Design and Synthesis of Metallopeptide Sensors: Tuning Selectivity with Ligand Variation

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We chose a fluorescent pentapeptide sensor (-CPGHE) containing a dansyl fluorophore as a model peptide and investigated whether the selectivity and sensivity of the peptides for heavy and transition metal ions could be tuned by changing amino acid sequence. In this process, we developed a selective peptide sensor, **Cp1-d** (-HHPGE, K_d = 670 nM) for detection of Zn^{2+} in 100% aqueous solution and a selective and sensitive peptide sensor, **Cp1-e** (-CCHPGE, K_d = 24 nM) for detection of Cd^{2+} in 100% aqueous solution. Overall results indicate that the selectivity and sensitivity of the metallopeptide sensors to specific heavy and transition metal ions can be tuned by changing amino acid sequence.

Key Words: Peptides, Zn^{2+}, Cd^{2+}, Peptide sensor, Chemical sensor

Introduction

Many metalloproteins that was complexed with heavy and transition metal ions are essential in various types of biological processes.[1-2] Design of biopolymers provides the bioinorganic chemist a powerful tool toward the goal of addressing interesting questions. For example, how is metal-binding affinity of proteins and peptides tuned by primary amino acid sequences? How does the structural context of a metal binding site of the biopolymers affect metal binding ability?

Previously, we reported that a small peptide sensor (**Cp1**) that mimicked the zinc binding sites of metalloproteins.[3] The peptide sensor containing His, Glu, and Cys residues as a ligand showed a turn on response to the specific transition metal ions (Zn^{2+} and Cd^{2+}) in 100% aqueous solution. Furthermore, we synthesized several peptide sensors that had different secondary structures and found out that metal-binding affinity of the peptide sensors could be tuned by specific secondary structures.[4,5]

In the present study, we investigated whether or not metal-binding affinity of the small peptide sensors could be tuned by modulating amino acid sequence. To investigate the role of amino acids for selective monitoring of Zn^{2+} ions, we chose **Cp1** (-CPGHE) as a model peptide and synthesized several peptide sensors, as shown in Table 1. We measured the emission spectra of the peptide sensors in the presence of various metal ions in HEPES buffer solution at pH 7.4. Overall results indicated that the position of the amino acids (His, Cys and Glu) in the peptide moiety in the sensors plays an important role in the binding affinity to specific metal ions rather than the selectivity to various metal ions. Furthermore, we confirmed the selectivity and sensitivity of the peptide sensors for heavy and transition metal ions could be tuned by changing amino acids. **Cp1-d** (-HHPGE) among the peptide series showed the great selectivity for Zn^{2+} over Cd^{2+}. Specially, **Cp1-e** (-CCHPGE) unlike the other peptide sensors shows more potent binding affinity to Cd^{2+} than Zn^{2+} ions.

Results and Discussion

The peptide sensors (**Cp1-a** to **Cp1-c**) were synthesized to

elucidate the relationship between the metal binding affinity and the position of specific amino acids (His, Cys, and Glu). As a pre-organization of the ligand site of the peptides is required for the selective monitoring of metal ions,[3,5] all peptide sensors employed in this study commonly contained Pro-Gly amino acids to have pre-organized structure.[6] As shown in Scheme 1, 1,5-dimethylaminonaphthalene sulfonamide (dansyl) group as a fluorophore was introduced to the N-terminal of the peptides because the dansyl group can display a large Stokes shift along with varying quantum yield by changing its local environment and the dansyl fluorophore of various chemical sensors has been used to effectively detect metal ions by chelation enhanced fluorescence (CHEF) effect.[7]

We synthesized three peptides consisting of the same amino acid compositions (Cys, Pro, Gly, His, and Glu), as shown in Table 1. **Cp1-d** containing two His residues and Glu as a ligand
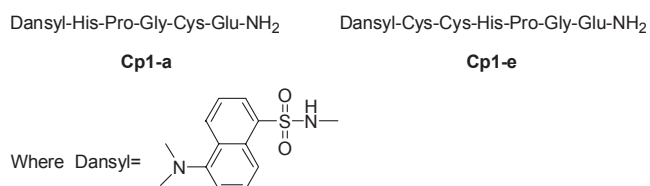

