A Fluorescent Zinc Probe Based on Metal-Induced Peptide Folding

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A Fluorescent Zinc Probe Based on Metal-Induced Peptide Folding

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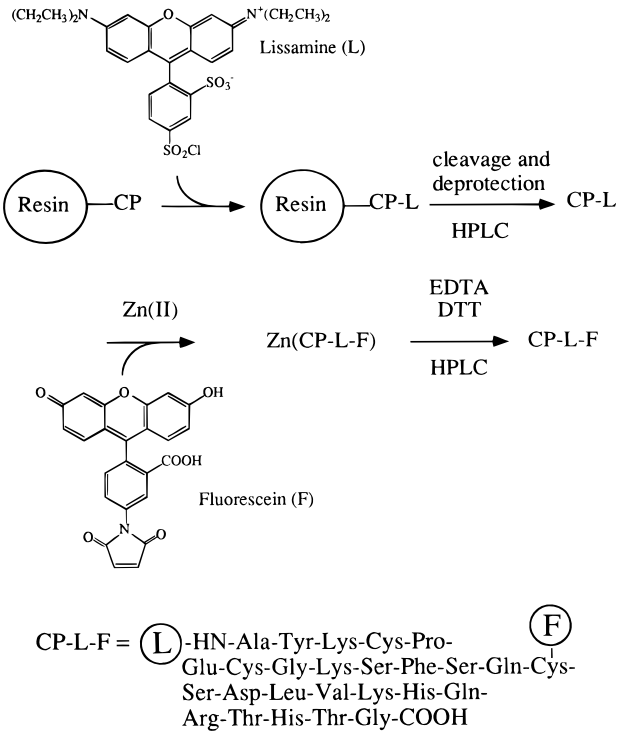
Fluorescent indicators have provided valuable insights into the physiological effects of changes in the concentrations of ions in biological systems.[1] Ratioable fluorescent dyes, such as the fura probes for Ca(II),[2,3] allow the analyte to be quantitated independent of the concentration of the dye, the efficiency of the instrumentation, and the thickness of the sample, and thus have revolutionized fluorescence microscopy.[4] Recently, Tsien and co-workers have reported such an indicator for adenosine 3′,5′-cyclic monophosphate (cAMP) based on intermolecular fluorescence resonance energy transfer and have used this probe to image levels of cAMP in living cells.[5]

Because zinc is an important structural component of many proteins that bind to DNA and because it has recently been proposed that Zn(II) plays a role in signaling in mitosis and suppressing apoptosis in cells,[6] there is a need for sensitive and selective ratioable fluorescent probes for Zn(II). Zinc levels are difficult to monitor, both because Zn(II) (d^{10}) is a closed shell ion and because few Zn(II) indicators exist. Herein, we report a new ratioable fluorescent probe for Zn(II) that is based on the zinc finger consensus peptide, CP. The activity of this dye is based on changes in fluorescence energy transfer due to the metal-induced folding of this peptide.

Zinc finger peptides typically bind zinc tightly and selectively and hence provide an ideal framework for a selective zinc probe. The zinc finger consensus peptide (CP) binds Zn(II) with a K_d of 5.7 ($\pm$1.3) $\times$ 10^{-12} M at pH 7.0.[7] CP discriminates well against other metal ions by factors of 1.1 $\times$ 10^4, 4.4 $\times$ 10^5, and 2.8 $\times$ 10^5 for Co(II), Fe(II), and Ni(II), respectively.[7,8] Furthermore, the affinity for metal ions can be modulated by changes in the amino acid sequence.[7,9] Because of the spectroscopic properties of Zn(II), unmodified CP is not an effective zinc probe. Eis and Lakowicz have modified a zinc finger peptide with Trp and a single fluorescent dye and demonstrated zinc-dependent changes in fluorescence.[10] However, this peptide requires excitation in the ultraviolet making it unsuitable for *in vivo* applications. We have modified CP with two fluorescent dyes, fluorescein (F) as the donor and lissamine (L) as the acceptor, to "visualize" zinc binding. In the absence of Zn(II), the peptide is unfolded and the dyes are relatively far apart; the amount of intramolecular energy transfer between the chromophores is small. Upon binding Zn(II), the peptide folds, bringing the fluorophores closer together and increasing the amount of intramolecular energy transfer.[10,11]

Scheme 1. Labeling of Consensus Zinc Peptide, CP, with the Fluorescent Dyes Lissamine (L) and Fluorescein (F)[a]



[a] While the peptide was still attached to the resin, lissamine sulfonyl chloride (L) was conjugated to the amino terminus. The singly labeled peptide was cleaved off the resin and purified by reverse phase HPLC. The peptide (CP-L) was treated with Zn(II) to protect the metal-binding cysteine residues (positions 4 and 7) and was then reacted with fluorescein maleimide (F), which conjugated to the unprotected Cys residue at position 14. The doubly labeled peptide (CP-L-F) was demetalated using EDTA, reduced using dithiothreitol (DTT), and purified by HPLC.

The zinc finger peptide was synthesized via solid phase peptide synthesis, using Fmoc chemistry;[12] the dyes were subsequently conjugated to the peptide (Scheme 1). The identity of the probe, CP-L-F, was confirmed using mass spectrometry.[13] The spectrum of CP-L-F contains two strong absorptions (495 and 578 nm) corresponding to conjugated fluorescein and lissamine, respectively. The fluorescence emission spectrum contains two maxima (521 and 596 nm) that likewise correspond to fluorescein and lissamine as shown in Figure 1. Because the fluorescence spectrum at these two wavelengths changes differentially upon addition of zinc, the probe is ratioable, making it suitable for fluorescence ratio imaging microscopy.[4] Furthermore, the absorbance and fluorescence of CP-L-F are in regions of the visible spectrum that are not obscured by normal cellular components, making this probe an excellent candidate for *in vivo* studies.

