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The Thermodynamics of Proteins Interactions with Essential First Row Transition Metals

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Abstract

Background—The binding of metal ions to proteins is a crucial process required for their catalytic activity, structural stability and/or functional regulation. Isothermal titration calorimetry provides a wealth of fundamental information which when combined with structural data allow for a much deeper understanding of the underlying molecular mechanism.

Scope of review—A rigorous understanding of any molecular interaction requires in part an in-depth quantification of its thermodynamic properties. Here, we provide an overview of recent studies that have used ITC to quantify the interaction of essential first row transition metals with relevant proteins and highlight major findings from these thermodynamic studies.

General significance—The thermodynamic characterization of metal ions-proteins interactions is one important step to understanding the role that metal ions play in living systems. Such characterization has important implications not only to elucidating proteins' structure-function relationships and biological properties but also in the biotechnology sector, medicine and drug design particularly since a number of metal ions are involved in several neurodegenerative diseases.

Major conclusions—Isothermal titration calorimetry measurements can provide complete thermodynamic profiles of any molecular interaction through the simultaneous determination of the reaction binding stoichiometry, binding affinity as well as the enthalpic and entropic contributions to the free energy change thus enabling a more in-depth understanding of the nature of these interactions.

Introduction

Metalloproteins represent one of the most diverse classes of proteins that play important roles in key biological processes from DNA synthesis to energy production. Approximately one third of all proteins have a bound metal and almost half of all enzymes require the presence of a specific metal ion to function (1, 2, 3). The binding of metal ions to proteins is a crucial process required for the protein's catalytic activity, structural stability and functional regulation. Metal coordination by proteins essentially occurs via the proteins' side

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chain functional groups which include histidine, methionine, cysteine, tyrosine, aspartic and glutamic acids. Amino acids with simple hydroxo or amino functions, such as serine, threonine, lysine and tryptophan are less well suited for metal-ion coordination.

While the affinity and selectivity of a protein to a particular metal ion is the result of unique protein features in addition to metal coordination chemistry, metalloproteins are dynamic molecules and incorporation of a correct metal ion is rather a question of biology. According to the Irving-Williams series (4), the affinity of divalent metal ions to ligands follows the order of $Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} < Zn^{2+}$ but holds generally well for metal binding sites in proteins although deviations from this affinity trend can occur (5). In animals and humans, Mn^{2+} and Fe^{2+} bind rather weakly to proteins whereas tighter binding is often observed with Cu^{2+} and Zn^{2+} although redox-active metal ions (i.e. Mn, Fe, Cu, Ni) can modulate binding affinities. The undisputable role of metal ions in the folding and misfolding of proteins and the interplay between metal homeostasis and protein dynamics, structural and conformational changes is illustrated by their relevance in numerous neurodegenerative diseases including Menke's syndrome, Wilson's disease, Alzheimer's disease and Parkinson's disease. These diseases are characterized by elevated levels and mis-compartmentalization of either iron, copper or zinc or a combination of them (6). The focus of this review concerns the thermodynamics of proteins interactions with the first row transition metals or the d-block elements which occupy the central block of the periodic table. Of those elements, only the most essential metal ions in humans (i.e. V, Mn, Fe, Co, Ni, Cu, and Zn) will be considered. The occurrence of these trace metals and some of the essential functions and symptoms associated with their deficiency and overdose in humans are shown in Table 1. Because too much or too little is often harmful and even lethal, the concentrations and distributions of metals is tightly regulated by proteins and enzymes in cells so as to provide essential amount of metal ions while preventing toxicity.

Isothermal titration calorimetry (ITC) has been widely used to study the thermodynamics of metal ions-proteins interactions. Like with most binding events, enthalpic (ΔH) and entropic (ΔS) changes provide insights into the structure and dynamics of this recognition event. Thus, unraveling the thermodynamic driving forces that govern the interactions of metal ions with proteins is critical to understanding metal trafficking pathways, metal homeostasis and metal detoxification. This review briefly discusses the chemical and biochemical properties of essential first row transition metals and then provides a thermodynamic quantification (binding stoichiometry, binding affinity and enthalpic and entropic contributions to the binding free energy) of their interactions with representative proteins using isothermal titration calorimetry (ITC). A thorough and comprehensive review paper by N. Grosseoheme on metal-based ITC experiments highlighting common complications and solutions to unavoidable metal ions solution chemistry and different linked equilibria is included in this special volume and will not be revisited here. In that study, the author reviewed ITC experimental concerns and pitfalls and offered guidance to scientists wishing to perform ITC titrations that involve metal ions.

Chemical, Biochemical and Binding Properties of Essential First Row Transition Metals

Zinc (Zn)

Zinc is the second most abundant trace element in the human body, following iron, and is generally thought of as non-toxic. It is an essential element for humans and animals and required for growth and development and fertility. It is also important in digestion, nucleic acid synthesis and the immune system. It is known to activate parts of the brain that governs taste and smell and has been proposed to have therapeutic benefits against infectious diseases including a shortening of the common cold in humans. Although a trace element, it is hard to underestimate the impact of zinc on human physiology given that an estimated 10% of the human genome encodes zinc proteins amounting to at least 3000 proteins (10). There are several hundred kinds of zinc-containing enzymes where zinc plays a catalytic and/or a structural role (8–10). The six fundamental classes of enzymes where zinc is found include oxidoreductases, hydrolases, transferases, lyases, ligases, isomerases. These enzymes catalyze the metabolic conversion or degradation of proteins, nucleic acids, lipids, porphyrin precursors and other bioorganic compounds. Additionally, there exist numerous superfamilies of zinc finger domains which are ubiquitous structural elements known to bind DNA, RNA and protein whereby zinc plays a critical role in the regulation of the transcription and translation of the genetic message (8–10).

From a chemical point of view, in the active sites of protein and enzymes, zinc is usually a Lewis acid that lowers the pK of coordinated water (from about 10 for free $[\text{Zn}(\text{H}_2\text{O})_6]^{2+}$ to less than 7 in enzymatic systems) and/or activates substrates (10, 11). Because it is redox inactive, zinc does not have d-d transitions (therefore no absorption spectroscopy) and lacks the ligand field stabilization phenomenon which means that zinc can in principle adopt flexible coordination number and geometry (from tetrahedral to distorted trigonal bipyramidal) that is dictated by the size and charge of its ligands. Two examples of zinc interaction with small peptides (i.e. zinc-fingers) and proteins (i.e. human carbonic anhydrase), whereby zinc serves a structural role in one instance and a catalytic role in the other, will be discussed in this section.

Zinc finger transcription factors are one of the largest classes of eukaryotic proteins that require one or more Zn^{2+} ions to stabilize a protein structure. They are generally recognized for their DNA/RNA binding ability and use different combinations of Cys and His residues to coordinate the zinc ions (12). Zinc finger proteins are composed of several modular zinc finger domains each containing a single zinc-binding site that recognizes a specific base pair segment of DNA (or RNA). Zinc fingers are typically unfolded in the absence of zinc (apo state) and folded into biologically active three-dimensional structures upon Zn binding (13–15). The coordination of the zinc ion to the cysteine and histidine residues is essential to drive the folding process of the zinc finger structure which otherwise would not be obtained if these ligands are switched within the sequence (16). Carbonic anhydrases (CAs) are a family of zinc dependent metalloenzymes that catalyzes the hydrolysis of carbon dioxide to bicarbonate ion. Depending on the class type (α , β or γ), the catalytic zinc ions in CAs is bound by either three histidine residues in the α - and γ -classes or by one histidine and two

cysteine residues in the β -class (17–18). Interestingly, zinc fingers and carbon anhydrase represent the extremes of metalloprotein design in terms of protein-folding thermodynamics due to the fact that the former are unfolded while the latter are folded in the absence of zinc. In the case of carbonic anhydrase, the measured zinc ion binding free energy represents the actual free energy of metal-ligand binding because the protein is already in the folded state in the absence of zinc (i.e. binding of zinc occurs between the apo-folded state and the holo-folded state). With zinc fingers, the apo-protein is unfolded which means that the apo-folded protein has a higher energy (i.e. a positive free energy cost to protein folding). Thus, the actual free energy of metal ion binding is the sum of the measured thermodynamic contribution of zinc binding plus the free energy due to protein folding.

