METAL-BINDING PROPERTIES OF SYNTHETIC METALLOPROTEINS

Olesya A. Kharenko

A Dissertation
Submitted to the Graduate College of Bowling Green
State University in partial fulfillment of
the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2005

Committee:
Michael Y. Ogawa, Advisor
Verner P. Bingman
Graduate Faculty Representative
Felix N. Castellano
George S. Bullerjahn
ABSTRACT

Michael Y. Ogawa, Advisor

This dissertation describes the metal-binding properties of synthetic self-assembled metalloproteins. A family of the synthetic metalloproteins was prepared based on the de novo designed peptide C16C19-GGY having the sequence

Ac-K(IEALEGK)2(CEACEGK)(IEALEGK)GY-amide.

This sequence is based on the IEALEKG heptad repeat known to form two-stranded α-helical coiled coils, but was modified to contain the Cys-X-X-Cys thiolato metal binding motif found in a variety natural of metalloproteins. This cysteine-containing random coil apopeptide is capable of binding a variety of soft metal ions such as, Cu(I), Cd(II), Ag(I), Hg(II), Au(I), and Pt(II) which results in the formation of a metal-bridged self-organized α-helical bundles. It has been shown that such binding produces metal-specific oligomerization states of the resulting metalloproteins: synthetic Cu(I), Ag(I), Au(I), and Pt(II)-metalloproteins have an oligomerization state which differs from the one predicted by original design. These inorganic cofactors not only induce peptide self-assembly, but direct and transform the oligomerization state of the peptide. This illustrates how the structures of metalloproteins may be controlled by the coordination chemistry of their inorganic cofactors. A 1:1 metal:peptide stoichiometry is observed for the Cu(I) and Ag(I) adducts but the Cd(II) and Hg(II) complexes show a metal:peptide stoichiometry of 1:2.

It is also shown that the Cu(I)-metalloprotein described here displays an intense room temperature long-lived (microsecond) luminescence at 600 nm. Such incorporated chemical functionality allows using this synthetic metalloprotein as a photoinduced electron-transfer agent in future studies.
This dissertation is dedicated to my parents,

Lyudmila and Anatoliy Kharenko and

to my sister, Olga Kharenko
ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisor, Dr. Michael Y. Ogawa, for his continuous support during every stage of my research work. His outlook on science motivated my growth as a scientist. He taught me to see a broader perspective and think independently. His creative ideas, great personality, positive-thinking attitude, constant feedback and guidance had a significant impact on my scientific work.

I would like to thank the member of my committee Prof. Felix N. Castellano, Prof. George S. Bullerjahn, and Prof. Verner P. Bingman for the kind support, encouragement, thought-provoking conversations, and for revisions and comments about my dissertation. My special thanks go to Prof. Felix N. Castellano for the use of his laser facilities. I would like to acknowledge Prof. Brian Gibney and Amy Petros from Columbia University for teaching me the redox potentiometry techniques. I would like also to acknowledge our collaborators Dr. David C. Kennedy and Prof. Michael J. Maroney from University of Massachusetts and Prof. Borries Demeler University of Texas Health Science Center.

My special thanks go to my friends and co-workers for creating friendly working environment.

Finally, my sincere appreciation is expressed to my family, my Mom, Dad, and my Sister for giving me love, unconditional support, and encouragement without of which I would not be able to achieve my goals.

I want to thank everyone at the Center for Photochemical Sciences for giving my an opportunity to work here and creating wonderful working atmosphere.
TABLE OF CONTENTS

CHAPTER I. INTRODUCTION AND BACKGOUND................................................................. 1
   I.1. Metal-Induced Folding of Metalloproteins: Zinc Finger Peptides......................... 4
   I.2. Metalloprotein Design......................................................................................... 8
      I.2.1. Structural Design..................................................................................... 9
      I.2.2. Functional Design ................................................................................. 19
REFERENCES TO CHAPTER I.................................................................................... 24

