J|A|C|S
COMMUNICATIONS

Published on Web 12/24/2003

Emission Ratiometric Imaging of Intracellular Zinc: Design of a Benzoxazole Fluorescent Sensor and Its Application in Two-Photon Microscopy

Masayasu Taki,[†] Janet L. Wolford,[‡] and Thomas V. O'Halloran*[,†,‡]

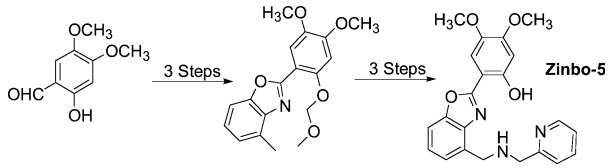
Department of Chemistry and Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, 2145 Sheridan Road, Evanston, Illinois 60208

Received October 15, 2003; E-mail: t-ohalloran@northwestern.edu

The inorganic physiology of intracellular zinc is poorly understood but of emerging importance in understanding a variety of disorders in humans. Histochemical studies of mammalian tissues including prostate,[1] insulin secreting pancreatic islets,[2] and granule neurons of the hippocampus[3−5] reveal patterns of Zn(II) accumulation that are disrupted in some types of prostate cancer, diabetes, and neurodegenerative disorders, respectively. The function of zinc in these tissues or even within single-cell organisms such as *S. cerevisiae* remains controversial.[5−8] Confocal fluorescence microscopy has proved to be a central tool in understanding calcium biology and has the potential to resolve these issues in zinc biology. Two-photon excitation (TPE) fluorescence microscopy provides significant advantages over standard laser confocal approaches by providing deeper sectioning, less phototoxicity, and selective excitation of a smaller focal volume (i.e., femtoliter), thus decreasing background fluorescence.[9] Such advances can be applied to zinc physiology; however, this requires the parallel development of new zinc-specific chemical probes that operate within cells.

Protein-based zinc probes are useful in a variety of physiological experiments,[10,11] but several families of cell permeable organic compounds that undergo a change in fluorescence intensity upon metal binding can be used without microinjection into each cell.[2,12−23] Compounds that also exhibit a shift in the fluorescence excitation or emission maxima upon formation of a 1:1 complex can be used as probes in ratiometric imaging experiments, allowing quantitative measurements of intracellular changes in metal ion concentration. Photophysical studies of bidentate, zinc-responsive benzazoles show promise in fluorescence ratio approaches.[20] Benzofuran-based zinc probes such as ZnAF-R1 and ZnAF-R2 have been used for excitation ratio imaging of a zinc loaded cell,[19] and the coumazin compound also has some excitation ratiometric properties.[23] Here, we describe an emission ratiometric probe for intracellular zinc and demonstrate its utility in TPE microscopy of mammalian cells. The fluorescent zinc reporter Zinbo-5 is cell permeable, binds free Zn^{2+} with a K_d in the nanomolar range, and shows significant zinc-induced changes in quantum yield and in both the excitation and the emission maxima. We further show that Zinbo-5 is well suited for two-photon experiments that readily reveal changes in intracellular zinc within single cells.

The Zinbo family compounds are built around the highly fluorescent benzoxazole core substituted with a variety of Zn-chelating groups.[24] The synthesis of Zinbo-5, which employs the aminomethyl pyridine moiety, involves reaction of a MOM-protected phenol aldehyde derivative with amino-*m*-cresol and oxidation with barium manganate to provide the benzoxazole derivative. Bromination of the methyl group using NBS, coupling with 2-aminomethyl pyridine, and deprotection by *p*-toluenesulfonic acid yielded Zinbo-5.



A mixture of aqueous buffer (50 mM HEPES, pH 7.20, 0.1 M KNO_3) and DMSO (5%, v/v) was used for the UV titration of Zinbo-5 with Zn^{2+}. A significant decrease in the absorption band of the apo at 337 nm ($\epsilon = 2.3 \times 10^4\ M^{-1}\ cm^{-1}$) and an increase of a new band at 376 nm ($\epsilon = 1.9 \times 10^4\ M^{-1}\ cm^{-1}$) was observed with a distinct isosbestic point at 356 nm (Figure S1). The absorption bands at 337 and 376 nm linearly decreased and increased, respectively, up to a 1:1 $[Zn^{2+}]$/[Zinbo-5] ratio, indicating formation of a 1:1 complex (Figure S1).

