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Ternary Complex Formation and Competition Quench Fluorescence of ZnAF Family Zinc Sensors

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Our current understanding of the intracellular thermodynamics and kinetics of Zn(II) ions is largely based on the application of fluorescent sensor molecules, used to study and visualize the concentration, distribution and transport of Zn(II) ions in real time. Such agents are designed for high selectivity for zinc in respect to other biological metal ions. However, the issue of their sensitivity to physiological levels of low molecular weight Zn(II) ligands (LMWLs) has not been addressed. We followed the effects of eight such compounds on the fluorescence of ZnAF-1 and ZnAF-2F, two representatives of ZnAF family of fluorescein-based zinc sensors containing the *N*,*N*-bis(2-pyridylmethyl)ethylenediamine chelating unit. Fluorescence titrations of equimolar Zn(II)/ZnAF-1 and Zn(II)/ZnAF-2F solutions with acetate, phosphate, citrate, glycine, glutamic acid, histidine, ATP and GSH demonstrated strong fluorescence quenching. These results are interpreted in terms of an interplay of the formation of the [ZnAF-Zn(II)-LMWL] ternary complexes and the competition for Zn(II) between ZnAF and LMWLs. UV-vis spectroscopic titrations revealed the existence of supramolecular interactions between the fluorescein moiety of ZnAF-1 and ATP and His, which, however, did not contribute to fluorescence quenching. Therefore, the obtained results show that the ZnAF sensors, other currently used zinc sensors containing the *N*,*N*-bis(2-pyridylmethyl)ethylenediamine unit, and, in general, all sensors that do not saturate the Zn(II) coordination sphere, may co-report cellular metabolites and Zn(II) ions, leading to misrepresentations of the concentrations and fluxes of biological zinc.

Introduction

Zinc is an essential metal ion, playing several major roles in biology. Coordinated Zn(II) ions are necessary for the function of many proteins involved in crucial life processes, such as DNA replication and repair, gene expression and cellular metabolism. Catalytic Zn(II) ions are present in active sites of hydrolytic enzymes, while structural Zn(II) ions enable structure-specific protein-nucleic acid and protein-protein interactions via formation of zinc fingers and related domains.[1] Zinc fluxes provide intra- and intercellular signaling, in particular in the central nervous system.[2,3] The signaling functions are commonly ascribed to free Zn^{2+} ions. The information about their intracellular and extracellular levels and distributions is obtained by using fluorescent zinc sensors, chelating agents whose fluorescence is strongly modified (usually activated) by specific Zn(II) chelation.[4,5]

Both intracellular and extracellular biological fluids are rich in low molecular weight ligands (LMWLs), small molecules with known chemical abilities to form complexes with metal ions, including Zn(II). Despite this well-known fact, the issue of interactions of fluorescent zinc sensors with physiological levels of LMWLs has not been addressed, although the ability of some zinc sensors to form ternary complexes has been reported.[6-10] We studied the effects of eight such compounds: acetate, phosphate, citrate, glycine, glutamic acid, histidine, ATP, and GSH (Scheme 1), on the fluorescence of ZnAF-1 and ZnAF-2F, two representative compounds of the ZnAF family of fluoresceine-based zinc sensors, which bind Zn(II) with a 1:1 stoichiometry.[11] These compounds contain a *N*,*N*-bis(2-pyridylmethyl)ethylenediamine (dipicolylethylenediamine) chelating unit (Scheme 2), which has also been employed in several other series of zinc sensors developed recently.[3,12,13] All LMWLs presented in Scheme 1 are present intracellularly at substantial, millimolar or submillimolar, yet often highly variable concentrations. Citrate, many other organic acids, glutamate, and last, but not least, ATP, are controlled or influenced by the Krebs cycle.[14] In this study we used acetate and inorganic phosphate as representatives of the variable pool of these acids. Histidine and glycine as representative amino acids of the protein biosynthesis pool, vary according to cell cycle and metabolism.[15,16] GSH is synthesized and used up according to various cellular stresses and assaults.[17,18]

These compounds also represent a variety of Zn(II) binding modes (their Zn^{2+} binding groups are marked in Scheme 1). We studied their effects on the performance of ZnAF-1 and ZnAF-2F using fluorescence spectroscopy, under conditions used previously to determine the Zn(II) binding properties of these sensors.[11]