CHAPTER II. EXPERIMENTAL...................................................................................... 28

CHAPTER III. RESULTS AND DISCUSSION: SYNTHESIS, CHARACTERIZATION AND
METAL-BINDING PROPERTIES OF SYNTHETIC METALLOPROTEINS
   III.1. Peptide Design............................................................................................. 38
   III.3. Metal-Binding Properties of C16C19-GGY..................................................... 50
   III.4.1. Investigation of the Metal-Binding Properties of the Synthetic Cu(I)/C16C19-GGY
            Metalloprotein .............................................................................................. 62
   III. 4.2. Investigation of the Metal-Binding Properties of the Synthetic Cd(II)/C16C19-GGY
            Metalloprotein.............................................................................................. 111
   III. 4.3. Investigation of the Metal-Binding Properties of the Synthetic Hg(II)/C16C19-GGY
            Metalloprotein.............................................................................................. 120
   III. 4.4. Investigation of the Metal-Binding Properties of the Synthetic Ag(I), Pt(II), and Au
            (I)/C16C19-GGY Metalloproteins..................................................................... 131
CONCLUSIONS.......................................................................................................... 147
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.1. Schematic views of synthetic coiled-coils. Top: primary amino acid sequence of the two helices of the coiled-coil. Left: Helices in parallel orientation. Right: helical wheel diagram of the coiled coil; view down helical axes of the coiled-coil</td>
<td>10</td>
</tr>
<tr>
<td>I.2. Helical wheel diagram for parallel three, and four stranded coiled coil peptides. Adapted from reference 1</td>
<td>11</td>
</tr>
<tr>
<td>I.3. Helical wheel diagram of the coiled coil; view down helical axes of the coiled-coil Adapted from reference 51</td>
<td>16</td>
</tr>
<tr>
<td>III.1. Wheel representation showing the sequence of C16C19-GGY peptide</td>
<td>40</td>
</tr>
<tr>
<td>III.2. Analytical chromatogram of the purified C16C19-GGY peptide</td>
<td>41</td>
</tr>
<tr>
<td>III.3. ESI-MS of the purified C16C19-GGY (m/z observed 1137, 853, calculated 1137 (M+3H&lt;sup&gt;+&lt;/sup&gt;)&lt;sup&gt;3+&lt;/sup&gt;, 853 (M+4H&lt;sup&gt;+&lt;/sup&gt;)&lt;sup&gt;4+&lt;/sup&gt;)</td>
<td>42</td>
</tr>
<tr>
<td>III.4. MALDI-TOF MS spectrum of the C16C19-GGY (32-mer) (m/z observed: 3408, calculated 3408)</td>
<td>43</td>
</tr>
<tr>
<td>III.5. Circular dichroism spectra of aqueous solutions of reduced C16C19-GGY (46 µM) (●) and oxidized apopeptide (▲) (pH ~5-6, 298)</td>
<td>44</td>
</tr>
<tr>
<td>III.6. Circular dichroism spectra of the reduced C16C19-GGY (157 µM) taken in 1 : 1 ethanol / methanol mixture (298 K)</td>
<td>45</td>
</tr>
<tr>
<td>III.7. SDS-PAGE electrophoresis of the C16C19-GGY and Pal14C9 oxidized samples</td>
<td>46</td>
</tr>
<tr>
<td>III.8. High performance size exclusion chromatography (HPSEC) chromatogram of the H21-mer and C16C19-GGY monitored at 230 nm. The sample was eluted with 0.2 M sodium acetate buffer pH 5.4, 0.3 ml/min</td>
<td>47</td>
</tr>
</tbody>
</table>
III.9. Analytical chromatogram of the oxidized C16C19-GGY peptide…………………………… 49

III.10. The circular dichroism spectra of a 100 µM solution of C16C19-GGY peptide after
0, 19, 25, 96, 120, and 196 hours of oxidation by exposure to ambient air....................... 49

III.11. The oxidation course of C16C19-GGY monitored by HPLC. 100 µM solution of
C16C19-GGY oxidized under aerobic ambient conditions by exposure to air
with the following injection to HPLC after 0 – 7 hours............................................... 50

