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Design and Evaluation of a Peptidyl Fluorescent Chemosensor for Divalent Zinc

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The rapid analysis of trace metal cations for environmental and biomedical applications is particularly demanding since it requires the specific recognition of a particular element in the presence of numerous closely related species. While remarkable progress has been achieved for inorganic analytes, such as Ca^{2+},[1] there is still a significant need for the genesis of new fluorosensors.[2] In light of the selectivity and avidity with which proteins bind divalent metal cations,[3] we have chosen to use a polypeptide architecture as the framework for metal ion recognition.[4] In addition, our ability to manipulate synthetic polypeptides allows the coordinated integration of fluorescent reporters for signal transduction within a metal-binding peptidyl construct. Herein we report the design, synthesis, and evaluation of a selective fluorescent chemosensor, sensitive to nanomolar concentrations of Zn^{2+}.

The classical method of transducing a metal-binding event into a fluorescent signal has generally relied on the fluorophore being directly involved in metal coordination.[5,6] In contrast, the design presented here consists of a synthetic polypeptide template and a covalently attached fluorescent reporter that is sensitive to metal-induced conformational changes of the supporting framework, yet remote from the metal-binding site. This approach combines the advantages of optical signaling[7] with the selective metal-binding properties of an appropriate peptide. Furthermore, the modular design enables an approach wherein the fluorescent signaling mechanism is decoupled from the composition of the coordination sphere. We have employed the metal dependent modulation of the zinc finger domains to effect changes in the emission properties of a covalently attached fluorophore. Peptides of this family show selective binding to divalent zinc[8] with reported dissociation constants as low as 5.7 pM.[9] These domains are synthetically accessible and may be described by the consensus sequence ($\underline{F}$/$\underline{Y}$)-X-C-X_{2-4}-C-X_3-$\underline{F}$-X_5-$\underline{L}$-X_2-H-X_{3-5}-H-X_{2-6}.[10,11] In the absence of metal ions, zinc fingers are disordered.[12] In contrast, when appropriate metal cations are present, coordination to the histidine and cysteine residues nucleates the formation of a defined tertiary structure including a cluster of conserved hydrophobic residues (underlined in the consensus sequence). We have replaced of one of the latter residues with a microenvironment-sensitive fluorophore[13] to monitor the metal-binding event. A schematic[14] drawing of the sensing peptide is shown in Figure 1.

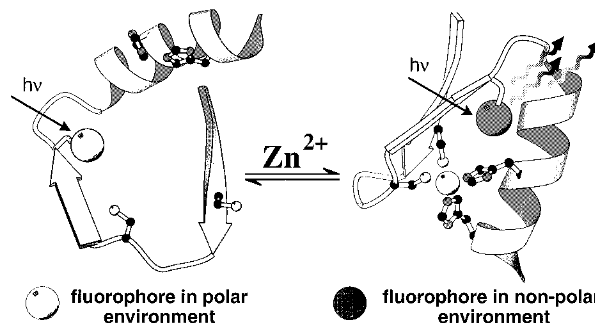

Figure 1. Schematic drawing of the chemosensor design. Metal binding induces peptide folding, shielding a fluorophore from solvent and increasing emission intensity.

To allow the selective elaboration of a side chain amine at variable positions within a synthetic polypeptide, a differentially protected β-amino alanine (Baa) derivative, (*S*)-2,3-diamino-N^{α}-(9-fluorenylmethyloxycarbonyl)-N^{β}-(allyloxycarbonyl)propanoic acid (Fmoc-L-Baa(alloc)-OH), was utilized.[15] Compatibility of this residue with Fmoc solid phase peptide synthesis allows for orthogonal deprotection with a palladium catalyst.[16] The newly liberated amine is then available for fluorophore attachment, while leaving the remainder of the protected peptide intact until the final deprotection and cleavage from the resin.

Judicious placement of the fluorophore is required for the generation of a significant change in fluorescence upon metal binding; the scaffold must be able to accommodate the chromophore within the hydrophobic cluster. In order to optimize the structure of the peptidyl template for this purpose, the solution structures of several *individual* zinc finger domains were examined.[17] Particular attention was given to domains in which mutations had been made in the residues which comprise the hydrophobic cluster. As a result of these studies, the zinc finger domain "ZFY-swap"[18] was selected as the template, and Phe_{10} of that sequence was chosen for replacement with the dansylated Baa amino acid derivative. Consequently, the peptide ZNS1 was synthesized[19] (Scheme 1) and assayed for the ability to selectively bind and report divalent zinc.