ITC of Zinc Binding to Zinc Finger Domains

As reported earlier, zinc plays a major role in stabilizing protein structures and the best known example is the Cys₂His₂ zinc-finger domains which were first identified in transcription factor IIIA (19). These zinc finger motifs adopt a specific fold ($\beta\beta\alpha$ arrangement) that enables them to be recognized within the major groove of DNA. Whereas only zinc ions bind very tightly to zinc-finger peptides and are able to activate them at low metal-ion concentrations for specific DNA binding activity, other first row divalent metal ions such as Co(II), Ni(II), Cd(II) and Fe(II) are also able to bind with significant affinity (i.e. free energy change varied between – 33 to – 50 kJ/mol with the free energy of zinc binding to the Cys₂His₂ zinc-finger domain being close to –66 kJ/mol (20). Such selectivity for zinc ions explains why zinc fingers have such tight affinity for zinc over redox active metals that might otherwise cause oxidative damage to the nucleic acids that are bound by the proteins in vivo. Nonetheless, several studies using the Cys₂His₂ zinc finger as a model to understand the thermodynamics of zinc-coupled protein folding reported that the enthalpy of the reaction is dominated by zinc-peptide bond formation and that the dehydration of the metal ion had a significant and favorable contribution to the overall entropy of the reaction. A more recent study using ITC to quantify the thermodynamics of Zn²⁺ binding to peptides corresponding to three naturally occurring Zn-binding sequences in transcription factor proteins supported these early findings and concluded that zinc binding to the classical Cys₂His₂ zinc finger is both enthalpically and entropically favored but less so (i.e. more enthalpically disfavored and entropically favored with increasing Cys ligands) with two derivatives containing three Cys and one His (Cys₂HisCys) or four Cys (Cys₄) metal coordinating residues (21). Additionally, the study found that upon binding, Zn²⁺ displaces a proton from each coordinated Cys residue negating the notion that Cys thiols in zinc fingers and corresponding proteins remain protonated upon Zn²⁺ binding. Fig. 1 is a representation of the ITC raw data and integrated heats for the titrations of zinc ions into a Cys₂His₂ motif (a structurally well characterized classical zinc finger domain referred to as Sp1–3), a Cys₂HisCys motif (or MyT1–2) lacking Zn-stabilized secondary structure and a lower propensity to form α helical and β sheet secondary structures, and finally a Zn-binding sequence that coordinates the metal ion with four cysteines (Cys₄ motif or GR-2) with little Zn-stabilized secondary structure. Table 2 shows the best fit parameters for ITC data for Zn²⁺ binding to Sp1–3 and two other peptide derivatives (MyT1–2 and GR-2, a Cys₂HisCys and a Cys₄ peptide derivatives, respectively).

The data presented in Table 2 indicate a similar affinity of the three different peptides for Zn^{2+} but very different enthalpy-entropy contributions to the binding free energy. For instance, the presence of more cysteines in the peptide lead to less favorable enthalpy and more favorable entropy with zinc binding to the Cys₄ peptide being completely driven by entropy with an enthalpic penalty (i.e. positive ΔH). This trend is attributed to an increased enthalpic penalty to deprotonate the thiols of peptide with increasing number of cysteines. This is accompanied by a concomitant entropic gain due to protons release and this enthalpic-entropic compensation process is perhaps a common feature of tetrahedral Zn^{2+} coordination in these types of peptides (21).

ITC of Zinc Binding to Carbonic Anhydrase

Carbonic anhydrase (CA) is considered the prototype of zinc enzymes and has been the subject of intense studies for the past seven decades (22). The zinc ion is coordinated by three histidine residues at the active site of the protein with the remaining tetrahedral site occupied by a water molecule which is hydrogen-bonded to a threonine residue. Deprotonation of the water molecule generates a hydroxyl ion (OH^-) which carries out a nucleophilic attack on the carbon dioxide substrate to form a zinc-bound bicarbonate intermediate which is then displaced by H_2O to release bicarbonate and complete the catalytic cycle. The charge on the zinc ion makes the water molecule more acidic than free H_2O which acts as the source of the nucleophilic zinc-bound hydroxide ion.

Given that carbonic anhydrase is an excellent model protein to study, a recent detailed and thorough thermochemical analysis of zinc association with the protein found that the equilibrium constant for zinc binding to CA is about 3 orders of magnitude lower than previously reported (i.e. 2×10^9 vs. $1 \times 10^{12} \text{ M}^{-1}$). The higher value was estimated using equilibrium dialysis methods (23–24) whereas the revised value was based on a more direct method using sensitive isothermal titration calorimetry (25). Figure 2 is an illustration of ITC binding isotherms where buffer interferences and serious dilution issues are shown (right panel). The measured thermodynamic values of Zn^{2+} binding to apoCA in various buffers and the corrected thermodynamic values (buffer-independent) are reported in Table 3.

The high value of the equilibrium constant K_a of $\sim 2 \times 10^9$ (Table 3) is consistent with the notion that an essential metalloenzyme like CA should strongly bind the native zinc ion. This association is primarily enthalpy-driven with an average value of 16.2 kcal/mol consistent with the formation of three Zn^{2+} His bonds (~ 5 kcal/mol per bond). The unfavorable entropy could be partially accounted for by structural changes upon zinc binding including ordering of the hydrophilic region of the protein's active site and stabilization of the side-chain residues of α -helices.

Copper (Cu)

Copper is present in more than a dozen copper-dependent enzymes many of which are involved in electron transfer, activation of oxygen and other small molecules like nitrogen, methane, and carbon monoxide, and deactivation of toxic intermediates of oxygen reduction (26). Some copper-containing proteins have similar functions to their iron-dependent

analogues suggesting that in the early evolution of life, iron and copper may have co-existed. Examples of such synergy is illustrated by ceruloplasmin and cytochrome c oxidase with the former being a copper binding protein with an important role in iron metabolism and the latter being one of the complexes on the mitochondrial electron-transport chain which requires heme iron for its activity. Additionally, both copper and iron are redox-active metals that are essential for many biological functions but potentially harmful due to their roles in catalyzing Fenton or Haber–Weiss type reactions leading to the production of hydroxyl radicals and oxidative damage. Fortunately, cells have developed intricate machinery to ensure tight control of these redox metal ions during acquisition and distribution.

From a chemical point of view, as with ferrous ions, the reduced form of copper, Cu(I), can catalyze Fenton chemistry and this is one of the reasons that intracellular concentration of “free” copper is extremely low where the metal is highly controlled by chaperone proteins. While Cu(II) forms complexes with coordination numbers 2, 3 or 4 with the latter generally adopting a square-planar or tetragonal geometry, Cu(I) complexes prefer coordination numbers 4, 5 or 6 and are tetrahedral (7). Generally speaking, and from a spectroscopic and structural point of view, there exist three main classes of copper centers in biological molecules (Type 1, 2, and 3), although several other types exist but do not have the exact characteristics of classical copper centers such as N₂O reductase of the nitrogen cycle and cytochrome c oxidase of the respiratory chain. Type 1 are blue copper centers with a reversible electron transfer and oxidation functions (Ex. Azurin, plastocyanin, laccase, ascorbate oxidase and ceruloplasmin); type 2 and type 3 are normal non-blue copper centers (monomeric and dimeric copper centers) with an O₂ transport and oxidation functions (Ex. Hemocyanin, nitrite reductase, Cu,Zn-superoxide dismutase, mono- and di-oxygenases, and oxidases) (8). Here, we will discuss two types of copper-protein interactions, one involving copper catalysis through a superoxide dismutase (SOD1) and the other involving copper transport through a cytoplasmic Cu-chaperone (Atox 1).

ITC of Copper (also Cobalt and Zinc) Binding to Cu/Zn/SOD1

Copper-zinc superoxide dismutase (CuZnSOD1 or SOD1) is a metalloenzyme that catalyzes the disproportionation of the metastable superoxide ions into dioxygen and hydrogen peroxide through oxidation-reduction chemistry at the metallic catalytic center. The enzyme is present in the cytoplasm and in the intermembrane space of mitochondria at micromolar concentrations, as well as in the nucleus and in the peroxisomes at lower levels. It is a homodimeric metalloprotein harboring one Cu²⁺ and one Zn²⁺ ion in each of its subunits. There is renewed interest in the metal-binding properties of the cytosolic and the plasma CuZn superoxide dismutase (CuZnSOD1) due to its implication in familial amyotrophic lateral sclerosis (ALS) (27). More than 100 different mutations in the gene encoding CuZnSOD1 are known to cause ALS and it has been suggested that some of these mutations might affect the zinc- and copper-binding sites of the protein (28). Additionally, altered affinity for zinc ions has been postulated to play a role in the disease (29) but aggregation of the SOD1 protein is considered to be the primary mode of pathogenesis.

