Highly Zinc-Selective Fluorescent Sensor Molecules Suitable for Biological Applications

Tomoya Hirano, Kazuya Kikuchi, Yasuteru Urano, Tsunehiko Higuchi, and Tetsuo Nagano*

Graduate School of Pharmaceutical Sciences The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku Tokyo 113-0033, Japan

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Zinc (Zn^{2+}) is the second most abundant heavy metal ion after iron, and it is an essential component of many protein scaffolds (e.g., carbonic anhydrase and zinc finger proteins).[1] Chelatable Zn^{2+} is released from nerve terminals by excitatory signals,[2] and binds to the *N*-methyl-D-aspartate (NMDA) receptor, changing its function.[3] Zn^{2+} is co-stored with insulin in secretory vesicles of pancreatic β-cells, and is released when insulin is secreted.[4] Zn^{2+} also suppresses apoptosis,[5] and induces the formation of β-amyloid,[6] which is thought to be related to the etiology of Alzheimer's disease.

Although Zn^{2+} has many important cellular roles, little is known about the cellular regulation of Zn^{2+} in comparison with other cations such as Ca^{2+}, Na^{+}, K^{+}, etc. Therefore, several chemical tools for measuring Zn^{2+} in living cells have recently been developed to clarify its physiological significance.[7−14] There are two types of fluorescent sensor molecules for Zn^{2+}, one based on a quinoline structure excitable with UV light (TSQ,[7] Zinquin,[8] and TFLZn[9]) and the other based on fluorescein excitable with visible light.[10−12] A cell-permeable Zn^{2+} sensor molecule based on fluorescein (Zinpyr-1) was reported recently.[10] Zinpyr-1 fluoresces strongly upon addition of Zn^{2+} to cells. However, it has the disadvantages that the basal fluorescence is high (quantum yield, 0.39) and is pH-sensitive with a pK_a of 8.3. Thus, the fluorescence can be changed by intracellular pH changes under physiological conditions, and such pH changes are observed in many cells exposed to certain biological stimuli.[15]

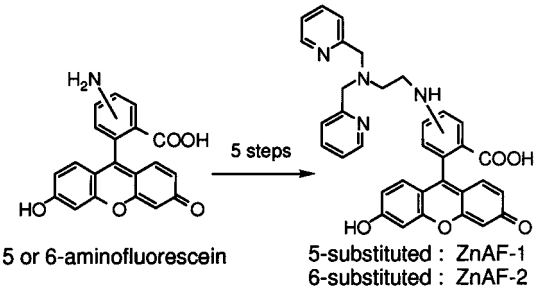

Figure 1. Structure of newly synthesized fluorescent Zn^{2+} sensor molecules.

Fluorescein is a convenient fluorophore for biological experiments, since it has a high fluorescence quantum yield in aqueous solution, and its excitation wavelength is in the visible range, which does not cause severe cell damage. This wavelength is free from interference by autofluorescence from biological molecules and is also suitable for fluorescence confocal microscopy with an Ar laser. We have explained the fluorescence quenching of aminofluorescein in terms of photoinduced electron transfer (PET).[11] Aminofluorescein itself does not fluoresce due to the high HOMO level of its electron-donating group. If this electron donation is hindered by complex formation with a cation using the lone pair of nitrogen or by chemical conversion to a less electron-donating group, the HOMO level is lowered, resulting in fluorescence with a high quantum yield. On the basis of this approach, we have developed several fluorescent sensor molecules for nitric oxide,[16] singlet oxygen,[17] and Zn^{2+}.[11] This method is advantageous for providing a wide range of sensor molecules, because if we can design and synthesize a suitably specific reactive moiety which is linked to aminofluorescein, we can obtain sensor molecules suitable for different types of analytes. It is also advantageous that the fluorescence quantum yield of aminofluorescein is very low, so if the sensor molecule is converted to fluorescent form, the measured fluorescence intensity is essentially entirely due to the analyte.