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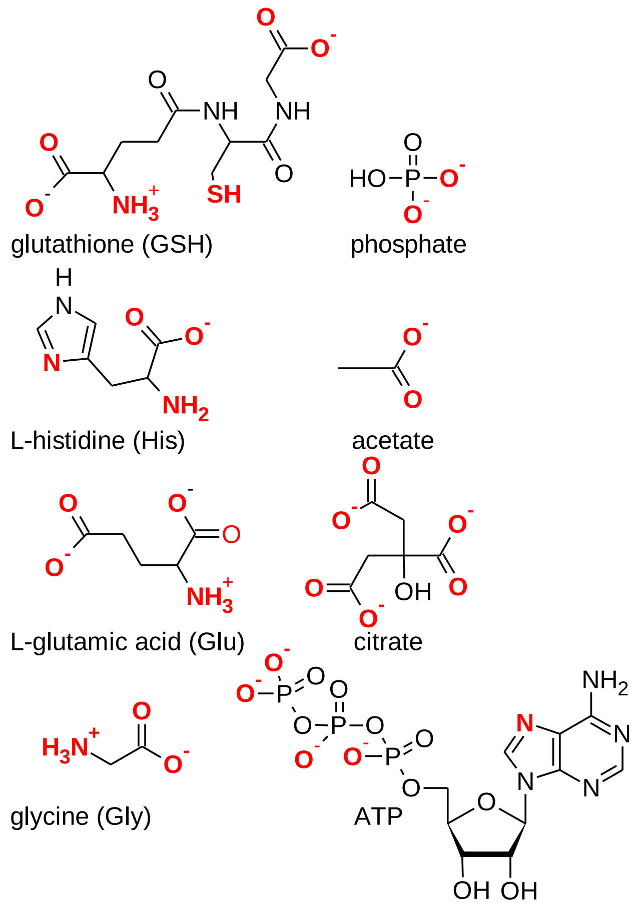
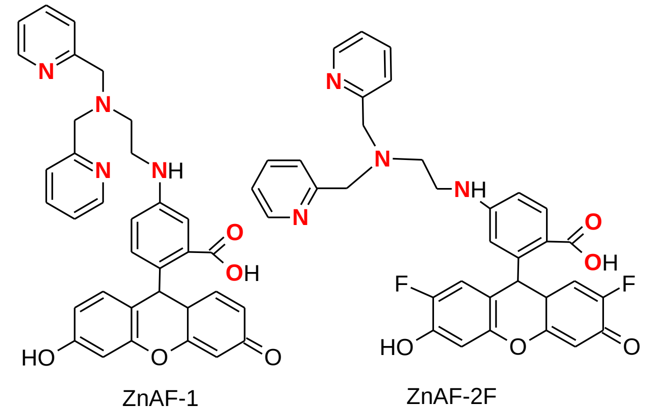


Scheme 1 Low Molecular Weight Ligands (LMWLs) studied in this work. Major protonation states for pH 7.4 are shown. Potential Zn(II) binding sites are marked red

Experimental

Materials

The reagents were obtained from the following sources: ZnAF-1, ZnAF-2F, L-glutamic acid, 99.5%, ethylene glycol-bis(2-aminoethylether)-*N,N,N',N',*-tetraacetic acid, 99% (EGTA), and standard 0.1 M $ZnCl_2$ solution from Fluka, sodium acetate (anhydrous), sodium phosphate dibasic, 98.5%, glycine hydrochloride, 99%, ethylenediaminetetraacetic acid, ACS reagent (EDTA), adenosine 5'-triphosphate disodium salt hydrate, 99% (ATP) and L-glutathione reduced (GSH), 99% from Sigma, L-histidine, 98%, from Aldrich, Hepes, 99.5 %, from Roth, citric acid hydrate, 99%, from Standard, Poland, $MgCl_2$ hexahydrate, 99%, from Merck. All solutions were prepared using water purified to the resistivity of 18.2 MΩ with a Milli-Q (Millipore, Bedford, MA) reverse osmosis system. The ATP stock solutions were kept on ice in order to prevent its hydrolysis and were controlled by UV spectroscopy. No hydrolysis was detected.



Scheme 2 Structures of Zn^{2+} sensors studied in this work.[11,19]

Fluorescence spectroscopy

The fluorescence spectra of ZnAF1 and ZnAF2F were recorded at 25 °C on a Cary Eclipse spectrofluorimeter (Varian) in 1 cm cells, using the fluorophore excitation at 492 nm and following its emission in the range of 500-700 nm. Excitation and emission bandwidths of 2.5 nm were used. The initial concentrations of fluorophore and Zn^{2+} ($ZnCl_2$) were 1 μM in 100 mM Hepes, pH 7.4, unless stated otherwise. Titrations were performed for LMWL concentrations from 0 to 20 mM (GSH, His, citric acid) or from 0 to 60 mM (all other LMWLs). For ATP, the experiments were performed in the absence and presence of equimolar amounts of $MgCl_2$. In order to account for dilution effects, each LMWL titration was accompanied by a parallel control titration with equal volumes of the Hepes buffer, and results are expressed as ratios of sample to control fluorescence. The titrations were performed in three to six repetitions, and averaged prior to calculations. In separate experiments the effect of the mixture of GSH, ATP, $MgCl_2$ and Gly (all 2 mM), and 0.1 mM citric acid on the fluorescence of both zinc sensors was tested. In all cases, the addition of LMWL affected solely the fluorescence intensity. The shapes of the spectra and their maximum emission wavelengths were not affected during titrations. The effect of LMWLs on the residual fluorescence of the sensors in the absence of Zn(II) was negligible.