Scheme 1. Chemical structures of representative peptide sensors

Table 1. Sequences, mass, and retention time of peptide sensors

S. No.	Name	Sequence	Cal. Mass/ Obs. Mass $(M+H^+)$	Retention time (min.)
1	**Cp1-a**	Dansyl-HPGCE-NH₂	773.26/773.97	23.85
2	**Cp1-b**	Dansyl-CHPGE-NH₂	773.26/773.95	24.05
3	**Cp1-c**	Dansyl-EPGCH-NH₂	773.26/773.84	23.08
4	**Cp1-d**	Dansyl-HHPGE-NH₂	807.25/807.95	14.57
5	**Cp1-e**	Dansyl-CCHPGE-NH₂	876.20/876.91	18.55

Solvent A was water containing 0.1% (v/v) TFA and solvent B was acetonitrile containing 0.1% (v/v) TFA. The peptides were analyzed with a linear gradient of 5-50% B over 45 min.
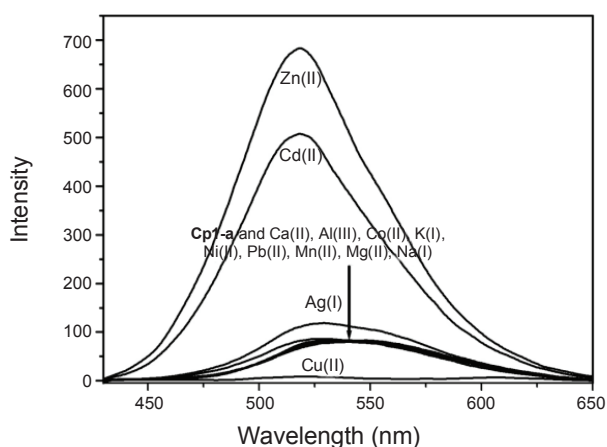
Figure 1. Fluorescence spectra of **Cp1-a** (5 μM) in 10 mM HEPES buffer solution (pH = 7.4) in the presence of various metal ions (1 equiv).

was synthesized because His is frequently found as a ligand in metal binding site of several metalloproteins. We synthesized **Cp1-e** in which one more Cys residue was appended in the peptide moiety of **Cp1-e**. The peptide sensors were synthesized in solid-phase peptide synthesis with Fmoc chemistry.[8] After cleavage of the crude product from resin, each peptide was purified using semi-preparative HPLC with a C_{18} column. The successful synthesis and purity (> 95%) were confirmed by analytical HPLC with a C_{18} column and an ESI mass spectrometer.

As the peptides are fully water soluble, the fluorescence spectrum of the peptides was measured in 100% aqueous media. Fluorescence spectra ranging from 350 to 650 nm were recorded with excitation at a wavelength of 330 nm. To investigate metal selectivity of the peptides, we measured the fluorescence spectrum of the peptides in the presence of various metal cations (Ca^{2+}, Cd^{2+}, Co^{2+}, Pb^{2+}, Cu^{2+}, Ag^+, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+} as perchlorate anion and Na^+, Al^{3+}, K^+, as chloride anion). Figure 1 shows fluorescent emission spectra of **Cp1-a** (5 μM) in 10 mM HEPES buffer solution at pH 7.4 containing each metal ion (1 equiv).

Cp1-a like **Cp1** shows a considerable response with Zn^{2+}, Cd^{2+}, and Cu^{2+}. The emission intensity at 515 nm increased in the presence of Zn^{2+} and Cd^{2+}, whereas the emission intensity at 515 nm decreased in the presence of Cu^{2+}. Figure 2 shows

emission intensity change of **Cp1-a**, **Cp1-b** and **Cp1-c** in 10 mM HEPES buffer solution at pH 7.4 containing each metal ion (1 equiv). **Cp1-b** and **Cp1-c** also exhibited turn on response to Zn^{2+} and Cd^{2+}, but showed turn off response to Cu^{2+}.