We have already reported fluorescent Zn^{2+} sensor molecules, ACF-1 and ACF-2, which are excitable with visible light, for biological applications.[11] However, improvements are desirable in two respects, i.e., the slow complex formation rate and the small quantum yield. ACFs require about 100 min for completion of the complex formation due to the properties of the macrocyclic polyamine ring, which is the acceptor of Zn^{2+}. We therefore designed ZnAF-1[18] and ZnAF-2, utilizing *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) as the acceptor of Zn^{2+} (Figure 1). Fluorescein was employed as a fluorophore instead of 6-hydroxy-9-phenylfluorone, the fluorophore of ACFs, because of its larger quantum yield.

ZnAF-1 and ZnAF-2 were synthesized from the corresponding aminofluoresceins by using 4-nitrobenzenesulfonyl chloride for alkylation of the amine.[19] At pH 7.5 (100 mM HEPES buffer, $I = 0.1$ ($NaNO_3$)), both compounds showed almost no fluorescence. However, upon addition of Zn^{2+}, the fluorescence intensity was increased by 17-fold for ZnAF-1 and 51-fold for ZnAF-2 (Table 2). The fluorescence intensity of the free bases was little influenced by pH. Thus, the basal fluorescence intensity should be little affected by physiological pH changes. The excitation and

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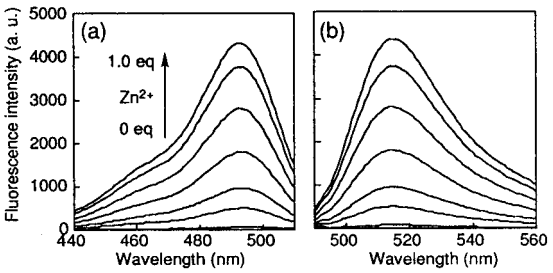

Figure 2. (a) Excitation spectra (emission at 514 nm) and (b) emission spectra (excitation at 492 nm) of ZnAF-2 (5 μM) in the presence of various concentrations of Zn^{2+} ranging from 0 to 5.0 μM. These spectra were measured at pH 7.5 (100 mM HEPES buffer, $I = 0.1$ ($NaNO_3$)).

Table 1. Chemical Properties of ZnAFs with and without Zn^{2+} [a]

	free		Zn^{2+}		
compd	ϵ[b]	Φ[c]	ϵ[b]	Φ[c]	Kd (M)
ZnAF-1	7.4×10^4 (489)	0.022	6.3×10^4 (492)	0.23	7.8×10^{-10}
ZnAF-2	7.8×10^4 (490)	0.023	7.6×10^4 (492)	0.36	2.7×10^{-9}

[a] All data were obtained at pH 7.5 (100 mM HEPES buffer, $I = 0.1$ ($NaNO_3$)). [b] ϵ stands for extinction coefficient (M^{-1} cm^{-1}). Measured at each λ_{max}, which is shown in parentheses (nm). [c] Φ stands for quantum yield, determined using Φ of fluorescein (0.95) in 0.1 N NaOH as a standard.[20]

Table 2. Selectivity of ZnAF-1, ZnAF-2, and Me-ZnAF-1 toward Other Cations[a]

compd	free	Zn^{2+} [b]	Cd^{2+} [b]	Co^{2+} [b]	Ni^{2+} [b]	Ca^{2+} [c]	Mg^{2+} [c]
ZnAF-1	110	1881	181	118	218	104	115
ZnAF-2	80	4105	1462	173	741	89	116
Me-ZnAF-1	61	4039	2398	121	225	90	82

[a] All data were obtained at pH 7.5 (100 mM HEPES buffer, $I = 0.1$ ($NaNO_3$)) and expressed as arbitrary units. [b] Those ions (5 μM) were added to 5 μM sensor molecule. [c] Those ions (5 mM) were added to 5 μM sensor molecule.

emission maxima did not change upon addition of Zn^{2+}: excitation at 492 nm and emission at 514 nm for both ZnAF-1 and ZnAF-2 (Table 1 and Figure 2). The apparent dissociation constant, K_d, was determined as 0.78 nM (ZnAF-1) or 2.7 nM (ZnAF-2) using Zn^{2+} and pH-buffered solutions (Table 1).[21] These values indicate that these molecules can be used in the sub-nM range, which is the same as that for Zinquin in undifferentiated eukaryotic cells.[22] The detection limit of ZnAFs is also in the sub-nM range, which affords sufficient sensitivity for application in mammalian cells.

