Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 1963–1968

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

PEPTIDYL CHEMOSENSORS INCORPORATING A FRET MECHANISM FOR DETECTION OF Ni(II)

Dierdre A. Pearce, Grant K. Walkup, and Barbara Imperiali*

Division of Chemistry and Chemical Engineering, California Institute of Technology, 1200 E. California Blvd., Pasadena CA 91125, U.S.A.

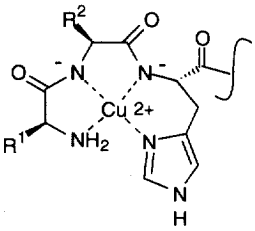
Received 14 April 1998; accepted 16 June 1998

Abstract: Hexapeptides incorporating two fluorophores flanking a tripeptide sequence that binds Ni(II) and Cu(II) with high affinity have been synthesized. While Cu(II) quenches the fluorescence of the resulting peptides, coordination of Ni(II) enables enhanced FRET (fluorescent resonance energy transfer) from one fluorophore to the other.

Introduction

Interest in developing fluorescent chemosensors for the detection of transition metal ions in complex aqueous solutions has been stimulated by the importance of such species in biological systems, particularly in maintaining the integrity of physiological and cellular systems[1] and in monitoring environmental conditions.[2,3] A number of fluorescent chemosensors for Cu(II) and Ni(II) have been described in the literature.[4-6] These generally consist of purely synthetic polyamine or polyamide ligands attached to a fluorophore that is quenched on binding of the target metal ion to the ligand.

In this study the ATCUN (**A**mino **T**erminal **Cu**(II)- and **N**i(II)-binding) motif of the serum albumins has been recruited for the development of sensors that selectively recognize these species in the presence of other metal ions. This motif consists of a simple tripeptide sequence that binds the metal ions in a slightly distorted square planar geometry through the terminal amine nitrogen, two deprotonated amide nitrogens of the peptide backbone and the imidazole-δ-nitrogen of the histidine residue at the third position, **1**.[7] Appending a fluorophore to the ATCUN-motif generates highly sensitive chemosensors that selectively bind Cu(II) and Ni(II) and signal the presence of these analytes by a decrease in fluorescence emission from the fluorophore.[8] Importantly, solid phase synthesis and the modular nature of the peptide structure allows non-native residues to be incorporated readily into the binding motif, altering the relative selectivity of the resulting peptidyl ligand. For example, replacing the second α-amino acid of the motif with a β-amino acid generates a highly selective chemosensor for Cu(II).[8]



Cu(II)-ATCUN complex

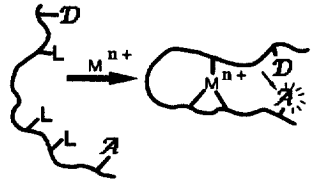
1

A concern with the current sensor design is the reduction in the fluorescence intensity of the chemosensor that occurs in that the presence of the target metal ion. In particular, interaction of the sensor with the target analyte would be difficult to distinguish from destruction of the sensor. Moreover, signaling methods involving fluorescence enhancement are more sensitive than those involving fluorescence quenching.[9] To

0960-894X/98/$ - see front matter
PII: S0960-894X(98)00354-0

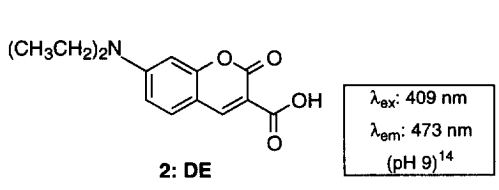
address this concern peptidyl chemosensors have been investigated which incorporate a FRET (fluorescence resonance energy transfer) mode of signaling, Figure 1. A pair of fluorophores with suitable photophysical properties are anchored to either end of the ATCUN binding motif; upon binding of a metal ion by the peptide the fluorophores are brought closer together and enhanced transfer of engergy from the donor (D) to the acceptor (A) may occur. This communication reports the synthesis of a series of fluorescent peptidyl chemosensors and their response upon addition of Cu(II), Ni(II), and a number of other divalent metal ions.

