is bound. For example, the zinc excess sensor SmtB and related proteins are repressors in their Zn-free apo form, and de-repression occurs upon zinc binding. In contrast, the Zn-bound form of Zur, an uptake regulator responsive to zinc deficiency, is a repressor, inactivating gene transcription in the presence of sufficient Zn²⁺. The Zn-bound form of ZntR, an excess sensor, is an activator of gene transcription. In the most simple cases, sensor proteins bind to specific DNA sequences in the upstream region of genes that code for proteins involved in zinc homeostasis. Such recognition sequences on the DNA have different names in different phyla. In bacteria, they usually carry the names of their cognate sensor proteins. In some animals, “metal-response elements” (MREs) and zinc transcriptional response elements (ZTRES)⁷⁸ are known, both of which mediate response to high zinc. 11-Base-pair ZREs (zinc-responsive elements) that mediate response to zinc deficiency are known for baker's yeast,⁷⁹ and in plants, zinc-deficiency response elements (ZDREs) have been identified recently.⁸⁰ In addition, putative MREs have also been predicted in plants, albeit without the associated protein(s) that recognise them.⁸¹ The sensor proteins from different phyla also differ significantly from each other. In mammals and insects, MREs with the core consensus sequence TGCRNC (N = any nucleotide, R = A or G) are recognised by the zinc finger protein MTF-1;⁷⁴ the 10-base-pair plant ZDREs are recognised by basic leucine zipper (bZIP) proteins,⁸⁰ and in bacteria, a variety of sensor families recognise different inverted repeat sequences.⁸²

Zinc sensing by transcriptional regulators almost always involves allostery:^{73,77} in the most simple cases, the binding of zinc elicits a conformational change, or stabilises a particular protein conformation, which increases or decreases the affinity to DNA. Many bacterial DNA-binding proteins exert their function as dimers, especially when interacting with inverted repeats or palindromic DNA sequences. In some cases, dimerisation is also influenced by Zn binding. The Zn²⁺ affinities of several zinc sensors have been measured by competition with metallochromic dyes⁸³ and isothermal titration calorimetry,⁸⁴ with most values around log *K* = 12–13 (i.e. picomolar *K*_D).⁷⁷ *In vivo* reporter assays in *E. coli* yielded femtomolar values for activation of the sensors Zur and ZntR.⁴⁸ Some of the related protein–DNA interactions have also been quantified, allowing the quantitative description of the various coupled equilibria (metal binding–DNA binding).⁷³

In structural terms, one unifying feature of the zinc binding sites in sensor proteins is their high solvent exposure. This presumably facilitates fast binding kinetics, although these have not been measured for any zinc sensor. Regarding specificity, scrutiny of the structures for different metal sensor proteins reveals that metal sites are “optimised” for the cognate metal ion,⁶⁷ both in terms of coordination geometries and of the HSAB principle (Pearson's principle of hard and soft acids and



bases; see ref. 85 for an overview). Cu^+ sensors harbour 2- and 3-coordinate sites, often comprising cysteines, Zn^{2+} sensors have 4-coordinate sites comprising mixtures of Cys, His and Asp, and Mn^{2+} , Fe^{2+} , Co^{2+} , and Ni^{2+} sensors have 6-coordinate sites, with the Mn^{2+} sensors displaying an abundance of carboxylate residues. However, this will not, per se, prevent e.g. Cu^+ binding to a Zn^{2+} sensor and *vice versa*. The first mechanism to prevent wrong sensing relates to the idea that intracellular metal concentrations and dissociation constants of the relevant metal-protein combinations are correlated.⁵⁴ This may however not be sufficient – especially under conditions where the more competitive metal ion is in excess. In this case, allosteric changes can be metal-specific: other metals may bind to a particular sensor, but without eliciting the conformational change required to alter DNA-binding affinity. The coordination chemistry principle that is exploited here is preferred coordination geometry: for example, Zn^{2+} does bind to the Ni/Co excess sensor NmtR from *Mycobacterium tuberculosis*, but adopts tetrahedral rather than the required octahedral coordination geometry, and hence does not trigger the sensing mechanism.⁸⁶

2.2 Proteins for uptake and efflux

Several proteins that mediate the transmembrane transport of zinc, along with transporters for other metal ions, have been discovered and characterised in the past decade.⁶⁵ All transmembrane transport proteins are catalogued in the Transporter Classification Database (TCDB).⁸⁷ This classification system works similarly to the EC system for enzyme classification, giving each protein a TCDB number. Channels and pores are class 1, electrochemical potential-driven transporters are class 2, and primary active transporters are class 3. The most important membrane transport protein classes for zinc and their TCDB numbers are compiled in Table S1 (ESI[†]); it should however be acknowledged that in some cases, information on transport mechanisms is still too limited for a conclusive classification.

Because of its inherently stronger tendency for complex formation, and hence reduced mobility,⁸⁸ transport mechanisms for Zn^{2+} (and those for other 3d row metal ions[‡]) differ significantly from those of the more mobile alkali and earth alkali metal ions.⁶⁵ The higher affinities are also the likely reason for the fact that transmembrane transport of 3d row metal ions is considerably slower than that of the group Ia and IIa metal ions, with typically less than 10 ions per second.⁶⁵ In principle, zinc transport can be active or passive, against or with Zn^{2+} gradients, although most systems that have been characterised in some detail tend to be active transporters. Fig. 1 shows cartoons for the most important types of efflux and uptake transporters. ATP-driven class 3 active transporters will be discussed first (Sections 2.2.1 and 2.2.2), followed by class 2 porters (Sections 2.2.3–2.2.5), with representatives from class 1 and others highlighted in Section 2.2.6.

[‡] Frequently misquoted in current literature, zinc is strictly speaking not a transition metal, as the only ionic form occurring in nature, Zn^{2+} , does still have a completely filled 3d shell.²⁶⁶

2.2.1 ATP-binding cassette (ABC) transporters. The by far most common bacterial uptake systems for zinc are members of the ATP-binding cassette transporter families of the ZnuABC and AdcABC/ZitSPQ types (TC 3.A.1.15). These may play a role in the virulence of at least some pathogens.⁸⁹ They usually consist of three proteins (Fig. 1 and 2): a cytosolic dimeric ATPase, a membrane-spanning dimeric permease, and one monomer of a substrate-binding protein (SBP) which provides selectivity. In Gram-positive bacteria (Tro/Adc/Zit systems), the latter protein is located at the cell surface (e.g. AdcA from *Streptococcus pneumoniae*), whilst in Gram-negative bacteria (Znu systems), the ZnuA SPBs are periplasmic – albeit often anchored to the inner membrane by an N-terminal hydrophobic tail. ABC transporters exist for the transport of organic substrates, inorganic ions, and metal chelates including siderophores and porphyrins.⁹⁰ 3D structures for complete ABC transporters are available albeit not for zinc; a backbone trace for the entire assembly for BtuCD-F,⁹¹ an ABC system for the uptake of cobalamin, is shown in Fig. 2. The protein folds for the various components of BtuCD-F and zinc-transporting ABC systems are either known or expected to be similar.

The SBP forms a complex with the permease as shown in Fig. 2, which allows the delivery of the substrate into a channel formed by the permease.⁹⁰ How ATP hydrolysis and Zn^{2+} transport are coupled for ZnuABC systems has not yet been determined, but it is likely that this will involve structural rearrangements at the permease dimer interface, with this interface providing a cavity with alternating access to outside and inside.⁹²

SBPs all adopt a venus-fly trap structure, with the substrate-binding site between the two domains. Substrate binding may elicit a conformational change in the SBP, but this is not always

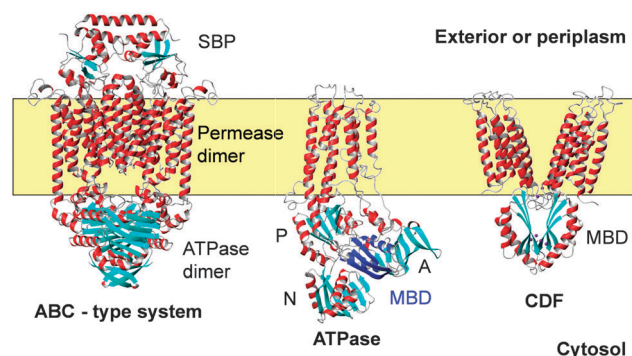


Fig. 2 Backbone traces for examples for three membrane (yellow) transporters for metal ions including Zn^{2+} . Helices are shown in red and grey, and β -strands are shown in cyan. The ABC transporter shown is BtuCD-F (pdb 4fi3)⁹¹ for cobalamin uptake. A detailed structure for a ZnuA substrate binding protein (SBP) is shown in Fig. 3. The ATPase is CopA from *A. fulgidus*. The structure is based on cryo-electron microscopy (pdb 3j09),¹⁰⁸ and was chosen as it illustrates the position of the cytosolic MBD (highlighted in dark blue). Actuator (A), phosphorylation (P), and nucleotide-binding (N) domains are also shown. The metal-binding loop of the MBD is in close proximity to the actuator domain. The CDF protein is YiiP (FieF) from *E. coli* (pdb 3h90).¹²⁸ The functional dimer is shown. The cytosolic domains have the same ferredoxin-like fold as MBDs from ATPases. A comparison of MBD folds and metal sites is provided in Fig. 5.



the case. Furthermore, the sequences of many ZnuA proteins contain long His-rich stretches, which is perhaps the best hallmark to distinguish a “true” ZnuA from the related Mn-binding proteins, which are otherwise closely similar, including the location and identity of metal-binding His residues.²⁴ The structure and role of these loops is a challenging puzzle in understanding the mode of action of ZnuA proteins. Several structures for ZnuA from *E. coli* are available (Fig. 3),^{93–95} with some striking variations in metal stoichiometry and coordination modes between these structures. The most recent study established that not just one, but two Zn^{2+} ions can be bound with significant affinity. Although the purified form contained only 1 mol. equiv. of Zn, it was possible to partially populate a second site by adding excess Zn^{2+} , followed by gel filtration chromatography which in general removes any weakly bound metal ions. The presence of a second site with a dissociation constant below 20 nM was also corroborated by titration studies with Mag-Fura-2 as competing chelator.⁹⁵ Only one of the protein-derived Zn ligands for this second site is visible in the structure, but some weak electron density suggests the presence of other ligands, most likely from the His-rich loop (residues 117–137), which is not resolved in any of the published structures. The latter indicates that these stretches are dynamic and/or disordered.

The primary zinc binding site in *E. coli* ZnuA is composed of three His residues and one Glu residue in two of the structures (2osv⁹⁴ and 2ps0⁹⁵), or the same three His residues plus a water molecule, which is hydrogen-bonded to the Glu residue, in another (pdb 2ogw⁹³) – in essence, the structures differ by inner- and outer-sphere coordination of the Glu residue. A further interesting variation is seen in the structure of ZnuA from *Salmonella enterica* (pdb 2xqv⁹⁶); here, His60 is not coordinated to the zinc ion; instead, another His from the His-rich loop has taken its place. The latter structure was obtained by soaking apo-crystals with Zn^{2+} , therefore it is not clear whether this mode would also be adopted in solution.

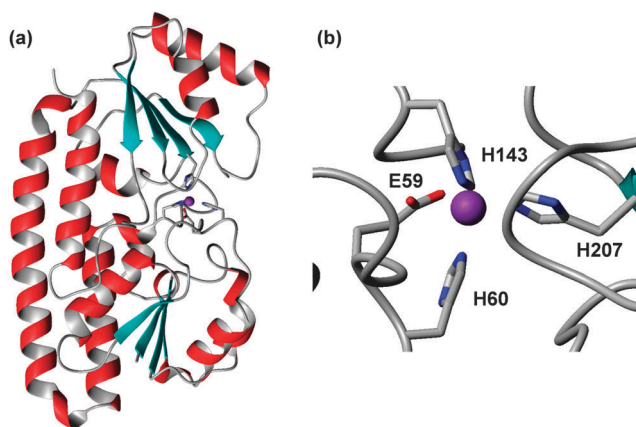


Fig. 3 Overall structure (a) and zinc-binding site (b) in *E. coli* ZnuA (pdb 2osv).⁹⁴ The Zn^{2+} ion is shown as a purple sphere, and is coordinated to three His and one Glu residue, the latter in a monodentate fashion. Variations to this motif are known (see text). The residues are numbered according to the UniProt sequence; the 2osv pdb file deviates from this by 18 residues.

ZnuA from the cyanobacterium *Synechocystis* adopts the same fold as the enterobacterial ZnuA's.⁹⁷ However, Glu59 is replaced by a Pro residue, and the fourth coordination site is occupied by a water molecule, which is not hydrogen-bonded to another residue. A deletion mutant lacking the His-rich loop (residues 138–173) has been compared to the wild-type.⁹⁸ Isothermal titration calorimetry (ITC) experiments established that the wild-type harboured two classes of zinc binding sites with affinities that differed by 2 orders of magnitude ($K_{\text{D(ITC)}} = 10$ and 1000 nM; $\log K_{\text{ITC}} = 8$ and 6). About up to 3 zinc ions could be bound with the weaker affinity, and the His-loop deletion mutant only retained the stronger binding site. Based on the fact that zinc loading of the high-affinity site was not affected by the loop deletion, a role of the loop in “chaperoning” zinc to the high-affinity site has been dismissed; instead, it was proposed that the loop may have a sensing/regulatory function that may slow down zinc uptake through the ABC system when periplasmic concentrations become 100 times higher than normal.⁹⁸

Most of the work on the substrate-binding components of Zn-transporting ABC systems has been carried out for proteins from Gram-negative bacteria, but there are also candidate systems in Gram-positive bacteria, most prominently AdcA and AdcAII⁹⁹ from Streptococci. Furthermore, a related periplasmic protein is present in the spirochaete *Treponema pallidum* and is the founding member of the TroA proteins.¹⁰⁰ However, the metal specificity of proteins designated TroA is unclear, and *in vitro* TroAs are capable of binding a range of metal ions with significant affinity – as expected. It is very likely that some TroAs are, *in vivo*, Mn or Fe binding proteins, whilst others are truly orthologous to ZnuAs, but prediction of metal specificity is non-trivial.²⁴ In the case of AdcA and AdcAII from *Streptococcus pneumoniae*, zinc specificity has been established *in vivo*.¹⁰¹

Recent work has demonstrated that the substrate-binding components may receive Zn^{2+} from other proteins. In some Gram-negative bacteria, the periplasmic zinc-binding protein ZinT (see Section 2.3.3) may deliver Zn^{2+} to ZnuA.¹⁰² In Streptococci, poly-His-triad (PhtD; see Section 2.2.6) proteins are located at the cell surface and have been proposed to deliver zinc to AdcAII.¹⁰³ Significantly, although AdcA and AdcAII are functionally equivalent, they differ structurally: whilst AdcAII overall resembles typical ZnuAs, AdcA is a fusion protein of a ZnuA-like portion and a ZinT-like portion, thus it appears that the ZinT-like protein can either be independent or fused to its partner protein.

2.2.2 P-type ATPases. P-type ATPases (TC 3.A.3) are a large family of transporters for the full complement of biologically relevant metal ions. Most are composed of 6–10 transmembrane helices and several cytosolic domains, including nucleotide-binding (N), actuator (A), and phosphorylation (P) domains (Fig. 1 and 2).¹⁰⁴

$\text{P}_{1\text{B}}$ -ATPases transport d-block metal ions (Table S1, ESI[†]), and are found in all kingdoms of life. No Zn-transporting ATPases are known for mammals, but several examples from plants and bacteria have been studied. Most Zn-transporting $\text{P}_{1\text{B}}$ -ATPases are involved in Zn^{2+} efflux from the cytosol, which in plants may also include transport into organelles.



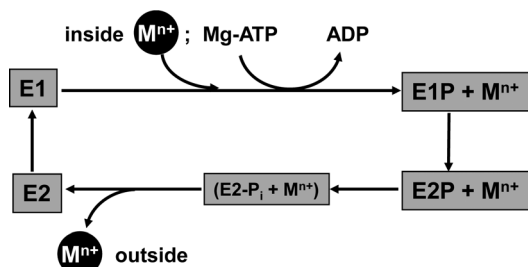


Fig. 4 Major states in the catalytic cycle of metal-effluxing P-type ATPases. The “P” signifies the occurrence of a phosphorylated enzyme intermediate (E1P and E2P states). The E2-P_i state refers to a state in which the hydrolysed P_i group is still bound to the protein; this is usually modelled by AlF₄[−], and also informs on the E2 state. E2P states are commonly mimicked by BeF₃[−] adducts.