This result indicates that the position of Cys, His, and Glu in the peptide moiety of the peptide sensors did not play a critical role in the selectivity for Zn^{2+}, Cd^{2+}, and Cu^{2+}. Generally, chemical or peptide sensors are rarely able to differentiate between Zn^{2+} and Cd^{2+} due to the similar size and the same positive charge.[9] However, the sensitivity for specific metal ions seemed to depend on the position of the amino acid of the peptide sensors. **Cp1-a** and **Cp1-b** like **Cp1** showed more sensitive response to Zn^{2+} than Cd^{2+}, whereas **Cp1-c** exhibited similar response to Zn^{2+} and Cd^{2+}, as shown in Figure 2.

Emission spectra of **Cp1-d** (-HHPGE) were measured in the presence of various metal ions, as shown in Figure 3. Interestingly, **Cp1-d** containing two His residue and a Glu residue as a ligand exhibited a great selectivity for Zn^{2+} over Cd^{2+} in comparison to the other peptides (**Cp1**, **Cp1-a**, **Cp1-b**, and **Cp1-c**). Considering the fact that almost all chemical sensors suffer from the selectivity between Zn^{2+} and Cd^{2+}, this result shows important information for the design of chemical sensors that have a great selectivity for Zn^{2+} over Cd^{2+}. The result strongly suggests that a Cys residue as a soft ligand plays an important role in the selective and sensitive response of the peptides to Cd^{2+} due to the more softness of Cd^{2+} than Zn^{2+}. To confirm this suggestion, we synthesized **Cp1-e** (-CCHPGE) containing two Cys residues, His and Glu as a ligand and measured emission spectra in the presence of various metal ions. As we expected, **Cp1-e** shows more sensitive response to Cd^{2+} than Zn^{2+}. Overall results revealed that the selectivity and sensitivity of the metallo-peptides to specific heavy and transition metal ions can be tuned by changing amino acid sequence.