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(11) An analog in which the acceptor was texas red (TR) was also synthesized (CP-TR-F). Texas red (absorption maximum 600 nm in CP-TR-F) has a smaller spectral overlap with fluorescein than does lissamine (absorption maximum 578 nm in CP-L-F). Less intramolecular energy transfer was observed for CP-TR-F than for CP-L-F. As a result, CP-L-F shows larger changes in fluorescence in response to zinc binding.

(12) The sequence of CP is the following: NH_2-Ala-Tyr-Lys-Cys-Pro-Glu-Cys-Gly-Lys-Ser-Phe-Ser-Gln-Cys-Ser-Asp-Leu-Val-Lys-His-Gln-Arg-Thr-His-Thr-Gly-COOH. This peptide differs from the previously reported consensus zinc finger peptide (see refs 7 and 8) by only two amino acid residues (^{1}Pro → Ala and ^{14}Lys → Cys).

(13) ElectroSpray mass spectrometry of CP-L-F was performed by PeptidoGenic Research & Co., 5031 Preston Ave., Livermore, CA 94550, using a Sciex *API* I ElectroSpray Mass Spectrometer.

S0002-7863(96)01184-5 CCC: $12.00

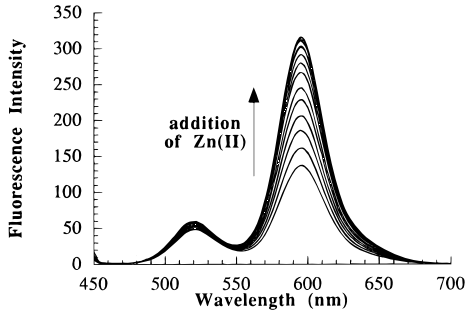

Figure 1. Zn(II) titration of CP-L-F.[14] Fluorescence emission spectra for excitation at 430 nm. The initial concentration of CP-L-F was 3.7 μM; Zn(II) was added in aliquots of 0.1 molar equiv relative to CP-L-F (11.3 nmol).

Zinc binding to CP-L-F was monitored by observing the fluorescence spectrum as a function of zinc concentration[14] as shown in Figure 2. The high affinity of CP-L-F was confirmed via competition experiments with unmodified CP. The dissociation constant for Zn(CP-L-F) was estimated to be 1×10^{-12} M at pH 7.1. These results suggest that the incorporation of the fluorescent dyes has not adversely affected the metal binding and folding properties of the zinc finger peptide. Thus, the new probe takes advantage of the highly efficient, specific, and tunable zinc binding characteristics of these peptides while introducing intense, ratioable fluorescence above 450 nm. With the advent of new fluorescent probes for zinc such as the one presented herein, a variety of studies investigating the role of zinc in cellular processes should now be feasible.

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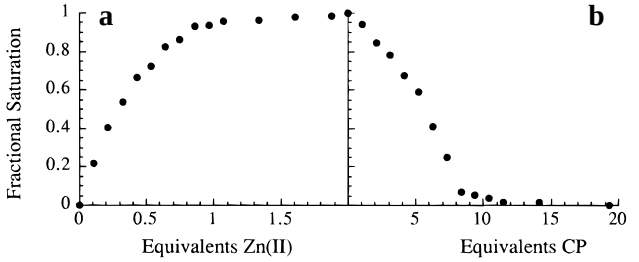

Figure 2. (a) Fractional saturation of Zn(CP-L-F) as a function of Zn(II) added, calculated from the spectra presented in Figure 1. The initial slope and the curvature revealed the formation of both 1:1 and 2:1 peptide to metal complexes. The formation of 2:1 peptide to metal complexes has been observed previously for other zinc finger peptides.[15] Only 1:1 complex persists in the presence of excess zinc(II). (b) The effect of unlabeled CP on the fluorescence due to CP-L-F (4.7 μM).[16] Equivalents given are relative to total moles of CP-L-F (14.6 nmol). These data indicate a dissociation constant of approximately 1×10^{-12} M for Zn(CP-L-F).

Note Added in Proof: A report of an independently developed zinc sensor based on similar principles has recently been reported (Walkup, G. K.; Imperiali, B. *J. Am. Chem. Soc.* **1996**, *118*, 3053−3054).

Supporting Information Available: Figure depicting the ratio of fluorescence emission intensities (595 nm/560 nm) of the probe as a function of total zinc concentration (1 page). See any current masthead page for ordering and Internet access instructions.

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(14) These spectra are obtained by exciting into the fluorescein absorption (430 nm) of CP-L-F (3−5 μM in 100 mM HEPES, 50 mM NaCl, pH 7.1 buffer) and examining the fluorescence emission as a function of wavelength. All peptide manipulations were performed under an atmosphere of 95% nitrogen−5% hydrogen to avoid peptide oxidation and quenching of the fluorescence by molecular oxygen.

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(16) A solution of CP was added to the sample containing CP-L-F (4.7 μM) and 2 equiv of Zn(II) (relative to CP-L-F). The resulting fluorescence spectra, which reflect a linear combination of the spectra of CP-L-F and Zn(CP-L-F), were deconvoluted to yield the fractional saturation of Zn(CP-L-F) as a function of the number of equivalents of CP (relative to CP-L-F). These fractional saturation data were used to determine the dissociation constant for Zn(CP-L-F) assuming that the dissociation constant for ZnCP is 5.7×10^{-12} M. The samples were equilibrated for 15 min at 37 °C at each point.