Briefly, for P-type ATPases involved in metal efflux, the general Post-Albers reaction cycle involves four principal states (Fig. 4). In E1 and E1P states, an intra-membrane transport site is accessible from the cytosol, whereas in E2P and E2 states, this site is exposed to the exterior. The E1P state is reached by binding of Mg-ATP to the N domain and hydrolysis to ADP, with the remaining phosphate group transferred to an Asp residue in the P domain. It is thought that a concomitant conformational change prevents back-flow of the transported ion(s). A further, large conformational change leads to the E2P state, from which the metal substrate is released to the exterior. Hydrolysis of the covalently bound phosphate group gives the intermediate E2-P_i state; release of P_i yields the E2 state. A further conformational change closes the exit towards the exterior, and renders the inter-membrane metal binding site(s) accessible to metal substrate from the interior again (E1 state).⁹⁰ The various conformational changes alter both the mutual orientation of the cytosolic domains, and that of the trans-membrane helices, and are triggered by binding and releasing the species involved.

X-ray crystal structures of ZntA from *Shigella sonnei* in its Zn-free E2P and E2-P_i states have been determined recently (Fig. 5).¹⁰⁵ Structures for the closely related Cu⁺-transporting ATPase, CopA from *Legionella pneumophila*,^{106,107} and a model based on cryo-electron microscopy of CopA from *Archaeoglobus fulgidus* (Fig. 2) are also available.¹⁰⁸ Transmembrane helix 6 of many P_{1B}-ATPases harbours a CPC motif where both Cys residues may provide a metal binding site. In bacterial Zn-ATPases, this site comprises at least one further conserved Asp residue from the C-terminal helix 8, as shown by metal binding studies in mutated ZntAs.¹⁰⁹ The crystal structures of *S. sonnei* ZntA indeed show this residue (D714) close to the CPC motif (Fig. 5a). In the E2-P_i state only, which corresponds to a state after metal release, the carboxylate of D714 is hydrogen-bonded to the conserved K693; it is proposed that this switch may block/inhibit zinc binding to the intra-membrane site. Crucially, both Asp714 and Lys693 are absolutely essential for ATPase activity.¹¹⁰

Many of these ATPases also contain an N-terminal, cytosolic metal binding domain (MBD, sometimes also referred to as

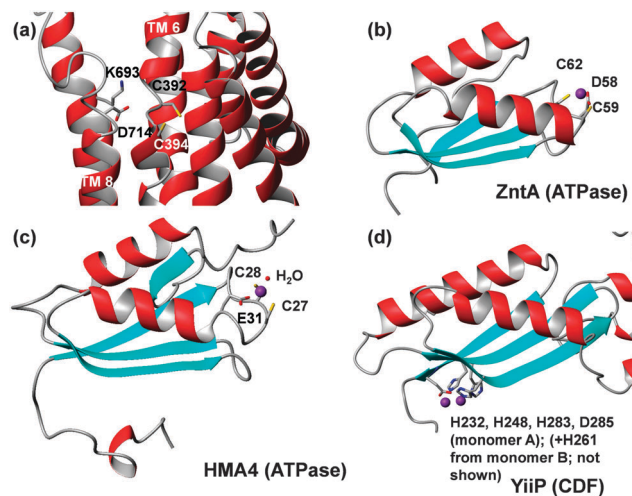


Fig. 5 Metal binding residues and sites in ATPases and ferredoxin-like MBDs. (a) Transmembrane site of *E. coli* ZntA in the E2P state (pdb 4umv).¹⁰⁵ Besides the two Cys residues, Asp714 is also suggested to be involved in metal binding. Alternatively, it can form a salt bridge with Lys693. ATPase MBDs contain a flexible metal-binding loop with DCxxC ((b), bacteria) or CCxxE ((c), plants) motifs between strand 1 and helix 1. In contrast, the metal-binding sites in the MBD of CDFs (d) are located at the opposite end of the domain, and coordinating residues are located in relatively rigid β -strands. Also see Fig. 6.

HMA, for heavy-metal associated domain). In none of the X-ray structures of the full-length proteins were these domains visible, likely due to disorder. The cryo-EM model of CopA has enabled location of this domain in the Cu-free E2 and E2P forms, where it interacts with N, P and A domains (Fig. 2). Contrastingly, the MBDs in both CopA and ZntA have also been docked in a different location,^{105,106} close to the membrane surface where an interaction with an amphipathic helix may take place. This helix is part of a proposed “platform” for metal entry into a funnel leading to the intra-membrane site.

Isolated MBDs of *E. coli* ZntA,¹¹¹ *Synechocystis* ZiaA,¹¹² and *A. thaliana* HMA4⁵² have been studied by solution NMR spectroscopy, but Zn-bound structures are only available for ZntA (pdb 1mwz¹¹¹) and HMA4 (pdb 2kkh⁵²) (Fig. 5b and c). All MBDs adopt a ferredoxin-like fold, and both ZntA and ZiaA MBDs bind Zn through a DCxxC motif, whilst the plant MBDs have a CCxxE motif. Thus, whilst the protein folds are very similar, the metal binding motifs are not, yet the overall composition (Cys, Cys, carboxylate) of the binding sites is still the same. It is thought that a fourth ligand is provided by water, and that the carboxylate binds in a monodentate fashion. Furthermore, the presence of three negatively charged residues may be sufficient to suppress the Lewis acidity of bound Zn²⁺.⁶⁵

The MBD is not required for transport or ATPase activity, and mutating the two Cys in the DCxxC motif of ZntA to Ala did not negatively affect transport either, suggesting that the MBD did not block access to the intra-membrane site, and was also not required for Zn²⁺ delivery. However, mutation of the two Cys to Ser in *E. coli* ZntA reduced ATPase activity by ca. 50%,¹¹⁰ suggesting a regulatory function, with the apo-MBD inhibiting the enzyme in the absence of the substrate metal ion.



E. coli ZntA lacking the MBD has been studied by EXAFS, providing structural information on the transmembrane (TM) site.¹¹³ The best fit was obtained with 2 S ligands at 2.30 Å and 2 N/O ligands at 2.0 Å, hence this site is likely to be four-coordinate. Affinities have also been determined for both MBD and TM site, and were found to be similar,¹⁰⁹ consistent with the idea that both sites are formed by two Cys and one carboxylate ligand.

Both HMA4⁵² and ZiaA¹¹² MBDs bind Cu⁺ more tightly than Zn²⁺. In either organism, there are additional ATPases with higher affinity towards Cu⁺,^{52,112} and these systems have been used to illustrate that “specificity” can be achieved through “relative affinities”:¹¹⁴ as long as there is sufficient Cu-binding capacity provided by dedicated proteins, other proteins meant to bind or deal with less competitive metal ions are left free to do so. Furthermore, the cytosolic copper chaperone ScAtx1 cannot interact with ZiaA, but direct protein–protein interactions, as well as Cu⁺ exchange, occur with two copper-transporting ATPases, PacS and CtaA.¹¹² Similar dedicated interactions between ATPase and copper chaperone are common for Cu⁺ pathways, but unknown for zinc. It is thus possible that at least some MBDs provide an additional selectivity filter – both through their tailored metal sites, as well as through their protein surfaces.

Finally, Zn-transporting ATPases from plants (e.g. HMA2 and HMA4 from *A. thaliana*) also harbour Cys- and His-rich C-terminal stretches that are located in the cytosol.¹¹⁵ The Cys residues are arranged in characteristic patterns including CC and CC_xC motifs, and some of the His residues occur consecutively with no intervening other residues – resembling engineered His-tags. Like the His-rich stretches in ZnuAs, these sections are likely to be structurally disordered, and their role is unclear, although it is known that Zn binding elicits a conformational change that may influence interactions with the other cytosolic domains and affect their activity.¹¹⁶ Indeed, mutant *A. thaliana* HMA4 lacking its Cys/His-rich C-terminal tail was more efficient at pumping Zn²⁺ and Cd²⁺, consistent with an inhibitory function of this portion when not fully occupied.¹¹⁷ The same study also reported that up to 10 Zn²⁺ ions could be bound to this section.

2.2.3 Cation-diffusion facilitator (CDF) proteins. The name of this protein superfamily (TC 2.A.4; Table S1, ESI†) is somewhat misleading, as its members are secondary active transporters, *i.e.* the transport process is not passive diffusion, but active and requires energy, in the form of an electrochemical potential. CDF transporters use the proton-motive force,¹¹⁸ function as M²⁺/H⁺ antiporters,¹¹⁹ and predominantly transport metal cations out of the cytosol, either outside the cell or into another compartment, for example the periplasm in Gram-negative bacteria, the vacuole in plants and yeast, mitochondria in eukaryotic cells, and the endoplasmic reticulum, Golgi, lysosomes, endosomes and other vesicles, including synaptic vesicles and various secretory granules in mammalian cells.^{120,121} The CDF proteins in mammals are named ZnT (also Slc30), and several of these have been implicated in various diseases. Mutations in ZnT8, the protein which transports zinc into

secretory granules in pancreatic beta cells for insulin packaging, have been linked to some cases of both type 1 and type 2 diabetes,¹²² although a very recent study involving the sequencing or genotyping of 150 000 patients has shown that loss-of-function mutation of ZnT8 in fact reduced the risk for diabetes.¹²³ Non-functional ZnT4 is responsible for the “lethal milk” syndrome,¹²⁴ due to a failure to load secretory vesicles in mammary epithelial glands with zinc, whilst mutant ZnT2 can lead to transient neonatal zinc deficiency.¹²⁵ ZnT3 in the brain loads Zn²⁺ into pre-synaptic vesicles of zingergic neurons, and its deficiency is associated with cognitive dysfunction.¹²⁶

The structure of a zinc- and iron-transporting CDF protein, YiiP (FieF) from *E. coli*, has been determined (Fig. 2, 3 and 6).¹²⁷ YiiP is a homo-dimer with 6 transmembrane helices per monomer. Each monomer also comprises a cytosolic metal-binding domain (MBD) with a ferredoxin-like fold, similar to that found in many P_{1B}-ATPases – but with completely different metal-binding sites (Fig. 5).¹¹⁵ In the dimer, the two cytosolic MBD are linked by two bridging zinc ions (Fig. 6), the binding residues of which are conserved in other Zn/Fe-CDFs.¹¹⁵ It has been proposed that the two zinc ions are necessary to keep the two cytosolic domains together, which otherwise would be driven apart by charge repulsion.¹²⁸ The isolated cytosolic domain of CzrB from *Thermus thermophilus* also formed dimers, with similar Zn-binding sites.¹²⁹ Size-exclusion HPLC analysis indicated that for the full-length protein, zinc did not change the oligomerisation state, but FRET analysis demonstrated that a conformational change, probably involving “en-bloc” movement of

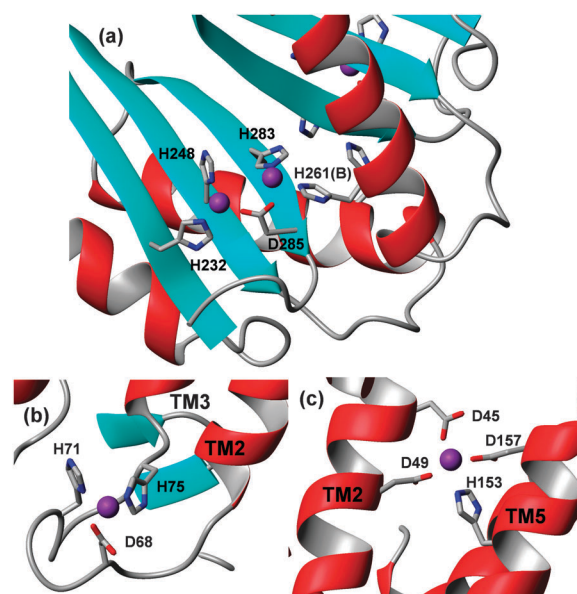


Fig. 6 Details of Zn-binding sites in *E. coli* YiiP. (a) The binuclear, inter-protomer binding site in the cytosolic MBD. Four metal-binding residues are provided by all three β -strands, giving a fairly rigid scaffold for Zn²⁺ coordination. The fifth ligand (H261) comes from helix 2 of the second monomer. The tetrahedral coordination for both Zn²⁺ ions is completed by two water molecules (not shown). (b) A further cytosolic site is located in a loop between TM2 and TM3; the fourth ligand is a water molecule (not shown). (c) The four-coordinate transmembrane transport site is formed by two residues from helix TM2 and two from TM5.



entire domains, occurred upon zinc binding. This is also consistent with a recent study using cryo-electron microscopy.¹³⁰ The 13 Å structure of Zn-free YiiP revealed a conformation different to that found in the Zn-bound structure, consistent with pivoting and/or scissoring of the transmembrane domains. An outward-facing conformation in the presence of Zn, and an inward-facing conformation in its absence was proposed. It is possible that these domain movements are at least partially mediated by the cytosolic MBDs. An ITC study suggested that a CDF protein from the bacterium *Maricaulis maris* lacking the MBD was still capable of binding Zn²⁺ and Cd²⁺ with micromolar affinity.¹³¹ Truncated versions of *Cupriavidus metallidurans*§ CzcD and *E. coli* ZitB lacking the MBD also were still capable of metal transport *in vivo*, but less so – hence the C-terminal MBDs were required for full functionality.¹³² All observations are consistent with the notion that the cytosolic domains may act as regulatory sensors for excess cytosolic zinc, and allosterically promote Zn²⁺ efflux by the transport domains.

In the YiiP structure, two further zinc ions were bound per monomer; one in the cytosolic loop connecting TM helices 2 and 3, formed of two His, one Asp, and one water ligand, and one intra-membrane site. The latter is the actual transport site; helix 2 provides two Asp ligands, and helix 5 an Asp and a His residue (Fig. 6). The second cytosolic site may modulate the packing of helices, and hence influence the transport site.

The binding of Zn²⁺, Cd²⁺ and Hg²⁺ to YiiP were studied by ITC.¹³³ At least two sets of binding sites for Zn²⁺ were evident, with $K_{D(ITC)} = 3 \mu\text{M}$ and $159 \mu\text{M}$, and there were also indications for a further binding site with higher affinity that was not adequately captured by the ITC experiments. It is possible that this high-affinity site corresponds to at least one of the interprotomer sites located in the MBD, and that one of the weaker sites corresponds to the transport site. An ITC study of a CDF protein from the hyperthermophile *Aquifex aeolicus* indicated that both Zn²⁺ and Cd²⁺ binding was endothermic,¹³⁴ suggesting that structural changes were required to accommodate the ions. Again, this would fit with the metal binding sites at the protomer interface, and be consistent with a regulatory role.

Contrary to their exclusive use for zinc in mammals, members of the CDF family in other phyla (plants, invertebrates, fungi, bacteria) also transport Fe²⁺ and Mn²⁺. Understanding the selectivity of CDF proteins is an active research area. Sequence analysis has suggested that these can be organised into three groups,¹³⁵ Zn-CDF, Fe/Zn-CDF, and Mn-CDF according to the principal transported metal ion(s). Hallmarks for metal specificity identified in the latter study included likely metal-coordinating residues in TM helices 2 and 5, as well as presence or absence of a His-rich cytosolic segment – similar to those stretches found in ZnuA's and Zn-transporting P-type ATPases (see Sections 2.2.1 and 2.2.2). *E. coli* YiiP belongs to the Fe/Zn-CDF group, and does not contain such a segment. A recent study on human ZnT5 and ZnT8 demonstrated that neither are capable of transporting Cd²⁺, but that a single His-to-Asp mutation in TM2 resulted in proteins with similar Zn²⁺ transport activity as the wild-type, but that could also promote the efflux of

Cd²⁺.¹³⁶ Metal specificity in CDF proteins from plants (termed metal tolerance proteins, MTPs) has also been investigated, mainly by complementation assays using wild-type and mutant proteins in Zn-sensitive yeast. One study identified several non-coordinating residues in the His-rich loop as well as in TM helix 3 of *A. thaliana* MTP1 as determinants for Zn selectivity over Co²⁺.¹³⁷ Another study on *A. thaliana* MTP1 identified N-terminal Cys residues as essential for conferring Zn tolerance to a sensitive yeast strain.¹³⁸ Mutation of several non-coordinating residues in TM helices 2 and 5 enhanced Zn transport, as did deletion of 12 N-terminal residues. Deletion of 28 residues from the N-terminus made no difference, but deletion of 55 N-terminal residues comprising two conserved Cys residues led to a non-functional protein. A range of other mutations, including deletion of the His-rich loop, led to loss of Zn selectivity. We have shown that the C-terminal cytosolic metal-binding domain (MBD) for plant MTPs is sufficient for achieving a similar phylogenetic clustering as that obtained when using the full length proteins, and sequence comparisons focusing on the likely MBD metal-binding residues suggested that at least part of the selectivity may be mediated by the MBDs.¹¹⁵ Great caution always has to be applied when inferring substrate selectivity from protein sequence data, and our proposition has not been experimentally tested yet.