III.12. Circular dichroism spectrum of C16C19-GGY (107 µM) taken in the absence (■)
and presence (●) of a fivefold excess of cadmium chloride (5 mM sodium
acetate buffer, pH 5.5-5.6, 298 K)............................................................................. 53

III.13. Circular dichroism spectrum of C16C19-GGY (107 µM) taken in the absence
(■) and presence (●) of a fivefold excess of tetrakis(acetonitrile) copper(I)hexaflouro-
phosphate, (5 mM sodium acetate buffer, pH 5.5-5.6, 298 K)..................................... 53

III.14. Circular dichroism spectrum of C16C19-GGY (107 µM) taken in the absence
(▲) and presence (■) of a fivefold excess of silver nitrate (5 mM sodium acetate
buffer, pH 5.5-5.6, 298 K)......................................................................................... 54

III.15. Circular dichroism spectrum of C16C19-GGY (107 µM) taken in the absence (●) and
presence (■) of a fivefold excess of sodium aurothiomalate hydrate, (5 mM sodium acetate
buffer, pH 5.5-5.6, 298 K).......................................................................................... 54

III.16. Circular dichroism spectrum of C16C19-GGY (138 µM) taken in the absence (●) and
presence (■) of a fivefold excess of Pt(en)(NO3)2, (5 mM sodium acetate buffer, pH 5.5-
5.6, 298 K)............................................................................................................... 55

III.17. Circular dichroism spectrum of C16C19-GGY (107 µM) taken in the absence (●)
and (■) presence of a fivefold excess of mercury chloride (5 mM sodium acetate buffer,
III.18. UV-vis spectrum of C16C19-GGY/Cd(II) ([C16C19-GGY]=140 µM) taken in the presence of a twofold excess of Cd(II) in 0.2 M sodium acetate buffer pH 5.4-5.5……57

III.19. UV-vis spectrum of C16C19-GGY/Cu(I) taken in the presence of a twofold excess of Cu(I), [C16C19-GGY]=84 µM, 5-fold excess of TCEP in 0.2 M sodium acetate buffer pH 5.4-5.5 (298 K)………………………………………………………………………..58

III.20. UV-vis spectrum of C16C19-GGY/Hg(II) taken in the presence of a twofold excess of Hg(II), [C16C19-GGY]=111µM in 0.2 M sodium acetate buffer pH 5.4-5.5, (298 K)………………………………………………………………………..58

III.21. UV-vis spectrum of the aqueous solution of C16C19-GGY/Ag(I) taken in the presence of a 1.5-fold excess of Ag(I), [C16C19-GGY]=111µM (298 K)…………………………………...………………….59

III.22. UV-vis spectrum of C16C19-GGY/Pb(II) in aqueous solutions, ([C16C19-GGY]=100 µM) 298 K………………………………………………………………………..61

III.23. Determination of molecular weight of the Cu(I)/C16C19-GGY by sedimentation equilibrium ultracentrifugation…………………………………………………………...64

III.24. SDS-PAGE electrophoresis of the Cu(I)/C16C19-GGY complex, apopeptide monomer, SDS-PAGE polypeptide standards……………………………………………………….65

III.25. Emission spectrum obtained upon addition of Cu(I) the to the peptide solution. Conditions: 120 µM C16C19-GGY in 0.2 M acetate buffer (pH 5.4) containing 730 µM tris-(2-carboxyethyl)phosphine (TCEP) as a reducing agent……………………………………….68

III.26. Excitation spectrum obtained upon addition of Cu(I) the to the peptide. Conditions: 200µM C16C19-GGY in the presence of twofold excess of Cu(I)………………………………………..68

III.27. Quenching of emission observed upon addition of urea to the solution of the
III.28. Quenching of emission observed upon the exposure of the solution of the Cu(I)/C16C19-GGY adduct to air. The solution of the argon-saturated solution of Cu(I)/C16C19-GGY was bubbled with compressed air for 17-20 min and de-aerated with argon for 15-20 min with the following recording of the emission spectra.

III.29. Stability time course at aerobic conditions: the change in molar ellipticity at 222 nm as a function of time measured for the C16C19-GGY/Cu(I) at room temperature in 33 mM sodium acetate buffer, pH 5.4-5.4. (concentrations: 53 μM C16C19-GGY, 265 Cu(I) μM).