Figure 1A shows the emission spectra of Zinbo-5 when excited at the isosbestic point of the absorption titration spectrum (356 nm), at various free Zn^{2+} concentrations. The apo form exhibits a characteristic band at 407 nm ($\Phi = 0.02$) that shifts to 443 nm ($\Phi = 0.10$) upon binding with Zn^{2+}. Comparisons within the Zinbo family indicate that the hydroxyl function gives rise to a larger shift relative to the unsubstituted forms (data not shown) consistent with the model studies of Farhni and co-workers.[25] In contrast to those model compounds which exhibit a blue shift in emission upon zinc binding,[20] Zn^{2+} binding to Zinbo-5 leads to a red shift in the emission, suggesting a different photophysical mechanism for the polydentate Zinbo complexes.

The apparent dissociation constant ($K_d = 2.2 \pm 0.1$ nM at pH 7.20) for Zn^{2+} was determined by plotting the fluorescence intensity ratio between 395 and 443 nm (or UV absorbance) against $\log[Zn^{2+}]_{free}$ and fitting these data as previously described[14] (Figures 1B and S2). The corresponding pZn value ($-\log[Zn^{2+}]$)[14] is 9.3, comparable to the affinity of Zinquin,[14] Zinpyr,[26] and ZnAF[19] probes (Table S1). The Zn^{2+} affinity of Zinbo-5 is much higher than that of di(2-picolyl)amine (DPA), indicating that the phenolate oxygen and benoxazole nitrogen are likely chelating the metal as the third and fourth ligands.

A survey of physiologically relevant metal ions reveals that only Zn^{2+} and Cd^{2+} induced an emission shift as shown in Figure 1A, whereas other heavy metal ions, Mn^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}, and Cu^{2+}, quenched the fluorescence of Zinbo-5 (Figure S3). Na^+, K^+, Ca^{2+}, and Mg^{2+} afforded no fluorescent response even at metal concentrations as high as 5 mM. Neither high nor low concentrations of alkali metal and alkaline earth metal ions altered the fluorescent response of Zinbo-5 to Zn^{2+}, suggesting that this probe may be useful in a wide range of biological and microscopic applications.

Two-photon images of mouse fibroblast cells treated with Zinbo-5 show that intracellular zinc can be readily gauged with this probe. Given that no differences are observed between one- and two-photon-excited fluorescence emission or excitation spectra

[†] Department of Chemistry.
[‡] Department of Biochemistry, Molecular Biology, and Cell Biology.

10.1021/ja039073j CCC: $27.50

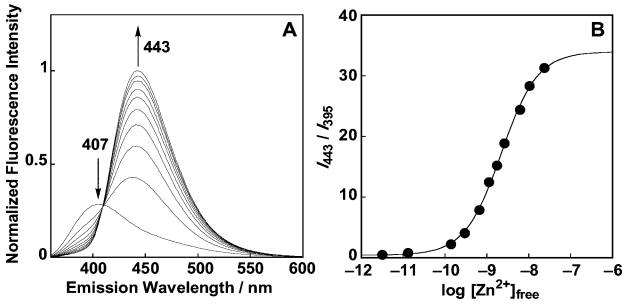


Figure 1. (A) Emission spectra of Zinbo-5 with the excitation at 356 nm in Zn^{2+}/EGTA buffered system (50 mM HEPES, pH 7.20, 0.1 M KNO_3; 10 mM EGTA, 1−9 mM zinc sulfate) at 0, 0.14, 0.29, 0.66, 1.1, 1.8, 2.6, 4.0, 6.1, and 11 nM free Zn^{2+}, respectively. (B) Plots of the fluorescence intensity ratio between 395 and 443 nm (I_{443}/I_{395}) with a best curve for a dissociation constant of 2.2(1) ± 0.1 × 10^{-9} M.