Figure 1. FRET-based Sensing Strategy

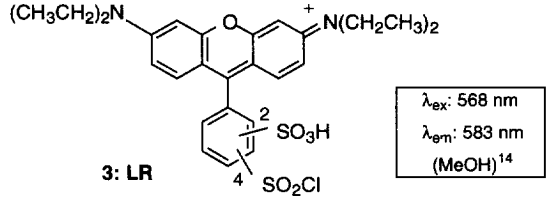


Synthesis of Chemosensors

A number of factors were considered when identifying potential fluorophore pairs to incorporate into the peptidyl chemosensors. The absorption spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor to facilitate FRET, and the fluorophores themselves should be resistant to photodegradation and display significant fluorescence in the chosen solvent system (pH 7.0, 50 mM [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (HEPES), 150 mM NaCl). Moreover, the donor should be excited at a long wavelength to avoid potential problems of background fluorescence from biological solutions or from fluorescent amino acids such as tryptophan.[10] Coumarin and rhodamine derivatives, used to probe macromolecular structure[11] and membrane-membrane interactions[12,13] in some previous FRET-based applications, were found to fulfill the required criteria. Of these, 7-diethylaminocoumarin-3-carboxylic acid (DE, **2**) and lissamine rhodamine B sulfonyl chloride (LR, **3**) were selected for incorporation within the chemosensors explored in this study.



2: DE

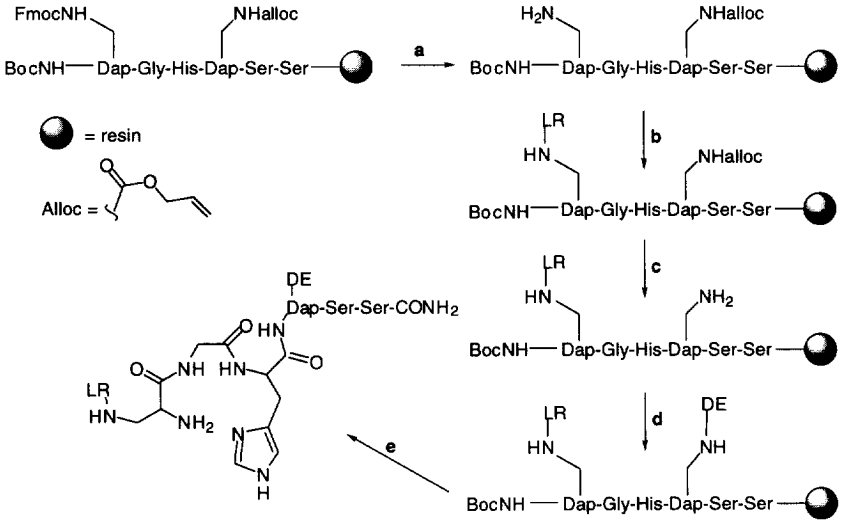


3: LR

A family of ATCUN-motif peptides was prepared, each containing the general sequence Dap(F1)-Xaa-His-Dap(F2)-Ser-Ser-$CONH_2$, where Dap denotes (*S*)-2,3-diaminopropanoic acid and Xaa either glycine or L-aspartic acid, with LR and DE positioned at F1 or F2. The *C*-terminal serine residues were included in the sequence to improve the solubility of the peptides.

All peptides were synthesized using standard Fmoc-based solid-phase protocols on Fmoc-PAL-PEG-PS resin (0.21 mmol/g, Perseptive Biosystems) to afford *C*-terminal carboxamides. The *N*-terminal amino acid residue was the orthogonally protected (*S*)-N^{α}-Boc-N^{β}-Fmoc-diaminopropanoic acid; the first fluorophore was attached to the deprotected β-amine of this residue and the second fluorophore attached to the free β-amine of a second Dap residue after removing the allyloxycarbonyl (alloc) protecting group with

tetrakis(triphenylphosphine)palladium(0)[15,16] (Scheme 1). The fluorophores DE and LR were obtained from Molecular Probes. The donor, DE, was coupled to the peptide with 1,3-diisopropylcarbodimide (DIPCDI), triethylamine (TEA) and 4-*N,N*-dimethylaminopyridine, (DMAP). Couplings involving the acceptor, LR, were afforded using DMAP and diisopropylethylamine (DIEA). The fluorophores and coupling reagents were dissolved in a minimum volume of dimethylformamide (DMF) before being added to the resin-bound peptide and the coupling reactions were monitored using a Kaiser test.[17] The completed peptides were deprotected and cleaved from the resin using 5% (v/v) triisopropylsilane (TIPS) in trifluoroacetic acid (TFA). The resulting mixture was reduced to a low volume by removing excess solvent with a stream of nitrogen and the crude products were isolated following trituration with a 1:1 mixture of ether and hexane. Peptides were collected, suspended in water, and lyophilized before purification by reversed-phase (C18) high performance liquid chromatography (HPLC). The fluorophore LR was available only as a mixture of isomers and, when incorporated into the peptides, yielded products that were a mixture of regioisomers. These were separated by HPLC and the composition of each isomer confirmed by electrospray mass spectroscopy, Table 1, before being characterized individually by UV-visible and fluorescence spectroscopy.