2.2.4 ZIP proteins. Zinc-iron permeases (ZIP; the abbreviation also stands for ZRT/IRT-like proteins, where ZRT or IRT stands for zinc-regulated or iron-regulated transporter; TC 2.A.5) mediate metal uptake into the cytosol, either from the extracellular space or from organelles. They were first discovered in plants,¹³⁹ but are now known to occur in all kingdoms of life including archaea.¹⁴⁰ Humans have 14 ZIP proteins (also classified as Slc39), and the plant *Arabidopsis thaliana* has at least 18 ZRT/IRT proteins.¹¹⁵ Some ZIP proteins seem to be constitutively expressed, and some, like yeast Zrt1 and Zrt2, are inducible by zinc limitation through the ZAP1 transcription factor.⁷² Mechanisms for ZIP induction in multicellular eukaryotes have only recently, with the discovery of zinc-responsive bZIP proteins in plants,[¶] begun to be elucidated, but no corresponding information is as yet available for ZIPs in animals. At least some ZIP proteins in mammals may be regulated by manifold post-transcriptional processes,^{58,140} as well as by trafficking and rate of degradation.^{58,59} Zip11 and Zip63 from the freshwater cyanobacterium *Nostoc punctiforme* are inducible and are required for maintenance of intracellular zinc levels,¹⁴¹ whilst the ZIP protein ZupT in *Salmonella enterica* is constitutively expressed and required for maximum virulence.¹⁴²

Their wide distribution and importance in human diseases notwithstanding,^{120,140,143} very little biophysical data and no X-ray structures for any ZIP protein are available. Despite the tremendous progress in the study of other membrane proteins, including those for zinc transport, in recent years, ZIP proteins have proven to be extremely difficult to express and purify in a functional form. Consequently, the mechanisms of transport and determinants of metal specificity are not yet well understood, although a recent breakthrough, described in more

§ Previously known as *Ralstonia metallidurans*, and *Alcaligenes eutrophus*.

¶ Note that the “ZIP” abbreviation in these two families is coincidental.



detail below, has begun to remedy this situation.¹⁴⁴ Most ZIP proteins are predicted to have 8 transmembrane helices, and many, irrespective from which phylum, comprise histidine-rich cytosolic loops. Extracellular loops tend to be short, with the exception of some animal ZIPs (e.g. human ZIP5, 6 and 10) which have large (ca. 200–300 amino acid residues) ectodomains which are evolutionarily related to prion proteins.¹⁴⁵ Due to the difficulties with studying full length ZIP proteins, some *in vitro* metal binding studies have been carried out on fragments. The peptide PHGHGHGHP from the large intracellular histidine-rich loop of IRT1 from *A. thaliana* has been studied by ITC.¹⁴⁶ IRT1 is regulated by a requirement for iron, and can transport Fe²⁺, but also Mn²⁺, Zn²⁺, Co²⁺ and Cd²⁺.¹⁴⁷ Thermodynamic parameters measured for the 2+ ions from Mn through to Zn, plus Cd²⁺ and Fe³⁺, followed the same trends as small-molecule chelators such as trien, but the loss of entropy was considerably larger. The pH- and buffer-independent stability constant for Zn²⁺ (log *K* = 6.21–6.69) was surprisingly low. *A. thaliana* IRT1 also contains an extracellular metal-binding loop. The Ac-(95)MHVLPDSFEMLSICLENPWVK(117)-NH₂ peptide derived from this loop, and an N-terminal fragment of human ZIP13 have been studied by NMR, mass spectrometry (MS) and potentiometry.¹⁴⁸ The latter technique yielded stability constants of log *K* = 14.75 for the IRT1 loop, which contains two His, one Cys, and several carboxylate groups, and 17.85 for the ZIP13 fragment that contained four Cys residues. These numbers are stoichiometric, not conditional constants, *i.e.* the conditional constants valid at neutral pH will be lower. MS analysis demonstrated that only 1:1 complexes were observed, and ¹H and ¹³C NMR spectroscopy showed that both metal-free and metal-bound peptides were disordered. The selective broadening of a number of resonances supported the coordination of Zn²⁺ by His, Cys and Glu in the IRT1 peptide. It is not yet known whether these protein regions contribute to selectivity; this will require studies of intact mutant proteins in a membrane environment.

Biochemical transport assays have shown that transport of Zn²⁺ by human ZIP1 and ZIP2 is stimulated by HCO₃[−],¹⁴⁰ as is that of human ZIP8.¹⁴⁹ Most recently, the bacterial ZIPB from *Bordetella bronchiseptica* has been purified and reconstituted in proteoliposomes, providing the so far most detailed biophysical study of a ZIP protein.¹⁴⁴ Purified ZIPB was dimeric. Zinc fluxes were monitored by stopped-flow fluorometry utilising various metallochromic dyes, which demonstrated that zinc transport by this protein obeyed a first order rate law. This suggests passive electrodiffusion. Zn²⁺ was the only divalent cation that elicited an electrogenic effect. Electric membrane potentials generated by K⁺ were able to drive Zn²⁺ transport in both directions, *i.e.* in a voltage-dependent manner. In addition, transport was shown to be pH dependent, but not driven by the proton-motive force.¹⁴⁴ No enhancement of transport was observed in the presence of HCO₃[−], and other anions inhibited transport. Taken together, these results demonstrated that ZIPB functions as a Zn²⁺ (and Cd²⁺) selective channel, and suggested that transport is driven by *in vivo* Zn²⁺ gradients. Since the free, hence electroactive, concentrations of Zn²⁺ inside the cytosol are extremely low (pico- to femtomolar),

gradient-driven electrodiffusion through a selective channel can provide an efficient uptake mechanism – providing that the free Zn²⁺ concentration in the extracellular (or organellar) medium is higher than the cytosolic concentration. This may often be the case in multicellular eukaryotes, but depending on their environment, is perhaps less common for unicellular organisms. It is also noteworthy that even though ZIPB works as a channel, Zn²⁺ transport was very slow – even slower than that measured for the secondary active transporter YiiP (Section 2.2.3). Furthermore, if more ZIP proteins turn out to be channels rather than porters, a re-classification will be required.

Human ZIP4, the protein linked to the skin disease Acrodermatitis enteropathica, also has an N-terminal extracellular domain which is rich in His and Cys residues.¹⁵⁰ hZIP4 was heterologously expressed in *Xenopus* oocytes, and zinc transport was monitored using radioactive ⁶⁵Zn²⁺. Analysis of the kinetics of uptake yielded two *K_M* values of 76 nM and 1.4 μM. The only metal ions that could compete with Zn²⁺ were Cu²⁺ and Ni²⁺, but in contrast to other ZIP proteins, not Cd²⁺ when present at micromolar concentrations. *E. coli* ZupT was shown to also promote the uptake of Fe²⁺, Co²⁺, and Mn²⁺.¹⁵¹ Plant ZIP proteins have also been reported to be relatively non-selective, promoting the uptake of Zn²⁺, Cd²⁺, and Fe²⁺.¹⁵² It is becoming clear that the metal selectivities of ZIP proteins may vary quite considerably, and it will be interesting to see how and where this selectivity is determined.

2.2.5 Tripartite RND-type pumps. In Gram-negative bacteria, ATPases and CDF proteins transport zinc ions from the cytosol into the periplasm, where they can either become bound to various periplasmic proteins, including ZraP (see Fig. 1 and Section 2.3.3), or presumably diffuse through porins into the exterior. In addition, a number of proteobacteria also have systems that actively promote efflux into the exterior medium. These systems belong to the tripartite resistance-nodulation-cell-division (RND) superfamily (TC 2.A.6), representatives of which are found in bacteria, archaea, and eukaryotes. One sub-group is involved in the efflux of organic substrates, and another in that of both mono- (e.g. *E. coli* CusCBA, which transports Cu⁺ and Ag⁺ (ref. 153)) and divalent metal ions (*Caulobacter crescentus* CzcCBA,¹⁵⁴ *Cupriavidus metallidurans* CzcCBA and ZncCBA,^{155,156} *Pseudomonas aeruginosa* CzcCBA¹⁵⁷).

RND systems are composed of three proteins: in the case of metal-transporting RND pumps, the “A” component is trimeric, spans the inner membrane and reaches into the periplasm; the “C” component is also trimeric, spans the outer membrane and also reaches into the periplasm; and six periplasmic “B” adaptor proteins are necessary to link these two trimers (Fig. 1 and 7).^{158,159}

The inner membrane CusA protein can use the proton-motive force for metal translocation, even in the absence of the “B” and “C” components,¹⁶⁰ although there is debate as to whether RND-mediated transport from the cytosol (“transenvelope”) is of major importance, or whether the main compartment from

|| In its narrower sense, the “RND” label refers to the inner-membrane-associated component of the tripartite pumps only.



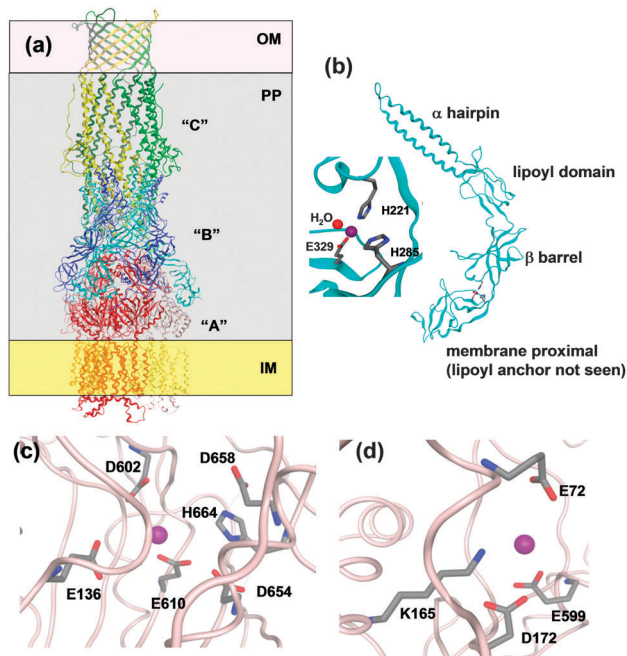


Fig. 7 Overall structure of an RND efflux system and resolved zinc binding sites in ZneA and ZneB. (a) Sketch using CusBA and C (pdb 3ne5¹⁶² and 3pik¹⁶¹). The three CusC monomers are drawn in yellow, light and dark green, the six CusB monomers in blue and cyan, and the three CusA monomers in red, dark pink, and light pink. OM = outer membrane, IM = inner membrane, and PP = periplasm. (b) Overall structure and zinc binding site in ZneB (pdb 3lnn).¹⁵⁵ (c) Residues within a 4.5 Å radius of the “proximal” Zn site in one of the ZneA protomers (pdb 4k0j). In addition, E610 and D658 which have also been implicated in Zn binding¹⁵⁶ are shown. (d) Residues within a 4.5 Å radius of the “distal” Zn site in one of the ZneA protomers.

which metals are transported is the periplasm.¹⁵⁸ Several findings support the latter proposition, not least the fact that the expression of RND systems is often regulated by a two-component sensor system.¹⁵⁷ The inner-membrane-spanning sensor histidine kinases of these systems sense the composition of the periplasm, not the cytosol (as illustrated in Fig. 1).

Several structures of RND systems are available, including ZneA¹⁵⁶ and ZneB¹⁵⁵ from *Cupriavidus metallidurans*. The structures of CusC¹⁶¹ and the CusBA complex¹⁶² from *E. coli* also have been determined recently, permitting insights on the entire tripartite complex. Trimeric CusC forms an α/β -barrel (Fig. 7). The outer-membrane spanning portion consists of a porin-like 12-stranded β -barrel, whilst the 100 Å long periplasmic tube is formed from 12 α -helices. The “A” component (CusA or ZneA) comprises three domains. 3×12 α -helices span the inner membrane; the periplasmic pore or porter domain consist of four sub-domains, and the “outer-membrane factor docking” domain has a similar diameter to that of the “C” component, likely enabling direct interactions between the two. This domain is also thought to be the exit funnel for substrates. The pore domain is accessible from the periplasm. For the related RND protein AcrB, three conformational states have been observed in crystal structures: “open access”, “bound”, and “extrusion”, with each “A” type protomer adopting one of

these conformations.¹⁶³ It is thought that this enables “functional rotation” and is related to energy coupling of zinc transport. In the ZneA structure determined at low pH (5.2), two of the three protomers were partially occupied by Zn at a site comprising E136, D602, E610, D645, and D658, termed the proximal site, located in the centre of the pore domains. In the structure at higher pH (7.5), all three proximal sites were occupied, and one second, distal site (D172, E599 from one protomer and E72 from another), located near the exit funnel, was also detectable (Fig. 7). Both sites were thought to mediate transfer to ZneC.¹⁵⁶ No channel for zinc transport was detected in the ZneA inner-membrane domain, contrary to a methionine-lined channel in CusA.¹⁶⁰ Therefore, the ZneA structure is compatible with export from the periplasm only.

Finally, the periplasmic adaptor protein “B” (e.g. CusB and ZneB) consists of four domains, namely an α -hairpin (or 3-helix bundle in the case of CusB) domain which interacts with the helices of the periplasmic portion of the “C” protein, a lipoyl domain consisting of two 4-stranded β -sheets, a small 6-stranded β -barrel, and the membrane-proximal domain which is anchored to the inner membrane through an N-terminal lipoyl tail (Fig. 7). The ZneB structure contains Zn²⁺ bound to a His₂Glu site between the membrane-proximal and β -barrel domains (Fig. 7), and Zn²⁺ binding induces conformational changes, observed in both crystal and solution states.¹⁵⁵ The location of the zinc site enables a hinge-like movement, and thus the adaptor protein could alter the distance between the “A” and the “C” periplasmic funnels. For the CusBA complex, a direct transfer of the metal ion from the “B” to the “A” component has been dismissed, as in the assembled complex, the metal sites in the two proteins are too far apart.¹⁶² Instead, it can be envisaged that Zn²⁺ binding to sites in ZneB and ZneA stabilise conformations that allow the collection of Zn²⁺ in the ZneA pore, followed by extrusion of excess Zn²⁺ through ZneC from the periplasm. This suggests that both ZneA and ZneB have an active role in Zn²⁺ flux control, with ZneA providing energy through proton antiport coupled to conformational changes, and ZneB also operating as part of a “switch” mechanism.¹⁵⁸ The apparent dissociation constant of Zn²⁺–ZneB has been reported as 3 μ M; it is possible that this corresponds to a set point at which the transport efficiency of the RND system changes.

2.2.6 Other membrane proteins implicated in zinc transport. Apart from the most prevalent systems described above, a number of novel zinc-transporting and -binding proteins have been discovered in recent years – in particular in pathogenic bacteria. Some representatives for which structural data are available will be briefly discussed in this section.

ZntB in γ -proteobacteria. A third zinc efflux protein found in enterobacteria and other pathogenic γ -proteobacteria, such as *Vibrio cholerae* and *Yersinia pestis*, is ZntB,¹⁶⁴ which belongs to the CorA family of metal transporters (TC 1.A.35). ZntB is an inner-membrane protein like the ATPase ZntA. Although it is classified as a channel, ZntB can export Zn²⁺ against its concentration gradient. It has been suggested that this process may be driven by H⁺ antiport, but no data to support this hypothesis have been published yet. If true, then ZntB will need to be reclassified as a class 2 porter.



CorA family proteins form homo-pentamers. The first 266 amino acids of *Salmonella typhimurium* ZntB form a large cytosolic domain, with the remaining 61 residues forming two transmembrane helices.¹⁶⁵ The structure of the large cytosolic soluble domains of *Vibrio parahaemolyticus* (pdb 3ck6)¹⁶⁶ and *S. typhimurium* ZntB (pdb 3nwi¹⁶⁷) (Fig. 8) have been determined. The two pdb entries show different structures of the “funnel” formed between the five monomers: the cylindrical channel with a diameter of 12 Å of the *S. typhimurium* structure is thought to represent an open form, whilst the conical shape of the pore in the *V. parahaemolyticus* structure is thought to show a closed conformation. The differences in channel structure come about by using alternative interaction surfaces between the monomers. Each monomer in the *S. typhimurium* structure, which was obtained by crystallisation in the presence of 1 mM ZnCl₂, contains two zinc ions, bound by one Cys and one His residue (Fig. 8). Site 3 also has a Glu residue nearby, but its sidechain atoms were not resolved. ITC measurements of both cytosolic domain and full-length ZntB indicated apparent Zn²⁺ dissociation constants in the micromolar range, similar to numbers found for other Zn²⁺ transporters.

