CHAPTER TEN

# Measuring and Imaging Metal Ions With Fluorescence-Based Biosensors: Speciation, Selectivity, Kinetics, and Other Issues

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## Contents



## Abstract

Fluorescence-based biosensors have shown themselves to be a powerful tool for the study of a variety of chemical species in biological systems, notably including metal ions. This chapter provides an overview of several important issues in using such sensors to study metallobiochemistry. These issues include selectivity for the analyte over potential interferents, including those that do not themselves induce a signal, the different forms in which metal ions are found (speciation), the utility of metal ion buffers, and the importance of kinetics in studying metal ion binding reactions. Finally, the chapter briefly discusses some of the issues in understanding whole-organism distribution of metal ions and its control.

## 1. INTRODUCTION

Understanding metallobiochemistry, particularly in cells and living systems, has been greatly aided by the development of indicators (also called

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sensors or probes) that transduce the presence or level of analytes, such as metal ions, as a change in fluorescence (reviewed in Carter, Young, et al., 2014; Que, Domaille, et al., 2008; Thompson, 2006; White & Argauer, 1970). These indicators have been especially useful in biology because of their ready adaptation to microscopy and imaging, correlating the chemical information they provide to the structure of the cell or organism (Negrin & Contag, 2006; Ntziachristos, 2006). A valuable subset of these indicators include those employing biological molecules, to provide high affinity, high selectivity, and other desirable features for these applications; they are the subject of this volume.

However, use of fluorescent biosensors (and other indicators) requires care to obtain accurate, valid data from which biological insight can be derived. Particularly for analytes such as metal ions, consideration of issues such as selectivity, speciation, kinetics, and others are important. This chapter is devoted to these considerations; we and others have previously discussed some of these issues (Bozym, Hurst, et al., 2008; Donat & Bruland, 1990; Stumm & Morgan, 1996). We note that these issues apply in varying degree to measurements with other indicators (such as small molecules), and for other analytes, such as reactive oxygen species. We also note that a considerable proportion of the scientific literature focused on the biology of metal ions neglects these issues to a degree, reducing confidence in observations and conclusions. A key goal of this chapter is not only to provide guidance on the use of fluorescence biosensors to determine metal ions, but also to support valid experiments in this field.

## 2. SPECIATION OF METAL IONS OF BIOLOGICAL INTEREST

Since a large fraction of biological chemistry takes place in solutions (particularly aqueous solutions) and other condensed phases, metal ions of biological interest rarely are seen in an unliganded form; i.e., without a solvent molecule, counterion, or other ligand molecule closely associated with them. While these ligand associations may be weak or transient, the high concentrations of water, chloride, carbonate/bicarbonate, and other species normally present in biological media assure that virtually all metal ions are bound to one or more such ligands. The identity, number, and distribution of these and other ligands bound to the metal ion are referred to as the

metal's "speciation" in solution (Stumm & Morgan, 1996) which depends on the chemical properties of both the metal ion and the ligands. Thus, $Zn^{2+}$ prefers to coordinate nitrogen or sulfur ligands compared to oxygen, frequently in a tetrahedral arrangement around the metal ion; $Cu^{2+}$ prefers sulfur ligands in a trigonal bipyramidyl or octahedral arrangement (Cotton & Wilkinson, 1988). Speciation also refers to the ionization state of the metal for elements that ordinarily exhibit multiple oxidation states like copper or iron. Inside cells, metals such as zinc, copper, and iron are found almost exclusively bound tightly to multidentate ligands such as proteins, where they often serve as enzyme cofactors essential for catalysis or for stabilizing protein structure.

We can distinguish ligands based on the strength (affinity) of their association, expressed as the stability of the complex(es) they make with particular metals. The association reaction in the simplest case of one ligand L binding to the metal M to form a complex LM is expressed as:

$$\mathrm{L} + \mathrm{M} \leftrightarrow \mathrm{LM} \tag{1}$$

The equilibrium dissociation constant $K_D$ (inverse of the association constant, $K_A$) is expressed in the usual way:

$$K_D = [\mathrm{L}][\mathrm{M}]/[\mathrm{LM}] \tag{2}$$

Since such equilibrium constants depend strongly on pH, ionic strength, and ionization state, they are referred to as "conditional stability constants." If the stability constants, formation constants, concentrations, and p$K_a$'s of the various ligands with the metal ion(s) are known (Smith & Martell, 1973), the proportion of the metal ion(s) in each form can be calculated using computer programs such as MINEQL (Environmental Research Software, Hallowell, Maine). An example is shown in Fig. 1 of the pH dependence of zinc ion speciation in a fresh water (e.g., not physiological) medium (Stumm & Morgan, 1996).