Scheme 1. Addition of fluorophores to resin bound peptide (rbp) **P3**. **(a)** 20% v/v piperidine/DMF. **(b)** rbp/LR/DMAP/DIEA (molar ratio 1:5:2.5:5) in DMF, 36 h, 25 °C. **(c)** $[((C_6H_5)_3P)_4Pd]/CHCl_3/CH_3COOH$/morpholine.[15,16] **(d)** rbp/DE/DMAP/DIPCDI/DIEA (molar ratio 1:5:2.5:5:5) in DMF, 42 h, 25 °C. **(e)** 5% v/v TIPS/DMF, 1:1 ether:hexane, lyophilize, RP HPLC.

Table 1: Calculated and Observed Electrospray Mass Values of the Sensors **P1–P7**

Peptide	Sequence[a]	$[MH]^+_{calcd}$	$[MH]^+_{obsd}$
P1	Dap(DE)GlyHisDap(alloc)SerSer-NH_2	885.4	885
P2	Dap(LR)GlyHisDap(alloc)SerSer-NH_2	1182.4	1183
P3	Dap(LR)GlyHisDap(DE)SerSer-NH_2	1342.5	1341
P4	Dap(DE)GlyHisDap(LR)SerSer-NH_2	1342.5	1341
P5	Dap(LR)GlyHisDapSer(DE)Ser-NH_2	1342.5	1341
P6	Dap(LR)GlyHisSerSerDap(DE)r-NH_2	1342.5	1342
P7	Dap(LR)AspHisDap(DE)SerSer-NH_2	1342.5	1341

[a]Fluorophores or the protecting group 'alloc' attached to the β-amine of the Dap residue are listed in brackets.

Fluorescent Properties of Chemosensors

In an initial survey, UV-visible and fluorescence emission spectra were acquired from 1 μM aqueous solutions of peptides **P1** to **P7** (pH 7.0, 50 mM HEPES, 150 mM NaCl). The UV-visible spectra were dominated by peaks at 436 nm and 576 nm, corresponding to the absorbance maxima of DE and LR respectively; Figure 2a presents a representative example. In collecting fluorescence spectra, the peptide solutions were irradiated at 400 nm to obtain maximum excitation of the donor, DE, while minimizing excitation of the acceptor, LR. The resulting emission from both fluorophores was monitored between 425 and 750 nm. Emission spectra of peptide **P1**, containing only the DE fluorophore, featured a single peak at 470 nm whilst the related peptide **P2**, which contains only the LR fluorophore, featured a single peak at 588 nm. Fluorescence emission spectra of peptides **P3** to **P7** exhibited a small peak at 470 nm and a larger peak at 588 nm, corresponding to emission from both fluorophores. Spectra of regioisomers formed from coupling LR through *ortho-* and *para-* sulfonyl chloride substituents were not significantly different from each other, nor were spectra of the regioisomers **P3** and **P4**, in which the positions of the two fluorophores on the peptide chain were swapped. Moving the fluorophores further apart on the peptide backbone, as for **P5** and **P6**, did not measurably alter the relative intensity of emission from LR. In summary, in the absence of divalent metal ions, the measured fluorescence emission from LR in the peptides **P3–P7** is due to energy transfer from DE and to the direct absorption of light energy by LR at 400 nm.

In all cases, addition of Cu(II) to peptide solutions resulted in quenching of fluorescence emission from both fluorophores. By contrast, addition of Ni(II) to the solutions produced no change to spectra of **P1**, and small decreases in the emission spectra of **P2**. Since binding of Ni(II) does not result in quenching of DE (**P1**) or complete quenching of LR (**P2**), a signal due to FRET is possible and increases in the long wavelength emission (by LR) were observed in the spectra of **P3–P7** on addition of Ni(II).

Figure 2. UV-Visible and fluorescence spectra of peptide **P7**. **(a)** UV-visible spectrum of 1 μM solution of **P7**. **(b)** Response of 2 μM solution of **P7** on addition of 10 μM Ni(II). Spectra acquired at 0 m, 10 m, 20 m, 30 m, 60 m, 120 m after addition of Ni(II). **(c)** Response of a 2 μM solution of DE and LR (1:1) on addition of 10 μM Ni(II) (λ_{ex} = 400 nm). Spectra acquired at 0 m, 120 m. Spectra were acquired at ambient temperature at pH 7.0 (50 mM HEPES, 150 mM NaCl).