The zinc sites found in *S. typhimurium* ZntB are not conserved in ZntB from *V. parahaemolyticus* and no metal sites were resolved in its structure. Instead, many chloride ions were detected. The latter observations, together with electrostatic potential calculations, had previously led to the suggestion that metal transport in ZntB is mostly mediated by electrostatic forces – and hence perhaps would not require dedicated zinc binding sites. The likely 3-coordinate site 3 in *S. typhimurium* ZntB, together with its micromolar dissociation constant, suggests that ZntB from different bacteria may have different mechanisms.

Polyhistidine triad proteins. Polyhistidine triad (Pht) proteins are a family of surface proteins recently discovered in pathogenic Gram-positive Streptococci.¹⁶⁸ Pht proteins interact with components of the immune system; they can bind complement factor H, impairing the deposition of complement C3 on the bacterial cell surface, and are thus required for pneumococcal virulence. They are currently being considered for their potential use in novel vaccines. Their expression is zinc-regulated, *via* the AdcR sensor protein.¹⁶⁹ It has been suggested that zinc-dependent expression confers the ability to adapt protein expression to different host environments.

The name of these proteins stems from the presence of 5–6 instances of a HxxHxH motif. A crystal structure for a 54-amino acid segment of PhtA (pdb 2cs7)¹⁷⁰ and a solution NMR structure for a 137-amino acid segment of PhtD (pdb 3zff; Fig. 9),¹⁰³ homologous members of this family, are available. Both comprise one Zn²⁺ ion, coordinated by three His and one Glu residue.

The *phtD* gene occurs in an operon with *adcII*, one of the extracellular components of the AdcABC transporter (see Section 2.2.1). The proteins AdcAII and PhtD interact *in vivo* and *in vitro*, and directional transfer of Zn²⁺ from PhtD to AdcAII has been demonstrated.¹⁰³ This, together with the regulation by AdcR, would be concordant with a role in zinc

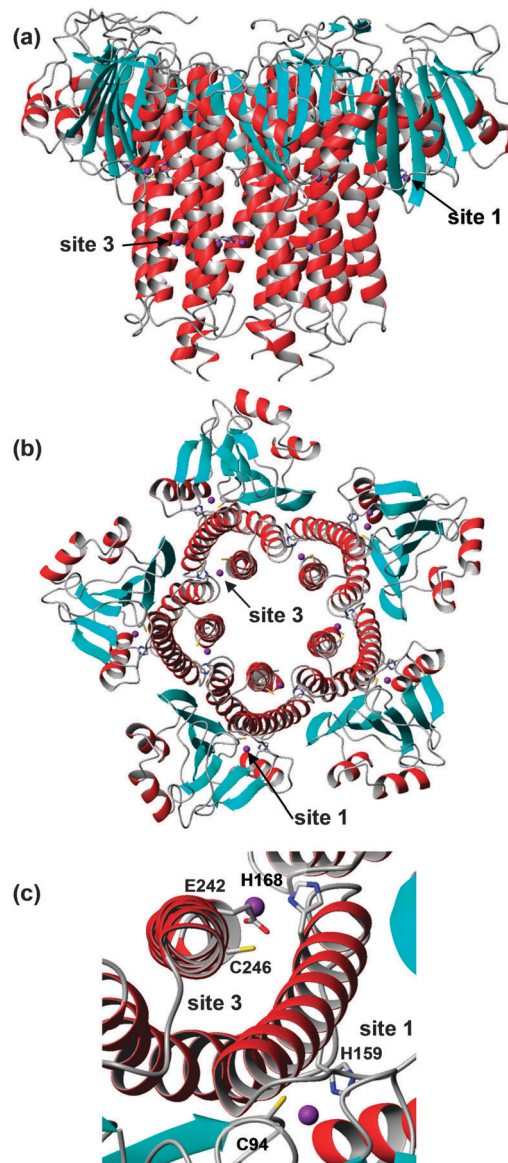


Fig. 8 Overall structure and zinc-binding sites of soluble domain of *Salmonella typhimurium* ZntB (pdb 3nwi). (a) Pentameric assembly; the transmembrane domain would be located below the lower end of the structure. (b) Pentameric assembly, seen from the membrane side and showing locations of sites 1 and 3. (c) Detail of sites 1 and 3. The sidechain of E242 is not resolved in the X-ray structure, but has been reconstructed using Swiss pdb viewer.

uptake at low zinc levels, rather than protection against toxicity, although the finding that excess zinc leads to the upregulation of Pht proteins seems to contradict this hypothesis.¹⁶⁹

Outer-membrane channels in Gram-negative bacteria. Whilst the substrate-binding proteins (AdcA/ZitS) in Gram-positive bacteria are essentially exposed to the cell exterior, this is not the case for the periplasmic ZnuA proteins. The presence of periplasmic zinc sensors, briefly mentioned in Section 2.2.5, points towards an at least partial regulation of metal levels within the periplasm, but this implies a certain level of control



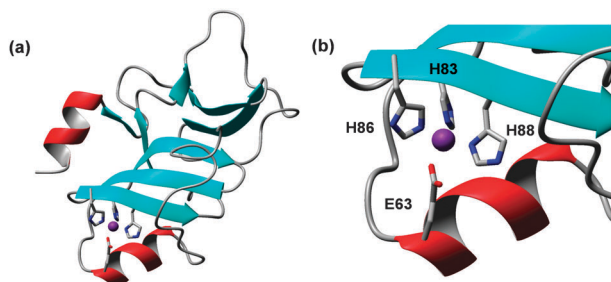


Fig. 9 Structure of PhtD from *Streptococcus pneumoniae* (pdb 3zfj). (a) Overall fold of one Pht domain. (b) Details of zinc binding site.

over transport across the outer membrane. Very little is known about zinc uptake across this barrier; mostly, it seems to have been assumed that Zn^{2+} enters the periplasm by diffusion along a concentration gradient through non-specific pores. This may indeed be the case in zinc-replete conditions, but this notion becomes problematic when exterior free Zn levels become extremely low. Two of the most zinc-deprived environments are the open ocean (where free Zn^{2+} can be as low as single-digit picomolar), and mammalian host tissues and plasma invaded by pathogenic bacteria.

In support of a more active role of the outer membrane, several bacterial outer-membrane proteins have been shown to be zinc-regulated, including TonB-dependent receptors from the cyanobacterium *Anabaena* PCC 7120,²⁶ the soil γ -proteobacterium *Pseudomonas protegens*,¹⁷¹ the opportunistic pathogen *Acinetobacter baumannii*,¹⁷² and the pathogenic *Neisseria meningitidis*.¹⁷³ TonB-dependent receptors usually transport organic substrates, including siderophores for iron uptake. The upregulation of TonB-dependent receptors in response to zinc deprivation has led to suggestions that they may function in the transport of “zincophores” – biological zinc chelators analogous to siderophores. In addition, the expression of several porins of *Pseudomonas protegens*,¹⁷¹ that of the OprD porin in *Pseudomonas aeruginosa*,¹⁷⁴ and at least one cyanobacterial porin from *Synechococcus* WH8102¹⁷⁵ was shown to be zinc-dependent. In the latter case, zinc-binding ability was demonstrated by capturing the native protein on an immobilised zinc affinity chromatography column. Further work, including electrophysiological and structural studies, is required for a better understanding of the roles of bacterial outer-membrane proteins in zinc uptake.

2.3 Intracellular zinc trafficking

The past 15 years have seen an enormous increase in the identification and study of proteins necessary for the assembly of more or less complex metalloproteins such as Cu,Zn-superoxide dismutase, cytochrome *c* oxidase, iron-sulfur cluster proteins, nitrogenase and hydrogenase. The biosynthetic pathways for the latter proteins require an assembly line of proteins, some of which have the sole function of metal binding and transfer and/or final insertion into the metalloprotein. Whilst many players in these assembly lines have been identified for Mo, Fe, Co, Ni, and Cu ions, zinc chaperones had remained conspicuously absent.

This is not surprising, given the need to populate hundreds to thousands of different proteins: a dedicated chaperone per protein (as is the case for, e.g., copper) is clearly not a viable strategy. Instead, it is now thought that intracellular zinc is buffered and “muffled”, with the muffling at least partially mediated by metallothioneins (Section 2.3.1).⁵⁶ In addition, a new family branch of putative metallochaperones of the COG0523 cluster, from a variety of organisms, has been implicated in zinc homeostasis.¹⁷⁶ Finally, periplasmic zinc “chaperones” have been discovered, indicating a previously unexpected level of control over zinc concentrations in this bacterial compartment.

2.3.1 Metallothioneins (MTs) were amongst the first proteins discovered to have a role in metal homeostasis.^{177,178} Since then, proteins with similar properties (*vide infra*) to the original MT from horse kidney have been isolated from a variety of animals,¹⁷⁹ plants,^{180–184} fungi,¹⁸⁵ and bacteria,^{186,187} and several thousands of gene sequences for predicted MTs are known.¹⁸⁸ The lack of a clear evolutionary relationship between MTs from different phyla indicates that they may have evolved more than once.

The characteristic features of an MT are low molecular weight (usually less than 10 kDa), a high cysteine content (15–30%), scarcity or absence of aromatic residues, and spectroscopic evidence of metal-thiolate cluster formation. Fig. 10 shows some examples for zinc-binding MTs; it is clear that most of these feature very little secondary structure.

Indeed, MTs have only ordered structure when metals are bound – in essence, with respect to protein structure, the metal clusters fulfil the same stabilising role as a hydrophobic core would in other proteins. The presence of metal-thiolate clusters, relatively high solvent accessibility for at least some metal ions in the clusters, coupled with metal-dependent protein folding, leads to a peculiar combination of high thermodynamic stability with high kinetic lability – ideal prerequisites for intracellular zinc trafficking proteins. It is noted that the measurement of affinity constants of a protein with, e.g., 20 thiolate groups and seven metal binding sites is far from trivial, which might explain continuing disagreement about respective values,^{189,190} even though there is overall agreement on the conclusion that free thiols from partially metallated MTs are present *in vivo*, and participate in zinc homeostasis.^{191,192}

The way to the first 3D structures for an MT was long and took at least one wrong turn: the first X-ray structure published was in fact incorrect, and it needed the advent of protein NMR spectroscopy to set the record straight.¹⁹³ Besides requiring the development of the most important homonuclear ^1H , ^1H correlation experiments still in use today, heteronuclear ^1H , ^{13}C NMR experiments were essential for defining the correct metal-cysteine connectivities,¹⁹⁴ which in turn are indispensable for defining the complete structure of an MT. Most biological and biophysical work has been carried out on mammalian MTs, but the past decade has witnessed prolific research activity to understand MTs from other phyla.^{188,195–197} These studies have highlighted the enormous diversity in structures, properties and functions of these intriguing proteins. Up until 2001, two types of clusters were known for Zn-binding MTs: an M_3Cys_9



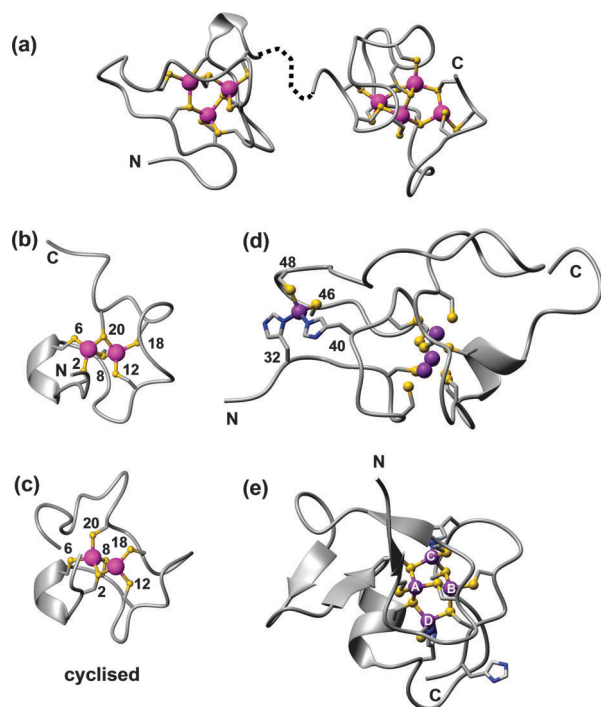


Fig. 10 Examples of zinc-binding metallothionein structures. (a) The two solution structures of the Cd₄-α (1mrt) and Cd₃-β (2mrt) domain of rat MT2, mapped on the X-ray structure (4mt2).¹⁹³ (b) One possible model for the γ domain of wheat E_C in its Cd₂ form (pdb 2l61).²¹¹ (c) Cyclised γ domain of wheat E_C (Cd₂ form; pdb 2mfp).²¹² (d) Domain 2 of wheat E_C (Zn₄ form, pdb 2kak),²⁰⁸ with residue numbers for the mononuclear His₂Cys₂ site. (e) SmtA from *Synechococcus* sp. PCC 7942 showing the Zn₄Cys₉His₂ cluster and the zinc finger fold (pdb 1jjd). The zinc finger site A is inert towards metal exchange, site C is the most reactive.

and an M₄Cys₁₁ cluster (Fig. 10a). These two general arrangements can be achieved by diverse primary sequences, and utilising different M-Cys connectivity patterns. It has remained impossible to predict these patterns from primary sequence alone. Despite all methodological progress, the dedicated ¹H, ^{111/113}Cd NMR experiments introduced in the 80ies are still required for their determination.

The structure determination of the first bacterial MT, SmtA from *Synechococcus* sp. PCC 7942, brought a new level of structural variation.¹⁹⁸ SmtA contains only nine Cys residues, but binds four Zn²⁺ or Cd²⁺ ions. Two further ligands are provided by the imidazole side chains of two His residues, giving an M₄Cys₉His₂ cluster (Fig. 10e), the structure of which closely resembles that of the M₄Cys₁₁ clusters in mammalian MTs,¹⁹⁹ but with replacement of two terminal Cys residues by His. The two histidines have different roles in the cluster. His40 is located at the division line between the N-terminal “zinc-finger-like” portion of SmtA, which contains significant secondary structure, and the C-terminal “MT-like” portion, which lacks secondary structure like other MTs. His40 is essential to order this C-terminal section, as shown by studying a His40Cys mutant, in which structural order in the C-terminal section was markedly decreased.²⁰⁰ His49 is part of the most solvent-exposed and reactive metal site C, and reduces the redox liability

of this site.²⁰¹ Histidines are present in many other MTs²⁰² but do not always participate in metal binding. For example, His55 in SmtA is not coordinated to a metal ion. The knowledge of important structural features has enabled the discovery of further bacterial MTs from cyanobacteria, pseudomonads and other α- and γ-proteobacteria, some of which have been characterised *in vitro*.²⁰³ The four Cys residues defining the zinc finger site A are strictly conserved, whilst a loop providing some of the residues for the most solvent-exposed site C is variable. Since this site governs the reactivity of the MT,²⁰¹ it seems likely that different bacterial MTs have evolved to operate in different conditions.

Whether the zinc finger in SmtA engages in any biomolecular interactions has remained unknown, but it has a crucial effect on the dynamic behaviour of the protein. Using high-resolution mass spectrometry and the ⁶⁷Zn isotope, the Zn²⁺ self-exchange reaction was studied, revealing that site A was inert towards metal exchange.²⁰⁴ This is due to the inaccessibility of this site, and indeed, the reaction of Zn₄SmtA with an excess of Cd²⁺ did not lead to the expected full exchange, but yielded a Cd₃ZnSmtA species. This product is entirely kinetically controlled; Cd₄SmtA can be readily generated from apo-SmtA by reconstitution with Cd²⁺, and adopts a well-ordered structure similar to that of Zn₄SmtA.²⁰⁵

Such isostructural replacement of Zn²⁺ by Cd²⁺, whilst common in mammalian and a few other structurally characterised MTs, is not a necessary occurrence; a notable exception is the plant type 4 MT E_C from wheat.^{206–210} Wheat E_C is a two-domain MT; the N-terminal 20-residue domain contains an M₂Cys₆ cluster,²¹¹ and folds equally well with either Zn²⁺ or Cd²⁺ (Fig. 10b). A designed cyclised version of this domain also adopts similar structures with Zn²⁺ and Cd²⁺, and allowed the unambiguous determination of ¹¹³Cd-Cys connectivities, with Cys2 and Cys8 as bridging residues (Fig. 10c).²¹² The C-terminal domain is with 47 residues considerably larger, and only adopts ordered structure in the presence of four Zn²⁺ ions, whilst the Cd₄ form is disordered.^{208,209} The reason for this differential behaviour is the presence of a mononuclear Cys₂His₂ site in this domain (Fig. 10d); occupation of this site is important for the folding of the entire domain. We have proposed that according to the HSAB principle, the four Cd²⁺ ions that can bind to this domain do not utilise the two His residues, but instead form alternative clusters with the remaining eleven Cys residues, which are sufficient for a Cd₄Cys₁₁ cluster as found in other MTs.²⁰⁹

Specificity for a less competitive metal ion can normally not be engendered by absolute thermodynamic stability. In the case of thiolate ligands, the order of stability is Zn²⁺ < Cd²⁺ < Cu⁺; this is evidenced by differences in pH stability and metal-replacement titrations.¹⁷⁸ Nevertheless, the example of wheat E_C has shown that differential behaviour towards similar metal ions may be achieved by different protein folding and dynamics, mediated by the most favourable metal-ligand combinations. A more dynamic structure may have implications for *in vivo* protein stability; indeed, proteolysis as a mechanism for metal release has been recently demonstrated for other plant



MTs,²¹³ and in general, less well-folded proteins are more prone to proteolytic degradation. It remains to be seen whether protein-folding-mediated metal discrimination has any consequences *in vivo*.