It is well established that apo-human SOD1 protein is an extremely stable dimer in solution due to the presence of disulfide bonds. Therefore, the ITC data shown below represent the binding of metal ions (zinc, cobalt and copper) to the apo dimeric form. The affinity of metal ions for both binding sites is highly pH-dependent and it is documented that acidic pHs (< 6.5), zinc (or cobalt) ions preferentially bind to the zinc site but other metals (i.e. Cu, Cd, Hg) can be substituted for zinc in vitro suggesting that the zinc site has little specificity for what metals it can bind (30). However, zinc and copper ions binding to SOD1 is lost at pH 4.5 and 3.0, respectively due to protein denaturation. Earlier studies evaluated the four binding constants of zinc(II) ions to apo-bovine superoxide dismutase using equilibrium dialysis. The binding constants of zinc ions binding to the native zinc sites of SOD (average value of $\sim 1 \times 10^{11} \text{ M}^{-1}$) were much larger than those to the native copper sites (average value of $\sim 3 \times 10^7 \text{ M}^{-1}$) at pH 6.25 and 4 °C (31). The native copper sites of bovine superoxide dismutase selectively react with copper ions since zinc binding to these sites were much weaker ($\sim 10^6$ times weaker affinity) although silver (Ag^{+1}) had equal affinity to the second copper site as determined in a competition experiment involving the displacement of Ag^{+1} by Cu^{+2} in a bovine Cu/Ag/Zn₂SOD sample (32). Figure 3 illustrates representative ITC titrations for Zn^{2+} and Cu^{2+} binding to bovine and human SOD1, respectively with the apparent thermodynamic parameters for several metal ions binding to apo-SOD1 and its derivatives shown in Table 4.

The reason that low pHs are used in these titrations is because of the very high affinity of metal ions for SOD1 at neutral pHs (i.e. the apparent K value for the first Cu(II) ion binding to dimeric CuZnSOD at pH 7.0 is $\sim 10^{15} \text{ M}^{-1}$, a value beyond the detection range of ITC, assuming direct titration in the absence of Cu(I) competing ligands). While the binding of metal ions to SOD1 is enthalpically favorable, the data in Table 4 indicate that both human and bovine SOD1 proteins bind approximately one Zn^{2+} or one Co^{2+} per dimer despite the fact that the apoprotein dimer contains two equivalent zinc binding sites. An earlier study by Hirose et al. (31) found that copper ions bind to all four sites with an equal affinity of $K \sim 2 \times 10^6 \text{ M}^{-1}$. The values of K varied from $\sim 10^6 \text{ M}^{-1}$ for binding of Cu^{2+} to the apoprotein, to 10^5 M^{-1} for binding to SOD1 pre-equilibrated with Zn^{2+} , to 10^4 M^{-1} for SOD pre-equilibrated with Cu^{2+} . The discrepancy in the thermodynamics values of these metal ions binding to SOD1 is unclear although differences in buffer and experimental conditions are noted between the two studies and a dramatic decrease in metal-binding affinity has been reported at pHs below 5.5 (32, 34). Additionally, and despite the fact that the two empty zinc sites are identical in the apo dimer, a significant structural change is observed upon binding of the first zinc ion to the protein as evidenced by a large and negative change in heat capacity ($\sim -650 \text{ cal.mol}^{-1} \cdot \text{K}^{-1}$) (33). This large conformational change introduces a negative cooperativity between sites affecting the binding of the second zinc or other metal ions to other sites on the protein. These observations are consistent with a similar ITC investigation of Mn^{2+} binding to homodimeric tartrate dehydrogenase where thermodynamic asymmetry in the binding of metal ions to the protein was reported (35). Interestingly, when the ITC experiments were repeated with higher concentrations of SOD1, the stoichiometry of binding increased to $\sim 2 \text{ Zn}^{2+}$ or 2 Co^{2+} per dimer, as expected (30, 33, 36).

ITC of Copper Binding to Atox1

The inherited Menkes and Wilson disorders are human diseases of copper metabolism associated with defects of two copper transport ATPases, ATP7A and ATP7B, respectively. These two ATPases accept copper from Atox1, a human cytosolic metallo-chaperone (also known as Hah1) involved in Cu(I) transport that itself acquires copper from the copper transport protein Ctr1. Atox1 features a classical $\beta\alpha\beta\beta\alpha\beta$ fold with a CXXC motif able to bind Cu(I) with high affinity and the two ATPases (ATP7A and ATP7B) have unique six soluble N-terminal copper-binding domains which according to genetic and biochemical studies are not functionally equivalent (37, 38). The copper transport proteins Ctr1 and ATP7A/B have been implicated in cellular resistance of cisplatin (CisPt) which is one of the most common anticancer drugs used against many severe forms of cancers. The majority of the side effects and cell resistance are believed to be due to CisPt interactions with proteins that detoxify the drug prior to reaching the nucleus and the DNA target. Recent in vitro work showed that CisPt also interacts with the cytoplasmic Cu-chaperone Atox1 (39, 40). In order to quantify the binding affinities of Cu(I) to proteins involved in intracellular copper trafficking and ultimately delineate the mechanisms by which metal ions are brought into cells and delivered to metal-requiring proteins, and to have a better understanding of metal homeostasis related diseases, a number of studies have examined the thermodynamics of copper interactions with these various proteins using isothermal titration calorimetry, among other methods (37, 38, 41–43).

Even though the structures and metal-binding sites of these copper binding and transport proteins are very similar, the literature values for binding affinities are wildly scattered and differ by as much as 13 orders of magnitude (K values ranging between 10^5 and 10^{18} M^{-1}) (38, 41). Possible interpretations for such large variations include the source of raw materials and the different experimental approaches used in these studies. Furthermore, the intrinsic instability of free Cu^+ ions in aqueous solution despite stringent precautionary steps to minimize its oxidation and that of cysteine ligands added additional complications and uncertainties to the experimental results and subsequent data analysis. Nonetheless, we present here a case study to illustrate the ubiquitous usage of ITC without disputing or endorsing a specific protocol or method. Sorting out these discrepancies or attempting to resolve these fundamental issues is beyond the scope of this review.

The Atox1 metallochaperone and its homologs, both eukaryotic and prokaryotic, contain a single CXXC soluble N-terminal motif whereas both WND (Wilson disease protein) and MNK (Menkes disease protein) contain six such domains which are believed to play discrete roles in the function and regulation of WND. Although all six metal-binding domains can bind copper, domains 1 to 4 are most important for interaction with Atox1 whereas domains 5 and 6 are required for copper transport. ITC was therefore used to measure the association constant and stoichiometry of Cu(I) binding to various constructs of the Wilson disease protein (WND) and its copper chaperone (Atox1). Table 5 shows the apparent values of the binding affinity (K) and enthalpy change (ΔH) and also the measured stoichiometries which are in good agreement with the number of CXXC motifs in each polypeptide. For instance, Cu(I) binding stoichiometries of ~ 1 for Atox1, ~ 2 for WD34 and WD56 and ~ 4 for WD14 is consistent with the presence of one, two and four CXXC motifs on these peptides,

respectively. It is unclear though why WD12 binds only one Cu(I) ion and WD16 binds 5 Cu(I) ions and not the expected 2 and 6 Cu(I), respectively. It is possible that in these variants, different conformations are adopted by certain domains such as either one domain does not bind Cu(I) or that two domains arrange in such a way to bind one Cu(I) ion as observed in the x-ray structure of Atox1 at high concentration.

The overall similarity in Cu(I) binding constants for Atox1 and the WND domains suggests that copper transfer between Atox1 and WND is under kinetic control and not governed by a thermodynamic gradient. Assuming Atox1 has a slow rate of dissociation (k_{off}) for copper, complexation between Atox1 and WND could alter k_{off} and lower the kinetic barrier for transfer between metal-binding sites as suggested previously for Atx1 and Ccc2 (44). Because all 5 Cu(I) binding sites on WD16 have the same K value, it was determined that the discrete functions of the WND metal-binding domains are not defined by copper-binding affinities but instead by the domains conformational structures and electrostatic surface properties.

Iron (Fe)

With the exception of lactobacilli, iron is an essential trace element for almost all organisms and is involved in myriad biological processes from photosynthesis and respiration to oxygen transport and DNA biosynthesis. The extreme biological importance of iron is illustrated by the following two simple examples; (1) the identity and distribution of algal species when blue-green algal blooms occur in lakes is determined by its ability and efficacy to chelate iron and (2) the growth of malignant cells depends on the concentration of transferrin receptors which are required for iron uptake for cellular proliferation. The uptake, transport and storage of iron is controlled by complex yet very tight regulatory mechanisms (45). Although iron is the most abundant transition metal on Earth, it is highly insoluble ($K_{sp} \sim 10^{-39} \text{ M}^4$ for $\text{Fe}(\text{OH})_3$ at pH 7.0 corresponding to a theoretical concentration of $[\text{Fe}^{3+}] = 10^{-18} \text{ M}$) and not readily bioavailable. To survive, living organisms have developed highly sophisticated processes to acquire iron which include high iron-affinity proteins and special low-molecular weight compounds (i.e. siderophores) to solubilize iron for transport, storage and incorporation into proteins and other biomolecules. In humans and higher organisms, iron acquisition, transport and storage are accomplished by non-heme iron proteins (i.e. the transferrins for transport and the ferritins for storage) and are fully reversible processes under physiological conditions. The tight regulation ensures virtually no “free iron” on the generation of harmful and reactive oxygen species.