III.30. Circular dichroism spectra recorded before and after exposure to air for 7 hours of the Cu(I)-C16C19-GGY. (53 μM peptide, 265 μM Cu(I), 33 mM sodium acetate buffer, pH = 5.4-5.5).

III.31. Stability time course at aerobic conditions: the change of molar ellipticity at 222 nm as a function of time measured for the Cu(I)/C16C19-GGY/TCEP at room temperature in 30 mM sodium acetate buffer, pH 5.4-5.4. (concentrations: 87 μM C16C19-GGY, 174 Cu(I) μM, 435 μM TCEP).

III.32. Circular dichroism spectra recorded before and after oxidation course of the Cu(I)–C16C19-GGY. (87 μM peptide, 174 μM Cu(I), 435 μM TCEP, 30 mM sodium acetate buffer, pH = 5.4-5.5).

III.33. Thermal melting curve of 55 μM C16C19-GGY in the presence of two fold excess of Cu(I) and five fold excess of TCEP.

III.34. Emission spectra recorded upon the addition of ferricyanide to a solution of the Cu(I)/C16C19-GGY complex. Cu(I)/C16C19-GGY complex was formed by the equimolar
addition of the tetrakis(acetonitrile)Cu(I)hexafluorophosphate without reducing agent in 0.2 M sodium acetate buffer, pH=5.4, [C16C19-GGY]=197 μM, λ_{exc}= 300 nm………78

III.35. Emission spectra recorded upon the addition of ferricyanide and the sequential addition of TCEP to a solution of the Cu(I)/C16C19-GGY complex. Cu(I)/C16C19-GGY complex was formed by the equimolar addition of the tetrakis(acetonitrile)Cu(I)hexafluorophosphate in 0.2 M sodium acetate buffer, pH=5.4, [C16C19-GGY]=58 μM, λ_{exc}= 300 nm……………………………………………………………………………...79

III.36. Circular dichroism spectra recorded upon the addition of ferricyanide and TCEP to a solution of the Cu(I)/C16C19-GGY complex. Cu(I)/C16C19-GGY complex was formed by the equimolar addition of the tetrakis(acetonitrile)Cu(I)hexafluorophosphate, [C16C19-GGY]=10 μM, 1 mm cell……………………………………………………..80

III.37. Near UV Circular dichroism spectrum of C16C19-GGY (109 µM) taken in the presence of a twofold excess of tetrakis(acetonitrile) copper(I)hexafluorophosphate and fivefold excess of TCEP in 0.2 M sodium acetate buffer pH 5.4…………………………………..81

III.38. Emission titration of C16C19-GGY by [Cu(CH3CN)4]PF₆. Emission spectra obtained upon addition of Cu(I) to the 120 μM peptide solution 0.2 M acetate buffer (pH 5.4) containing 730μM tris-(2-carboxyethyl)phosphine (TCEP) as a reducing agent………82

III.39. Emission titration of C16C19-GGY by [Cu(CH3CN)4]PF₆. Titration plot of emission intensity (600 nm) vs. equivalents of Cu(I) added………………………………………………83

III.40. UV titration of 100 µM C16C19-GGY by Cu(I) in 0.2 M acetate buffer pH 5.4; 500 μM TCEP. Difference spectra of the Cu(I)/C16C19-GGY solutions………………………………..84

III.41. UV titration of 100 µM C16C19-GGY by Cu(I) in 0.2 M acetate buffer pH 5.4. The titration plot of A₂₉₆ vs. equivalents of Cu(I): growth of new absorption band upon
Cu(I) addition……………………………………………………………………………………..85

III.42. Far UV Circular Dichroism titration Far UV of C16C19-GGY (236 µM) in 0.2 M sodium acetate buffer, pH 5.4 in the presence of 1 mM TCEP. Inset: Plot of $[\theta]_{222\text{ nm}}$ as a function of added Cu(I)………………………………………………………………………. 86