In this fresh water example (total concentrations $[Zn^{2+}] = 10$ n*M*, $[Mg^{2+}] = 3$ m*M*, $[Ca^{2+}] = 1$ m*M*, $[CO_3{}^{2-}] + [HCO_3{}^{-}] = 2$ m*M*, $[SO_4{}^{2-}] =$ 0:3 m*M*, $[Na^{+}] = 0.25$ m*M*, $[Cl^{-}] = 0.25$ m*M*), it is noteworthy that at pH7.5 more than a third of the zinc ion is bound to carbonate; the physiological concentration of bicarbonate/carbonate (26 m*M* in serum) is nearly 10-fold higher than in this example. Also noteworthy is the increase in the forms of zinc ion containing hydroxide ligands ($ZnOH^{+}$ and $Zn(OH)_2$)

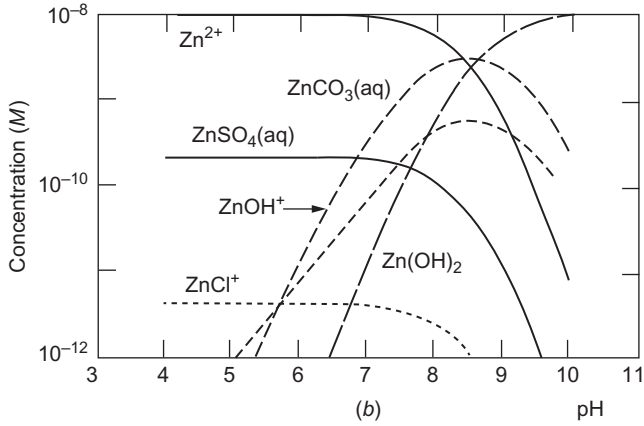


**Fig. 1** Zinc ion speciation in fresh water as a function of pH. *Reproduced with permission from Stumm, W., & Morgan J. J. (1996).* Aquatic chemistry: Chemical equilibria and rates in natural waters. *New York: Wiley-Interscience.*

with increasing pH, this contributes to the formation of insoluble precipitates in millimolar zinc solutions at or above neutral pH (Stumm & Morgan, 1996).

As we and others have discussed previously (Bozym et al., 2008; Colvin, Holmes, et al., 2010; Krezel & Maret, 2006), in biological systems, only a small proportion of many of the active, multivalent metal ions such as Zn, Fe, Cu, Mg, Ca, Mn, Co, and Ni, are in complexes with weak, rapidly exchangeable ligands like chloride or water, rather than in high-affinity complexes with multidentate ligands. In the case of zinc at pH 7.5, rapidly exchangeable complexes include at least $ZnHCO_3^+$, $ZnCl^+$, $ZnOH^+$, ZnOHCl, and $ZnCl_2$. We term this fraction of the total zinc ion operationally as "free zinc"; the concept is probably similar to what others have described as "labile," "mobile," or "bioavailable." These zinc species are capable of rapidly exchanging ligands to bind to proteins, other biological ligands and fluorescent indicators. In some cases, protein-binding sites recognize and bind metal complexes (Franklin, Ma, et al., 2003); for example, transferrin binds an iron-carbonate complex (Harris, 2012). Although the total zinc concentration in cells is 0.1–0.5 m*M* (Eide, 2006), the high concentrations of fairly tight metal ion ligands (e.g., ~3 m*M* reduced glutathione, 2–20 m*M* glutamic acid, etc.) in addition to proteins that tightly bind zinc (Auld, 2001; Christianson, 1991) decrease the "free" concentration of zinc to the picomolar range (Bozym, Thompson, et al., 2006;

Dittmer, Miranda, et al., 2009; Krezel & Maret, 2006; Van Dongen, Evers, et al., 2007). The pool of zinc ligands in the cell may function as a "zinc buffer" to maintain the free zinc concentration. However, the cellular free zinc concentration can alter dramatically (p*M* to n*M*) with modest changes in the total zinc concentration, as the buffering capacity is overwhelmed by increased zinc (Wang, Hosteen, et al., 2012).

## 3. METAL ION BUFFERS AND THEIR FORMULATION

Determining the effects in vitro of metal ions present at low concentrations (and particularly low *free* concentrations) in biological media on various processes is challenging, for two major reasons. First, there are many sources of potential contamination with the metal ion of interest, or similar ones. For instance, a physiological buffer containing 125 m*M* NaCl made with "ACS reagent grade" sodium chloride likely contains significant quantities of potential interferents: the American Chemical Society specifies <5 ppm heavy metals (determined as lead) and <2 ppm iron. Thus the buffer containing NaCl might have *several hundred nanomolar* total zinc in addition to whatever else is present, making it difficult to prepare a buffer that contains 5 n*M* total zinc with this much NaCl. We note that other chemical constituents of the media are also likely to be contaminated with zinc (or other interfering metals like copper); borosilicate glassware, some plastic ware, Kimwipe tissues, paper, dust, and human skin are also well-known sources of contaminating metal ions. Second, in most biological media, there are abundant ligands that bind many metal ions with medium to high affinity, effectively lowering the concentration of "free metal" (see Section 2). For these reasons, accurately preparing solutions of metal ions at low concentrations is usually impractical.