In order to account for the Zn(II) contamination of Hepes buffered solutions of ZnAF-1 and ZnAF-2F, we titrated 1.0 μM sensors with Zn(II) ions ($ZnCl_2$) in the range from 0.01 to 0.9 μM. The titrations were done in duplicate for each sensor. In all cases we observed a linear increase of fluorescence. The slope of such line is dictated by the Zn(sensor) complex, while its intercept is the sum of fluorescence of the free sensor and that complexed with the Zn^{2+} impurity. The stock solutions of sensors were calibrated as follows: first, the solutions were titrated with $ZnCl_2$ to obtain saturation of the complex formation (100%). Next, these solutions were titrated with EDTA up to a 20-fold excess of this chelator, to assure full removal of Zn(II) from the

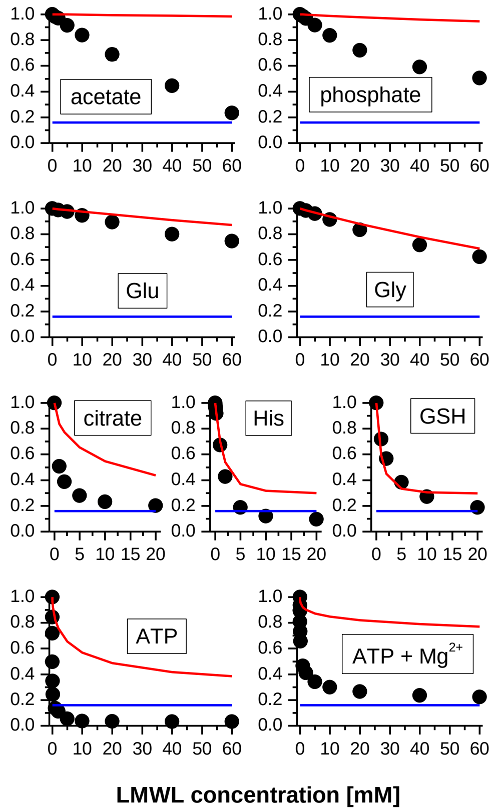


Fig. 1. Fluorescence titrations of the Zn(II) complex of ZnAF-1 (1 μM) with LMWLs. Black circles – experimental fluorescence at 515 nm relative to that in the absence of LMWL (F/F_0); red lines – titrations simulated according to a competition-only model, blue lines – fluorescence of zinc-free sensors.

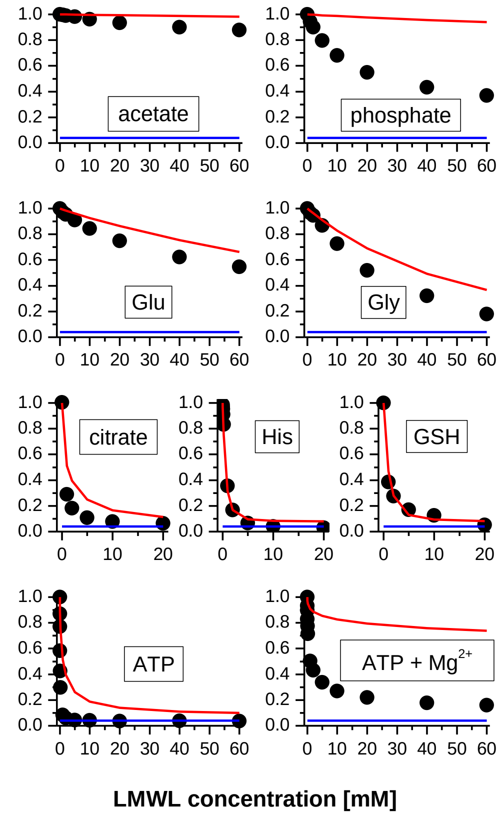


Fig. 2. Fluorescence titrations of the Zn(II) complex of ZnAF-2F (1 μM) with LMWLs. Black circles – experimental fluorescence at 515 nm relative to that in the absence of LMWL (F/F_0); red lines – titrations simulated according to a competition-only model, blue lines – fluorescence of zinc-free sensors.

sensor. The remaining fluorescence of zinc-free sensors, corrected for dilution, was expressed as fraction of the Zn(sensor) complex. Additionally, the Zn^{2+} content of the 0.1 M Hepes buffer was controlled by ICP-MS, with a satisfactory agreement with the results obtained using sensors.

The published conditional Zn(II) binding constants of ZnAF-1 and ZnAF-2F were determined by competitive fluorescence titrations with NTA.[9,17] We additionally confirmed them by analogous competitive titrations, but using EGTA as a competitor,[18] in the 100 mM Hepes at pH 7.4.