III.43. Emission decay of the of Cu(I)-metalloprotein excited at 357 nm monitored at 600 nm. The red line represents the fit of the data to Equation as described in the text. (The Cu(I)/C16C19-GGY complex was formed in 1.5-fold excess of tetrakis (acetonitrile)copper(I) hexafluorophosphate in argon-saturated 0.2 M sodium acetate buffer, pH 5.4, the peptide concentration is 132 µM)………………………..87

III.44. Stability time course at aerobic conditions: the change in molar ellipticity at 222 nm as a function of time measured for the C16C19-GGY/Cu(I) at room temperature in 33 mM sodium acetate buffer, pH 5.4-5.4. (concentrations: 53 µM C16C19-GGY, 265 Cu(I) µM)……………………………………………………………………………..91

III.45. Emission decay of the copper/C16C19-GGY complex formed by the addition of 2.5-fold excess of CuCl$_2$ to 41 µM of C16C19-GGY excited at 357 nm and monitored at 600 nm……………………………………………………………………..91

III.46. Circular dichroism spectrum of C16C19-GGY (50 µM) taken in the presence (■) of a tenfold excess of CuCl$_2$……………………………………………………………………………………………………..92

III.47. Analytical chromatogram of the C16C19-GGY/copper complex formed by the addition of CuCl$_2$……………………………………………………………………………………………………..92

III.48. Copper K-edge XAS data of Cu(I)-C16C19-GGY. Top: The XANES spectrum bottom: Fourier-transformed; and insert: Fourier-filtered EXAFS spectrum data
III.49. Synthetic Cu₄S₄ cluster containing Cu(I) in a trigonal NS₂-donor environment Schematic representation of synthetic 4-helix Cu(I)-metalloprotein with Cu₄S₄-cofactor

III.50. Schematic representation of synthetic 4-helix Cu(I)-metalloprotein with Cu₄S₄-cofactor

III.51. UV-visible spectra of 186 μM Cu(I)-metalloprotein in 0.2 M sodium acetate buffer pH 5.5 in anaerobic cell upon the oxidation by titration with K₂IrCl₆

III.52. Redox titration curve of Cu(I)-metalloprotein monitored by optical spectroscopy.
Absorbance at 310 nm is plotted against the measured redox potential vs. SCE and the obtained data were fit to the single Nernst equation curve (n=1)

III.53. The difference spectrum of the Cu(II)-adduct

III.54. Emission spectra of 112 μM Cu(I)-metalloprotein in 0.2 M sodium acetate buffer pH 5.5 in anaerobic cell upon the oxidation by titration with K₂IrCl₆

III.55. Redox titration curve of Cu(I)-metalloprotein. Plot of the emission intensity at 600 nm is plotted against the measured redox potential vs. SCE and the obtained data were fit to the single Nernst equation curve (n=1)

III.56. Emission spectra of 120 μM Cu(I)-metalloprotein in 0.2 M sodium acetate buffer pH 5.5 in anaerobic cell upon the oxidation by titration with K₂IrCl₆

III.57. Redox titration curve of Cu(I)-metalloprotein. Plot of the emission intensity at 600 nm is plotted against the measured redox potential vs. SCE and the obtained data were fit to the single Nernst equation curve (n=1)

III.58. The UV-vis titration of C16C19-GGY by tetrakis(acetonitrile)Cu(I) hexaflourophosphate in the presence and absence of TCEP
III.59. The comparison of the emission intensity of the Cu(I)-C16C19-GGY in the absence or presence of TCEP, λ<sub>exc</sub>=600 nm.................................................................108

III.60. Emission decay of the of Cu(I)-metalloprotein excited at 357 nm monitored at 600 nm. The red line represents the fit of the data to Equation as described in the text. (The Cu(I)/C16C19-GGY complex was formed in argon-saturated aqueous solution in 5-fold excess of tetrakis(acetonitrile)copper(I) hexafluorophosphate in the presence of TCEP, the peptide concentration is 114 μM).................................................................109

III.61. SDS-PAGE electrophoresis shows the that the Cd(II)/C16C19-GGY complex exists as a monomer and a dimer peptide, respectively.................................................................112