A better approach is to prepare metal ion buffers wherein the amount of "free" metal ion changes less following addition or subtraction (by becoming bound to a ligand) of the metal ion than it would in the buffer's absence (Baker, 1988). The effect is closely analogous to the behavior of a pH buffer, where addition of acid or base results in a smaller change in pH than in the buffer's absence. For metal ion buffers, a suitable, fairly strong ligand is added to the solution in excess under conditions where it binds >99% of the metal ion present; importantly, the proportion of metal ion bound by the added ligand is constant under a given set of conditions, such that over a broad range the concentration of free metal ion rises linearly with added metal

ion, but much less steeply. For example, suppose we formulate a zinc ion buffer using 2 m*M* nitrilotriacetic acid (NTA, log $K = 10.4$) as the ligand at pH 7.0, 25°C, and low ionic strength (Bozym et al., 2008). If 0.5 μ*M* total zinc ion is present, the free zinc ion concentration will be 1.06 p*M* with essentially all of the rest bound to NTA. If additional 1.5 μ*M* total zinc is added as a contaminant, the free concentration only increases to 4.26 p*M*: i.e., the deleterious effects of the contaminating zinc are minimized. As shown in Fig. 1, metal speciation is pH dependent, so it is important to include a pH buffer in the metal buffer solution. We typically use the sulfonated Good's buffers (HEPES, MOPS, MES, etc.) as pH buffers as they generally have low affinity for metal (Mash, 2003). Other pH buffers, including Tris, phosphate, Bicine, and citrate, have higher metal ion affinity (Dawson, Elliott, et al., 1989); phosphate is additionally problematic due to the low solubility of its zinc salts ($K_{sp} \approx 10^{-33}$). Furthermore, in some cases, the protein can bind a metal–ligand complex (such as Me-NTA) rather than the metal alone. In this case, the concentration of the complex is significantly higher than the concentration of the "free metal ion." Therefore if the metal affinity is calculated using the free metal ion, rather than the complex, the calculated metal affinity will likewise be incorrect. To check for this, measure fraction bound metal at a constant "free metal" but varying metal buffer concentration. Additionally, binding of the metal to the protein should not significantly alter the free metal concentration.

## 4. SELECTIVITY OF METAL ION BINDING TO INDICATORS AND ITS ASSESSMENT

Due to the many different metal ions normally present in biological media, selectivity is an essential characteristic of indicators designed to quantify them. Thus while an indicator may have high sensitivity for a particular metal ion due to binding it with high affinity, it is equally important that other metal ions present do not compete for that binding site, or they will interfere with the measurement. Thus a sensor useful for determining free zinc ion in cells not only must have picomolar affinity but also must not bind metal ions such as $Mg^{2+}$ or $Ca^{2+}$, which may be present at billion-fold higher concentrations. A major reason for adapting biological molecules as sensors for metal ions and other analytes is their high selectivity (Thompson, 2006), as they evolved to bind specific metal ions in the presence of other analytes in the cell.

Many fluorescent indicators act by the analyte inducing a change in fluorescence upon binding. It is important to demonstrate both that competing analytes do not induce a change in fluorescence and that the interferent does not bind to the indicator, competing with the analyte binding to the indicator, and inducing a spectral change. Furthermore, a compound or ion can also interfere if it perturbs the affinity of the binding site even if it does not occupy the same site.

To correctly assess interference, one needs to determine whether the compound/ion affects the affinity and/or the signal change upon binding of the analyte to the indicator. The simplest way to detect this is to assess the response of the indicator to a relevant concentration of the analyte (i.e., when [analyte] = $K_D$) in the presence and absence of a relevant concentration of the prospective interferent. The fractional occupancy, $\nu_i$ of the indicator binding site by each compound $i$ (analyte and interferent) can be calculated if their concentrations [$M_i$] and dissociation constants $K_i$ are known:

$$\nu = [M_i]/K_i/1 + \Sigma([M_i]/K_i) \tag{3}$$

For instance, suppose, we wish to know what fraction of human carbonic anhydrase II active sites will be occupied by $Zn^{2+}$ and $Cu^{2+}$ present at free concentrations of 5 and 0.5 n*M*, respectively; this presumes that binding to the protein does not deplete the free concentration of either metal ion. Since the $K_D$'s for copper and zinc are roughly $10^{-13}$ and $10^{-12}$ *M*, respectively (Huang, Lesburg, et al., 1996; McCall & Fierke, 2004), we can calculate that the binding site is 50% bound with copper and 50% bound with zinc ion under these conditions. Even though its concentration is lower than zinc, the copper ion interferes significantly because its affinity is 10-fold higher. In this example, it is evident that potential interferents with high affinities can interfere dramatically even if their concentrations are quite low and irrespective of whether they induce any spectroscopic change at all. If the interferent does not induce a fluorescence change, it may be necessary to determine its ability to interfere by competing with the analyte; for metal ions this means formulating buffers with the desired range of free interferent metal ions as well as analyte ions.