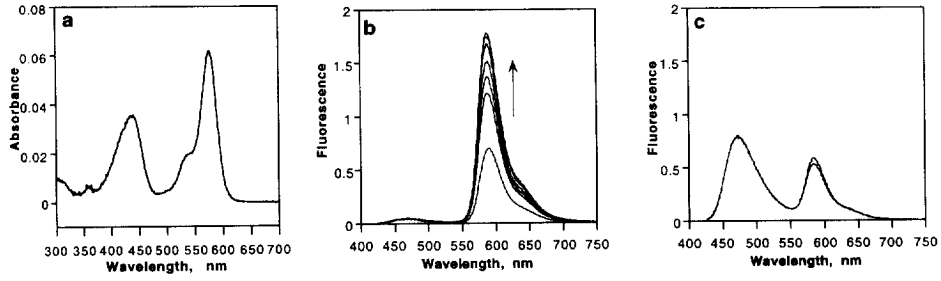
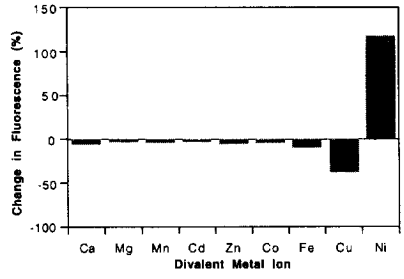


Figure 2b illustrates the response of peptide **P7** on the addition of an equivalent of Ni(II). In this spectrum the peak due to emission at 588 nm increased significantly following addition of Ni(II). By contrast, solutions containing equal quantities of the free DE and LR fluorophores show no significant response to

additions of Ni(II), Figure 2c. The fluorescence response to Ni(II) is relatively slow, reflecting the slow water exchange rate of Ni(II)aq compared to Cu(II)aq.[18] However, the rate of change in the emission maxima at 588 nm is also dependent on the identity of the second risidue (Xaa) in the peptide sequence. Following addition of ten equivalents of Ni(II), fluorescence emission at 588 nm from peptides **P3** (in which Xaa is glycine) and **P7** (in which Xaa is aspartic acid) reach a steady state in 180 minutes and 45 minutes, respectively. Addition of excess ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) to all peptide solutions slowly reversed the changes to the fluorescence spectra that occurred as the result of additions of Cu(II) and Ni(II). The apparent dissociation constant, Kd, for the Ni(II)–**P7** complex was estimated from fluorescence data acquired from titrations of Ni(II) into the peptide solution, is better than 100 nM.

The fluorescence response of **P7** to a number of divalent metal ions is presented in Figure 3. Only changes to the intensity of fluorescence emission from LR are reported because, with the exception of Cu(II), the fluorescence emission of DE is unchanged upon addition of the metal ions. Small responses were observed on the addition of up to ten equivalents of Ca(II), Mg(II), Mn(II), Cd(II), Zn(II), Fe(II) or Co(II) to the peptide, the magnitude of which are somewhat greater than those reported in previous studies of peptidyl chemosensors.[8] Addition of Cu(II) to the peptide solution resulted in significantly greater quenching of fluorescence emission from LR in the peptide; ten equivalents of Cu(II) caused 40% quenching compared with less than 10% quenching caused by addition of other divalent metal ions. Only addition of Ni(II) generated a FRET response. Peptide **P3** exhibited similar responses to additions of the divalent metal ions used in this study (data not shown).

Figure 3. Relative change in fluorescence emission at 588 nm upon addition of 10 μM divalent metal ions to 1 μM **P7** at pH 7.0 (50 mM HEPES, 150 mM NaCl). Data have been scaled such that, in the absence of metal ions, the fluorescence of **P7** is equal to unity, with metal-induced changes in fluorescence calculated relative to that value.



