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Variation of Linker Length in Ratiometric Fluorescent Sensor Proteins Allows Rational Tuning of Zn(II) Affinity in the Picomolar to Femtomolar Range

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Research over the past decade has shown that transition-metal homeostasis is a tightly regulated process in which the acquisition, distribution, and excretion of metals is controlled by specific chaperones, pumps, and metal-sensitive transcription factors.[1] The free cellular concentration of Cu(I) has been estimated to be less than 10^{-18} M in yeast,[2] while the concentration of free Zn(II) has been estimated to range from ~1 fM in *Escherichia coli* to 5 pM−1 nM in mammalian cells.[3,4] Further understanding of transition-metal homeostasis and its role in, for example, neurodegenerative diseases requires the ability to directly image the concentration of these metals in living cells in real time.

The development of fluorescent probes that bind transition-metal ions with high affinity and selectivity has become an area of active research.[5] Of those, genetically encoded sensors based on fluorescence resonance energy transfer (FRET) offer important advantages such as ratiometric detection, noninvasiveness, and control over subcellular localization.[3,6,7] In these sensors, conformational changes in a sensor domain are translated into a change in energy-transfer efficiency between donor and acceptor fluorescent domains, which is detected by a change in the ratio of donor and acceptor emission. Finding the optimal domain organization and peptide linkers for the construction of FRET-based sensors is often a process of trial-and-error. A more rational design would require a quantitative understanding of the factors that determine energy-transfer efficiencies in these fusion proteins. We recently reported a FRET-based sensor with a subnanomolar affinity for Zn(II) based on the Zn(II)-induced dimerization of two metal binding domains, Atox1 and the fourth domain of ATP7B (WD4).[7] We reasoned that the Zn(II) affinity of the reported sensor system could be further improved by fusion of its two components, cyan fluorescent protein fused to Atox1 (CFP−Atox1) and yellow fluorescent protein fused to WD4 (WD4−YFP), via a flexible peptide linker (Figure 1). Here, we show that a very high and tunable Zn(II) affinity can be obtained in this way and that both the Zn(II) affinity and the ratiometric change that is observed upon Zn(II) binding can be quantitatively understood by considering the distribution of linker conformations that are available to the fusion protein in the unbound state.

A recently reported strategy of partial digestion and re-ligation of linker DNA was used to obtain fusion proteins of CFP−Atox1 and WD4−YFP with linker lengths ranging from 2 to 9 GGSGGS repeats (CA−L*n*−WY, with n = number of repeats).[8] This series of fusion proteins allowed us to systematically study the effect of linker length on both the Zn(II) affinity and the change in emission ratio. Figure 2 shows Zn(II) titrations for sensor proteins with 2, 5, and 9 repeats (see Supporting Information for proteins with other linker lengths). A much higher Zn(II) affinity was found for all

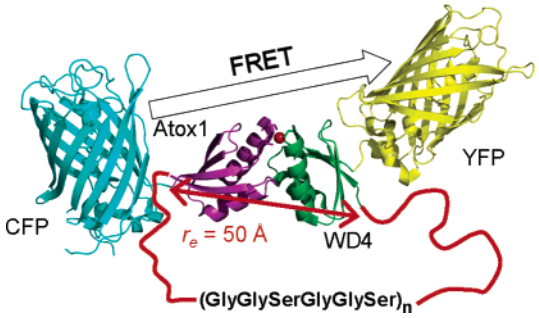

Figure 1. FRET-based Zn(II) sensor proteins consisting of CFP, Atox1, a flexible peptide linker, WD4, and YFP. A series of sensor proteins was constructed with peptide linkers containing 2−9 GGSGGS repeats.

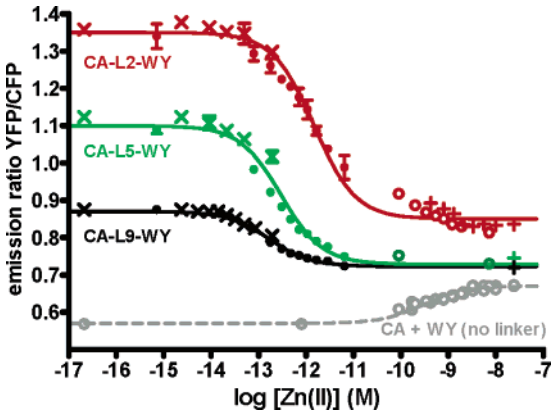

Figure 2. Zn(II) titration for CFP−Atox1−linker−WD4−YFP proteins with 2, 5, and 9 GGSGGS repeats. Titrations were done in 50 mM Tris, 100 mM NaCl, 1 mM DTT, and 10% glycerol (pH 7.5) using EDTA (X), HEDTA (●), EGTA (○), and Ca−EDTA (+) as Zn(II) buffers. Also shown is a titration for a 1:1 mixture of CFP−Atox1 and WD4−YFP (2 μM each).

single protein sensors compared to the previously reported system. The Zn(II) affinity was found to depend on the linker length with K_d values ranging from 170 fM for the protein with the longest linker (CA−L9−WY) to 1.4 pM for the protein with the shortest linker (CA−L2−WY). Mutation of the conserved threonines in both MTCXXC metal binding motifs into aspartates yielded a sensor (T252D/T385D CA−L9−WY) with an even lower K_d of 34 fM (Figure S5). The same mutations have been shown to also increase the Zn(II) affinity when CFP−Atox1 and WD4−YFP were not linked, but the previously observed disruption of the Atox1−WD4 complex at higher Zn(II) concentration was not observed in the presence of the peptide linker.[7] Remarkably, Zn(II) binding resulted in a decrease in energy transfer for the single protein sensors, whereas an increase in FRET was observed when CFP−Atox1 and WD4−YFP were not linked.[9] Similar emission ratios were observed