For example, we wish to determine if calcium or magnesium ions at levels found in serum interfere with zinc determination using the fluorescent zinc indicator FuraZin-1. Upon binding Zn, FuraZin-1 exhibits a change in the ratio of its emission observed at 510 nm when excited at 325 nm, to that

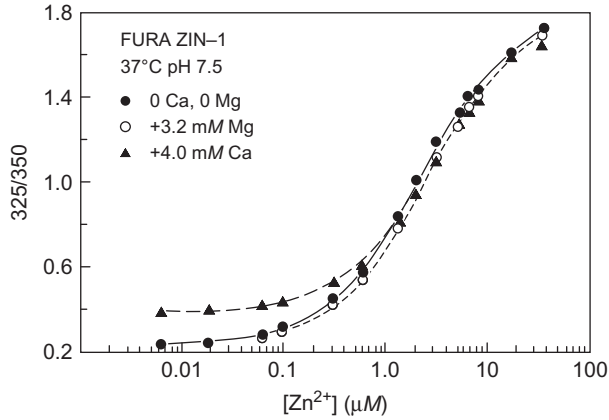


**Fig. 2** Zinc-dependent fluorescence excitation ratios (exc 325 nm/350 nm, em 510 nm) for FuraZin-1 in the absence (●) and presence of $Mg^{2+}$ (○) or $Ca^{2+}$ (▲), and best-fit binding isotherms. *Reproduced from Thompson, R. B., Peterson, D., et al. (2002). Fluorescent zinc indicators for neurobiology.* Journal of Neuroscience Methods, 118*, 63–75, with permission.*

when excited at 350 nm; this is termed excitation ratiometric (Haugland, 2005). We formulated zinc buffers (124 m*M* NaCl, 1.75 m*M* KCl, 10 m*M* MOPS, 10 m*M* Bicine, pH 7.4 at 37°C) in the absence and presence of relevant calcium (4 m*M*) and magnesium (3.2 m*M*) concentrations (Thompson, Peterson, et al., 2002) having the same range of free zinc concentrations (6 n*M* to 40 μ*M*), and measured the excitation ratio. When the measured ratios were fit to a simple binding isotherm (Fig. 2), the apparent $K_D$'s were $2.1 \pm 0.1$, $2.9 \pm 0.09$, and $2.52 \pm 0.05$ μ*M* for no Ca or Mg, with Ca, and with Mg, respectively. As the figure and derived values make clear, both potential interfering cations exert a modest change on the apparent $K_D$ for zinc that would result in a small error, particularly at lower free zinc concentrations. For most purposes, however, these small errors would be viewed as negligible for measuring free zinc ion in this concentration range. For an example of a substantial interference, see Fig. 4.

## 5. ADVANTAGES OF BIOMOLECULES FOR METAL ION SENSING

Among the important advantages of biological molecules, such as proteins, as metal ion sensors (e.g., biosensors) is that their metal ion binding properties can be modified and improved either by design, or evolution in vitro. In particular, the binding affinity, selectivity, and binding kinetics

of metal ion binding to the biosensor based on human carbonic anhydrase II have all been improved by site-directed mutagenesis (reviewed by Fierke & Thompson, 2001; Hurst, Wang, et al., 2010). Similar optimization has been carried out on other protein-based zinc sensors (Van Dongen et al., 2007), as well as for sensors for other analytes. By comparison, small molecule fluorescent zinc indicators have shown less adaptability in affinity, selectivity, or kinetics since most use variants of one of three zinc-binding moieties: di-(2-picolyl) amine, iminodiacetate, or tetraazacyclododecane (reviewed in Burdette & Lippard, 2001; Kikuchi, Komatsu, et al., 2004; Que et al., 2008). Iminodiacetate has higher affinity for zinc and copper(II) than calcium or magnesium ions, but often levels of the latter two are high enough to interfere. The di-(2-picolyl) amine and tetraazacyclododecane binding moieties give adequate selectivity against Ca and Mg which may be present at micromolar to millimolar ranges, and have nanomolar or tighter affinity for zinc (Aoki, Kaido, et al., 2003; Burdette, Walkup, et al., 2001). Other binding moieties such as 8-aminoquinoline (Nolan, Jaworski, et al., 2005) give faster response and adequate selectivity against intracellular (e.g., high nanomolar) calcium levels, but have an affinity that is too weak for measuring most physiologically relevant (subnanomolar) zinc levels.