From a chemical point of view, iron can exist in various oxidation state (from -2 to $+6$) with $+2$ and $+3$ being the most common forms of iron although high-valent iron species ($+4$ and $+5$) are common intermediates of iron-dependent monooxygenases. The high reactivity of iron is related to an easily tunable $\text{Fe}^{2+}/\text{Fe}^{3+}$ redox potential by appropriate choice of ligands encompassing almost the entire biological spectrum of redox potentials (from -0.5 V to $+0.6 \text{ V}$). While Fe^{3+} prefers oxygen ligands such as in phenolates and carboxylates, Fe^{2+} has a preference for nitrogen and sulfur ligands (in addition to oxygen ligands) like histidine, protoporphyrin and cysteine. In this section, we will cover the thermodynamics of $\text{Fe}^{2+}/\text{Fe}^{3+}$

binding to the iron transport protein, transferrin and the iron storage protein, ferritin in humans.

ITC of Fe²⁺/Fe³⁺ Binding to Human Transferrin

Transferrins (Tf) are a family of single-chain bilobal glycoproteins that play a crucial role in iron homeostasis. They bind reversibly but very tightly ($\sim 10^{21} \text{ M}^{-1}$) two Fe(III) ions, one in each lobe, in the presence of a synergistic and physiological carbonate (or bicarbonate) anion, although other synergistic anions have been shown to be as effective. Although transferrin is the primary iron-binding and transport protein in humans, it is only about 30% saturated with iron in normal human serum and up to 40 different metals have been shown to bind to transferrin (46 and references therein). Iron-loaded transferrin binds with high affinity to the transferrin receptor (TfR) on the cell surface followed by the internalization of the transferrin/transferrin receptor via receptor mediated endocytosis into an acidic endosome where iron is released. Of the two known homodimeric type II cell transmembrane glycoprotein receptors (TfR1 and TfR2), TfR1 is more extensively characterized receptor and has the highest affinity for iron-loaded transferrin (dissociation constants of $\sim 1 \text{ nM}$ for TfR1 and $\sim 30 \text{ nM}$ and TfR2).

While numerous literature reports exist on the interaction of ferric ions with transferrins, a recent study from our lab investigated the binding of ferrous ions to human transferrin (hTf) by ITC under strictly anaerobic conditions in the presence and absence of the synergistic anion bicarbonate. The data showed the presence of one class of strong Fe²⁺ binding sites with $\sim 70\%$ occupancy (i.e. a binding stoichiometry of $\sim 0.7 \text{ Fe}^{2+}$ per hTf molecule) and an association constant of $\sim 4 \times 10^7 \text{ M}^{-1}$ (Fig. 4). A second weak class of binding sites did not permit the accurate determination of binding stoichiometry, affinity or enthalpy change of the reaction. The strong association of Fe²⁺ with hTf was achieved with favorable enthalpy and entropy changes (Table 6) where the large and positive entropy value was attributed to changes in the hydration of the protein and of the metal ions upon interaction (46). Similar binding thermodynamics were observed when Zn²⁺ was titrated into an apo-hTf sample albeit with a much higher affinity and a binding stoichiometry close to unity (Table 6). Whereas the mechanism of iron removal from transferrin at the acidic pH of the endosome is debatable, it is still unknown whether reduction of Fe³⁺ bound to transferrin occurs before or after iron is released from the transferrin–transferrin receptor complex. Based on our ITC results and our calculated formal redox potential of the Fe³⁺–hTf complex ($\sim -127 \text{ mV}$), a value well within range of physiological reducing agents, it was suggested that iron removal from transferrin might proceed via a reduction process whereby iron is released from the protein as Fe²⁺ (46). The thermodynamics of Fe³⁺ binding to a variety of transferrins including ovotransferrin, melanotransferrin and human transferrin as well as holo-transferring interaction with its receptor (TfR1) were reviewed elsewhere (47).

ITC of Fe²⁺ Binding to Human (and *E. coli*) Ferritin

Ferritins are ubiquitous and well-characterized iron storage and detoxification proteins (48–50). In bacteria, Archaea, diatom and plants, ferritins are homopolymers composed of H-type subunits, while in vertebrates, they typically consist of 24 similar subunits of two types, H and L. In amphibian red cells, three types of subunits (H, M, and L) form hetopolymeric

ferritins where H and M subunits appear to have similar functional properties. The H-subunit is responsible for the rapid oxidation of Fe(II) to Fe(III) at a dinuclear center, whereas the L-subunit appears to help iron clearance from the ferroxidase center of the H-subunit and support iron nucleation and mineralization. The mechanism by which ferritins form a mineral core inside their hollow structure has been studied for over four decades. Despite their overall similar structures, ferritins from different origins markedly differ in their iron binding, oxidation, detoxification, and mineralization properties. A proposed universal mechanism by which ferritins function (50) has been challenged by a number of studies from different labs (51–53). A number of inconsistencies in the literature data remain and do not support a unified mechanism for all ferritins. What is clear is that iron uptake, oxidation and deposition in ferritins involve a complex series of events including the passage of Fe²⁺ ions through specific channels, binding at the ferroxidase centers followed by oxidation of bound Fe²⁺ to Fe³⁺ by either molecular oxygen or hydrogen peroxide, and finally movement of the resulting hydrolyzed Fe³⁺ products to mineralization sites within the protein's cavity where a mineral ferric oxide/hydroxide core forms. In this section, we will focus on the thermodynamic properties of Fe²⁺ binding to recombinant human H-chain apoferritin (HuHF) and to bacterial apoferritin (EcFtnA) in order to determine the location of the primary ferrous ion binding sites on the protein and the principal pathways by which the Fe²⁺ travels to the dinuclear ferroxidase center prior to its oxidation to Fe³⁺.

Figure 5 illustrates the ITC results for the anaerobic titration of Fe²⁺ into apo-HuHF and apo-EcFtnA at pH 7 and 25.00 °C while the derived binding parameters are summarized in Table 6. In both cases, the calorimetric titrations indicate that the dinuclear (in case of HuHF) or trinuclear (in case of EcFtnA) ferroxidase centers are the principal loci for Fe²⁺ binding. In HuHF, only one site of the ferroxidase center is found occupied by Fe²⁺ at pH 7 (i.e. ~ 24 Fe²⁺ per ferritin molecule) suggesting that Fe²⁺ oxidation to form the diFe(III) species might occur in a stepwise fashion. When two of the ferroxidase center residues (E62K & H65G) are mutated, Fe²⁺ binding to the center was abolished, a result pointing to the importance of one or both residues in Fe²⁺ binding. Further ITC results showed a reduction in the Fe²⁺ binding capacity of the protein in the 3-fold hydrophilic channel variant S14 (D131I+E134F) confirming that the primary avenue by which Fe²⁺ gains access to the interior of ferritin is through these channels. The fact that these 3-fold channel mutations do not completely prevent Fe²⁺ binding to the ferroxidase center suggests that iron might gain access to the center by other pathways. Overall the ITC data indicated that the first step in the mechanism of iron deposition in ferritin corresponds to the binding of a single Fe²⁺ ion at the ferroxidase center (i.e. the histidine-containing site). The binding of a second Fe²⁺ may be facilitated by molecular oxygen which could oxidize the di-ferrous-protein complex to form the di-ferric-protein complex

As to Fe²⁺ binding to EcFtnA, the ITC data revealed the presence of two main classes of binding sites (~ 24 Fe²⁺ per site) which were ascribed to Fe²⁺ binding at two of the three trinuclear sites (most likely A and B sites). Some degree of inter- and intra-subunit negative cooperativity between the three sites was also suggested by the ITC results. Overall, the complex binding isotherms for Fe²⁺ binding to EcFtnA were described and fitted using a developed binding model for three classes of independent binding sites. The data helped clarify the initial Fe²⁺ binding sites and molecular events prior to iron oxidation. The third

binding site in EcFtnA (C site) does not appear to be involved in strong Fe^{2+} binding, at least initially, but seems to modulate Fe^{2+} binding at the adjacent dinuclear iron sites A and B.