UV-vis spectroscopy

The absorption spectra of ZnAF1 were recorded at 25 °C on a Cary 50 Bio spectrophotometer (Varian) in 1 cm cells, using the range of 250-800 nm. The concentrations of fluorophore and Zn^{2+} ($ZnCl_2$) were 10 μM in 100 mM Hepes, pH 7.4. Titrations were performed separately for His concentrations from 0 to 50 mM and for ATP concentrations from 0 to 100 mM. Control spectra were recorded in the absence of $ZnCl_2$. All samples were prepared separately from stock solutions, thereby eliminating dilution effects, and measured within 15 min of preparation.

Calculations

The calculations of theoretical competition for Zn(II) between the sensor and the LMWL were performed with the use of a Newton-Raphson algorithm implemented under Microsoft Excel. The output of these calculations is equivalent to that of the Species module of the SolEq[21] software suite. The literature stability constants for respective binary complexes were used, as cited below.

The calculations of the apparent stability constants of Zn(sensor)(ATP) complexes were done using the same algorithm, with an assumed formula of the ternary complex presented in Equation 1 (E1):

$$K_{tern} = [\text{Zn(sensor)(ATP)}]/([Zn^{2+}][\text{sensor}][\text{ATP}]) \qquad \text{(E1)}$$

The K_{tern} values were determined by recalculating the relative fluorescence ($F_R = F/F_0$) in each titration point as a weighted sum of concentrations of fluorescing species, and then averaging over all titration points. The following F_R values were used: 1 for Zn(ZnAF-1) and Zn(ZnAF-2F) complexes, 0.16 for ZnAF-1, and 0.04 for ZnAF-2F alone. The calculations were performed for several F_R values of Zn(sensor)(ATP) complexes within the range of 0.02 to 0.04 for both ZnAF-1 and ZnAF-2F. The values that resulted in the lowest K_{tern} standard deviations were accepted.

Results

Selection of experimental conditions for LMWL fluorescence titrations

The goal of this study was to establish whether fluorescent zinc indicators are responsive to the presence of other zinc binding biomolecules. We chose 0.1 M Hepes at pH 7.4 as the medium for all experiments, because Hepes does not form complexes with Zn(II) ions under these "physiological" conditions.[22]

ATP is known to require Mg^{2+} ions for its biological activity, and the association constant of the Mg(ATP) complex is sufficiently high to assure that the majority of ATP molecules will be complexed *in vivo* if adequate amounts of Mg^{2+} ions are available.[21] On the other hand, the levels of ATP may be much higher from those of Mg^{2+} ions locally.[24] Therefore, experiments with ATP were performed in the absence and presence of equimolar amounts of $MgCl_2$.

Even highly purified water often contains traces of Zn^{2+} ions, e.g. leaking from the container or tubing walls. In order to account for this impurity, and for a possible contamination of buffer and sensors with traces of Zn(II), samples of 1 μM ZnAF-1 and ZnAF-2F in 0.1 M Hepes were titrated with stock solutions of $ZnCl_2$. The slope of the resulting linear dependence of fluorescence on Zn(II) is dictated by the Zn(sensor) complex, while its intercept is the sum of fluorescence of the sensor free of zinc and that complexed to the Zn^{2+} impurity The background concentration of Zn^{2+} ions in sensor samples, prior to $ZnCl_2$ addition, was 15-30 nM, confirmed by ICP-MS The relative fluorescence of zinc-free sensors ($F_R = F/F_0$, where F_0 is the fluorescence of the 1 μM Zn(II)-sensor complex), determined in the presence of a 20-fold molar excess of EDTA, was 0.16 for ZnAF-1 and 0.04 for ZnAF-2F, in a good agreement with the published data.[11]

The extent of Zn(II)-sensor complex formation at the beginning of LMWL fluorescence titrations (1 μM of both sensor and $ZnCl_2$), being 93% for ZnAF-2F and 98% for ZnAF-1, was calculated using the published conditional K_d values for 100 mM Hepes, pH 7.4.[11] These constants, originally determined by competitive titrations with NTA, were confirmed by us by analogous titrations with EGTA (K_d = 1.35 nM for the ZnEGTA complex)[20], and were used in calculations described below.

LMWL fluorescence titrations

Figures 1 and 2 present LMWL titrations of ZnAF-1 and ZnAF-2F, respectively. In all cases the decrease of fluorescence intensity was observed in the course of titrations, but the extent of this effect varied strongly among LMWLs, and also, for the same LMWL, between ZnAF-1 and ZnAF-2F.