Selectivity toward other cations was next examined (Table 2). Although the Zn^{2+}-induced fluorescence augmentation was smaller with ZnAF-1 than with ZnAF-2, the selectivity of ZnAF-1 against Cd^{2+} was much higher than that of ZnAF-2 (Table 2). Upon addition of Cd^{2+}, the fluorescence intensity of ZnAF-1 almost did not increase. However, other fluorescent sensor molecules for Zn^{2+} fluoresced upon addition of Cd^{2+}, for example, 10-fold for Zinquin[8] and 6-fold for Newport Green.[12] ZnAF-1 is the first Zn^{2+} sensor molecule that can distinguish Cd^{2+} from Zn^{2+}. This selectivity may originate from the carboxylic acid moiety on the benzene ring. We speculate that the mechanism may be as follows. When Zn^{2+} is added to ZnAF-1, the four nitrogen atoms of the acceptor, *N*,*N*-bis(2-pyridylmethyl)ethylenediamine, form a four-coordinate complex, resulting in strong fluorescence augmentation. However, the ionic radius of Cd^{2+} is larger than that of Zn^{2+}, so carboxylate coordinates instead of nitrogen adjacent to the benzene ring. This blocks the on−off mechanism of fluorescence, resulting in no fluorescence augmentation, because there is no large HOMO change. In the case of ZnAF-2, the carboxylate cannot participate in complex formation because of its geometry. To test this hypothesis, the carboxylate of ZnAF-1 was derivatized to its methyl ester using methanol and concentrated sulfuric acid[23] to hinder the participation of the carboxylate in complex formation. The selectivity of ZnAF-1 methyl ester (Me-ZnAF-1) against Cd^{2+} was almost the same as that of ZnAF-2, supporting the idea that the carboxyl group is important for the selective augmentation of fluorescence intensity by Zn^{2+}. Neither Fe^{2+} nor Fe^{3+} enhanced the fluorescence intensity. Cu^{2+} formed complexes with ZnAF-1 and ZnAF-2, resulting in quenching of fluorescence. Other cations, which exist at high concentration under physiological conditions, Ca^{2+}, Mg^{2+}, Na^+, and K^+, did not enhance the fluorescence intensity even at very high concentration (5 mM) (Table 2). It was also confirmed that these ions did not interfere with Zn^{2+}-enhanced fluorescence (data not shown).

The complexes of ZnAFs and Zn^{2+} were formed immediately upon Zn^{2+} addition, as determined from the time course of fluorescence intensity.[24] This characteristic is convenient for biological applications. The fluorescence intensity of the Zn^{2+} complex was dependent on pH, with a pK_a value of 6.2 for both ZnAF-1 and ZnAF-2,[24] but was almost stable over the physiological pH range.

To determine the cell permeability of ZnAFs, cultured macrophage (RAW 264.7) were incubated with PBS (Phosphate Buffered Saline) containing ZnAF-2. As a result, the cells did not stain, that means ZnAF-2 could not permeate through the cell membrane. So we prepared a diacetyl derivative of ZnAF-2 (ZnAF-2 DA), which was synthesized from ZnAF-2, acetic anhydride, and cesium carbonate.[16] ZnAF-2 DA was more lipophilic, and once ZnAF-2 DA permeates into the cell, it will be transformed into ZnAF-2 by esterase in the cytosol. RAW 264.7 were incubated with PBS containing ZnAF-2 DA, and the cells stained by ZnAF-2 (data not shown). These data indicate that ZnAF-2 DA can be used as a cellular probe for Zn^{2+}.

In conclusion, we have developed new fluorescent Zn^{2+} sensor molecules, ZnAFs, which possess the characteristics of improved selectivity and faster complex formation. Since these sensor molecules only fluoresce after the coordination with Zn^{2+}, they should be useful for studies on the biological functions of Zn^{2+}.

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Supporting Information Available: Synthesis, experimental details, and characterization of ZnAF-1 and ZnAF-2 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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