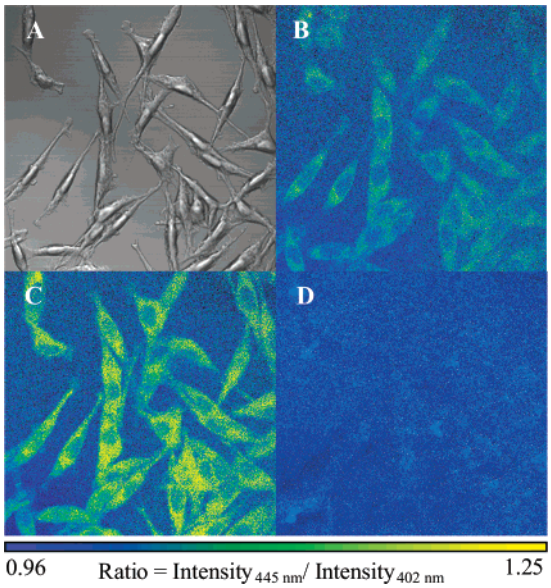


Figure 2. Emission ratio images of fibroblast [L(TK)$^-$] cells in Zinbo-5. (A) Brightfield transmission image; (B) ratio of images collected at 445 and 402 nm; (C) ratio image following a 30 min treatment with 10 μM zinc sulfate and 20 μM pyrithione at pH 7.4, 25 °C, followed by wash with Zinbo-5 stock; (D) ratio image of the same field after a 15 min treatment with 1 mM TPEN.

for molecules lacking a center of symmetry[27] such as Zinbo-5, the one-photon spectra serve as a guide in the TPE experiment. A mode-locked TiSa laser tuned to 710 nm provided optimal intensity for two-photon excitation near the isosbestic point in the UV−vis titration (356 nm) (Figure S1), exciting both the free and the metal-bound forms of the probe. Cells were imaged in the presence of Zinbo-5 using a Zeiss LSM 510 with a META detector allowing data collection in 10.7 nm windows centered at 445 and 402 nm (Figure S4). The image produced by taking the ratio of emission intensities at 445 and 402 nm was multiplied by 30 to increase brightness and scaled in color using NIH Image (Figure 2). For cells grown in standard conditions,[15] the ratio image reveals very low levels of available Zn^{2+} (Figure 2B). The ratio image changes in a manner dependent upon the availability of Zn^{2+} within the cell: the ratio is clearly higher when the intracellular Zn^{2+} is increased by addition of 10 μM zinc sulfate and 20 μM pyrithione, a zinc-selective ionophore (Figure 2C). This corresponds to an average intracellular increase in the ratio of ca. 15%. The signals in these ratio images were shown to originate from the Zn^{2+}/Zinbo-5 complex by treatment with a tighter binding competitor. Treatment of cells with an excess of the cell-permeable, high affinity zinc chelator, *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), decreases the intracellular ratio to equal that of the unbound probe (Figure 2D). This and control experiments on untreated cells indicate that autofluorescence does not contribute to the ratio images.

These emission ratio imaging experiments reveal that Zinbo-5 can readily reveal changes in intracellular zinc availability. The photophysical data further suggest that Zinbo compounds can also be used in epifluorescence excitation ratio imaging approaches to studying zinc in tissues and cells. These methods are particularly amenable to investigation of live tissue samples in real time and are being applied to studies of hippocampus, where physiological fluctuations in synaptic zinc concentration are estimated to be as high as 100−300 μM or as low 2 nM.[5]

Acknowledgment. We thank NIH DK52627 and GM38784 (T.V.O.), JSPS (M.T.), the Robert H. Lurie Comprehensive Cancer Center of Northwestern University, the Rice Foundation, and NSF (CHE-9810378) for financial support, and N. Brown and E. Kawamoto for advice.