We compared the experimental titration curves to theoretical LMWL competition-only curves, according to Reaction 1 (R1), where *i* includes all stoichiometries of complexes:

$$\text{Zn(sensor)} + i\ \text{LMWL} \Leftrightarrow \text{(sensor)} + \text{Zn(LMWL)}_i \qquad \text{(R1)}$$

These curves, shown in Figures 1 and 2 as red lines, were calculated using the published protonation and stability constants for Zn(II) complexes with LMWLs: acetate,[25] phosphate,[26] Glu,[27] Gly,[28] citrate,[29] His,[30] GSH,[31] and ATP.[23,32] The apparent agreement between the model of interaction described by R1 and the experimental data was found only for the interaction of Gly with ZnAF-1 (Fig. 1). Deviations were small, but significant for interactions of citrate with ZnAF-1 (Fig. 1) and His with ZnAF-2F (Fig. 2). In all other cases the differences between this competition-only model and the experimental titration curves were very significant, thus strongly suggesting the formation of ternary complexes.

Calculations of stability constants of ternary complexes with ATP from fluorescence data

Table 1 Conditional binding constants (100 mM HEPES, pH 7.4), characterizing the ternary Zn(II) complexes with ZnAF sensors and ATP

sensor	K_d (M)[a] (-log K_d)	K_{tern} (M^{-2})[b] (log K_{tern})	K_{ATP} (M)[c] (-log K_{ATP})
ZnAF-1	7.8×10^{-10} (9.11)	$2.6 \pm 1.0 \times 10^{13}$ (13.4 ± 0.2)	$4.9 \pm 1.9 \times 10^{-5}$ (4.3± 0.2)
ZnAF-2F	5.5×10^{-9} (8.26)	$2.9 \pm 0.5 \times 10^{12}$ (12.45 ± 0.08)	$6.3 \pm 1.1 \times 10^{-5}$ (4.2± 0.1)

[a] $K_d = ([Zn^{2+}][\text{sensor}])/[\text{Zn(sensor)}]$ – literature values[9,17]

[b] $K_{tern} = [\text{Zn(sensor)(ATP)}]/([Zn^{2+}][\text{sensor}][\text{ATP}])$

[c] $K_{ATP} = 1/(K_{tern} \times K_d)$

ATP exhibited the strongest, nearly complete quenching of fluorescence of both ZnAF-1 and ZnAF-2F. The residual fluorescence was consistently constant for ATP concentrations of 10 mM and higher for both sensors. As a result we were able to calculate conditional binding constants for ternary complexes of the assumed Zn(sensor)(ATP) stoichiometry, which are presented in Table 1. Figure 3 presents the corresponding species distributions of binary and ternary complexes in Zn(II)/sensor/ATP systems.

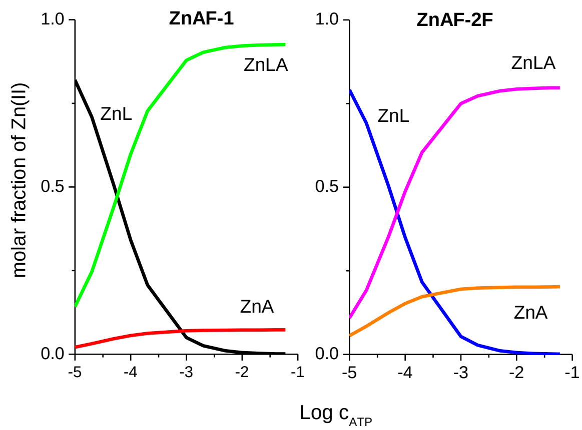


Fig. 3 The species distribution of Zn(II) complexes of ATP (A) and sensors (L) (top, ZnAF-1; bottom, ZnAF-2F, as indicated). Total concentrations of Zn(II) and sensor, 1 μM; ATP varied from 0.01 to 60 mM.

The effect of LMWL mixture.

The significant quenching of both zinc sensors by all LMWLs studied encouraged us to simulate intracellular conditions, by reacting ZnAF-1 and ZnAF-2F with the mixture of GSH, ATP, $MgCl_2$ and Gly (all 2 mM), and 0.1 mM citric acid. The component concentrations were chosen to represent typical physiological LMWL levels, with Gly representing the overall amino acid pool. The results of these experiments are shown in Figure 4. The LMWL mixture quenches the sensor fluorescence strongly, diminishing the intensities of emission bands by a factor of four for ZnAF-1 and five for ZnAF-2F.