The concept of “relative affinities”¹¹⁴ in action has been illustrated by the pair of MTs from *C. elegans*.^{214–216} The genome of this soil nematode harbours exactly two genes for MTs. *Mtl-1* is constitutively expressed in the pharynx of the worm, whilst *mtl-2* is strongly induced by external Cd²⁺, which is quite abundant in many soils. Exposure of an equimolar mixture of Zn₇MTL-1 and Zn₆MTL-2 to sub-stoichiometric Cd²⁺ led to preferential incorporation of Cd²⁺ into MTL-2.²¹⁶ This was in full accordance with affinity data measured for Zn- and Cd-loaded MTL-1 and MTL-2.²¹⁵ Whilst both MTs display almost equal affinity towards Zn ($K_{DZn} = 10^{-12}$ M), their affinities towards Cd²⁺ differ by almost two orders of magnitude ($K_{DCd} = 7.9 \times 10^{-14}$ M (MTL-1) and 10^{-15} M (MTL-2)). This is only partially due to the presence of three Zn-coordinating His residues in MTL-1, but in the absence of structural data, the full molecular basis for reduced Cd²⁺ affinity of MTL-1 remains to be elucidated.

Major recent advances in understanding the reactions of MTs, including those described above, were possible by employing native Electrospray Ionisation Mass Spectrometry (ESI-MS),²¹⁷ the only technique that can simultaneously detect all metallo-species present in a mixture. This has been employed for characterising metallo-species resulting from recombinant expression, and led to several discoveries, including the formation of mixed and undermetallated metallo-species if the growth media contain an excess of the “wrong” metal ion,^{218,219} and that under such circumstances, often sulfide ions are recruited in the *E. coli* cytosol to complete the metal–thiolate clusters.²²⁰ Similar cluster expansion can also be achieved by chemical means.²²¹

Apart from work on composition, metal affinities, metal “preferences”, and structures, the most important biophysical studies of MTs concern their reactivity in reactions with likely physiological relevance: metal uptake (metallation),²²² metal release/metal transfer^{201,217} and metal exchange.²²³ Thioneins (metal-free, or apo-MTs) can acquire metal ions from other proteins, for example the zinc finger transcription factor TF-IIIa,^{224,225} Cd-loaded carbonic anhydrase²²⁶ and the Zn-bound insulin hexamer.²²⁷ In turn, metallated MTs can also transfer their cargo to other molecules, including chelators such as EDTA²⁰¹ and apo-proteins.²²⁸ In the case of zinc-dependent enzymes, Zn-MTs can activate these, and zinc transfer from Zn-MT to several enzymes has been shown to occur in cell extracts from mouse heart.²²⁹ Metal release may be elicited by oxidation of the cysteine thiols,^{230,231} and whether or not a protein may acquire Zn²⁺ in the presence of MT depends, amongst other factors, on the redox state of the cell, and the thionein/metallothionein ratio.²³²

A prevailing question regarding the metal-binding properties of MTs concerns cooperativity within individual clusters. Various earlier experiments had indicated that metal release, *e.g.* to EDTA, occurs in a cooperative fashion. Providing that the species observed are in equilibrium and hence thermodynamic products, this would indicate that partially formed clusters are,

relative to other available alternatives (for example mixtures of fully formed clusters and “empty” proteins), less stable. If that is the case, then species formed at sub-stoichiometric levels of Zn²⁺ added to apo-proteins should also reflect this. Curiously, most ESI-MS studies have demonstrated a lack of cooperativity in metal uptake reactions. An early study of rabbit MT-3 indicated non-cooperative binding, with no preference for either 3- or 4-metal species observed, which would be expected if the Zn₃Cys₉ or Zn₄Cys₁₁ cluster was formed cooperatively.²³³ Over-metallated species with 8 and 9 Zn ions bound were also observed; this could be due the fact that at least the β domain of MT-3 is optimised for binding Cu⁺ rather than a divalent ion.²³⁴ Subsequently, rabbit MT-2 was shown to also not form either cluster preferentially, but the fully metallated Zn₇ form was dominant.²³³ Finally, titrations of human apo-MT-1a with various metal ions including Zn²⁺ using a stopped-flow approach showed that this MT also binds these metal ions non-cooperatively, as yet again the observed metallo-species did not indicate a preference for complete clusters.^{235,236}

Since MTs can be populated by cadmium *in vivo*,^{177,178} it is of interest to study the replacement of Zn²⁺ by Cd²⁺. Spectrophotometric analysis of the kinetics of Cd²⁺/Zn²⁺ exchange in rabbit MT-2 as well as the isolated α domain revealed that metal exchange occurs *via* an associative mechanism, with the incoming Cd²⁺ initially binding to the fully metallated MT, followed by exchange with a bound Zn²⁺.²³⁷ This is consistent with the observation of a Cd₅ species for the α domain of MT-2.²²³ Analysis of the products of exchange by ¹¹¹Cd NMR spectroscopy indicated preferential occupation of particular binding sites with Cd²⁺.²³⁷ One of the sites in *C. elegans* MTL-1 did not react at all, leading to the formation of a stable Cd₆Zn₁MTL-1 species,²¹⁶ which is also the major form isolated when MTL-1 is expressed in the presence of excess Cd²⁺.²¹⁵ In contrast to the kinetic origin of the Cd₃Zn species for SmtA, the preferential formation of Cd₆Zn₁MTL-1 has thermodynamic roots; the site in question is likely a His₃Cys site, with very low affinity towards the soft Cd²⁺.²¹⁵

Inter-protein metal exchange reactions of particular current interest concern the reactions of zinc-loaded mammalian MT-3 with copper-loaded proteins and peptides present in the brain, all with relationships to neurodegenerative diseases.²³⁸ The amyloid-beta peptide forms toxic oligomers in Alzheimer's disease. Thought to be due to redox cycling between Cu⁺ and Cu²⁺, the associated generation of reactive oxygen species, and modulation of aggregation, amyloid-beta cytotoxicity is exacerbated by copper, but overall toxicity can be alleviated by extracellular Zn₇MT-3.²³⁹ The mechanism for this protective effect involves “swapping” zinc with copper, leading to the formation of Zn-amyloid-beta, and mixed, oxidised Zn,Cu-MT-3, in which copper is redox-silent. Metal swapping is rapid, even though it probably occurs not by direct interaction between the proteins, but through free Cu²⁺.²⁴⁰ Zn²⁺ transfer from Zn₇MT-3 to amyloid-beta can also be promoted by hydrogen peroxide.²⁴¹ Either way, Zn²⁺ release from MT-3 leads to the formation of fibrillar aggregates of amyloid-beta, with different morphology to those formed in the absence of Zn²⁺. The physiological



significance of these zinc-induced changes in aggregation and morphology is not yet clear, and the role of MT-3 in the pathogenesis of Alzheimer's disease has remained controversial.²⁴² The redox activity of copper-loaded alpha-synuclein²⁴³ and prion protein²⁴⁴ is also silenced by Zn₇MT-3.

In summary, many MTs have clear roles in intracellular zinc homeostasis and are crucially involved in maintaining adequate intracellular Zn²⁺ levels. They also provide a link between these levels and the cellular redox balance. No other metalloprotein family has been studied so extensively regarding possible metal uptake, release, and transfer reactions. However, although this is not within the scope of this review, it should not be forgotten that MTs are not restricted to these roles.

2.3.2 Cytosolic zinc chaperones? Up until recently, it was thought that the role of keeping zinc safe but available was performed exclusively by MTs (Section 2.3.1). This notion was fundamentally changed by the discovery that an entire sub-family of putative metallochaperones was regulated by the zinc sensor protein Zur – the COG0523 family of small GTPases.¹⁷⁶ “COG” stands for “cluster of orthologous groups”, and it is implied that the COG classification allows inferring biological function. “Older” members of COG0523 function in cobalamin synthesis (CobW), although their precise mode of action is not clear. Other related G3E GTPases work in the assembly of Ni-dependent urease (UreG) and hydrogenases (HypB), and Fe-dependent nitrile hydratase (Nha3). In contrast, no specific pathway has been inferred for Zur-regulated COG0523 proteins; hence, their exact role in zinc homeostasis is as yet unclear.

The *yciC* gene from *Bacillus subtilis* was the first representative of a Zur-regulated COG0523 member,²⁴⁵ and *yciC* expression is important to support bacterial growth under zinc starvation conditions. How the YciC protein achieves this is not known, but one hypothesis is that the protein could help to optimise zinc usage by rapid re-distribution between old and new proteins – a strategy that, for iron, has been dubbed as metal “hot-bunking”.²⁴⁶

Two COG0523 proteins from *E. coli*, YeiR²⁴⁷ and YjiA,²⁴⁸ have been studied recently *in vitro*. Like *B. subtilis* YciC, YeiR also protects the bacterium from the effects of zinc starvation, whilst YjiA has not been allocated a particular metal specificity, but is thought to have a role in the response to carbon starvation. Both proteins bind various metal ions, including Zn²⁺, Ni²⁺ and Co²⁺. Metal ions modulate the GTPase activity of COG0523 proteins; Zn²⁺ enhanced the GTPase activity of YeiR, but inhibited that of YjiA. Quantitative metal-binding studies of these proteins can be quite complex, as metal binding induces dimerisation. Up to three Zn²⁺ ions per monomer were found to bind to YeiR, as measured by inductively-coupled plasma atomic emission spectroscopy (ICP-AES), whilst a two-metal species dominated the ESI-MS spectrum. Dissociation constants of << 100 nM, 43 nM and 408 nM were estimated (for the highest affinity site) or determined by competition with the metallochromic dye Mag-Fura-2. The location and identity of the zinc-binding sites on YeiR has not been comprehensively determined. Upon mutation of the three Cys residues in a CXCC motif conserved in COG0523 members (but not other G3E GTPases),

the zinc content only decreased to 2.6 molar equivalents, suggesting that at least two unperturbed sites remained.

YjiA was shown to bind Ni²⁺, Co²⁺, and Zn²⁺ with micromolar affinities, but with different stoichiometries: one Co, two Ni, and up to four Zn ions. GDP decreased the stoichiometry in each case, by about one molar equivalent. YjiA did not crystallise in the presence of metal ions; therefore, metal-binding sites were determined by soaking apo-crystals in 3 mM ZnSO₄. Four sites were observed in the structure (pdb 3ixm;²⁴⁸ Fig. 11a and b) – one site bridging two monomers, two surface sites, and one internal site involving one of the Cys residues of the CXCC motif and two Glu residues. Mutation of the residues of this internal site decreased the Co and Ni stoichiometry in solution, but confoundingly not that of Zn. Mutation of the CXCC motif in YjiA altered metal sensitivity of the GTPase activity: whilst any response to Ni was lost, Zn still inhibited the mutant proteins – in parallel to the findings regarding metal:protein stoichiometry. Further studies are needed to elucidate the biological and the molecular function of these intriguing proteins.

2.3.3 Periplasmic zinc chaperones. The periplasm of Gram-negative bacteria can be considered as a separate cellular compartment. Mounting evidence suggests that the (free) metal concentrations in this compartment are also subject to active regulation. As in general cellular metal homeostasis, mechanisms are in place to safeguard against both too high and too low concentrations.

Exposure of *E. coli* to excessive zinc leads to high expression levels of the periplasmic protein ZraP.^{28,64} Its expression is

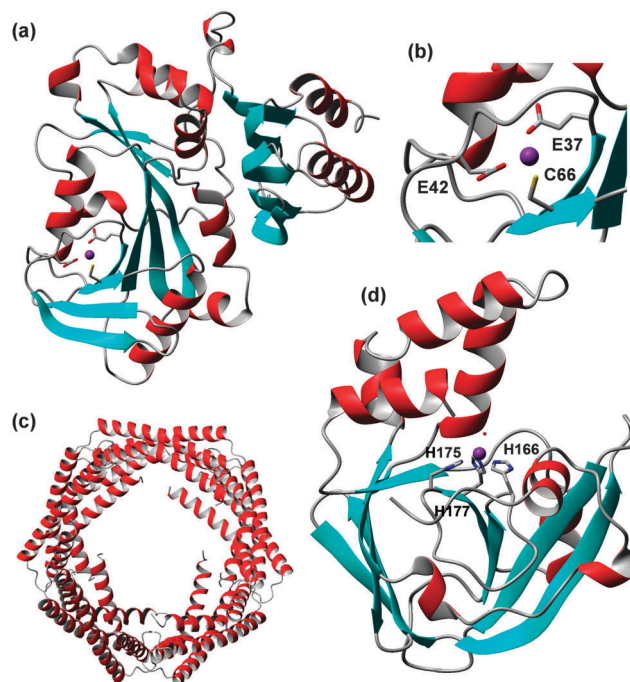


Fig. 11 Proteins with possible “zinc chaperone” function. (a) Overall fold of YjiA from *E. coli* (pdb 4ixm) (b) Detail of the internal zinc binding site. (c) Decameric ZraP from *Salmonella typhimurium* (pdb 3lay); no zinc-bound structure is available. (d) Overall structure of ZinT from *E. coli* (pdb 1txl), with the His₃–H₂O site shown.



regulated by the ZraSR two-component system (Fig. 1).²⁴⁹ ZraP has been shown to bind zinc,²⁵⁰ and a metalloproteomic study demonstrated that in zinc excess conditions, the large majority of cellular zinc in *E. coli* was bound to ZraP.²⁵¹ The catalytic molecular chaperone activity of ZraP is Zn-dependent, and Zn also affects its oligomerisation state, with higher oligomers (10–20) observed in the presence of zinc. A structure for ZraP from *Salmonella typhimurium* is available, but without bound Zn (pdb 3lay; Fig. 11c).²⁵² Despite its expression being upregulated in response to zinc excess and its demonstrated sequestration properties, ZraP is not required for zinc resistance.²⁴⁹ In contrast, ZraP, Spy and CpxP, all molecular chaperones, are all required for resistance against polymyxin B, a host-generated antibacterial cationic peptide.

Extreme zinc starvation experiments in *E. coli* have demonstrated a role for the periplasmic protein ZinT (formerly known as YodA).^{28,253} The expression of ZinT is Zur-regulated. ZinT is thought to facilitate zinc acquisition in extreme depletion conditions, and may be able to supply Zn^{2+} to the ZnuABC system.²⁵⁴ Structures for ZinT proteins are available (Fig. 11d).^{102,255,256} The protein adopts a lipocalin-like fold, and the zinc binding site bears resemblance to those found in some ZnuA proteins: three His residues plus one water molecule. Zinc binding has very little effect on the structure. In addition, ZinTs contain a His-rich N-terminal sequence (HGHHAHG), which was not resolved in any structure. Competition experiments of *E. coli* ZinT and Mag-Fura-2 indicated a K_D below 20 nM,²⁸ and a K_D of 2.2 nM was determined for *Salmonella enterica* ZinT.¹⁰² The interaction between *Salmonella* ZinT and ZnuA has been studied. Mixing a Zn–ZinT complex with apo-ZnuA led to the formation of a ternary complex observable by analytical ultracentrifugation. The complex could not be crystallised, but a model was generated based on small-angle X-ray scattering data. The model suggested that the ZnuA His-rich loop may be capable of helping to extract Zn^{2+} from the ZinT binding site.¹⁰²

ZinT proteins are not present in all Gram-negative bacteria, but homologues are present at the cell surface of several Gram-positive bacteria, including *Bacillus subtilis* and Streptococci.⁷⁵ In the latter, ZinT is one domain of the AdcA protein, the second domain being homologous to ZnuA. Together with the evidence obtained for ZinT–ZnuA interactions in Gram-negative bacteria, it seems likely that ZinT is one of the surface or periplasmic proteins (or domains) that supply either extra-cellular or periplasmic Zn^{2+} to AdcA (Streptococci) or ZnuA (other Gram-positive and Gram-negative bacteria).