The fluorescence emission properties of ZNS1 are sensitive to the presence of nanomolar concentrations of Zn^{2+} (Figure 2). The addition of Zn^{2+} results in increased emission upon excitation at 333 nm, and the response (at 475 nm) is linear

(1) For a review, see: Tsien, R. Y. In *Fluorescent Chemosensors for Ion and Molecule Recognition*; Czarnik, A. W., Ed.; American Chemical Society:, Washington, DC, 1993; pp 130−146.
(2) Czarnik, A. W. *Chem. Bio.* **1995**, *2,* 423−428.
(3) Fraústo da Silva, J. J. R.; Willams, R. J. P. *The Biological Chemistry of the Elements*; Clarendon Press: New York, 1993; pp 223−435.
(4) For a protein-based sensor that uses a diffusable fluorophore to report divalent zinc, see: (a) Thompson, R. B.; Patchan, M. W. *Anal. Biochem.* **1995**, *227,* 123−128. (b) Thompson, R. B.; Jones, E. R. *Anal. Chem.* **1993**, *65,* 730−734.
(5) Czarnik, A. W. *Acc. Chem. Res.* **1994**, *27,* 302−308.
(6) Zalewski, P. D.; Forbes, I. J.; Seamark, R. F.; Borlinghaus, R.; Betts, W. H.; Lincoln, S. F.; Ward, A. D. *Chem. Bio.* **1994**, *1,* 153−161.
(7) Czarnik, A. W. in *Fluorescent Chemosensors for Ion and Molecule Recognition*; Czarnik, A. W., Ed.; American Chemical Society: Washington DC, 1993; pp 1−9.
(8) Berg, J. M.; Merkle, D. L. *J. Am. Chem. Soc.* **1989**, *111,* 3759−3761.
(9) Krizek, B. A.; Merkle, D. L.; Berg, J. M. *Inorg. Chem.* **1993**, *32,* 937−940.
(10) (a) Berg, J. M. *Acc. Chem. Res.* **1995**, *28,* 14−19. (b) Klug, A.; Schwabe, J. W. R. *FASEB J.* **1995**, *9,* 597−604.
(11) An alternate consensus sequence is observed for a subset of the zinc finger domains. Weiss, M. A.; Mason, K. A.; Dahl, C. E.; Keutmann, H. T. *Biochemistry* **1990**, *29,* 5660−5664.
(12) (a) Frankel, A. D.; Berg, J. M.; Pabo, C. O. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84,* 4841−4845. (b) Eis, P. S.; Lakowicz, J. R. *Biochemistry* **1993**, *32,* 7981−7993.
(13) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Plenum Press: New York, 1983; pp 189−218.
(14) This figure was generated using MOLSCRIPT. Kraulis, P. J. *J. Appl. Crystallogr.* **1991**, *24,* 946−950.
(15) Sinha Roy, R.; Imperiali, B. Unpublished results.
(16) Kates, S. A.; Daniels, S. B.; Albericio, F. *Anal. Biochem.* **1993**, *212,* 303−310.
(17) (a) Lee, M. S.; Gippert, G. P.; Soman, K. V.; Case, D. A.; Wright, P. E. *Science* **1989**, *245,* 635−637. (b) Omichinski, J. G.; Clore, G. M.; Appella, E.; Sakaguchi, K.; Gronenborn, A. M. *Biochemistry* **1990**, *29,* 9324−9334. (c) Kochoyan, M.; Keutmann, H. T.; Weiss, M. A. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88,* 8455−8459. (d) Hoffman, R. C.; Horvath, S. J.; Klevit, R. E. *Protein Sci.* **1993**, *2,* 951−965.
(18) Zinc finger domains with single-residue replacements have been structurally characterized, showing that the hydrophobic cluster is tolerant of substitution at position 10 (i.e., the second underlined residue in the consensus sequence). Weiss, M. A.; Keutmann, H. T. *Biochemistry* **1990**, *29,* 9808−9813.
(19) The peptide was purified by reversed phase (C_{18}) HPLC. The identity of ZNS1 was confirmed by electrospray mass spectroscopy (ZNS1 $[MH^+]$ calcd = 3230.7, obs = 3231.0).

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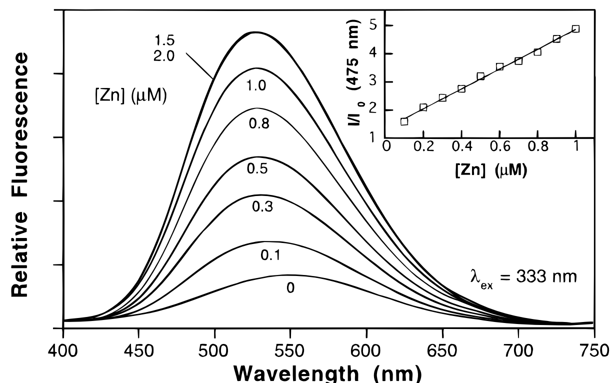
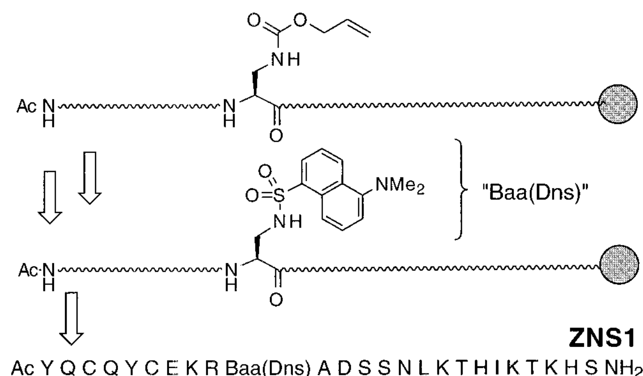