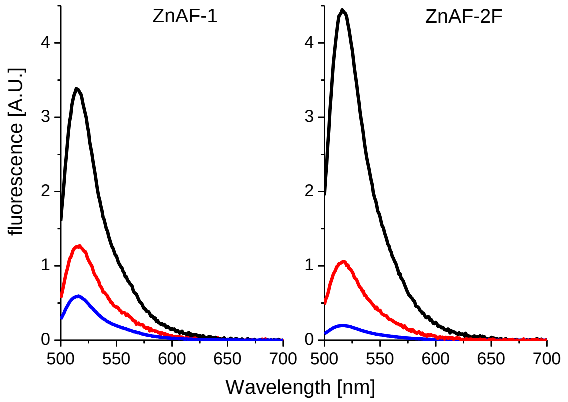


Fig. 4 The effect of the LMWL mixture (2 mM GSH, 2 mM ATP, 2 mM $MgCl_2$, 2 mM Gly and 0.1 mM citric acid) on the fluorescence of 1 μM Zn(II) complexes of ZnAF-1 and ZnAF-2F. Black – initial emission spectra of complexes of sensors; red – the experimental spectra in the presence of LMWL mixture; blue – the spectra of sensors in the absence of Zn(II).

Interactions of Zn(ZnAF-1) with LMWLs followed by UV-vis spectroscopy.

Titrations of ZnAF-1 with His and ATP were also followed by UV-vis spectroscopy. A higher, 10 μM sensor concentration was chosen for better detection of the spectrum. These experiments were limited to ZnAF-1, due to its higher Zn(II) affinity, resulting in a diminished competition effect. Figure 5 presents the ATP titration. Two effects could be seen. At lower ATP ratios, between 1 and 1000, there were no significant changes of the intensity of the observed band at 490-493 nm, but a systematic shift of the absorption maximum could be seen, from 492.8 to 491.1 nm. The increase of ATP ratio between 1000 and 10000 was accompanied by a large change of spectral change and intensity. The calculations of apparent p*K* values for these two processes are presented in Fig. 6. The first one was characterized by p*K* of 3.69 ± 0.03, with Hill coefficient[33] *n* of 1.7 ± 0.1, and the second by p*K* of 1.57 ± 0.03, with Hill coefficient *n* of 3.2 ± 0.7, both values in terms of ATP concentration. The control titrations in the absence of Zn(II) revealed the same spectral effects. In His titrations a slight blueshift of the maximum of ZnAF-1 absorption was the only effect detected.

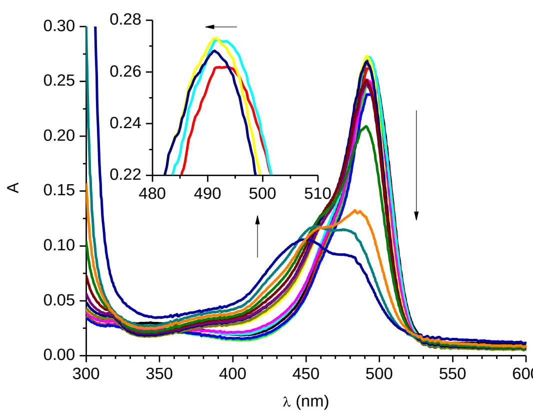


Fig. 5. UV-vis titration of 10 μM Zn(II) complex of ZnAF-1 with ATP (from 0 to 100 mM). Arrows indicate the direction of spectral changes.

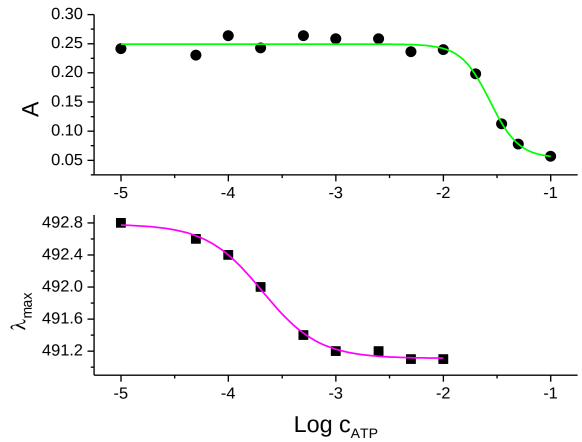


Fig. 6 Calculations of apparent affinity constants for interactions of fluorescein moiety in ZnAF-1 with ATP, obtained from absorption spectra. Lines represent best fits to Hill equation.[33]

Discussion

Formation of ternary Zn(sensor)(LMWL) complexes

The above presented data clearly demonstrate that the abilities of ZnAF-1 and ZnAF-2F to report Zn^{2+} ions depend strongly on the presence of common LMWLs. The universal effect of all LMWLs was the reporting of either less, or much less Zn^{2+} ions than the solution actually contained. Our simple test tube experiments, together with the numerical analysis based on the known abilities of LMWLs to form Zn(II) complexes, provide evidence for two concurrent mechanisms for this biased reporting. The formation of Zn(II)-LMWL complexes in a simple competition manner contributes significantly to the sensor fluorescence quenching for many of LMWLs tested, as seen in Figs. 1 and 2 (red lines). This is supported by the fact that, for six out of eight LMWLs studied, the quenching is systematically lower for ZnAF1, which binds the Zn(II) ion seven times stronger in terms of the conditional K_d at pH 7.4.[11]