For example, the kinetics of zinc ion binding to wild-type apocarbonic anhydrase II (CA II) is slow and it would be desirable to speed up zinc equilibration without significantly decreasing the affinity at the same time. Huang, Lesburg, et al. (1996) used a design process, whereas Hunt and Fierke (1997) used random mutagenesis followed by selection (e.g., evolutionary) process to identify mutants with faster zinc equilibration kinetics. Ultimately a mutant (E117A) was developed with an 800-fold faster zinc association rate constant than the wild type (e.g., nearly diffusion-controlled), which loses less than an order of magnitude in overall zinc affinity. An example of the improved response is shown in Fig. 3, which depicts zinc-binding kinetics to wild type and E117A human apocarbonic anhydrase II in the presence of ABD-N. This fluorophore binds to CA II with a concomitant increase in fluorescence anisotropy (polarization) if and only if zinc is bound to the active site (Thompson, Maliwal, et al., 1998). Under these conditions, equilibration of WT CA II with 0.9 n*M* zinc takes more than 90 min, whereas the E117A variant takes less than 20 min; the differences are more dramatic at lower free zinc concentrations. Since small molecule indicators use only a few different zinc-binding moieties to assure selectivity, it is difficult to see how the structure of the zinc-binding moieties can be altered to enhance kinetics without changing selectivity or affinity.

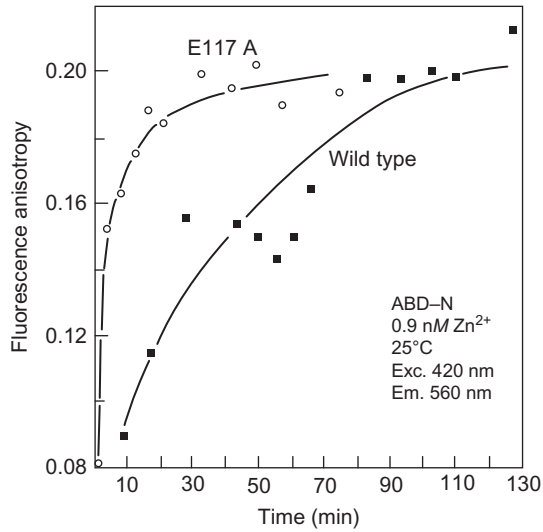


**Fig. 3** Fluorescence anisotropy of ABD-N as a function of time after addition of 0.9 n*M* free $Zn^{2+}$ and wild type (■) and E117A (○) apocarbonic anhydrase II.

The Fierke group has also demonstrated that the selectivity of CA-based metal ion sensors can be altered by mutagenesis. In particular, wild-type apocarbonic anhydrase exhibits only about 10-fold higher affinity for Cu(II) than Zn(II) (0.1 p*M* vs 1 p*M*), which makes it difficult to measure the low levels of free Cu(II) predicted to be present in most cells (Rae, Schmidt, et al., 1999) in the presence of physiological free zinc levels. This is illustrated in the upper panel of Fig. 4, which shows a fluorescence lifetime-based determination of Cu(II) in the presence and absence of 1.5 n*M* free zinc ion. It is apparent that the phase angles (inversely proportional to the fluorescence lifetime, or in this case the fraction of the apoprotein with Cu(II) bound) are substantially different in the presence and absence of this concentration of zinc. In particular, the measured phase angle at $10^{-14}$ *M* free copper in the presence of zinc is close to that measured at $10^{-12}$ *M* copper in its absence, an error of 100-fold. In this case zinc only competes with Cu(II) for binding to CA II, the bound zinc ion has little effect on the fluorescent signal. However, by use of the Cu(II)-selective Q92A variant, which has similar Cu(II) affinity to the wild type but 17-fold weaker zinc affinity, such that the selectivity is improved to 170-fold, the curves are practically superimposable (lower panel).

There are other key advantages of these protein-based biosensors. Some of ours and others incorporate the fluorescent label by fusing a Fluorescent