Manganese (Mn)

Mn plays many important roles in biology the most important of which is in oxygen production by photosynthetic plants, algae, and cyanobacteria. These organisms use an enzyme called photosystem II (PSII) and solar energy to carry out a water-splitting reaction that provides enough reducing equivalent to convert carbon dioxide to carbohydrate and water. Mn is also a cofactor in a number of mammalian enzymes such as catalase, peroxidase and superoxide dismutase whose main functions are to detoxify reactive oxygen species. Other important enzymes with di-Mn centers include arginases which catalyze the divalent cation-dependent hydrolysis of L-arginine to form L-ornithine and urea. Manganese also plays an important role in microbial metabolism and is often associated with microbial virulence (57). The reader is referred to a comprehensive chapter on Mn biological chemistry and its role in oxygen generation and detoxification that appeared in a recent book by R. R. Crichton (7).

From a chemical point of view, manganese can have three oxidation states (Mn^{2+} , Mn^{3+} and Mn^{4+}). Unlike iron, the +2 oxidation state of Mn is thermodynamically more stable than the +3 oxidation state due to the half-filled d^5 shell in both Mn^{2+} and Fe^{3+} . Whereas free Fe^{2+} (or other redox active metals such as Cu) can participate in the generation of reduced oxygen species, free Mn^{2+} is not harmful and does not undergo Fenton-type chemistry. Here, we cover the thermodynamics of Mn^{2+} binding to human calprotectin (CP), an innate immune factor that plays a key role in nutritional immunity and found in abundance in neutrophils. The antimicrobial activity of CP is due to the chelation of the essential nutrients Mn^{2+} and Zn^{2+} resulting in bacterial metal starvation and death.

ITC of Mn^{2+} Binding to Human Calprotectin (CP)

Calprotectin (CP) is a transition metal-chelating antimicrobial protein of the calcium-binding S100 family that is produced and released by neutrophils. It is the only identified mammalian Mn(II)-sequestering protein that inhibits the growth of various pathogenic microorganisms by sequestering the essential transition metal ions manganese and zinc. It does so by using a unique hexahistidine coordination motif. Several recent studies have looked at the biophysical and thermodynamic characterization of Mn(II) and Zn(II) binding to human CP to better elucidate the mechanism of CP in nutritional immunity (58–61).

Unlike most S100 proteins which are homodimers with two identical transition metal-binding sites, CP is a heterodimer of S100A8 and S100A9 subunits and two distinct binding sites that involve residues His17 and His27 from S100A8 and His91 and His95 from subunit S100A9 (Site I or S1) and residues His20 and Asp30 from S100A9 and His83 and His87 from S100A8 (Site II or S2) (58, 62). To determine which transition metal (Mn, Zn or both) gives CP its antimicrobial activity and the location of the metal-ions binding sites, ITC experiments were performed on wt CP and two of its variants (S_1 = CP mutant with the four S1 histidine ligands changed to asparagines and S_2 = CP mutant with the three S2

histidines changed to asparagines and the aspartic acid to serine). Figure 6 and Table 7 show that wt CP binds two Zn^{2+} ions per heterodimer with similar high affinity constants (average association constant K_a of $2.85 \times 10^8 \text{ M}^{-1}$) but only one with a high affinity constant K of $7.7 \times 10^8 \text{ M}^{-1}$ with ~ 3000 -fold weaker affinity for the second Mn^{2+} ion ($K \sim 2.7 \times 10^5 \text{ M}^{-1}$). Variant S2 retained its high affinity for both Zn^{2+} and Mn^{2+} ions whereas variant S1 bound one Zn^{2+} ion but not Mn^{2+} . In contrast, variant Zn/Mn in which all site I and site II residues are mutated (all His residues changed to Asn and Asp to Ser) exhibited no binding affinity to either Mn^{2+} or Zn^{2+} (58).

In addition to the fact that Zn/Mn did not increase the sensitivity of *S. aureus* to superoxide stress, the IC_{50} of wt Cp (139 mg/ml) was compared with that of Zn/Mn (9042 mg/ml) confirming that the antimicrobial activity of CP is indeed due to its metal chelation properties. All together, by sequestering manganese and zinc, the neutrophil protein calprotectin starves bacteria of these essential nutrients and thus plays a crucial role in host defense against bacterial and fungal pathogens. More recent studies suggested a role for calcium in enhancing the affinity and the specific binding of Mn(II) to human CP (59, 61).

Nickel (Ni) and Cobalt (Co)

Nickel and cobalt appear to have had important roles prior to the evolution of atmospheric oxygen as evidenced by their presence in many anaerobic bacteria. Despite the very low amounts in mammalian serum (< 100 -fold less than Zn, Fe or Cu), both Ni and Co are involved in a number of important enzymes and molecules such cobalamins (the coenzymatically active form of vitamin B12) which contains a Co atom inside a corrin ring and glyoxylase, a cytosolic enzyme that contributes to the detoxification of glycolic waste including methylglyoxal and other R-ketoaldehydes into lactate (7). Given that glyoxylase is inactivated by Zn(II) but activated by Ni(II) and Co(II), it is unclear whether it is a true Ni-enzyme especially since (1) zinc binds more tightly than nickel, (2) nickel proteins are virtually unknown in higher eukaryotes and (3) no known nickel homeostasis factors in humans have yet been found (63). With the exception of glyoxylase, all Ni-enzymes characterized to date are confined to microorganisms and are involved in the use and/or production of gases including NH_3 (Urease), CO (Acireductone dioxygenase), O_2 (Superoxide dismutase), H_2 (Hydrogenase), CO/ CO_2 (CO Dehydrogenase), CH_4 (Acetyl-CoA synthase/CO Dehydrogenase/methyl CoM reductase) (7). Due to its low occurrence and availability in natural environments and given its importance as a catalytic cofactor of enzymes found in eubacteria, archaebacteria, fungi, and plants, organisms have developed high affinity Ni-uptake systems including metallochaperones and other regulators of Ni homeostasis (63).

From a chemical point of view, Ni^{2+} and Co^{2+} (like Fe^{2+}) are electron rich and can participate in the generation of reactive free radicals such as the conversion of ribonucleotides into their deoxy-derivatives. Ni-sites in particular show significant adaptability in terms of coordination and redox chemistry spanning a wide range of redox potential and allowing Ni-centers in enzymes to carry out a rich array of redox reactions. For instance, redox potentials of the Ni-center in the Ni-superoxide dismutase ranges between +

890 mV and – 16 mV whereas those in CO dehydrogenase and methyl CoM reductase go as low as – 600 mV for a stunning total range of 1.5 V (7).

Given the rarity of Ni- and Co-depending enzymes in humans, the thermodynamic binding properties of these metal ions with their corresponding enzymes have not been characterized, to the best of our knowledge. However, an earlier study (64) and a more recent review by D. E. Wilcox (65) discussed ITC binding data of Ni^{2+} , Cu^{2+} , and Zn^{2+} Binding to two Ureases UreE (from *Klebsiella aerogenes* and *Bacillus pasteurii*) which are metalloenzymes with two Ni^{2+} binding sites found in microorganisms and plants that hydrolyzes urea to ammonia and carbamate. Overall, the ITC data (Fig. 7) revealed two chemically different Ni^{2+} coordination environment in the two proteins whereby Ni^{2+} binding to UreE from *K. aerogenes* exhibited an enthalpically favored process but was entropically driven in the case of *B. pasteurii* (64).

Vanadium (V)

Vanadium is an essential element for many organisms including tunicates (ascidians or sea squirts), brown algae (seaweeds, lichen and mushrooms (i.e. *Amanita muscaria*)). In tunicates, V accumulates inside blood cells in very acidic vacuoles at concentrations up to a million fold higher than the surrounding seawater concentration (i.e. ~ 35 mM vs. ~ 35 nM). Despite this high concentration, the precise role of V in these marine organisms remain unknown. In bacteria, vanadium (in the form of $\text{VO}(\text{SO}_4)_3$) interferes with siderophores-mediated iron transport suggesting that it may be transported by siderophores, similarly to the siderophore-like ligand (amavidine) found in the *A. muscaria* mushroom. In humans, vanadium is beneficial (i.e. potential anti-diabetic drug) but not yet proven to be an essential element although it elicits a number of physiological responses such as the inhibition of phosphate-metabolizing enzymes (i.e. phosphatases, ribonuclease, and ATPases). Whereas vanadium concentrations in tissues can be up in the μM range, human blood plasma concentrations range between 1 and 750 nM with the majority of it associated with plasma proteins (human serum albumin, HSA and human serum transferrin, hTf) and transported in the +IV oxidation state in the form of VO^{2+} . Several studies (66–71) have investigated the association of VO^{2+} with HSA and hTf and the overall consensus was that VO^{2+} ions are transported mainly by transferrin in the form of $(\text{VO})_2\text{hTf}$ with only a small amount present in the form of a binary $(\text{VO})_2\text{HSA}$ complex and other minor components of mixed complexes of VO^{2+} -hTf-lactate or VO^{2+} -hTf-citrate. A calorimetric study of the interaction between three types of vanadium compounds (vanadyl sulfate, $\text{VO}(\text{SO}_4)_3$, sodium metavanadate, NaVO_3 , and the insulin-enhancing drug candidate bis(maltolato)oxovanadium(IV), BMOV) with human serum transferrin was recently performed (72) and found that all of these vanadium species had similar affinities for human serum transferrin (Fig. 8, table 8). Because of the rapid dissociation of BMOV before binding and the rapid oxidation of vanadyl ions to vanadate ions, it was suggested that the observed association constant is that of the vanadate–transferrin complex. Thus, the formation constant may not be relevant to the physiological bio-distribution of BMOV but at least agrees with earlier studies of vanadate binding to transferrin (66).