The previous reported peptide sensors containing a His residue showed a turn off response to Cu^{2+} because GlyGlyHis and GlyHis are general peptide motifs originated from the amino terminal Cu^{2+} and Ni^+ binding (ATCUN) site.[10] Thus, it is not surprising that all peptide sensors containing at least one His residue employed in this study showed a turn off response to Cu^{2+}. Cu^{2+} as a paramagnetic metal ion quenches fluorescence emission.[11] **Cp1-d** and **Cp1-e** among the peptide series, shows most sensitive response to Cu^{2+}. This can be explained by the
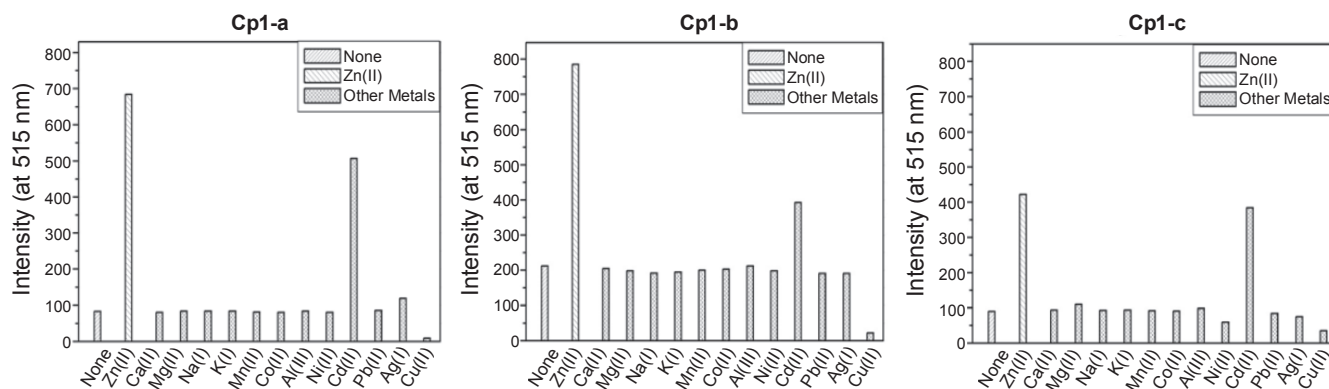
Figure 2. Emission intensity (I_{515nm}) change of peptide sensors (5 μM) in the presence and absence of various metal ions (1 equiv) in 10 mM HEPES buffer solution (pH 7.4), λ_{ex} = 330 nm.
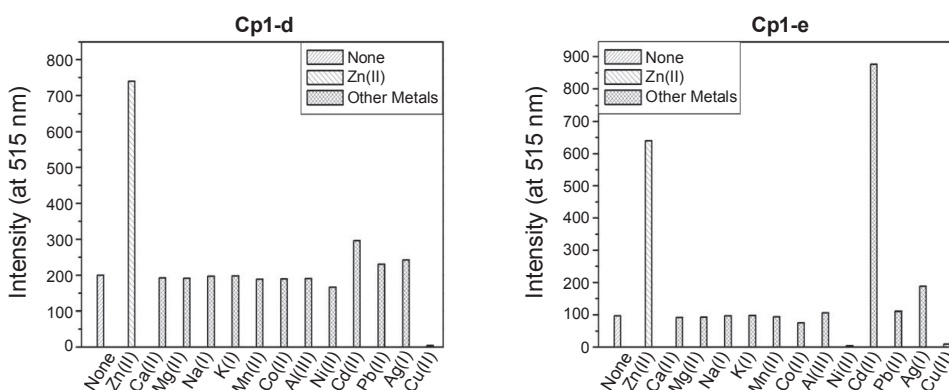
Figure 3. Emission intensity (I_{515nm}) change of peptide sensors (5 μM) in the presence and absence of various metal ions (1 equiv) in 10 mM HEPES buffer solution (pH 7.4), λ_{ex} = 330 nm.
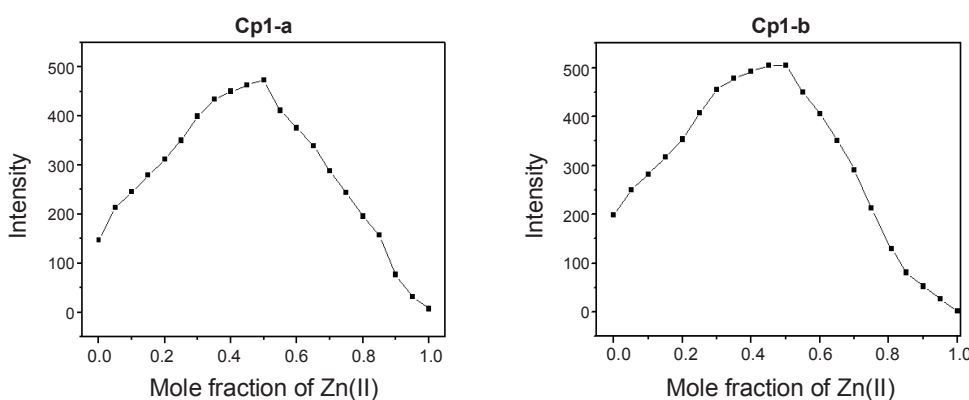

Figure 4. A Job plot for the representative peptides. The total concentration of peptide and Zn^{2+} is 2.5 μM.

fact that **Cp1-d** contained two His residues and **Cp1-e** contained His residues as well as two Cys residues for interaction with Cu^{2+}.

We measured the binding affinities of the peptides for Zn^{2+} because the model peptide, **Cp1** displayed a great sensitivity for Zn^{2+} and zinc ions play an important role in biological functions, such as gene expression, apoptosis, enzyme regulation, and amyloid fibril formation.[12] Previous reported **Cp1** formed a 1:1 complex in the interaction with Zn^{2+} and Cd^{2+}, respectively on the basis of Job's plot analysis. Thus, we did Job's plot analysis of the peptide sensors. As shown in Figure 4, the

emission intensity of the complex between the peptides and Zn^{2+} exhibits a maximum at 0.5 mol fraction, which supports that the peptides form 1:1 complex with Zn^{2+}.