3. Conclusions and outlook

Research regarding structures and thermodynamics of proteins involved in the homeostasis of essential metal ions including zinc has been flourishing over the past decade. The largest part of biophysical work has been carried out on prokaryotic systems, mainly because eukaryotic systems often tend to be technically more challenging and more complex. The comparable ease of combining *in vitro* and *in vivo* approaches, and of

genetic and genomic manipulations, for prokaryotic systems has particularly favoured rapid progress in our understanding of metal homeostasis. Some major players in zinc homeostasis, including ZIP and CDF transporters, are conserved from bacteria to man, and here, studies of bacterial representatives also greatly promote understanding of the eukaryotic proteins. In contrast, systems such as the ZnuABC zinc uptake transporters and the poly-histidine triad proteins are restricted to prokaryotes, and considering the importance of metal ions at the host–pathogen interface, this may open new opportunities in antibacterial therapy.

A survey of all structurally characterised metal binding sites considered in this review reveals that most are formed by either three or four amino acid side-chains, with coordination numbers of four and tetrahedral geometry prevailing – quite similar to structural and catalytic sites. Surprisingly, there are even examples for sites with the same ligand composition as catalytic sites, for example in *Synechocystis* ZnuA and *Cupriavidus metallidurans* ZneB, sites are formed by three His or two His and one carboxylate, respectively, with a fourth site free for an external ligand – motifs normally found in hydrolytic enzymes such as carbonic anhydrase and many metalloproteases. It remains unclear why these sites are not known to exhibit at least some degree of catalytic activity. In addition, apart from an obvious impact of the degree of surface-exposure of a particular site, our understanding of if and why most transport sites are considerably more labile than catalytic zinc sites is still patchy. At present, there is still a scarcity of kinetic data for zinc transporters, and zinc binding kinetics in general.²⁵⁷ It has however been noted that for example in the case of sensor proteins “the prevailing view on metal-mediated molecular regulation in terms of ‘on and off control’ might be oversimplified”.²⁵⁸

ATPases, CDF transporters and ZneCBA systems all seem to comprise regulatory zinc binding sites, the former two in the cytosolic domains, the latter in the periplasm, *i.e.* the compartments from which transport occurs. All three systems are active transporters, *i.e.* require energy. It would make sense that these pumps are inactivated as long as no zinc needs to be transported. Furthermore, many transmembrane zinc transporters from essentially all families harbour histidine-rich sequences which have been largely refractory to structural studies, and their mechanistic significance has remained mostly obscure.²⁵⁹ Large numbers of His-rich sequences and proteins have been identified in prokaryotes.²⁶⁰ Although many His-rich proteins may play roles unrelated to zinc homeostasis, similarities between these and MTs have been noted. Like for MTs, zinc binding to His-rich sequences has been observed to lead to conformational restriction.¹⁴⁶ It is conceivable that in the case of zinc transporters, this may impact on protein–protein or domain–domain interactions, and also play a role in metal-dependent regulation of transporter activity.

Excitingly, the very first example of a structurally characterised *de novo* designed transmembrane transporter is a zinc transporter involving a homo-tetrameric helical bundle forming two His_2Glu_4 di-zinc sites. Although structurally unrelated to any of the zinc transporters described above, “Rocker” is able to use a



proton gradient to transport Zn^{2+} against its concentration gradient.²⁶¹

Finally, it is clear that studies on isolated proteins are only one albeit substantial part of a much larger picture, and need to be combined with studies on cellular and organismal levels. Renewed efforts to quantitatively describe biological systems, and the emerging role of zinc as a signalling agent will require data on time-dependence of molecular events as well as an enhanced quantitative understanding of zinc probes in cellular environments. Recent studies of zinc chelators and several probes have shown that contrary to common assumptions, the probes TSQ (6-methoxy-8-*p*-toluenesulfonamido-quinoline),²⁶² Zinquin,²⁶³ sensors of the ZnAF family²⁶⁴ as well as the chelator TPEN²⁶⁵ are all capable to bind or sense protein-bound zinc besides the desired interaction with free or labile cellular zinc. This may lead to over-estimation of zinc concentrations and fluxes.

In summary, despite the tremendous acceleration of discovery, functional studies, and structure elucidation, ample scope remains for future studies on molecular mechanisms in zinc trafficking and homeostasis.

Acknowledgements

The author thanks the BBSRC (grant no. BB/J006467/1) and the Royal Society (Olga Kennard Fellowship to CAB 2004-9).

Notes and references

- J. L. Raulin, *Ann. Sci. Nat., Bot. Biol. Veg.*, 1869, **11**, 293–299.
- W. R. Todd, C. A. Elvehjem and E. B. Hart, *Am. J. Physiol.*, 1934, **107**, 146–156.
- A. S. Prasad, *Adv. Nutr.*, 2013, **4**, 176–190.
- I. Keilin and T. Mann, *Nature*, 1939, **144**, 442–443.
- W. Maret, *Adv. Nutr.*, 2013, **4**, 82–91.
- W. Maret, *J. Inorg. Biochem.*, 2012, **111**, 110–116.
- <http://www.copenhagenconsensus.com/copenhagen-consensus-2008/outcomes>.
- A. S. Prasad, *Curr. Opin. Clin. Nutr. Metab. Care*, 2009, **12**, 646–652.
- E. Mocchegiani, L. Costarelli, A. Basso, R. Giacconi, F. Piacenza and M. Malavolta, *Curr. Pharm. Des.*, 2013, **19**, 1753–1764.
- C. P. Wong and E. Ho, *Mol. Nutr. Food Res.*, 2012, **56**, 77–87.
- H. Kozłowski, M. Luczkowski, M. Remelli and D. Valensin, *Coord. Chem. Rev.*, 2012, **256**, 2129–2141.
- S. S. Leal, H. M. Botelho and C. M. Gomes, *Coord. Chem. Rev.*, 2012, **256**, 2253–2270.
- B. K. Y. Bitanhihrwe and M. G. Cunningham, *Synapse*, 2009, **63**, 1029–1049.
- A. S. Prasad, F. W. Beck, D. C. Snell and O. Kucuk, *Nutr. Cancer*, 2009, **61**, 879–887.
- M. Murakami and T. Hirano, *Cancer Sci.*, 2008, **99**, 1515–1522.
- R. B. Franklin and L. C. Costello, *J. Cell. Biochem.*, 2009, **106**, 750–757.
- H. Haase and L. Rink, *Annu. Rev. Nutr.*, 2009, **29**, 133–152.
- F. Chimienti, *Nutr. Res. Rev.*, 2013, **26**, 1–11.
- J. P. Barnett, C. A. Blindauer, O. Kassar, S. Khazaipoul, E. M. Martin, P. J. Sadler and A. J. Stewart, *Biochim. Biophys. Acta, Gen. Subj.*, 2013, **1830**, 5456–5464.
- I. Cakmak, *J. Trace Elem. Med. Biol.*, 2009, **23**, 281–289.
- H. H. Sandstead, *Adv. Nutr.*, 2013, **4**, 76–81.
- F. M. M. Morel, J. R. Reinfelder, S. B. Roberts, C. P. Chamberlain, J. G. Lee and D. Yee, *Nature*, 1994, **369**, 740–742.
- K. Hantke, *Curr. Opin. Microbiol.*, 2005, **8**, 196–202.
- C. A. Blindauer, *Chem. Biodiversity*, 2008, **5**, 1990–2013.
- J. P. Barnett, A. Millard, A. Z. Ksibe, D. J. Scanlan, R. Schmid and C. A. Blindauer, *Front. Microbiol.*, 2012, **3**, 142.
- M. Napolitano, M. A. Rubio, J. Santamaria-Gomez, E. Olmedo-Verd, N. J. Robinson and I. Luque, *J. Bacteriol.*, 2012, **194**, 2426–2436.
- D. H. Nies, *J. Bacteriol.*, 2012, **194**, 2407–2412.
- A. I. Graham, S. Hunt, S. L. Stokes, N. Bramall, J. Bunch, A. G. Cox, C. W. McLeod and R. K. Poole, *J. Biol. Chem.*, 2009, **284**, 18377–18389.
- E. K. LeGrand and J. Alcock, *Q. Rev. Biol.*, 2012, **87**, 3–18.
- B. D. Corbin, E. H. Seeley, A. Raab, J. Feldmann, M. R. Miller, V. J. Torres, K. L. Anderson, B. M. Dattilo, P. M. Dunman, R. Gerads, R. M. Caprioli, W. Nacken, W. J. Chazin and E. P. Skaar, *Science*, 2008, **319**, 962–965.
- S. Shafeeq, O. P. Kuipers and T. G. Kloosterman, *Mol. Microbiol.*, 2013, **88**, 1047–1057.
- D. Osman and J. S. Cavet, in *Adv. Microb. Physiol.*, ed. R. K. Poole, Academic Press Ltd-Elsevier Science Ltd, London, 2011, vol. 58, pp. 175–232.
- G. Porcheron, A. Garenaux, J. Proulx, M. Sabri and C. M. Dozois, *Front. Cell. Infect. Microbiol.*, 2013, **3**, 90.
- J. J. Braymer and D. P. Giedroc, *Curr. Opin. Chem. Biol.*, 2014, **19C**, 59–66.
- F. Li, I. Chvyrkova, S. Terzyan, N. Wakeham, R. Turner, A. K. Ghosh, X. J. C. Zhang and J. Tang, *Appl. Microbiol. Biotechnol.*, 2012, **94**, 1041–1049.
- R. A. Bozym, F. Chimienti, L. J. Giblin, G. W. Gross, I. Korichneva, Y. A. Li, S. Libert, W. Maret, M. Parviz, C. J. Frederickson and R. B. Thompson, *Exp. Biol. Med.*, 2010, **235**, 741–750.
- T. Fukada, S. Yamasaki, K. Nishida, M. Murakami and T. Hirano, *JBIC, J. Biol. Inorg. Chem.*, 2011, **16**, 1123–1134.
- W. Maret, *BioMetals*, 2011, **24**, 411–418.
- C. J. Frederickson, M. Hershfinkel and L. J. Giblin, in *Synaptic Plasticity and Transsynaptic Signaling*, ed. P. K. Stanton, C. Bramham and H. E. Scharfman, Springer, New York, 2005, pp. 123–137.
- A. M. Kim, M. L. Bernhardt, B. Y. Kong, R. W. Ahn, S. Vogt, T. K. Woodruff and T. V. O'Halloran, *ACS Chem. Biol.*, 2011, **6**, 716–723.
- M. M. Henary, Y. G. Wu and C. J. Fahrni, *Chem. – Eur. J.*, 2004, **10**, 3015–3025.
- M. Taki, J. L. Wolford and T. V. O'Halloran, *J. Am. Chem. Soc.*, 2004, **126**, 712–713.
- H. Woo, Y. You, T. Kim, G. J. Jhon and W. Nam, *J. Mater. Chem.*, 2012, **22**, 17100–17112.
- W. Chyan, D. Y. Zhang, S. J. Lippard and R. J. Radford, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, 143–148.
- R. J. Radford and S. J. Lippard, *Curr. Opin. Chem. Biol.*, 2013, **17**, 129–136.
- P. Chabosseau, E. Tuncay, G. Meur, E. A. Bellomo, A. Hessels, S. Hughes, P. R. V. Johnson, M. Bugliani, P. Marchetti, B. Turan, A. R. Lyon, M. Merckx and G. A. Rutter, *ACS Chem. Biol.*, 2014, **9**, 2111–2120.
- B. J. McCranor, R. A. Bozym, M. I. Vitolo, C. A. Fierke, L. Bambrick, B. M. Polster, G. Fiskum and R. B. Thompson, *J. Bioenerg. Biomembr.*, 2012, **44**, 253–263.
- C. E. Outten and T. V. O'Halloran, *Science*, 2001, **292**, 2488–2492.
- A. Krezel and W. Maret, *JBIC, J. Biol. Inorg. Chem.*, 2006, **11**, 1049–1062.
- C. Andreini, I. Bertini and A. Rosato, *Acc. Chem. Res.*, 2009, **42**, 1471–1479.
- M. Brylinski and J. Skolnick, *Proteins*, 2011, **79**, 735–751.
- M. Zimmermann, O. Clarke, J. M. Gulbis, D. W. Keizer, R. S. Jarvis, C. S. Cobbett, M. G. Hinds, Z. G. Xiao and A. G. Wedd, *Biochemistry*, 2009, **48**, 11640–11654.
- S. Tottey, K. J. Waldron, S. J. Firbank, B. Reale, C. Bessant, K. Sato, T. R. Cheek, J. Gray, M. J. Banfield, C. Dennison and N. J. Robinson, *Nature*, 2008, **455**, 1138–1142.
- A. W. Foster and N. J. Robinson, *BMC Biol.*, 2011, **9**, 3.
- D. Wang, O. Hosteen and C. A. Fierke, *J. Inorg. Biochem.*, 2012, **111**, 173–181.
- R. A. Colvin, W. R. Holmes, C. P. Fontaine and W. Maret, *Metalomics*, 2010, **2**, 306–317.
- D. H. Nies, *Science*, 2007, **317**, 1695–1696.
- B. P. Weaver, J. D. Beattie, T. Kambe and G. K. Andrews, *Biol. Chem.*, 2007, **388**, 1301–1312.
- J. Dufner-Beattie, Y. M. Kuo, J. Gitschier and G. K. Andrews, *J. Biol. Chem.*, 2004, **279**, 49082–49090.
- M. Pruteanu, S. B. Neher and T. A. Baker, *J. Bacteriol.*, 2007, **189**, 3017–3025.