Summary and Perspectives

Transition metal ions are ubiquitous in nature and almost exclusively constituents of proteins and/or enzymes. They are essential for life and can have diverse roles including structural, signaling, regulation, catalysis, oxido-reduction and electron transfer. Intricate and very sophisticated mechanisms have evolved in which cells tightly regulate metal accumulation, transport, distribution, recycling, and export. While great strides have been made in understanding the field of metal ions homeostasis, we still do not fully understand the details of these mechanisms and how metal ions are transported into cells, partitioned and incorporated in biomolecules. What is clear though is that the biology of metal ions is intricately linked to their chemistries (i.e. metal ion bioavailability and coordination chemistry could dictate the selectivity and affinity of metal ions to proteins and the specificity of protein-protein interactions).

The ITC studies presented in this review provided new insights in terms of the parsing of the Gibbs free energy change into enthalpic and entropic contributions and the molecular nature of the binding interactions that is not easily or directly available through other techniques. Due to its ability to provide all thermodynamic parameters in one single experiment, ITC is touted as the gold-standard in studying molecular interactions and is the preferred and most beneficial method particularly in the simple case of one-to-one interactions of moderate strength. The high precision, sensitivity and automation of commercially available ITC instrumentations have contributed to an exponential increase in the number of microcalorimetry publications and the diversity of ITC applications over the past decade. However, while microcalorimetry has obvious advantages (i.e. full in-solution thermodynamic characterization without the need for tagging, labeling or immobilization) over many other techniques, there are a number of disadvantages and drawbacks that could not be ignored. These limitations include difficulties in analyzing complex ITC data beyond the standard one independent binding site model, selection of suitable fitting models or lack thereof, time and sample consumption and the inability of ITC to reliably determine high or low binding affinities (i.e. $10^8 \text{ M}^{-1} < K < 10^3 \text{ M}^{-1}$). Nonetheless, the technique has become a routine method in the characterization of molecular interactions in diverse fields of studies ranging from biochemistry to environmental sciences, medicine and biotechnology. The development of novel, cutting-edge approaches and methodologies has expanded the use of calorimetry to more complex systems including living cells, cellular organelles, cell growth and metabolic activity as well as biofilms formation on medical devices. Furthermore, the improvement of ITC data analysis software for the quantitative analysis and interpretation of ITC isotherms has contributed to an enhancement in the precision of fitting thermodynamic parameters and the identification of best binding models (1–3). Due to the fact that calorimetric signals are direct measures of reaction rates and reaction enthalpies, calorimetry experiments and methods have also been introduced to determine kinetics of enzyme-catalyzed and ligand binding reactions (4–9).

In summary, the characterization of the binding thermodynamics of physiologically important transition metals is one important step in understanding the role that transition metals play in living systems. Due to the development of modern instrumentation and improvements in data analysis software, ITC is now routinely used in the characterization of

the energetics and molecular nature of non-covalent molecular interactions. The partitioning of the Gibbs free energy change into enthalpic and entropic contributions provides new insights into structure-function relationships and a more in-depth understanding of the nature of these interactions and is particularly useful in drug discovery studies. The ability to obtain kinetic parameters from ITC measurements adds another layer of information towards understanding biochemical pathways and catalytic mechanisms. Further studies at the interface of cellular biology, bioinorganic/biophysical chemistry and structural biology are needed to more fully understand how cells selectively respond to and regulate their environment in order to maintain a healthy level of essential metals while avoiding their inherent toxicity.

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Highlights

- Metal ions are essential for all living organisms and participate in a wide variety of cellular processes.
- Metal ion bioavailability and coordination chemistry could dictate the selectivity and affinity of metal ions to proteins
- The thermodynamics of metals ions binding to biological molecules can be quantified by ITC
- ITC is a unique technique able to quantify the enthalpic and entropic contributions of the binding interaction
- Carefully designed ITC experiments and proper controls are necessary for the accurate determination of all thermodynamic parameters

Figure 1.

Representative ITC binding isotherms and best fit for Zn^{2+} titrations into (A) Sp1-3, (B) MyT1-2, and (C) GR-2 zinc finger domains in 20 mM Hepes buffer, 100 mM NaCl, pH 7.4 and 25 °C (Reprinted with permission from Ref. 21).

Figure 2.

Representative ITC binding isotherms and best fits for Zn^{2+} titrations into apoCA in 100 mM ACES buffer at pH 7.4 (left panels) and in 100 mM PIPES buffer at pH 7.4 (right panels). Blue: integrated raw data. Red: integrated Zn^{2+} -buffer background titration. Black: corrected Zn^{2+} binding isotherm. The data was analyzed using a one-site binding model. (Reprinted with permission from Ref. 25).

Figure 3.

Representative ITC binding isotherms and best fits for Zn^{2+} titrations into bovine SOD1 (left panel) and Cu^{2+} into human SOD1 (right panel). The Zn^{2+} binding experiment was carried out in 100 mM MES buffer, pH 5.5 and 25 °C at a protein dimer concentration of 5 μM whereas the Cu^{2+} titration was performed in pivalic acid buffer at pH 5.0 and 25 °C. Note that pivalate buffer binds only weakly to Cu^{2+} and formation of insoluble hydroxo complexes is negligible. (Reprinted with permission from Refs. 31 and 33).

Figure 4.

Representative ITC binding isotherms and best fits for Fe^{2+} (left panel) and Zn^{2+} (right panel) titrations into human serum transferrin (hTf). (A) in the absence of 20 mM bicarbonate and (B) in the presence of 20 mM bicarbonate. The experiments were carried out under anaerobic conditions in the presence of 10 mM dithionite in 50 mM Mops, 50 mM NaCl, pH 7.01 and 25.00 °C. (Reprinted with permission from Ref. 46).

Figure 5.

Representative ITC binding isotherms and best fits for Fe^{2+} binding to HuHF (left panel) and EcFtnA (right panel). Both protein and Fe^{2+} solutions are in 50–100 mM Mops buffer, 50–100 mM NaCl, and 1 mM $\text{Na}_2\text{S}_2\text{O}_4$ at pH 7 and 25.00 °C. (A) panels are ITC raw data and (B) panels are plot of the integrated heats versus the Fe^{2+} /protein molar ratio. (Reprinted with permission from Refs. 54 and 55).

Figure 6.

Representative ITC binding isotherms and best fits for Mn²⁺ and Zn²⁺ binding to human wt Cp (left panels titrations were performed in 20 mM Tris, 100 mM NaCl, 5 mM β-mercaptoethanol, pH 7.5 and 30 °C) and CP mutants S1 and S2 (right panels titrations were performed in 20 mM Hepes, 75 mM NaCl, pH 7.5 and 25 °C). All protein and metal solutions were extensively degassed prior to titration. (Reprinted with permission from Refs. 58 and 60).

Figure 7. Average enthalpy and entropy contributions to the total Gibbs free energy change upon binding of Ni^{2+} to *K. aerogenes* and *B. pasteurii* urease. (Reprinted with permission from Ref. 64).

Figure 8. Representative ITC binding isotherm and best fit for NaVO_3 binding to apo-hTf in 50 mM Hepes, 160 mM NaCl, 5mM Na_2CO_3 , pH 7.4 and 25 °C. (Reprinted with permission from Ref. 72).

Table 1
Essential transition metals occurrence in humans with important functions and characteristic symptoms (7, 8, 9).