Figure 2. Fluorescence emission response of ZNS1 to increasing levels of Zn^{2+} (μM) in the presence of Mg^{2+} (50 mM) and Co^{2+} (100 μM) ions: excitation at 333 nm. Spectra were acquired with 1.4 μM ZNS1, 50 mM HEPES, pH 7.0, $\mu = 0.5$ (NaCl), and 3.5 μM EDTA. Addition of EDTA was required to complex adventitious metal and establish the initial base line prior to metal addition. The relative increase in fluorescence at 475 nm is linear between 0.1 and 1.0 μM Zn^{2+}.

Scheme 1. Synthesis of ZNS1



between 0.1 and 1 μM Zn^{2+}.[20] Since a potential application of the zinc sensor is for the analysis of sea water, preliminary studies were also carried out in the presence of metal cations including 0.5 M Na^{+}, 50 mM Mg^{2+}, and 100 μM Co^{2+}. It is notable that the chemosensor is compatible with these competing species.[21] Due to a blue shift which occurs upon metal binding, dual-wavelength ratiometric analysis of the fluorescence emission using 525 and 650 nm readings can be used for Zn^{2+} quantitation in solution.[22] An example of the response obtained by this method and the minimal effect of competing ions is shown in Figure 3. The addition of EDTA to the complexed material demonstrates that the signaling mechanism of this peptide is readily reversible. The presence of two cysteine residues in ZNS1 makes it incompatible with ions such as Cu^{2+}, due to complications from dithiol oxidation. However, the modular nature of the design and the ready access to a variety of metal-coordinating amino acids suggest strategies for remediating this limitation.

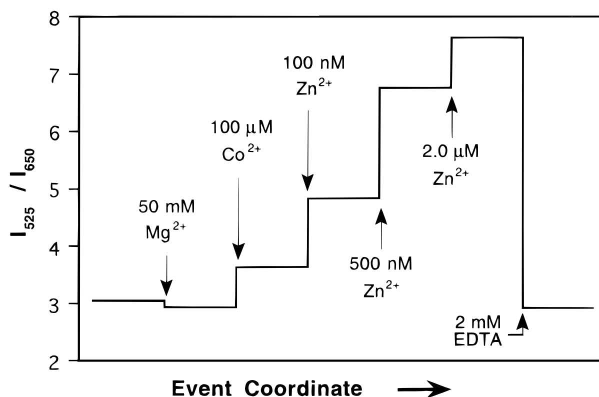


Figure 3. Ratiometric analysis of the fluorescence emission changes upon the addition of various divalent metals. Initial conditions are 1.4 μM ZNS1, 50 mM HEPES, pH 7.0, 3.5 μM EDTA, and $\mu = 0.5$ (NaCl). Arrows represent the addition of concentrated metal stocks to give the indicated composition.

The chemosensor described herein demonstrates the feasibility of exploiting metal-dependent structure modulation as the basis for trace analyte detection. ZNS1 shows excellent affinity for Zn^{2+} relative to the chemical probe Zinquin.[6] The modular design will allow future efforts to focus on the expansion of this concept for the development of new fluorosensors. For example, the metal-binding selectivities of the template can be manipulated by the modification of the metal-coordinating residues. Additionally, other fluorescent probes may be examined to improve the signal range and expand the versatility of the sensor design.

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Supporting Information Available: Amino acid and peptide synthesis protocols and fluorescence emission assay data (5 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(20) CD and UV−vis metal titration experiments verify that ZNS1 binds Co^{2+} with high affinity (K_D 1−5 μM). Competition analysis in the presence of 1.5 μM Zn^{2+} and 100 μM Co^{2+} reveals that less than 5% of the cobalt-bound species remains; therefore the K_D for Zn^{2+} is <1 nM.

(21) Bruland, K. W. In *Trace Elements in Sea Water*; Rilet, J. P., Chester, R., Eds.; Academic Press: London, 1975; pp 157−220.

(22) In the absence of metal ions the emission maximum is at 560 nm. The Zn^{2+} complex has an emission maximum at 525 nm. Since these maxima are not widely separated, ratiometric analysis is carried out at 525 and 650 nm. This analysis normalizes data against bias from sources such as varying instrument response.