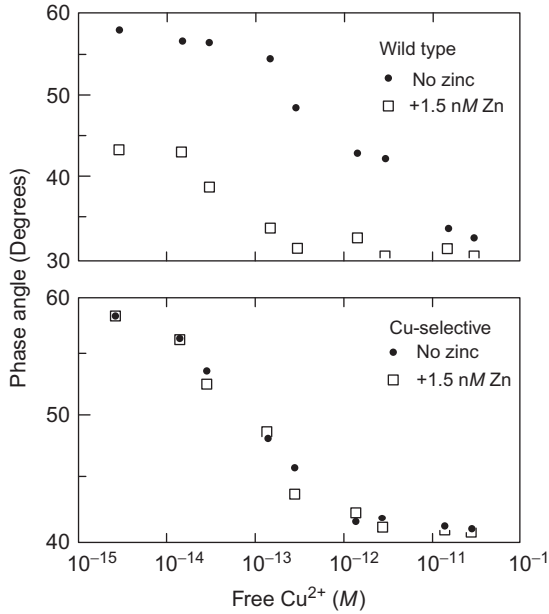


**Fig. 4** Cu(II)-dependent phase angles (inversely proportional to the fraction of protein with Cu(II) bound) in the absence (●) and presence (□) of 1.5 n*M* free Zn(II) ion for wild-type (*upper panel*) and Q92A copper-selective variant (*lower panel*). *Reproduced from McCranor, B. J., Szmacinski, H., et al. (2014). Fluorescence lifetime imaging of physiological free Cu(II) levels in live cells with a Cu(II)-selective carbonic anhydrase-based biosensor.* Metallomics *6(5), 1034–1042, with permission.*

Protein variant gene to the carbonic anhydrase variant gene and expressing the fusion protein. First, by expressing the sensor (or using a hybrid approach like ours; Bozym et al., 2006) inside the cell, one is spared the difficulty of chemically labeling the sensor protein with a fluorophore, with the attendant issues of homogeneity, yield, and getting it into the cell. Indeed, one of the key advantages of the expressible approach is the ability to target the sensor to various cell compartments as others (Qin, Dittmer, et al., 2011) and we have demonstrated (McCranor, Bozym, et al., 2012). By comparison, targeting small molecule sensors to organelles (apart from mitochondria with cationic dyes) remains challenging.

## 6. KINETICS AND MECHANISM OF METAL ION BINDING

An important factor in metallobiochemistry that is frequently overlooked is the kinetics of the metal ion binding reaction, which of course

is determined by the microscopic mechanism of the binding reaction. This factor is frequently overlooked in part because of the dearth of available kinetic data (compared with affinities) on many binding reactions of interest, as well as the fact that many such reactions may be thermodynamically feasible but are so slow they are often not observed in realistic time scales. One example is the dissociation of zinc ion from the active site of human carbonic anhydrase II (CA II) (Henkens & Sturtevant, 1968; Hunt & Fierke, 1997). The apparent equilibrium constant $K_{EQ}$ can be expressed as the ratio of the association rate constant $k_{ON}$ and the dissociation rate constant $k_{OFF}$, assuming a simple one-step binding mechanism:

$$K_{EQ} = k_{ON}/k_{OFF} \tag{4}$$

Thus the observed (net) rate, $k_{OBS}$, of equilibration to form the enzyme–metal complex is the sum of the off rate constant and the on rate constant times the concentration of metal, since this is a second-order reaction. This analysis assumes that the kinetics are measured under pseudo-first-order conditions where the concentration of the metal ion is higher than the protein and can be assumed to be constant during the reaction:

$$k_{OBS} = k_{OFF} + k_{ON}\left[Zn^{2+}\right] \tag{5}$$

such that the time it takes for the reaction to get halfway to equilibrium ($t_{1/2}$) is

$$t_{1/2} = \ln 2/k_{OBS} = 0.693/k_{OBS} \tag{6}$$

For zinc ion binding to human CA II, the apparent on and off rate constants are $1 \times 10^5\ M^{-1}\ s^{-1}$ and $8.4 \times 10^{-8}\ s^{-1}$, respectively (Hunt & Fierke, 1997). This apparent association rate constant is much slower than a diffusion-controlled second order rate constant for a small ion binding to a protein ($1 \times 10^8\ M^{-1}\ s^{-1}$) (Fersht, 1999), suggesting a two-step binding mechanism. The value for $k_{OFF}$ is measured by adding a high concentration of a high-affinity zinc ligand (like EDTA) that traps zinc upon dissociation from the protein; the half-time for dissociation is limited by the $k_{OFF}$ rate constant, so $t_{1/2} = 0.693/8.4 \times 10^{-8}\ s^{-1}$, or $8.25 \times 10^6$ s, or about 95 days. Assuming similar binding kinetics under cellular conditions, the off rate is slow enough that carbonic anhydrase will remain metallated for days, even if the zinc concentration is low. However, the association reaction will also be slow so that newly synthesized apo-CA could also be metallated and activated slowly. Note that while carbonic anhydrase II has a slow apparent $k_{ON}$,

even a newly transcribed, zinc-requiring metalloenzyme or transcription factor (Buchsbaum & Berg, 2000) with a picomolar affinity would not bind metal rapidly in the cell due to the low free zinc concentrations. For example, the zinc-binding kinetics for a zinc finger motif in the transcription factor Cp1 have been measured as $k_{ON} = 2.8 \times 10^7\ M^{-1}\ s^{-1}$ and $k_{OFF} = 1.6 \times 10^{-4}\ s^{-1}$ (Buchsbaum & Berg, 2000). For a free zinc concentration in cells of 10 p*M* (Bozym et al., 2006; Dittmer et al., 2009; Van Dongen et al., 2007), the observed zinc association rate ($k_{OBS}$) is estimated as $2.8 \times 10^7\ M^{-1}\ s^{-1}\ (1 \times 10^{-11}\ M) + (1.6 \times 10^{-4}\ s^{-1})$ or $4.4 \times 10^{-4}\ s^{-1}$, and thus $t_{1/2}$ will be ~30 min. If the free zinc concentration is 10-fold higher, the $t_{1/2}$ becomes ~3 min.