Element	Fraction of total body mass	Mass (for a 70 kg adult)	Functions	Deficiency symptoms	High exposure symptoms
Vanadium (V)	2 ppb	0.14 mg	Insulin-like effects, haloperoxidases in seaweeds, electron redox mediator	Impaired metabolism and/or growth	Severe eye, nose and throat irritation
Manganese (Mn)	0.2 ppm	14 mg	Glucose metabolism, Vitamin B1 function, Photosystem II complexes	Infertility, Impaired skeletal growth	Fatigue, anorexia, impotence
Iron (Fe)	60 ppm	4.2 g	DNA synthesis, oxygen transport	Anemia, immune system disorders, fatigue, impaired mental development	Iron overload, organs damage
Cobalt (Co)	20 ppb	1.4 mg	Vitamin B12 function,	Pernicious anemia	Dermatitis, Thyroid and heart damage, Suspected carcinogenic
Nickel (Ni)	0.2 ppm	14 mg	Growth, hydrogenases	Impaired growth/depression	Dermatitis, lung and nasal cancer
Copper (Cu)	1 ppm	70 mg	cytochrome oxidases, superoxide dismutases	Secondary anemia, liver disorder, energy deficiency, inadequate oxygen transport	Liver cirrhosis (in infant), death
Zinc (Zn)	33 ppm	2.3 mg	Metabolic conversion, growth, development and fertility, enzymes and transcription factors	Low sperm count, impaired immune system, lack of appetite	Fume fever, vomiting, stomach cramps, diarrhea, skin irritant

Table 2

ITC thermodynamic parameters for Zn²⁺ binding to Sp1-3, MyT1-2, and GR-2.

20 mM Hepes buffer, 100 mM NaCl, pH 7.4 and 25 °C	n	K^a (M ⁻¹) K_{ITC}^b (M ⁻¹)	H^a (kcal/mol) H_{ITC}^b (kcal/mol)	- T S ^a (kcal/mol)	G ^a (kcal/mol)
Sp1-3 (Cys ₂ His ₂) + Zn ²⁺	0.73 ± 0.01	(3 ± 1) × 10 ⁷ (1.2 ± 0.3) × 10 ⁶	- 5.0 ± 0.5 - 16.5 ± 0.2	- 5.2 + 8.2	- 10.2 - 8.3
MyT1-2 (Cys ₂ HisCys) + Zn ²⁺	0.58 ± 0.01	(1.6 ± 0.3) × 10 ⁷ (7 ± 1) × 10 ⁵	+ 0.1 ± 0.5 - 14.5 ± 0.3	- 9.9 + 6.6	- 9.8 - 7.9
GR-2 (Cys ₄) + Zn ²⁺	0.82 ± 0.01	(9 ± 6) × 10 ⁷ (4 ± 3) × 10 ⁶	+ 10 ± 2 - 10 ± 2	- 20.8 + 1	- 10.8 - 9.0

^a Average buffer-independent binding values;

^b ITC experimentally measured values (buffer-dependent values).

The low values of the binding stoichiometry (n < 1) are presumably due to potential cysteines oxidation which suppresses metal binding.

Table 3

ITC thermodynamic parameters for Zn²⁺ binding to apoCA in different buffers.

Zn ²⁺ binding to apoCA, 100 mM buffer, pH 7.4 and 25 °C	n	K ^a (M ⁻¹) K _{ITC} ^b (M ⁻¹)	H ^a (kcal/mol) H _{ITC} ^b (kcal/mol)	-T S ^a (kcal/mol)	G ^a (kcal/mol)
PIPES	0.97	(2.9 ± 0.3) × 10 ⁹ (3.3 ± 0.3) × 10 ⁶	- 16.4 ± 0.1 - 7.2 ± 0.1	3.5 ± 0.1 - 1.7 ± 0.1	- 12.9 ± 0.1 - 8.9 ± 0.1
MOPS	1.03	(1.7 ± 0.2) × 10 ⁹ (2.2 ± 0.3) × 10 ⁶	- 15.8 ± 0.1 - 8.1 ± 0.1	3.2 ± 0.1 - 0.6 ± 0.1	- 12.6 ± 0.1 - 8.6 ± 0.1
ACES	1.02	(1.9 ± 0.1) × 10 ⁹ (1.1 ± 0.1) × 10 ⁶	- 16.4 ± 0.1 - 7.4 ± 0.1	3.7 ± 0.1 - 0.8 ± 0.1	- 12.6 ± 0.1 - 8.2 ± 0.1
Tris	1.05	--- 7.3 ± 0.6 × 10 ⁶	--- - 12.3 ± 0.3	--- 3.0 ± 0.3	--- - 9.4 ± 0.1

^a Average buffer-independent binding values;

^b ITC experimentally measured values (buffer-dependent values).

Table 4

ITC thermodynamic parameters for Zn^{2+} , Cu^{2+} and Co^{2+} binding to SOD1 under the indicated buffer conditions and pH.

Zn^{2+} , Cu^{2+} and Co^{2+} binding to CuZnSOD1, 25 °C	n^a	K_{ITC}^b (M^{-1})	H_{ITC}^b (kcal/mol)	$-T \Delta S$ (kcal/mol)	G (kcal/mol)
Human apo-SOD1 + Zn^{2+} (100 mM Mes buffer, pH 5.5)	1.2 ± 0.2	$> 10^8$	-21.4 ± 0.6	> -10.43	< -10.9
Bovine apo-SOD1 + Zn^{2+} (100 mM Mes buffer, pH 5.5)	1.3 ± 0.1	$> 10^8$	-24.2 ± 0.1	> -13.42	< -10.9
Bovine apo-SOD1 + Co^{2+} (100 mM Mes buffer, pH 5.5)	1.1	$(1.8 \pm 0.1) \times 10^7$	-19.4	-9.51	-9.9
Human apo-SOD1 + Cu^{2+} (10 mM Pivalate buffer, pH 5.0)	4.0 ± 0.1	$(2.1 \pm 0.8) \times 10^6$	-7.5 ± 0.2	-1.1	-8.6
Human Zn_2 -SOD1 + Cu^{2+} (10 mM Pivalate buffer, pH 5.0)	1.9 ± 0.2	$(2.5 \pm 0.3) \times 10^5$	-5.0 ± 0.2	-2.4	-7.4
Human Cu_2 -SOD1 + Cu^{2+} (10 mM Pivalate buffer, pH 5.0)	- - -	$(5.1 \pm 0.3) \times 10^4$	-4.7 ± 0.2	-1.7	-6.4

^aThe values of the binding stoichiometry n for Zn^{2+} and Co^{2+} are on a per protein dimer basis and those for Cu^{2+} are on a protein monomer basis.

^bITC experimentally measured values (buffer-dependent values).

Zn_2 -SOD1 and Cu_2 -SOD1 are SOD1 samples pre-equilibrated with stoichiometric amounts of Zn^{2+} or Cu^{2+} (i.e. 2 metal ions per dimer).

Table 5

Apparent ITC thermodynamic parameters for Cu²⁺ binding to Atox1 and five variants of the Wilson protein, WD12, WD34, WD56, WD16, and WD14 in 100 mM MES, 100 mM NaCl pH 6.5 and 22 °C. The H values include contributions from Cu(I) binding and other associated events such as deprotonation of the two cysteines in the CXXC motif and changes in the buffer ionization state. Except for WD14 where an independent two-site model was used to fit the data, all other ITC data were best fitted with a single-site model. WD12, WD34, WD 56 and WD 16 represent WND metal-binding domains 1–2, 3–4, 5–6, and 1–6, respectively. The data are taken from ref. 37.

100 mM MES, 100 mM NaCl pH 6.5 and 22 °C	n	K _{ITC} (M ⁻¹)	H _{ITC} (kcal/mol)	- T S (kcal/mol)	G (kcal/mol)
Cu ²⁺ binding to Atox1	1.41 ± 0.22	(2.45 ± 1.23) × 10 ⁵	- 12.96 ± 1.20	+ 5.6	- 7.36
Cu ²⁺ binding to WD12	0.95 ± 0.38	(2.07 ± 1.57) × 10 ⁵	- 11.78 ± 4.17	+ 4.52	- 7.26
Cu ²⁺ binding to WD34	2.23 ± 0.19	(4.67 ± 2.72) × 10 ⁶	- 9.76 ± 3.66	+ 0.65	- 9.11
Cu ²⁺ binding to WD56	1.92 ± 0.02	(1.13 ± 0.21) × 10 ⁶	- 11.63 ± 0.04	+ 3.37	- 8.26
Cu ²⁺ binding to WD16	4.92 ± 0.66	(1.96 ± 0.56) × 10 ⁵	- 11.50 ± 0.38	+ 4.27	- 7.23
Cu ²⁺ binding to WD14	n ₁ =3.38 ± 0.04 n ₁ =1.22 ± 0.07	K ₁ =(1.89 ± 2.00) × 10 ⁶ K ₁ =(4.98 ± 5.46) × 10 ⁷	H ₁ = - 2.90 ± 2.25 H ₂ = - 9.33 ± 1.10	- 5.67 - 1.18	- 8.57 - 10.51

Table 6

Experimentally determined binding parameters for ITC measurements of Zn²⁺ and Fe²⁺ binding to human apotransferrin (hTf) at 25.00 °C. The reported quantities (average binding stoichiometry n, apparent binding affinity K, enthalpy change ΔH , entropy change ΔS and free energy change ΔG) are apparent average values and the standard errors from replicate determinations (5 N 6) are indicated.