However, the opposite effect could be seen for acetate, and ATP which quenched ZnAF-1 as effectively as ZnAF-2F. Also, for nearly all LMWLs the fluorescence quenching in the course of titrations was significantly stronger than that predicted by the competition model of interaction (experimental points systematically below the red prediction lines in Figs. 1 and 2). Furthermore, at higher His and ATP concentrations the fluorescence of the sensors was lower than that determined for Zn(II)-free molecules (blue lines). This effect can be seen clearly in Fig. 1 for ZnAF-1, because the fluorescence of its apo-form is higher, but is also true for ZnAF-2F. In general, the deviation between the predicted and the experimental performance of sensors is the highest for oxygen-only ligands, acetate, phosphate, citrate and ATP (Scheme 1). All these facts, taken together, indicate the formation of ternary complexes at least in some of the systems studied.

While the aim of our experiments was to screen a range of LMWLs and test the ternary complex formation hypothesis qualitatively, the saturation of fluorescence quenching at a level below that of the zinc-free sensor, seen for ATP, enabled us to perform a quantitative analysis, too. The assumed 1:1:1 stoichiometry of interaction between ATP, Zn^{2+} and the sensor, which found support in the low standard deviation of the calculated K_{tern} values, is also justified by the multidentate character of the triphosphate moiety of ATP in other known ternary complexes[34-36] (the ATP's N7 donor atom is unlikely to participate in the Zn(II) binding in the ternary complex.[34]). The dissociation constants for the process of release of the ATP molecule from the ternary complex, K_{ATP}, can be calculated from K_{tern} constants (Table 1). Their values are within the range established by other ternary ZnL(ATP) complexes, for L being amino acids[37,38] and synthetic nitrogen-based chelators,[39-41] and are only somewhat lower from those published for related binuclear dipicolylamine complexes, which serve as phosphate sensors.[35] The effectiveness of attachment of ATP to both sensors was identical within the experimental error, indicating the same structure of the ternary complex.

As shown in Figure 3, the binding of ATP to both sensors is so effective that the ternary complex could be a major species at the physiological, millimolar range of ATP concentrations. However, addition of equimolar Mg^{2+} to ATP reduced the quenching (Figs. 1 and 2), and this effect could not be reproduced quantitatively by a simple introduction of the ternary complex in the simulations, thus indicating the formation of additional complex species with the participation of Mg^{2+}.

The absorption spectroscopy titrations of ZnAF-1 with ATP presented in Figures 5 and 6 indicated that the studied sensors may be engaged in other kinds of interactions with LMWLs, not involving the bound Zn(II). Two types of interactions were detected via the modulation of the absorption spectrum of the fluorescein moiety. The subtle spectral effect with the apparent K_d of 0.2 mM and the Hill coefficient of 1.7 indicates that ZnAF family sensors may exist in the cells as bound to two ATP molecules. Further multiple ATP molecules bind to ZnAF-1 with the apparent K_d of 27 mM (n ~3). the first of these effects was also seen for His. However, these interactions are not responsible for fluorescence quenching. This is illustrated in Figure 7, which compares the profile of formation of the ternary ZnAL complex with that of the higher affinity absorption spectroscopic species.

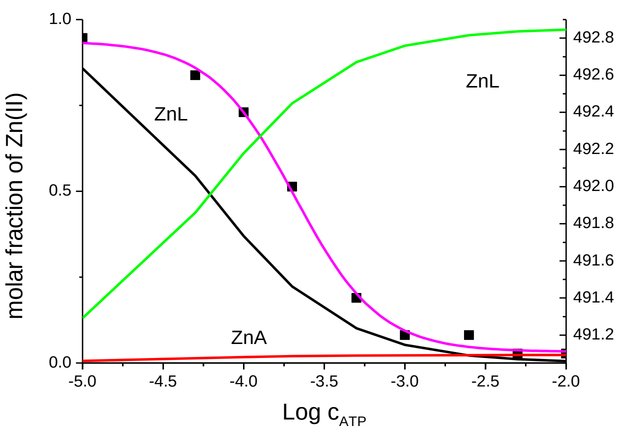
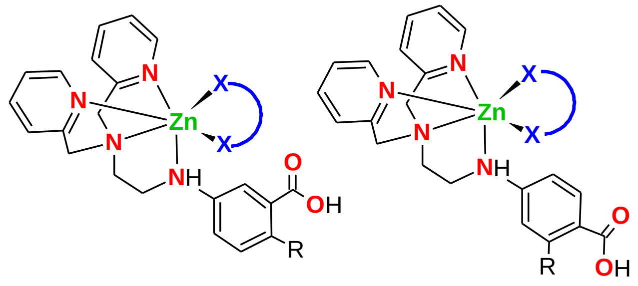


Fig. 7 Comparison of the profile of formation of the ternary complex of Zn(II), ZnAF-1 and ATP, calculated for 10 μM components (green line), with that of the higher-affinity effect observed in absorption spectra.