- 61 P. T. Chivers, *J. Bacteriol.*, 2007, **189**, 2953–2954.
- 62 C. D. Ellis, F. Wang, C. W. MacDiarmid, S. Clark, T. Lyons and D. J. Eide, *J. Cell Biol.*, 2004, **166**, 325–335.
- 63 D. Wang and C. A. Fierke, *Metallomics*, 2013, **5**, 372–383.
- 64 C. Appia-Ayme, A. Hall, E. Patrick, S. Rajadurai, T. A. Clarke and G. Rowley, *Biochem. J.*, 2012, **442**, 85–93.
- 65 J. M. Argüello, D. Raimunda and M. Gonzalez-Guerrero, *J. Biol. Chem.*, 2012, **287**, 13510–13517.
- 66 Z. Xiao and A. G. Wedd, *Nat. Prod. Rep.*, 2010, **5**, 768–789.
- 67 K. J. Waldron, J. C. Rutherford, D. Ford and N. J. Robinson, *Nature*, 2009, **460**, 823–830.
- 68 I. Zawisza, M. Rozga and W. Bal, *Coord. Chem. Rev.*, 2012, **256**, 2297–2307.
- 69 W. Maret, *J. Trace Elem. Med. Biol.*, 2005, **19**, 7–12.
- 70 W. Maret and B. L. Vallee, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 3478–3482.
- 71 L. A. Finney and T. V. O'Halloran, *Science*, 2003, **300**, 931–936.
- 72 S. Choi and A. J. Bird, *Metallomics*, 2014, **6**, 1198–1215.
- 73 A. J. Guerra and D. P. Giedroc, *Arch. Biochem. Biophys.*, 2012, **519**, 210–222.
- 74 V. Günther, U. Lindert and W. Schaffner, *Biochim. Biophys. Acta*, 2012, **1823**, 1416–1425.
- 75 E. M. Panina, A. A. Mironov and M. S. Gelfand, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 9912–9917.
- 76 H. Nanamiya and F. Kawamura, *Biosci., Biotechnol., Biochem.*, 2010, **74**, 451–461.
- 77 H. Reyes-Caballero, G. C. Campanello and D. P. Giedroc, *Biophys. Chem.*, 2011, **156**, 103–114.
- 78 L. J. Coneyworth, K. A. Jackson, J. Tyson, H. J. Bosomworth, E. van der Hagen, G. M. Hann, O. A. Ogo, D. C. Swann, J. C. Mathers, R. A. Valentine and D. Ford, *J. Biol. Chem.*, 2012, **287**, 36567–36581.
- 79 H. Zhao, E. Butler, J. Rodgers, T. Spizzo, S. Duesterhoeft and D. Eide, *J. Biol. Chem.*, 1998, **273**, 28713–28720.
- 80 A. G. Assunção, D. P. Persson, S. Husted, J. K. Schjørring, R. D. Alexander and M. G. Aarts, *Metallomics*, 2013, **9**, 1110–1116.
- 81 I. M. Evans, L. N. Gatehouse, J. A. Gatehouse, N. J. Robinson and R. R. D. Croy, *FEBS Lett.*, 1990, **262**, 29–32.
- 82 D. Osman and J. S. Cavet, *Nat. Prod. Rep.*, 2010, **27**, 668–680.
- 83 M. L. VanZile, X. H. Chen and D. P. Giedroc, *Biochemistry*, 2002, **41**, 9765–9775.
- 84 N. E. Grosseohme and D. P. Giedroc, *J. Am. Chem. Soc.*, 2009, **131**, 17860–17870.
- 85 C. Blindauer, *Biochemistry*, 2012, **34**, 4–13.
- 86 J. S. Cavet, W. M. Meng, M. A. Pennella, R. J. Applehoff, D. P. Giedroc and N. J. Robinson, *J. Biol. Chem.*, 2002, **277**, 38441–38448.
- 87 The Transporter Classification Database, M. H. Saier, V. S. Reddy, D. G. Tamang and A. Vastermark, *Nucleic Acids Res.*, 2014, **42**, D251–D258, <http://www.tcdb.org/>.
- 88 J. J. R. Fraústo da Silva and R. J. P. Williams, *The biological chemistry of the elements: the inorganic chemistry of life*, Oxford University Press, 2nd edn, 2001.
- 89 B. F. Weston, A. Brenot and M. G. Caparon, *Infect. Immun.*, 2009, **77**, 2840–2848.
- 90 J. S. Klein and O. Lewinson, *Metallomics*, 2011, **3**, 1098–1108.
- 91 V. M. Korkhov, S. A. Mireku and K. P. Locher, *Nature*, 2012, **490**, 367–372.
- 92 P. M. Jones, M. L. O'Mara and A. M. George, *Trends Biochem. Sci.*, 2009, **34**, 520–531.
- 93 B. R. Chandra, M. Yogavel and A. Sharma, *J. Mol. Biol.*, 2007, **367**, 970–982.
- 94 H. Li and G. Jögl, *J. Mol. Biol.*, 2007, **368**, 1358–1366.
- 95 L. A. Yatsunyk, J. A. Easton, L. R. Kim, S. A. Sugarbaker, B. Bennett, R. M. Breece, I. I. Vorontsov, D. L. Tierney, M. W. Crowder and A. C. Rosenzweig, *JBIC, J. Biol. Inorg. Chem.*, 2008, **13**, 271–288.
- 96 A. Ilari, F. Alaleona, P. Petrarca, A. Battistoni and E. Chiancone, *J. Mol. Biol.*, 2011, **409**, 630–641.
- 97 S. Banerjee, B. X. Wei, M. Bhattacharyya-Pakrasi, H. B. Pakrasi and T. J. Smith, *J. Mol. Biol.*, 2003, **333**, 1061–1069.
- 98 B. X. Wei, A. M. Randich, M. Bhattacharyya-Pakrasi, H. B. Pakrasi and T. J. Smith, *Biochemistry*, 2007, **46**, 8734–8743.
- 99 E. Loisel, L. Jacquamet, L. Serre, C. Bauvois, J. L. Ferrer, T. Vernet, A. M. Di Guilmi and C. Durmort, *J. Mol. Biol.*, 2008, **381**, 594–606.
- 100 Y. H. Lee, R. K. Deka, M. V. Norgard, J. D. Radolf and C. A. Hasemann, *Nat. Struct. Biol.*, 1999, **6**, 628–633.
- 101 L. Bayle, S. Chimalapati, G. Schoehn, J. Brown, T. Vernet and C. Durmort, *Mol. Microbiol.*, 2011, **82**, 904–916.
- 102 A. Ilari, F. Alaleona, G. Tria, P. Petrarca, A. Battistoni, C. Zamparelli, D. Verzili, M. Falconi and E. Chiancone, *Biochim. Biophys. Acta, Gen. Subj.*, 2014, **1840**, 535–544.
- 103 B. Bersch, C. Bougault, L. Roux, A. Favier, T. Vernet and C. Durmort, *PLoS One*, 2013, **8**.
- 104 J. M. Argüello, E. Eren and M. Gonzalez-Guerrero, *BioMetals*, 2007, **20**, 233–248.
- 105 K. T. Wang, O. Sitsel, G. Meloni, H. E. Autzen, M. Andersson, T. Klymchuk, A. M. Nielsen, D. C. Rees, P. Nissen and P. Gourdon, *Nature*, 2014, **514**, 518–522.
- 106 P. Gourdon, X. Y. Liu, T. Skjorringe, J. P. Morth, L. B. Møller, B. P. Pedersen and P. Nissen, *Nature*, 2011, **475**, 59–65.
- 107 M. Andersson, D. Mattle, O. Sitsel, T. Klymchuk, A. M. Nielsen, L. B. Møller, S. H. White, P. Nissen and P. Gourdon, *Nat. Struct. Mol. Biol.*, 2014, **21**, 43–48.
- 108 G. S. Allen, C. C. Wu, T. Cardozo and D. L. Stokes, *Structure*, 2011, **19**, 1219–1232.
- 109 S. J. Dutta, J. Liu, Z. Hou and B. Mitra, *Biochemistry*, 2006, **45**, 5923–5931.
- 110 J. Okkeri and T. Haltia, *Biochim. Biophys. Acta, Bioenerg.*, 2006, **1757**, 1485–1495.
- 111 L. Banci, L. Bertini, S. Ciofi-Baffoni, L. A. Finney, C. E. Outten and T. V. O'Halloran, *J. Mol. Biol.*, 2002, **323**, 883–897.
- 112 L. Banci, I. Bertini, S. Ciofi-Baffoni, L. Poggi, M. Vanarotti, S. Tottey, K. J. Waldron and N. J. Robinson, *JBIC, J. Biol. Inorg. Chem.*, 2010, **15**, 87–98.
- 113 D. Raimunda, P. Subramanian, T. Stemmler and J. M. Argüello, *Biochim. Biophys. Acta, Biomembr.*, 2012, **1818**, 1374–1377.
- 114 K. J. Waldron and N. J. Robinson, *Nat. Rev. Microbiol.*, 2009, **1**, 25–35.
- 115 C. A. Blindauer and R. Schmid, *Metallomics*, 2010, **2**, 510–529.
- 116 E. Eren, D. C. Kennedy, M. J. Maroney and J. M. Argüello, *J. Biol. Chem.*, 2006, **281**, 33881–33891.
- 117 L. Baekgaard, M. D. Mikkelsen, D. M. Sørensen, J. N. Hegelund, D. P. Persson, R. F. Mills, Z. Yang, S. Husted, J. P. Andersen, M. J. Buch-Pedersen, J. K. Schjørring, L. E. Williams and M. G. Palmgren, *J. Biol. Chem.*, 2010, **285**, 31243–31252.
- 118 Y. Chao and D. Fu, *J. Biol. Chem.*, 2004, **279**, 12043–12050.
- 119 E. Ohana, E. Hoch, C. Keasar, T. Kambe, O. Yifrach, M. Hershfinkel and I. Sekler, *J. Biol. Chem.*, 2009, **284**, 17677–17686.
- 120 T. Kambe, *Biosci., Biotechnol., Biochem.*, 2011, **75**, 1036–1043.
- 121 L. P. Huang and S. Tepasamordech, *Mol. Aspects Med.*, 2013, **34**, 548–560.
- 122 F. Chimienti, *Nutr. Res. Rev.*, 2013, **26**, 1–11.
- 123 J. Flannick, G. Thorleifsson, N. L. Beer, S. B. R. Jacobs, N. Grarup, N. P. Burt, A. Mahajan, C. Fuchsberger, G. Atzmon, R. Benediktsson, J. Blangero, D. W. Bowden, I. Brandslund, J. Brosnan, F. Burslem, J. Chambers, Y. S. Cho, C. Christensen, D. A. Douglas, R. Duggirala, Z. Dymek, Y. Farjoun, T. Fennell, P. Fontanillas, T. Forsen, S. Gabriel, B. Glaser, D. F. Gudbjartsson, C. Hanis, T. Hansen, A. B. Hreidarsson, K. Hveem, E. Ingelsson, B. Isomaa, S. Johansson, T. Jorgensen, M. E. Jorgensen, S. Kathiresan, A. Kong, J. Kooner, J. Kravic, M. Laakso, J.-Y. Lee, L. Lind, C. M. Lindgren, A. Linneberg, G. Masson, T. Meitinger, K. L. Møhlke, A. Molven, A. P. Morris, S. Potluri, R. Rauramaa, R. Ribel-Madsen, A.-M. Richard, T. Rolph, V. Salomaa, A. V. Segre, H. Skarstrand, V. Steinthorsdottir, H. M. Stringham, P. Sulem, E. S. Tai, Y. Y. Teo, T. Teslovich, U. Thorsteinsdottir, J. K. Trimmer, J. Tuomi, J. Tuomilehto, F. Vaziri-Sani, B. F. Voight, J. G. Wilson, M. Boehnke, M. I. McCarthy, P. R. Njolstad, O. Pedersen, L. Groop, D. R. Cox, K. Stefansson, D. Altshuler and corporate authors: Go-T2D Consortium; T2D-GENES Consortium, *Nat. Genet.*, 2014, **46**, 357–363.
- 124 L. P. Huang and J. Gitschier, *Nat. Genet.*, 1997, **17**, 292–297.
- 125 I. Lasry, Y. A. Seo, H. Ityel, N. Shalva, B. Pode-Shakked, F. Glaser, B. Berman, I. Berezovsky, A. Gonczarenko, A. Klar, J. Levy, Y. Anikster, S. L. Kelleher and Y. G. Assaraf, *J. Biol. Chem.*, 2012, **287**, 29348–29361.
- 126 S. Ayton, P. Lei and A. I. Bush, *Free Radical Biol. Med.*, 2013, **62**, 76–89.
- 127 M. Lu and D. Fu, *Science*, 2007, **317**, 1746–1748.
- 128 M. Lu, J. Chai and D. Fu, *Nat. Struct. Mol. Biol.*, 2009, **16**, 1063–1067.



- 129 V. Cherezov, N. Hofer, D. M. E. Szebenyi, O. Kolaj, J. G. Wall, R. Gillilan, V. Srinivasan, C. P. Jaroniec and M. Caffrey, *Structure*, 2008, **16**, 1378–1388.
- 130 N. Coudray, S. Valvo, M. H. Hu, R. Lasala, C. Kim, M. Vink, M. Zhou, D. Provasi, M. Filizola, J. Tao, J. Fang, P. A. Penczek, I. Ubarretxena-Belandia and D. L. Stokes, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 2140–2145.
- 131 D. Russell and T. Soulimane, *FEBS Lett.*, 2012, **586**, 4332–4338.
- 132 A. Anton, A. Weltrowski, C. J. Haney, S. Franke, G. Grass, C. Rensing and D. H. Nies, *J. Bacteriol.*, 2004, **186**, 7499–7507.
- 133 Y. Chao and D. Fu, *J. Biol. Chem.*, 2004, **279**, 17173–17180.
- 134 D. Goswami, J. Kaur, S. Surade, E. Grell and H. Michel, *Biol. Chem.*, 2012, **393**, 617–629.
- 135 B. Montanini, D. Blaudez, S. Jeandroz, D. Sanders and M. Chalot, *BMC Genomics*, 2007, **8**, 16.
- 136 E. Hoch, W. Lin, J. Chai, M. Hershfinkel, D. Fu and I. Sekler, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 7202–7207.
- 137 D. Podar, J. Scherer, Z. Noordally, P. Herzyk, D. Nies and D. Sanders, *J. Biol. Chem.*, 2012, **287**, 3185–3196.
- 138 M. Kawachi, Y. Kobae, S. Kogawa, T. Mimura, U. Krämer and M. Maeshima, *FEBS J.*, 2012, **279**, 2339–2356.
- 139 M. L. Guerinot, *Biochim. Biophys. Acta*, 2000, **1465**, 190–198.
- 140 J. Jeong and D. J. Eide, *Mol. Aspects Med.*, 2013, **34**, 612–619.
- 141 L. Hudek, L. A. Pearson, A. Michalczyk, B. A. Neilan and M. L. Ackland, *Appl. Microbiol. Biotechnol.*, 2013, **97**, 8649–8662.
- 142 M. Cerasi, J. Z. Liu, S. Ammendola, A. J. Poe, P. Petrarca, M. Pesciaroli, P. Pasquali, M. Raffatelli and A. Battistoni, *Metallomics*, 2014, **6**, 845–853.
- 143 D. L. Knoell and M. J. Liu, *Int. J. Vitam. Nutr. Res.*, 2010, **80**, 271–277.
- 144 W. Lin, J. Chai, J. Love and D. Fu, *J. Biol. Chem.*, 2010, **285**, 39013–39020.
- 145 S. Ehsani, H. R. Huo, A. Salehzadeh, C. L. Pocanschi, J. C. Watts, H. Wille, D. Westaway, E. Rogaeva, P. H. St. George-Hyslop and G. Schmitt-Ulms, *Prog. Neurobiol.*, 2011, **93**, 405–420.
- 146 N. E. Grosseohme, S. Akilesh, M. L. Guerinot and D. E. Wilcox, *Inorg. Chem.*, 2006, **45**, 8500–8508.
- 147 G. Vert, N. Grotz, F. Dedaldechamp, F. Gaymard, M. L. Guerinot, J. F. Briat and C. Curie, *Plant Cell*, 2002, **14**, 1223–1233.
- 148 S. Potocki, D. Valensin, F. Camponeschi and H. Kozlowski, *J. Inorg. Biochem.*, 2013, **127**, 246–252.
- 149 Z. Liu, H. Li, M. Soleimani, K. Girijashanker, J. M. Reed, L. He, T. P. Dalton and D. W. Nebert, *Biochem. Biophys. Res. Commun.*, 2008, **365**, 814–820.
- 150 S. Antala and R. E. Dempsey, *Biochemistry*, 2012, **51**, 963–973.
- 151 G. Grass, S. Franke, N. Taudte, D. H. Nies, L. M. Kucharski, M. E. Maguire and C. Rensing, *J. Bacteriol.*, 2005, **187**, 1604–1611.
- 152 U. Krämer, I. N. Talke and M. Hanikenne, *FEBS Lett.*, 2007, **581**, 2263–2272.
- 153 F. Long, C. C. Su, H. T. Lei, J. R. Bolla, S. V. Do and E. W. Yu, *Philos. Trans. R. Soc., B*, 2012, **367**, 1047–1058.
- 154 E. Y. Valencia, V. S. Braz, C. Guzzo and M. V. Marques, *BMC Microbiol.*, 2013, **13**.
- 155 F. De Angelis, J. K. Lee, J. D. O'Connell, L. J. W. Miercke, K. H. Verschuere, V. Srinivasan, C. Bauvois, C. Govaerts, R. A. Robbins, J. M. Ruysschaert, R. M. Stroud and G. Vandenbussche, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 11038–11043.
- 156 J. E. Pak, E. N. Ekende, E. G. Kifle, J. D. O'Connell, F. De Angelis, M. B. Tessema, K. M. Derfoufi, Y. Robles-Colmenares, R. A. Robbins, E. Goormaghtigh, G. Vandenbussche and R. M. Stroud, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 18484–18489.
- 157 G. Dieppois, V. Ducret, O. Caille and K. Perron, *PLoS One*, 2012, **7**, 12.
- 158 E. H. Kim, D. H. Nies, M. M. McEvoy and C. Rensing, *J. Bacteriol.*, 2011, **193**, 2381–2387.
- 159 P. Hinchliffe, M. F. Symmons, C. Hughes and V. Koronakis, *Annu. Rev. Microbiol.*, 2013, **67**, 221–242.
- 160 F. Long, C. C. Su, M. T. Zimmermann, S. E. Boyken, K. R. Rajashankar, R. L. Jernigan and E. W. Yu, *Nature*, 2010, **467**, 484–488.
- 161 H. T. Lei, J. R. Bolla, N. R. Bishop, C. C. Su and E. W. Yu, *J. Mol. Biol.*, 2014, **426**, 403–411.
- 162 C. C. Su, F. Long, M. T. Zimmermann, K. R. Rajashankar, R. L. Jernigan and E. W. Yu, *Nature*, 2011, **470**, 558–562.
- 163 S. Murakami, R. Nakashima, E. Yamashita and A. Yamaguchi, *Nature*, 2002, **419**, 587–593.
- 164 A. J. Worlock and R. L. Smith, *J. Bacteriol.*, 2002, **184**, 4369–4373.
- 165 A. M. Caldwell and R. L. Smith, *J. Bacteriol.*, 2003, **185**, 374–376.
- 166 K. Tan, A. Sather, J. L. Robertson, S. Moy, B. Roux and A. Joachimiak, *Protein Sci.*, 2009, **18**, 2043–2052.
- 167 Q. Wan, M. F. Ahmad, J. Fairman, B. Gorzelle, M. de la Fuente, C. Dealwis and M. E. Maguire, *Structure*, 2011, **19**, 700–710.
- 168 C. D. Plumptre, A. D. Ogunniyi and J. C. Paton, *Trends Microbiol.*, 2012, **20**, 485–493.
- 169 A. D. Ogunniyi, M. Grabowicz, L. K. Mahdi, J. Cook, D. L. Gordon, T. A. Sadlon and J. C. Paton, *FASEB J.*, 2009, **23**, 731–738.
- 170 A. Riboldi-Tunnicliffe, N. W. Isaacs and T. J. Mitchell, *FEBS Lett.*, 2005, **579**, 5353–5360.
- 171 C. K. Lim, K. A. Hassan, A. Penesyan, J. E. Loper and I. T. Paulsen, *Environ. Microbiol.*, 2013, **15**, 702–715.
- 172 M. I. Hood, B. L. Mortensen, J. L. Moore, Y. F. Zhang, T. E. Kehl-Fie, N. Sugitani, W. J. Chazin, R. M. Caprioli and E. P. Skaar, *PLoS Pathog.*, 2012, **8**, e1003068.
- 173 M. Stork, M. P. Bos, I. Jongerius, N. de Kok, I. Schilders, V. E. Weynants, J. T. Poolman and J. Tommassen, *PLoS Pathog.*, 2010, **6**, e1000969.
- 174 M. C. Conejo, I. Garcia, L. Martinez-Martinez, L. Picabea and A. Pascual, *Antimicrob. Agents Chemother.*, 2003, **47**, 2313–2315.
- 175 J. P. Barnett, D. J. Scanlan and C. A. Blindauer, *Metallomics*, 2014, **6**, 1254–1268.
- 176 C. E. Haas, D. A. Rodionov, J. Kropat, D. Malasarn, S. S. Merchant and V. de Crecy-Lagard, *BMC Genomics*, 2009, **10**.
- 177 M. Margoshes and B. L. Vallee, *J. Am. Chem. Soc.*, 1957, **79**, 4813–4814.
- 178 J. H. R. Kägi and B. L. Vallee, *J. Biol. Chem.*, 1961, **236**, 2435–2442.
- 179 G. Isani and E. Carpena, *Biomolecules*, 2014, **4**, 435–457.
- 180 E. Freisinger, *Dalton Trans.*, 2008, 6663–6675.
- 181 E. Freisinger, *JBIC, J. Biol. Inorg. Chem.*, 2011, **16**, 1035–1045.
- 182 O. I. Leszczyszyn, H. T. Imam and C. A. Blindauer, *Metallomics*, 2013, **5**, 1146–1169.
- 183 X. Q. Wan and E. Freisinger, *Metallomics*, 2009, **1**, 489–500.
- 184 O. Schicht and E. Freisinger, *Inorg. Chim. Acta*, 2009, **362**, 714–724.
- 185 B. Dolderer, H.-J. Hartmann and U. Weser, in *Met. Ions Life Sci.*, ed. A. Sigel, H. Sigel and R. K. O. Sigel, RSC Publishing, Cambridge, UK, 2009, vol. 5, pp. 83–105.
- 186 C. A. Blindauer, in *Met. Ions Life Sci.*, ed. A. Sigel, H. Sigel and R. K. O. Sigel, RSC Publishing, Cambridge, UK, 2009, vol. 5, pp. 51–81.
- 187 C. A. Blindauer, *JBIC, J. Biol. Inorg. Chem.*, 2011, **16**, 1011–1024.
- 188 C. A. Blindauer and O. I. Leszczyszyn, *Nat. Prod. Rep.*, 2010, **27**, 720–741.
- 189 A. Krezel and W. Maret, *J. Am. Chem. Soc.*, 2007, **129**, 10911–10921.
- 190 M. A. Namdarghanbari, J. Meeusen, G. Bachowski, N. Giebel, J. Johnson and D. H. Petering, *J. Inorg. Biochem.*, 2010, **104**, 224–231.
- 191 A. Krezel and W. Maret, *Biochem. J.*, 2007, **402**, 551–558.
- 192 D. H. Petering, J. Y. Zhu, S. Krezoski, J. Meeusen, C. Kiekenbush, S. Krull, T. Specher and M. Dughish, *Exp. Biol. Med.*, 2006, **231**, 1528–1534.
- 193 W. Braun, M. Vašák, A. H. Robbins, C. D. Stout, G. Wagner, J. H. R. Kägi and K. Wüthrich, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 10124–10128.
- 194 M. H. Frey, G. Wagner, M. Vašák, O. W. Sorensen, D. Neuhaus, E. Wörgötter, J. H. R. Kägi, R. R. Ernst and K. Wüthrich, *J. Am. Chem. Soc.*, 1985, **107**, 6847–6851.
- 195 M. Capdevila, R. Bofill, O. Palacios and S. Atrian, *Coord. Chem. Rev.*, 2012, **256**, 46–62.
- 196 Metallothioneins: Chemical and Biological Challenges, *JBIC, J. Biol. Inorg. Chem.*, 2011, **16**, 975–1134.
- 197 *Metallothioneins and Related Chelators*, ed. A. Sigel, H. Sigel and R. K. O. Sigel, RSC Publishing, Cambridge, vol. 5 of Metal Ions Life Sci., 2009.
- 198 C. A. Blindauer, M. D. Harrison, J. A. Parkinson, A. K. Robinson, J. S. Cavet, N. J. Robinson and P. J. Sadler, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 9593–9598.
- 199 C. A. Blindauer and P. J. Sadler, *Acc. Chem. Res.*, 2005, **38**, 62–69.
- 200 C. A. Blindauer, M. T. Razi, D. J. Campopiano and P. J. Sadler, *JBIC, J. Biol. Inorg. Chem.*, 2007, **12**, 393–405.
- 201 O. I. Leszczyszyn, C. D. Evans, S. E. Keiper, G. Z. L. Warren and C. A. Blindauer, *Inorg. Chim. Acta*, 2007, **360**, 3–13.
- 202 C. A. Blindauer, *J. Inorg. Biochem.*, 2008, **102**, 507–521.