There are a variety of mechanisms to avoid slow metallation in cells. One possible mechanism is that zinc associates with carbonic anhydrase as it is folding. Solvent-induced denaturation of CA II in vitro occurs in two steps: native human CA II unfolds to form a stable intermediate that retains much of the secondary structure of the native protein but is inactive; at higher guanidine HCl concentrations, this intermediate further denatures to form an unfolded state which is not well defined but does retain some secondary structure (Maartensson, Jonsson, et al., 1993; Tweedy, Nair, et al., 1993). The zinc affinity of the intermediate state is ~100-fold lower than the native state (Hunt & Fierke, 1997) and the binding kinetics are also likely more rapid. The bound zinc may then be trapped in the active site upon folding to the native state.

A second potential mechanism is that the cellular free zinc concentration is higher in localized regions of the cell or that another zinc species in the cell, other than free zinc, binds to the protein or domain and transfers the metal. Since the total cellular zinc concentration is ~100 μ*M*, this mechanism can potentially substantially increase the concentration of zinc available to bind to the protein and, hence, the apparent association rate constant. Some examples of this mechanism are known: a dipicolinate (DPA)–zinc complex binds to and forms a ternary complex with carbonic anhydrase (Pocker & Fong, 1983). The apparent association rate constant increases since both free zinc and the higher concentration of the DPA–zinc complex bind to CA II. Additionally, DPA bound to CA II facilitates zinc dissociation as a DPA–Zn complex, with a dissociation half-time in the minute range. While the detailed speciation of zinc in the cell remains to be determined, it is estimated that a significant fraction of the 100 μ*M* concentration of total zinc in the cell is bound to small molecules that are relatively abundant like glutathione, glutamate, or histidine. In some cases, a protein chaperone can

act as the species that transfers a metal ion to a metalloprotein. For example, copper chaperones like Atx1 selectively transfer Cu(I) to particular apoenzymes, like superoxide dismutase and lysyl oxidase (Boal & Rosenzweig, 2009). Protein chaperones have also been identified for other metal ions, including $Fe^{2+}$ (Shi, Bencze, et al., 2008). To date, no protein chaperones have been well validated for incorporation of zinc into metalloproteins in cells.

## 7. DISTRIBUTION OF METAL IONS THROUGHOUT THE CELL AND ORGANISM

Recently our knowledge has grown regarding the Zip and Znt families of transporters that are mainly responsible for moving zinc ions into and out of the cell itself and its compartments (reviewed in Eide, 2006; Kambe, Tsuji, et al., 2015; Kambe, Yamaguchi-Iwai, et al., 2004). However, our overall understanding of how zinc is distributed among various tissues and organs is meager. Early views suggested that zinc deficiency presented basically the same phenotype as a broader starvation, but in fact the symptomology of zinc deficiency differs markedly under different circumstances. For instance, the classic work of Prasad et al. (reviewed in Prasad, 1985) showed that young men who were profoundly zinc deficient exhibited stunted growth, underdeveloped external genitalia and secondary sex characteristics, reduced muscular development, and mental deficits. By comparison, infants lacking Zip4 (an intestinal zinc transporter with a major role in dietary uptake) exhibit acrodermatitis enteropathica which manifests as extensive perioral and perianal skin infections: a "super diaper rash." Furthermore, diarrheal disease attributable to zinc deficiency is responsible for a million infant and toddler deaths every year in underdeveloped countries (Walker & Black, 2004). While newborns from Nepalese mothers who were zinc deficient were normal, the mothers were much more prone to death from postpartum infections, suggesting that under conditions of limiting zinc the mother's physiology favors the fetus.

One interpretation of these disparate observations is that there is some level of central control of zinc distribution to various needy tissues and organs. While our understanding is improving on many fronts, such as identification of zinc-dependent transcription factors that control transporter expression (Outten & O'Halloran, 2001) and, hence, zinc levels in individual cells and organelles, the differing symptoms of deficiency suggest a

prioritization of jobs for which zinc is required that changes with time and developmental stage. Such coordination of diverting zinc from some tissues or organs to others would imply communication between needy tissues (presumably hormonal) and some central controller. Moreover, there would also seem to be a need for a store of zinc, and/or a zinc-rich tissue which could be catabolized in times of need.