In addition, nonlinear least-squares fitting of the titration profiles based on the 1:1 binding model strongly supports that the peptide sensors forms 1:1 complex with Zn^{2+}.[3] The dissociation constant of the peptide sensors was calculated based on the titration curve with Zn^{2+} by non-linear least square fitting. Assuming 1:1 complex formation, the dissociation constant was calculated based on the titration curve with Zn^{2+} by non-linear least square fitting, as shown in Figure 5.
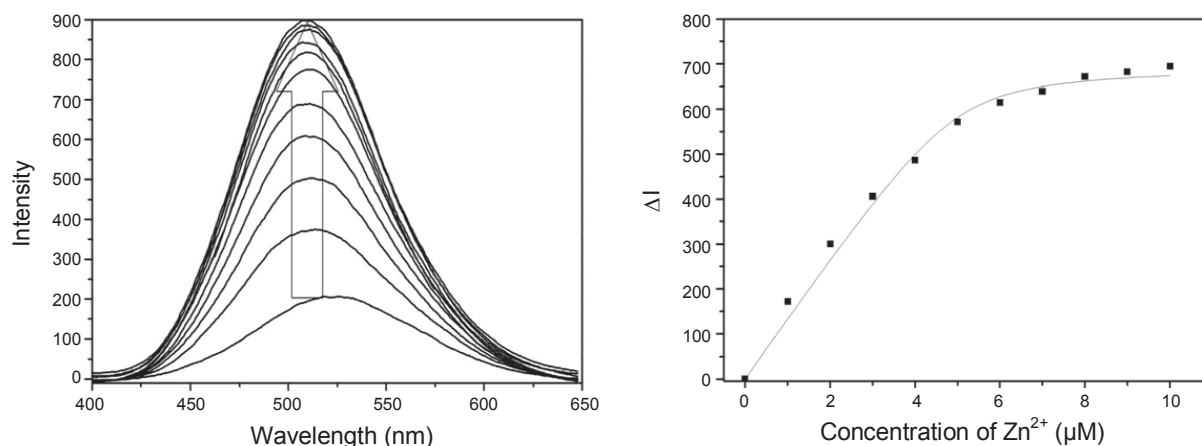
Figure 5. Emission spectra and titration curve of **Cp1-b** (5 μM) in 10 mM HEPES buffer solution (pH 7.4).

Table 2. Dissociation constants of the peptide sensors for specific metal ions

Name	Sequences	Metal Ion	K_d Value (M) (R_2)
Cp1	Dan-CPGHE-NH$_2$	Zn (II)	3.71×10^{-7} (0.97322)
Cp1-a	Dan-HPGCE-NH$_2$	Zn (II)	4.24×10^{-7} (0.98528)
Cp1-b	Dan-CHPGH-NH$_2$	Zn (II)	1.58×10^{-7} (0.99217)
Cp1-c	Dan-EPGCH-NH$_2$	Zn (II)	1.67×10^{-6} (0.82611)
Cp1-d	Dan-HHPGE-NH$_2$	Zn (II)	6.66×10^{-7} (0.97997)
Cp1-e	Dan-CCHPGE-NH$_2$	Cd (II)	2.38×10^{-9} (0.98947)
		Zn (II)	4.98×10^{-8} (0.96998)

Table 2 summaries the dissociation constants of the peptide sensors for Zn^{2+}. The K$_d$ values indicated the binding affinities of the peptide sensors in 100% aqueous solutions were more potent than those of the other chemical sensors for Zn^{2+}.[13] The binding affinity of the peptides was potent enough to detect micromolar range of Zn^{2+} in aqueous solution. Even though the peptides (**Cp1**, **Cp1-a**, **Cp1-b**, **Cp1-c** and **Cp1-d**) had the same amino acid composition, the K$_d$ values of the peptides were different. The K$_d$ value of **Cp1-b** for Zn^{2+} is the lowest among them. The calculated K$_d$ value of **Cp1-c** is the highest and fitting of the titration curve of emission intensity change *vs* concentration provided poor R^2 value. This result indicates that the position of the amino acids as a ligand in the peptide sensors plays an important role in the binding affinity to Zn^{2+}.

Cp1-d shows a great selectivity for Zn^{2+} over Cd^{2+}. The binding affinity for Zn^{2+} is comparable to those of the other peptide sensors. This shows a possibility of the development of selective and sensitive peptide sensors for Zn^{2+} over Cd^{2+} by changing amino acid sequence. Interestingly, **Cp1-e** preferentially binds Cd^{2+} over Zn^{2+} and has nanomolar affinity (K$_d$ = 24 nM) for Cd^{2+} in aqueous solution. Thus, the peptide has a great potential for monitoring the trace amounts of Cd^{2+} in aqueous solution due to its potent binding affinity for Cd^{2+}. Very low K$_d$ values of **Cp1-e** for Cd^{2+} and Zn^{2+} suggest that the increase of metal binding amino acids (Cys, His, and Glu) in the peptide moiety promotes a higher metal binding affinity but decrease selectivity for specific metal ions.