Supporting Information Available: Synthesis and characterization of Zinbo-5, UV−vis titration for Zn^{2+}, and experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Sorensen, M. B.; Stoltenberg, M.; Juhl, S.; Danscher, G.; Ernst, E. *Prostate* **1997**, *31*, 125−130.
(2) Gee, K. R.; Zhou, Z. L.; Qian, W. J.; Kennedy, R. *J. Am. Chem. Soc.* **2002**, *124*, 776−778.
(3) Danscher, G. *Histochemistry* **1981**, *71*, 81−88.
(4) Frederickson, C. J.; Suh, S. W.; Silva, D.; Thompson, R. B. *J. Nutr.* **2000**, *130*, 1471S−1483S.
(5) Kay, A. R. *J. Neurosci.* **2003**, *23*, 6847−6855.
(6) Li, L.; Kaplan, J. *J. Biol. Chem.* **2001**, *276*, 5036−5043.
(7) MacDiarmid, C. W.; Milanick, M. A.; Eide, D. J. *J. Biol. Chem.* **2003**, *278*, 15065−15072.
(8) Lee, J. Y.; Cole, T. B.; Palmiter, R. D.; Koh, J. Y. *J. Neurosci.* **2000**, *20*, RC79.
(9) Potter, S. M.; Wang, C. M.; Garrity, P. A.; Fraser, S. E. *Gene* **1996**, *173*, 25−31.
(10) Shults, M. D.; Pearce, D. A.; Imperiali, B. *J. Am. Chem. Soc.* **2003**, *125*, 10591−10597.
(11) Thompson, R. B.; Peterson, D.; Mahoney, W.; Cramer, M.; Maliwal, B. P.; Suh, S. W.; Frederickson, C.; Fierke, C.; Herman, P. *J. Neurosci. Methods* **2002**, *118*, 63−75.
(12) Zalewski, P. D.; Forbes, I. J.; Seamark, R. F.; Borlinghaus, R.; Betts, W. H.; Lincoln, S. F.; Ward, A. D. *Chem. Biol.* **1994**, *1*, 153−161.
(13) Koike, T.; Watanabe, T.; Aoki, S.; Kimura, E.; Shiro, M. *J. Am. Chem. Soc.* **1996**, *118*, 12696−12703.
(14) Fahrni, C. J.; O'Halloran, T. V. *J. Am. Chem. Soc.* **1999**, *121*, 11448−11458.
(15) Nasir, M. S.; Fahrni, C. J.; Suhy, D. A.; Kolodsick, K. J.; Singer, C. P.; O'Halloran, T. V. *J. Biol. Inorg. Chem.* **1999**, *4*, 775−783.
(16) Kimura, E.; Aoki, S. *Biometals* **2001**, *14*, 191−204.
(17) Burdette, S. C.; Walkup, G. K.; Spingler, B.; Tsien, R. Y.; Lippard, S. J. *J. Am. Chem. Soc.* **2001**, *123*, 7831−7841.
(18) Burdette, S. C.; Lippard, S. J. *Coord. Chem. Rev.* **2001**, *216*−*217*, 333−361.
(19) Maruyama, S.; Kikuchi, K.; Hirano, T.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2002**, *124*, 10650−10651.
(20) Henary, M. M.; Fahrni, C. J. *J. Phys. Chem. A* **2002**, *106*, 5210−5220.
(21) Hirano, T.; Kikuchi, K.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2002**, *124*, 6555−6562.
(22) Burdette, S. C.; Frederickson, C. J.; Bu, W.; Lippard, S. J. *J. Am. Chem. Soc.* **2003**, *125*, 1778−1787.
(23) Woodroofe, C. C.; Lippard, S. J. *J. Am. Chem. Soc.* **2003**, *125*, 11458−11459.
(24) Taki, M.; Hitomi, Y.; O'Halloran, T. V. Unpublished work. Northwestern University, Evanston, IL, 2003.
(25) Fahrni, C. J.; Henary, M. M.; VanDerveer, D. G. *J. Phys. Chem. A* **2002**, *106*, 7655−7663.
(26) Walkup, G. K. B., S. C.; Lippard, S. J.; Tsien, R. Y. *J. Am. Chem. Soc.* **2000**, *122*, 5644−5645.
(27) (a) Fan, G. Y.; Fujisaki, H.; Miyawaki, A.; Tsay, R. K.; Tsien, R. Y.; Ellisman, M. H. *Biophys. J.* **1999**, *76*, 2412−2420. (b) Xu, C.; Webb, W. W. *J. Opt. Soc. Am. B* **1996**, *13*, 481−491.

JA039073J