Apo-hTf + Metal ions ($\pm$ Bicarbonate = NaHCO ₃)	n	K _{ITC} (M ⁻¹)	H _{ITC} (kcal/mol)	- T ΔS (kcal/mol)	G (kcal/mol)
+ Fe ²⁺ (- NaHCO ₃); pH 7.01	0.68 $\pm$ 0.25	(1.38 $\pm$ 0.68) $\times 10^7$	- 10.01 $\pm$ 1.65	+103.10 $\pm$ 1.72	- 40.75 $\pm$ 0.49
+ Fe ²⁺ (+ NaHCO ₃); pH 7.01	0.69 $\pm$ 0.15	(3.97 $\pm$ 2.15) $\times 10^7$	- 11.64 $\pm$ 2.18	+106.42 $\pm$ 2.24	- 43.37 $\pm$ 0.54
+ Zn ²⁺ (- NaHCO ₃); pH 7.45	1.07 $\pm$ 0.18	(3.74 $\pm$ 2.68) $\times 10^7$	- 18.32 $\pm$ 3.05	+ 83.51 $\pm$ 3.13	- 43.22 $\pm$ 0.71
+ Zn ²⁺ (+ NaHCO ₃); pH 7.01	0.98 $\pm$ 0.12	(1.50 $\pm$ 0.82) $\times 10^8$	- 17.75 $\pm$ 2.43	+ 96.99 $\pm$ 2.49	- 46.67 $\pm$ 0.55
+ Zn ²⁺ (- NaHCO ₃); pH 7.01	0.87 $\pm$ 0.09	(7.45 $\pm$ 4.76) $\times 10^7$	- 18.72 $\pm$ 2.41	+ 87.91 $\pm$ 2.49	- 44.93 $\pm$ 0.63

Table 6

Experimentally determined parameters for Fe^{2+} (Zn^{2+} and Tb^{3+}) binding to HuHF and EcFtnA. The ITC data is collected under anaerobic conditions using 50–100 mM Mops buffer, 50–100 mM NaCl, and 1 mM $\text{Na}_2\text{S}_2\text{O}_4$ at pH 7 and 25.00 °C. The reported thermodynamic quantities are apparent values and include the contributions to the overall equilibrium from ferritin and buffer species in different states of protonation. Standard errors from replicate determinations are indicated. Only values for the strong sites are reported.

Apo-protein	n	$K_{\text{ITC}} (\text{M}^{-1})$	$H_{\text{ITC}} (\text{kJ/mol})$	S (J/K.mol)	G (kJ/mol)
HuHF + Fe^{2+}	25.03 ± 1.05	$(1.48 \pm 0.41) \times 10^5$	-8.19 ± 0.12	126.44 ± 0.73	-29.51 ± 0.72
HuHF + Zn^{2+}	$n_1 = 6.9 \pm 0.2$ $n_2 = 90.7 \pm 29.5$	$K_1 = (5.9 \pm 1.7) \times 10^6$ $K_2 = (3.1 \pm 0.6) \times 10^4$	$H_1 = -13.5 \pm 0.5$ $H_2 = 22.1 \pm 5.5$	$S_1 = 84.5 \pm 0.9$ $S_2 = 160.1 \pm 5.5$	$G_1 = -38.7 \pm 0.7$ $G_2 = -25.6 \pm 0.5$
HuHF + Tb^{3+}	$n_1 = 8.7 \pm 0.5$ $n_2 = 87.3 \pm 2.4$	$K_1 = (6.2 \pm 2.5) \times 10^6$ $K_2 = (6.7 \pm 1.1) \times 10^4$	$H_1 = 4.5 \pm 0.9$ $H_2 = 25.4 \pm 1.2$	$S_1 = 145.1 \pm 1.4$ $S_2 = 177.6 \pm 1.3$	$G_1 = -38.7 \pm 1.1$ $G_2 = -27.5 \pm 0.4$
EcFtnA + Fe^{2+}	$n_1 = 21.1 \pm 0.5$ $n_2 = 19.8 \pm 0.7$	$K_1 = (3.5 \pm 0.8) \times 10^6$ $K_2 = (1.8 \pm 0.4) \times 10^5$	$H_1 = 3.4 \pm 0.2$ $H_2 = 9.9 \pm 0.3$	$S_1 = 136.5 \pm 2.1$ $S_2 = 133.8 \pm 2.2$	$G_1 = -37.3 \pm 0.6$ $G_2 = -30.0 \pm 0.6$
EcFtnA + Zn^{2+}	$n_1 = 4.2 \pm 1.2$ $n_2 = 22.1 \pm 0.5$ $n_3 = 27.7 \pm 0.8$	$K_1 = (1.0 \pm 0.1) \times 10^7$ $K_2 = (3.7 \pm 0.2) \times 10^6$ $K_3 = (9.6 \pm 0.8) \times 10^3$	$H_1 = 1.2 \pm 0.7$ $H_2 = -24.4 \pm 0.2$ $H_3 = 47.9 \pm 1.3$	$S_1 = 138.2 \pm 2.5$ $S_2 = 44.0 \pm 0.7$ $S_3 = 236.9 \pm 1.6$	$G_1 = -40.0 \pm 0.2$ $G_2 = -37.5 \pm 0.1$ $G_3 = -22.7 \pm 0.2$

Table 7

Experimentally determined parameters for Mn^{2+} and Zn^{2+} binding to human CP and its variants. ITC titrations with were performed in 20 mM Tris, 100 mM NaCl, 5 mM β -mercaptoethanol, pH 7.5 and 30 °C (for wt CP and Zn/Mn) and in 20 mM Hepes, 75 mM NaCl, pH 7.5 and 25 °C (for CP variants S1 and S2).

Apo-protein	n	$K_{\text{ITC}} (\text{M}^{-1})$	H (kJ/mol)	S (J/K.mol)	G (kJ/mol)
CP + Mn^{2+}	$n_1 = 1$ $n_2 = 1$	$K_1 = 7.7 \times 10^8$ $K_2 = 2.7 \times 10^5$	---	---	$G_1 = -51.57$ $G_2 = -31.52$
CP + Zn^{2+}	$n_1 = 1$ $n_2 = 1$	$K_1 = 7.1 \times 10^8$ $K_2 = 1.8 \times 10^8$	---	---	$G_1 = -51.37$ $G_2 = -47.91$
S1 + Mn^{2+}	---	---	---	---	---
S1 + Zn^{2+}	1	1.2×10^8	---	---	-46.11
S2 + Mn^{2+}	1	1.7×10^8	---	---	-46.97
S2 + Zn^{2+}	1	2.9×10^8	---	---	-48.30
$\text{Zn/Mn} + \text{Mn}^{2+}$ (or Zn^{2+})	No Binding	No Binding	No Binding	No Binding	No Binding

Table 8

Experimentally determined parameters by ITC and DSC for different forms of vanadium complexes binding to human serum albumin in 50 mM Hepes, 160 mM NaCl, 5mM Na₂CO₃, pH 7.4.

Apo-hTf	n	K _{Apparent} (M ⁻¹)	H (kcal/mol)	-T S (J/cal.mol)	G (kcal/mol)
hTf + VOSO ₄	~ 2	K _{DSC} [*] = 1.2 × 10 ⁵	- 5.9 ± 0.6	- 1	G _{DSC} = - 6.9
hTf + BMOV	~ 2	K _{DSC} [*] = 5.5 × 10 ⁵	- 6.0 ± 0.5	- 3.2	G _{DSC} = - 9.2
hTf + NaVO ₃	1.94 ± 0.03	K _{ITC} = 4.2 × 10 ⁵ K _{DSC} [*] = 3.1 × 10 ⁵	- 9.1 ± 0.1	+ 1.4 + 1.6	G _{ITC} = - 7.7 G _{DSC} = - 7.5

* K_{DSC} is the average affinity constant determined by DSC for the C- and the N-lobe of transferrin.
The binding affinities of these lobes are not significantly different and thus the average binding enthalpies and affinity constants for these lobes are reported.