- 203 C. A. Blindauer, M. D. Harrison, A. K. Robinson, J. A. Parkinson, P. W. Bowness, P. J. Sadler and N. J. Robinson, *Mol. Microbiol.*, 2002, **45**, 1421–1432.
- 204 C. A. Blindauer, N. C. Polfer, S. E. Keiper, M. D. Harrison, N. J. Robinson, P. R. R. Langridge-Smith and P. J. Sadler, *J. Am. Chem. Soc.*, 2003, **125**, 3226–3227.
- 205 C. A. Blindauer, M. D. Harrison, J. A. Parkinson, N. J. Robinson and P. J. Sadler, in *10th International Symposium on Metal Ions in Biology and Medicine*, ed. P. Coltery, I. Maynard, T. Theophanides, L. Khassanova and T. Coltery, John Libby Eurotext, 2008, vol. 10, pp. 167–173.
- 206 E. A. Peroza and E. Freisinger, *JBIC, J. Biol. Inorg. Chem.*, 2007, **12**, 377–391.
- 207 O. I. Leszczyszyn, R. Schmid and C. A. Blindauer, *Proteins: Struct., Funct., Bioinf.*, 2007, **68**, 922–935.
- 208 E. A. Peroza, R. Schmucki, P. Güntert, E. Freisinger and O. Zerbe, *J. Mol. Biol.*, 2009, **387**, 207–218.
- 209 O. I. Leszczyszyn, C. R. J. White and C. A. Blindauer, *Mol. BioSyst.*, 2010, **6**, 1592–1603.
- 210 E. A. Peroza, A. Al Kaabi, W. Meyer-Klaucke, G. Wellenreuther and E. Freisinger, *J. Inorg. Biochem.*, 2009, **103**, 342–353.
- 211 J. Loebus, E. A. Peroza, N. Bluthgen, T. Fox, W. Meyer-Klaucke, O. Zerbe and E. Freisinger, *JBIC, J. Biol. Inorg. Chem.*, 2011, **16**, 683–694.
- 212 K. Tarasava, S. Johannsen and E. Freisinger, *Molecules*, 2013, **18**, 14414–14429.
- 213 E. A. Peroza, A. D. Cabral, X. Q. Wan and E. Freisinger, *Metallomics*, 2013, **5**, 1204–1214.
- 214 R. Bofill, R. Orihuela, M. Romagosa, J. Domenech, S. Atrian and M. Capdevila, *FEBS J.*, 2009, **276**, 7040–7069.
- 215 S. Zeitoun-Ghandour, J. M. Charnock, M. E. Hodson, O. I. Leszczyszyn, C. A. Blindauer and S. R. Stürzenbaum, *FEBS J.*, 2010, **277**, 2531–2542.
- 216 O. I. Leszczyszyn, S. Zeitoun-Ghandour, S. R. Stürzenbaum and C. A. Blindauer, *Chem. Commun.*, 2011, **47**, 448–450.
- 217 O. I. Leszczyszyn and C. A. Blindauer, *Phys. Chem. Chem. Phys.*, 2010, **12**, 13408–13418.
- 218 O. Palacios, S. Atrian and M. Capdevila, *JBIC, J. Biol. Inorg. Chem.*, 2011, **16**, 991–1009.
- 219 R. Bofill, M. Capdevila and S. Atrian, *Metallomics*, 2009, **1**, 229–234.
- 220 M. Capdevila, J. Domenech, A. Pagani, L. Tio, L. Villarreal and S. Atrian, *Angew. Chem., Int. Ed.*, 2005, **44**, 4618–4622.
- 221 T. Huber and E. Freisinger, *Dalton Trans.*, 2013, **42**, 8878–8889.
- 222 K. E. R. Duncan and M. J. Stillman, *J. Inorg. Biochem.*, 2006, **100**, 2101–2107.
- 223 K. E. Rigby Duncan, C. W. Kirby and M. J. Stillman, *FEBS J.*, 2008, **275**, 2227–2239.
- 224 J. Zeng, B. L. Vallee and J. H. R. Kägi, *Proc. Natl. Acad. Sci. U. S. A.*, 1991, **88**, 9984–9988.
- 225 M. Huang, C. F. Shaw and D. H. Petering, *J. Inorg. Biochem.*, 2004, **98**, 639–648.
- 226 J. Ejnik, A. Munoz, T. Gan, C. F. Shaw and D. H. Petering, *JBIC, J. Biol. Inorg. Chem.*, 1999, **4**, 784–790.
- 227 J. Zaia, D. Fabris, D. Wei, R. L. Karpel and C. Fenselau, *Protein Sci.*, 1998, **7**, 2398–2404.
- 228 C. Jacob, W. Maret and B. L. Vallee, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 3489–3494.
- 229 W. Feng, J. Cai, W. M. Pierce, R. B. Franklin, W. Maret, F. W. Benz and Y. J. Kang, *Biochem. Biophys. Res. Commun.*, 2005, **332**, 853–858.
- 230 W. Maret, *J. Nutr.*, 2000, **130**, 1455S–1458S.
- 231 S. Zeitoun-Ghandour, O. I. Leszczyszyn, C. A. Blindauer, F. M. Geier, J. G. Bundy and S. R. Stürzenbaum, *Mol. BioSyst.*, 2011, **7**, 2397–2406.
- 232 A. Krezel and W. G. Maret, *JBIC, J. Biol. Inorg. Chem.*, 2008, **13**, 401–409.
- 233 P. Palumaa, I. Tammiste, K. Kruusel, L. Kangur, H. Jorvall and R. Sillard, *Biochim. Biophys. Acta, Proteins Proteomics*, 2005, **1747**, 205–211.
- 234 E. Artells, O. Palacios, M. Capdevila and S. Atrian, *FEBS J.*, 2014, **281**, 1659–1678.
- 235 K. L. Summers, D. E. K. Sutherland and M. J. Stillman, *Biochemistry*, 2013, **52**, 2461–2471.
- 236 D. E. K. Sutherland, K. L. Summers and M. J. Stillman, *Biochemistry*, 2012, **51**, 6690–6700.
- 237 J. Ejnik, C. F. Shaw and D. H. Petering, *Inorg. Chem.*, 2010, **49**, 6525–6534.
- 238 G. Meloni, P. Faller and M. Vašák, *J. Biol. Chem.*, 2007, **282**, 16068–16078.
- 239 G. Meloni, V. Sonois, T. Delaine, L. Guilloureau, A. Gillet, J. Teissie, P. Faller and M. Vašák, *Nat. Chem. Biol.*, 2008, **4**, 366–372.
- 240 J. T. Pedersen, C. Hureau, L. Hemmingsen, N. H. H. Heegaard, J. Ostergaard, M. Vašák and P. Faller, *Biochemistry*, 2012, **51**, 1697–1706.
- 241 J. Durand, G. Meloni, C. Talmard, M. Vašák and P. Faller, *Metallomics*, 2010, **2**, 741–744.
- 242 Y. Manso, J. Carrasco, G. Comes, G. Meloni, P. A. Adlard, A. I. Bush, M. Vašák and J. Hidalgo, *Cell. Mol. Life Sci.*, 2012, **69**, 3683–3700.
- 243 G. Meloni and M. Vašák, *Free Radical Biol. Med.*, 2011, **50**, 1471–1479.
- 244 G. Meloni, A. Crameri, G. Fritz, P. Davies, D. R. Brown, P. M. H. Kroneck and M. Vašák, *ChemBioChem*, 2012, **13**, 1261–1265.
- 245 S. E. Gabriel, F. Miyagi, A. Gaballa and J. D. Helmann, *J. Bacteriol.*, 2008, **190**, 3482–3488.
- 246 M. A. Saito, E. M. Bertrand, S. Dutkiewicz, V. V. Bulygin, D. M. Moran, F. M. Monteiro, M. J. Follows, F. W. Valois and J. B. Waterbury, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 2184–2189.
- 247 C. E. Blaby-Haas, J. A. Flood, V. de Crecy-Lagard and D. B. Zamble, *Metallomics*, 2012, **4**, 488–497.
- 248 A. M. Sydor, M. Jost, K. S. Ryan, K. E. Turo, C. D. Douglas, C. L. Drennan and D. B. Zamble, *Biochemistry*, 2013, **52**, 1788–1801.
- 249 C. Appia-Ayme, A. Hall, E. Patrick, S. Rajadurai, T. A. Clarke and G. Rowley, *Biochem. J.*, 2012, **442**, 85–93.
- 250 M. Noll, K. Petrunkhin and S. Lutsenko, *J. Biol. Chem.*, 1998, **273**, 21393–21401.
- 251 A. M. Sevcenco, M. W. H. Pinkse, H. T. Wolterbeek, P. Verhaert, W. R. Hagen and P. L. Hagedoorn, *Metallomics*, 2011, **3**, 1324–1330.
- 252 E. V. Filippova, G. Minasov, L. Shuvalova, J. Winsor, I. Dubrovskaya, L. Papazisi and W. F. Anderson, *pdb 3lay*, released 19/01/2010.
- 253 M. P. Hensley, T. S. Gunasekera, J. A. Easton, T. K. Sigdel, S. A. Sugarbaker, L. Klingbeil, R. M. Breece, D. L. Tierney and M. W. Crowder, *J. Inorg. Biochem.*, 2012, **111**, 164–172.
- 254 P. Petrarca, S. Ammendola, P. Pasquali and A. Battistoni, *J. Bacteriol.*, 2010, **192**, 1553–1564.
- 255 G. David, K. Blondeau, M. Schiltz, S. Penel and A. Lewit-Bentley, *J. Biol. Chem.*, 2003, **278**, 43728–43735.
- 256 S. Eswaremoorthy, S. Swaminathan and S. K. Burley, New York SGX Research Center for Structural Genomics, *pdb 1txl*, released 20/07/2004.
- 257 C. A. Blindauer, *J. Inorg. Biochem.*, 2013, **121**, 145–155.
- 258 U. Heinz, L. Hemmingsen, M. Kiefer and H. W. Adolph, *Chem. – Eur. J.*, 2009, **15**, 7350–7358.
- 259 H. Kozłowski, S. Potocki, M. Remelli, M. Rowinska-Zyrek and D. Valensin, *Coord. Chem. Rev.*, 2013, **257**, 2625–2638.
- 260 T. F. Cheng, W. Xia, P. W. Wang, F. J. Huang, J. W. Wang and H. Z. Sun, *Metallomics*, 2013, **5**, 1423–1429.
- 261 N. H. Joh, T. Wang, M. P. Bhate, R. Acharya, Y. Wu, M. Grabe, M. Hong, G. Grigoryan and W. F. DeGrado, *Science*, 2014, **346**, 1520–1524.
- 262 J. W. Meeusen, H. Tomasiewicz, A. Nowakowski and D. H. Petering, *Inorg. Chem.*, 2011, **50**, 7563–7573.
- 263 A. B. Nowakowski and D. H. Petering, *Inorg. Chem.*, 2011, **50**, 10124–10133.
- 264 A. Staszewska, E. Kurowska and W. Bal, *Metallomics*, 2013, **5**, 1483–1490.
- 265 J. W. Meeusen, A. Nowakowski and D. H. Petering, *Inorg. Chem.*, 2012, **51**, 3625–3632.
- 266 A. D. McNaught and A. Wilkinson, IUPAC, *Compendium of Chemical Terminology*, Blackwell Scientific Publications, Oxford, 2nd edn (the “Gold Book”), 1997.