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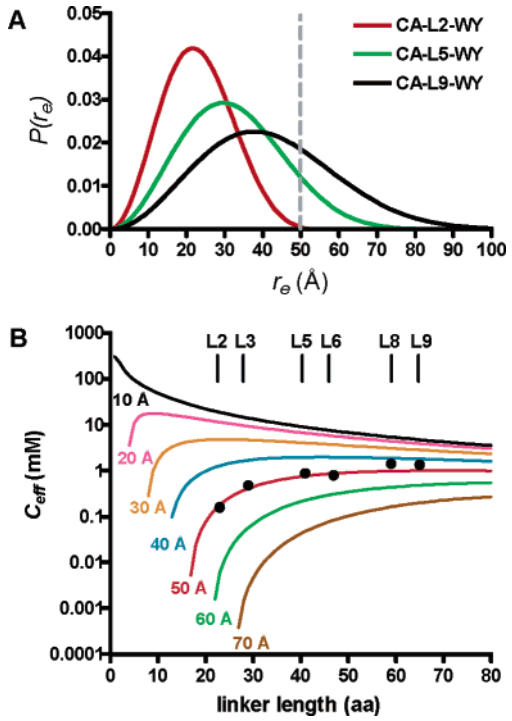

Figure 3. (A) Distribution functions showing the probability $P(r_e)$ of a certain end-to-end distance r_e for the peptide linkers used in CA−L2−WY, CA−L5−WY, and CA−L9−WY. (B) Calculated effective concentration (C_{eff}) as a function of linker length for different values of the end-to-end distance in the complex. For comparison, C_{eff} values that were experimentally determined for various CA−L*n*−WY proteins are shown as black circles.

for the Zn(II)-bound states of all protein sensors, although the ratio was somewhat higher for the Zn(II) complex of CA−L2−WY. This suggest that the structure of the Zn(II) complex is mostly independent of the presence of the peptide linker. The increased amount of FRET in the unbound state means that the CFP and YFP domains are, on average, closer together than those in the Zn(II)-bound state, an effect that becomes more pronounced for the shortest linker lengths.

The effect of linker length on the sensor properties can be understood by considering the conformational behavior of the peptide linker. We recently showed that the energy transfer between CFP and YFP domains separated by linkers containing GGSGGS repeats can be quantitatively described assuming a fully random-coil structure of the peptide linker.[8] These flexible peptide linkers assume a distribution of conformations that can be described using models originally developed to describe synthetic polymers such as the worm-like chain (WLC) model.[10] Figure 3A shows the distribution of end-to-end distances that is predicted by this WLC model for the linkers containing 2, 5, and 9 GGSGGS repeats using a persistence length of 4.5 Å. The distance between the C-terminus of Atox1 and the N-terminus of WD4 in the Zn(II)-bound complex can be estimated to be ∼50 Å based on the X-ray structure of the homologous Cd(II)-bridged Atox1−Atox1 homodimer.[11] Since the average end-to-end distances of the peptide linkers are all smaller than 50 Å, the random-coil model readily explains the observed decrease in FRET upon Zn(II) binding. In the Zn(II)-bound state, CFP and YFP are "fixed" at a relatively large distance, whereas many different conformations are possible in the absence of Zn(II), most of which have a smaller distance between CFP and YFP.

The WLC model can also be used to understand the linker length dependence of the Zn(II) affinity. The increased affinity for Zn(II) observed for the single protein sensor can be explained by an increase in the effective local concentration (C_{eff}) of the metal binding domains. This effective concentration can be calculated using the distance distribution for a certain linker length, as predicted by the WLC model, and the distance that the linker needs to bridge in the complex form.[12,13] Figure 3B shows the dependence of the effective concentration on the linker length for a variety of distances. The model predicts that an increase in linker length can result in either an increase or a decrease in effective concentration depending on the distance that the peptide needs to bridge in the complex. Experimental values for C_{eff} can be obtained by dividing the formation constant for intramolecular Zn(II) binding for CA−L*n*−WY by the formation constant for the ternary complex between CFP−Atox1, WD4−YFP, and Zn(II) that was determined previously ($\beta = 4.5 \times 10^{15}$ M^{-2}).[7] These experimental C_{eff} values agree very well with the values predicted by the WLC model assuming an end-to-end distance of 50 Å in the complex form. The distance distribution of the random-coil peptide linker thus fully explains not only the magnitude of the affinity enhancement but also its linker length dependence.

In conclusion, we have shown that variation of peptide linker length and metal binding site engineering allows rational tuning of both the Zn(II) affinity and the ratiometric change in fusion proteins of CFP−Atox1 and WD4−YFP. These sensor proteins cover a physiologically interesting range of Zn(II) concentrations and seem well-suited to probe intracellular Zn(II) concentrations in a variety of organisms and cell systems. These results also demonstrate the importance of considering conformational distributions in the design of multidomain proteins, in general, and FRET-based sensor systems, in particular.

Supporting Information Available: Experimental details regarding cloning, mutagenesis, protein production, Cu(I) binding, Zn(II) titrations, and calculation of $P(r_e)$ and C_{eff}. This material is available free of charge via the Internet at http://pubs.acs.org.

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