For organismal calcium homeostasis the roles of parathyroid hormone, vitamin D, and calcitonin are widely appreciated, as is that of the bone matrix as a store of calcium. For zinc, there has not been identified a clear, central locus for hormonal communications comparable to the parathyroid for integrated calcium control, nor an inert store from which zinc can be mobilized; we note that the 10–20 $\mu M$ total zinc in the serum represents 0.1% of the body's total zinc, and thus a very modest buffer for supplying tissue needs (Fig. 5). In principle, bone or skeletal muscle could serve as stores due to the high proportion of bodily zinc they contain, but it is far from clear under what circumstances this might be mobilized.

One of the potential roles of biosensors will be to help elucidate how zinc (and other metal ion) levels are controlled at the organismal level. Such studies will require whole animal studies of zinc levels and fluxes in vivo. Genetically expressed biosensors are well suited for such studies due to their targetability. In many cases, the salient species is likely to be a metal ion complex rather than a free metal ion, the former of which will be easier to detect using a biosensor than a small molecule. Moreover, such in vivo studies require long wavelength fluorescence excitation and emission to avoid background fluorescence, absorption, and scattering so as to be visible in animal models (Ntziachristos, 2006; Thompson, 1994); we and others have made long wavelength fluorescence sensors to meet these needs (Hirayama, Van de Bittner, et al., 2012; Kiyose, Kojima, et al., 2006; Thompson, Maliwal, et al., 2000; Zeng, Matveeva, et al., 2013). Of particular interest are bioluminescent sensors, since they do not require exciting light to penetrate the tissue to reach the sensor, nor are endogenous fluorophores such as flavins, pteridines, and porphyrins potential interferents because their fluorescence is not excited; these advantages have fueled bioluminescent sensors' popularity for in vivo imaging (Negrin & Contag, 2006). We (R. Thompson et al., in preparation; Thompson, Matveeva, et al., 2015) and others (Aper, Dierickx, et al., 2016) have developed bioluminescent sensors for zinc, and bioluminescent sensors for other metals are certain to be developed shortly.

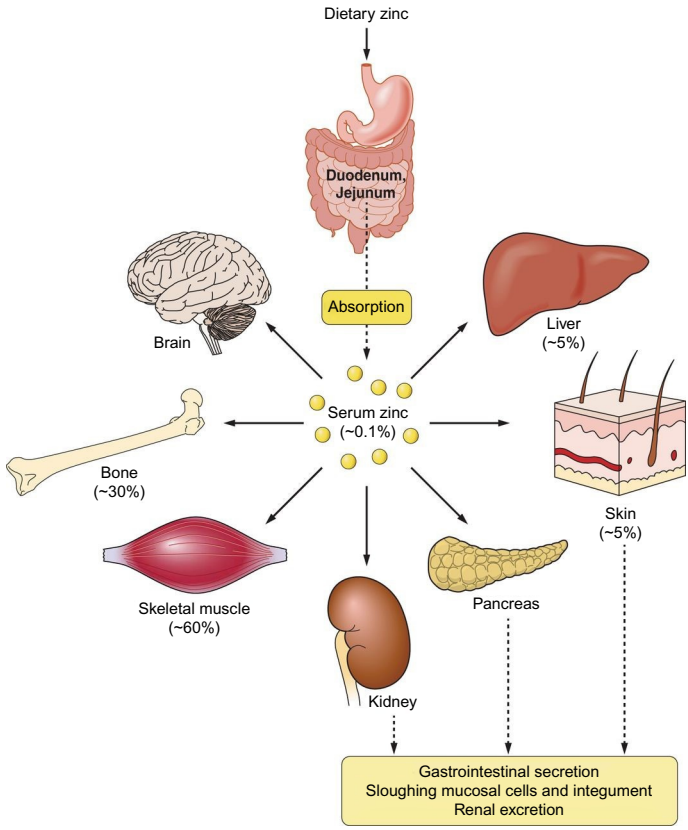


**Fig. 5** Distribution of zinc throughout the human body. *Reproduced from Kambe, T., Tsuji, T., et al. (2015). The physiological, biochemical, and molecular roles of zinc transporters in zinc homeostasis and metabolism.* Physiological Reviews, 95*(3), 749, with permission.*

## 8. CONCLUSION

Overall, biosensors have emerged as important tools in understanding the biology of metal ions, especially those classified historically as being at "trace" levels; with continued innovation by many investigators worldwide, we expect this trend to continue. The evident importance of metal ion fluxes in many disease processes in humans (Nriagu & Skaar, 2015), as also indicated by identification of transporter variants linked to various diseases (Hogstrand, Kille, et al., 2009; Li, Zhang, et al., 2007; Sladek, Rocheleau, et al., 2007; Taylor, Hiscox, et al., 2004), suggests it may be fruitful to track those fluxes in vivo, a job for which biosensors may be very well suited.

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