In conclusion, we show that the selectivity and sensitivity of the metallopeptide sensors to specific heavy and transition metal ions can be tuned by changing amino acid sequence. We successfully synthesize Zn^{2+} selective peptide sensor with a potent binding affinity (K$_d$ = 670 nM) and Cd^{2+} selective and sensitive peptide sensor with a potent binding affinity (K$_d$ = 24 nM).

Experimental

Reagents. Fmoc protected amino acids, *N,N'*-diisopropyl-carbodiimide, 1-hydroxybenzotriazole, and Rink Amide MBHA resin were from Novabiochem. Other reagents for peptide synthesis including trifluoroacetic acid (TFA), tri-isopropylsilane (TIS), dansyl chloride, triethylamine, diethyl ether, dimethyl sulfoxide (DMSO), *N,N*-dimethylformamide (DMF) and piperidine were purchased from Aldrich.

Peptide synthesis. The peptides were synthesized using Fmoc-chemistry by solid phase peptide synthesis with Fmoc chemistry.[8] The coupling of dansyl chloride was performed by applying the following procedure. To the resin bound peptide (65 mg, 0.05 mmol), dansyl chloride (40 mg, 0.15 mmol, 3 equiv) in DMF (3 mL) containing triethylamine (20 μL, 0.15 mmol, 3 equiv) were added and kept for 2 hour at room temperature. Deprotection and cleavage was achieved by treatment with a mixture of TFA/TIS/H$_2$O (9.5:0.25:0.25, v/v/v) at room temperature for 3 - 4 h. After cleavage of the product from resin, the peptides were purified by preparative-HPLC using a water (0.1% TFA)/acetonitrile (0.1% TFA) gradient (5 - 50% acetonitrile over 45 min). The peptide mass were characterized by ESI mass spectrometer (Platform II, Micromass, Manchester, UK). The homogeneity (> 95%) of the compound was confirmed by analytical HPLC on a C$_{18}$ column.

General fluorescence measurements. Fluorescence emission spectrum of a peptide probe in a 10 mm path length quartz cuvette was measured in 10 mM HEPES buffer solution (pH = 7.4) using a Perkin-Elmer luminescence spectrophotometer (model LS 55). Emission spectra (350-650 nm) of the peptide probes (5 μM) in the presence of various metal ions (Hg^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Pb^{2+}, Ag$^+$, Mg^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} as perchlorate anion; and Na$^+$, Al^{3+}, and K$^+$, as chloride anion) were measured by excitation with 330 nm for chelation enhanced fluorescence measurement. The slit size for excitation and emission was 5 nm, respectively. The concentration of peptide probe was confirmed by UV absorbance at 330 nm for dansyl group.

Determination of binding stoichiometry and binding constant. The binding stoichiometry of peptides with metal ions was determined by using Job's plot.[14] A series of solutions with varying mole fraction of metal ions were prepared by maintaining the total peptide and metal ion concentration constant (2.5 μM). The fluorescence emission was measured for each sample by exciting at 330 nm and spectra were measured from 350 to 650 nm. The fitting data were acquired by plotting a straight line through the maximum or minimum emission intensity in the titration curve and were plotted against the mole fraction of the metal ion versus emission intensity.

The dissociation constant was calculated based on the titration curve of the peptide with metal ion. The fluorescence signal, F, is related to the equilibrium concentration of the complex (HL) between peptide (H) and metal ion (L) by the following expression:

$$F = F_o + \Delta F * [HL]$$
$$[HL] = 0.5 \times [K_D + L_T + H_T - \{(- K_D - L_T - H_T)^2 - 4 L_T H_T\}^{1/2}]$$

Where F$_o$ is the fluorescence of the probe only and ΔF is the change in fluorescence due to the formation of HL. The dissociation constants were determined by a nonlinear least squares fit of the data with the equation.[15]

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