Significantly, fluorescence spectra of 1:1 solutions of DE and LR remain unchanged on addition of metal ions, in agreement with a model of static quenching of emission from the fluorophores by the peptide-bound metal ions rather than by a dynamic process of collisional quenching. The observed fluorescence response of the chemosensor to addition of a variety of metal ions is the cumulative result of a number of constraints, including the stability of the metal–peptide complex, quenching of the donor by the metal ion and FRET between the two fluorophores. The greatest responses, generated by Cu(II) and Ni(II), reflect both the

influence of the Irving–Williams series[19] and the original biological purpose for which the ATCUN motif evolved.[7] The small responses observed on addition of other metal ions such as Ca(II) may reflect the formation of complexes through other available donors such as peptide carbonyl groups and the carboxylate group of LR. Similar peptidyl chemosensors which did not contain a fluorophore with an ionizable carboxylate group have not shown sensitivity to other metal ions.[8]

In summary, the peptide-dependent FRET response of **P3–P7** to additions of Ni(II) is consistent with the fluorophores being brought into closer proximity as the peptide folds around the metal ion during coordination. The peptides **P3–P7** are capable of detecting the presence of both Ni(II) and Cu(II) in aqueous solution; the response to Ni(II) is slow but may be improved by selective design of amino acid sequences. Importantly, the fluorescence response to each species is distinctly different: binding of Cu(II) by the chemosensor generates fluorescence quenching of both fluorophores, whereas binding of Ni(II) by the same species produces a FRET signal. Finally, only minor interference is caused by other divalent metal ions such as Mg(II), Ca(II), Mn(II), Cd(II), Zn(II), Fe(II) and Co(II).

Acknowledgments

This research was supported by the NSF and the Gates Foundation. The award of an NIH pre-doctoral training grant fellowship (GM 07616) to GKW is also gratefully acknowledged.

References

1. Czarnik, A. W. *Chem. Biol.* **1995**, *2*, 423.
2. Jie, N.; Jiang, J. *Analyst* **1991**, *116*, 395.
3. Pal, B. K.; Ahmed, M. J. U.; Chakrabarti, A. K.; Chakraborty, D. *Ind. J. Chem. Techn.* **1997**, **4**, 191.
4. Fabbrizzi, L.; Licchelli, M.; Pallavicini, A.; Parodi, L. *Angew. Chem. Int. Ed.* **1998**, *37*, 800.
5. Desantis, G.; Fabbrizzi, L.; Licchelli, M.; Mangano, C.; Sacchi, D.; Sardone, N. *Inorg. Chim. Acta* **1997**, *257*, 69.
6. Corradini, R.; Dossena, A.; Galaverna, G.; Marchelli, R.; Panagia, A.; Sartor, G. *J. Org. Chem.* **1997**, *62*, 6283.
7. Harford, C.; Sarker, B. *Acc. Chem. Res.* **1997**, *30*, 123.
8. Torrado, A.; Walkup, G. K.; Imperiali, B. *J. Am. Chem. Soc.* **1997**, *120*, 609.
9. Chae, M.-Y.; Czarnik, A. W. *J. Am. Chem. Soc.* **1992**, *114*, 9704
10. Godwin, H. A.; Berg, J. M. *J. Am. Chem. Soc.* **1996**, *118*, 6514.
11. Mergny, J.-L.; Boutorine, A. S.; Garestier, T.; Belloc, F.; Rougee, M.; Bulychev, N. V.; Koshkin, A. A.; Bourson, J.; Lebedev, A. V.; Valeur, B.; Thuong, N. T.; Helene, C. *Nucleic Acids Res.* **1994**, *22*, 920.
12. Niles, W. D.; Silvius, J. R.; Cohn, F. S. *J. Gen. Physiol.* **1996**, *107*, 329.
13. Priyadarsini, K. I.; Mishra, B. K.; Manohar, C. *Chem. Phys. Lett.* **1991**, *182*, 608.
14. Haugland R. P. *Handbook of Molecular Probes*, 6th edition; Molecular Probes: Eugene, 1996; Chapter 1.
15. Walkup, G. K.; Imperiali, B. *J. Am. Chem. Soc.* **1997**, *119*, 3443.
16. Kates, S. A.; Daniels, S. B.; Albericio, F. *Anal. Biochem.* **1993**, *212*, 303.
17. Stewart, J. M.; Young, J. D. *Solid-Phase Peptide Synthesis*, 2nd Edition; Pierce Chemical: Rockford, 1984; p 152.
18. Wilkins, R. G. *Kinetics and Mechanism of Reactions of Transition Metal Complexes*, 2nd Edition; VCH: New York, 1991; p 199.
19. Frausto da Silva, J. J. R.; Williams, R. J. P. *The Biological Chemistry of the Elements: The Inorganic Chemistry of Life*; Clarendon: New York, 1993, p 34.