Figure 8 presents the hypothetical general structure of ternary complexes, which help rationalize experimental data. This structure proposal was inspired by the study of Lippard *et al.* on dual function DPA/porphyrin zinc sensors.[12] The only difference between ZnAF-1 and ZnAF-2F which may be relevant for Zn^{2+} binding is the location of the carboxyl group in the aromatic ring.



ZnAF-1 ZnAF-2F

Fig. 8 The overall structures of ternary complexes of ZnAF sensors (R denotes the rest of the fluorescein ring system) with LMWLs, denoted as X-X.

In the sensors studied this group cannot participate in the Zn(II) binding directly, but in ZnAF-1 it is located more favorably to interact with the LMWL bound to Zn(II) ion. Such interactions may explain secondary differences in interactions between ZnAF-1 and ZnAF-2F.

The fluorescence activation by Zn(II) in the ZnAF sensor series is based on the engagement of the lone electron pair of the amine substituent of the benzoic acid moiety in the coordination of the Zn^{2+} cation.[11,19] A ternary ligand able to neutralize the cation's charge, such as a hard carboxylate or phosphate anion, will decrease the delocalization of this lone pair, thus restoring self-quenching of a ZnAF molecule. Examples of fluorescence quenching by ternary complex formation are known in the literature.[42]

The results presented above can be discussed in terms of intracellular concentrations of LMWLs. Table 2 presents the extent of ZnAF-1 and ZnAF-2F quenching at these concentrations, calculated from our titrations. One can see that the most of LMWLs studied could quench these sensors significantly *in vivo*, with the largest effects exerted by ATP, GSH, and carboxylic acids. We also measured the quenching of both sensors with a mixture of GSH, ATP+$MgCl_2$, Gly and citric acid (Fig. 4). The effect was very significant, resulting in a severe underestimation of the Zn^{2+} concentration, fourfold by ZnAF-1 and fivefold by ZnAF-2F. Furthermore, levels of all these metabolites fluctuate due to the Krebs cycle[12] and redox homeostasis,[15] and respond strongly to a variety of stress stimuli. Therefore, the zinc signal reported by ZnAFs and any other zinc sensors capable of forming ternary complexes may strongly underestimate the actual intracellular levels of mobile zinc and report the physiological fluctuations of metabolites as modulations of zinc levels. The general character of the quenching mechanisms presented above indicates that this problem is likely to be shared by a vast majority of zinc sensors currently available. Nearly all are unable to saturate the Zn^{2+} coordination sphere completely, with one possible exception of a novel sensor characterized by NMR and X-ray studies to be 6-coordinate in a rare geometry of pentagonal pyramid.[5,43]

Table 2 The prediction of the effect of physiological concentrations of LMWLs on the fluorescence of ZnAF-1 and ZnAF-2F; components of the LMWL mixture used in the experiment illustrated in Fig. 4 are shown in bold.

LMWL	% of fluorescence at physiological concentration ranges		Physiological concentration ranges (mM)
	ZnAF-1	ZnAF-2F	
GSH	52-17	39-12	1-10[a]
His	96	88	0.08[b]
Citrate	95-92	83-72	0.1-0.16[c]
Gly	100-99	100-97	0.1-1[b]
ATP	35-4	42-4	0.1-10[d]
ATP + Mg^{2+}	73-30	87-8	
Glu	100	100	0.076-0.082[c]
phosphate	97	91	1.8[c]
Acetate	67	93	21.4[e]

[a] refs. 17 and 18

[b] refs. 15 and 16

[c] ref. 14

[d] refs. 14 and 35

[e] total concentration of all LMW carboxylic acids, ref. 14

Conclusions

Above, we presented the direct effects of eight low molecular weight bioligands (LMWLs), acetate, phosphate, citrate, glycine, glutamic acid, histidine, ATP and GSH on the ability of ZnAF-1 and ZnAF-2F, two representative fluorescein-based zinc sensors containing the *N*,*N*-bis(2-pyridylmethyl)ethylenediamine chelating unit, to detect Zn(II) ions in solution. We demonstrated that all these compounds quench the sensors' response, leading to a significant underestimation of Zn(II) concentrations in vitro. We also showed that this effect is partially due to competition for Zn(II) coordination between the sensor on the LMWL, and partially to the formation of ternary Zn(sensor)(LMWL) complexes. These quenching mechanisms indicate that the sensors which do not saturate the Zn(II) coordination sphere may co-report cellular metabolites and Zn(II) ions, rather than Zn(II) ions alone. This may lead to misrepresentations of the concentrations and fluxes of biological zinc. Since such sensors indeed constitute a vast majority of zinc sensors currently available, more attention should be paid to their abilities to form ternary complexes in vivo.

Notes and references

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