III.62. HPSEC results obtained by using series of peptide standards on Superdex 75 HPSEC column under non-denaturing conditions (0.1 M KCl / 0.05 M KH<sub>2</sub>PO<sub>4</sub>, pH 7), 0.4 ml/min flow rate. The distribution coefficients (Kd) were calculated according to the following expression: Kd =Ve-Vo/Ve-Vo, where Ve is the elution volume of the peptide, Vo is the void volume and Vt is the column bed volume..................................................113

III.63. UV–Vis titration in which successive additions of CdCl<sub>2</sub> were made to a 100 μM solution of C16C19 immobilized Cleland’s REDUCTACRYLTM reagent (Calbiochem).........114

III.64. The plot of A<sub>238</sub> vs. equivalents of Cd(II): growth of new absorption band upon Cd(II)addition.................................................................115

III.65. The Job plot obtained by monitoring the absorbance intensity at 238 nm as a function of the mole fraction of Cd(II).................................................................115

III.66. Plot of normalized ellipticity at 222 nm as a function of Cd(II) addition.........................116

III.67. Near UV Circular dichroism spectrum of C16C19-GGY (175 μM) taken in the presence
of a twofold excess of CdCl$_2$ in 20 mM sodium acetate buffer pH 5.4.

III.68. The UV-vis titration of C16C19-GGY by CdCl$_2$ in the presence and absence of TCEP.

III.69. HPSEC results obtained by using series of peptide standards on Superdex 75 HPSEC column under non-denaturating conditions (0.1 M KCl / 0.05 M KH$_2$PO$_4$, pH 7), 0.4 ml/min flow rate. The distribution coefficients (Kd) were calculated according to the following expression: $K_d = \frac{V_e - V_0}{V_t - V_0}$, where $V_e$ is the elution volume of the peptide, $V_0$ is the void volume and $V_t$ is the column bed volume.

III.70. UV–Vis titration in which successive additions of HgCl$_2$ were made to a 111 µM solution of C16C19-GGY, pH 5.4-5.5.

III.71. UV-vis Titration of 111 µM C16C19-GGY by HgCl$_2$ at pH 5.4-5.5.

III.72. Circular Dichroism titration of C16C19-GGY (103 µM) by HgCl$_2$.

III.73. Near UV Circular dichroism spectrum of C16C19-GGY (175 µM) taken in the presence of a twofold excess of HgCl$_2$ in 20 mM sodium acetate buffer pH 5.4.

III.74. CD of C16C19-GGY by HgCl$_2$ in the presence or absence of TCEP. Plot of normalized ellipticity at 222 nm as a function of Hg(II) addition.

III.75. The overlay of UV-vis titrations of C16C19-GGY by HgCl$_2$ in the presence or absence of TCEP.

III.76. The overlay of UV-vis titrations of C16C19-GGY by HgCl$_2$ performed in water or 0.2 M sodium acetate buffer pH 5.5.

III.77. HPSEC results obtained by using series of peptide standards on Superdex 75 HPSEC column under non-denaturating conditions (0.1 M KCl / 0.05 M KH$_2$PO$_4$, pH 7), 0.4 ml/min flow rate. The distribution coefficients (Kd) were calculated according to the
following expression: $K_d = \frac{V_e - V_o}{V_e - V_o}$, where $V_e$ is the elution volume of the peptide, $V_o$ is the void volume and $V_t$ is the column bed volume.

III.78. HPSEC of the Ag(I)-metalloprotein and Cu(I)/C16C19-GGY used as a standard

III.79. UV titration of 117 µM C16C19-GGY by Ag(I)

III.80. UV titration of 117 µM C16C19-GGY by Ag(I). The titration plot of $A_{230}$ vs. equivalents of Ag(I): growth of new absorption band upon Ag(I) addition

III.81. Circular Dichroism titration of 165 µM C16C19-GGY by AgNO$_3$. Plot of $[\theta]_{222}$ as a function of added Ag(I)

III.82. Near UV Circular dichroism spectrum of C16C19-GGY (165 µM) taken in the presence of a 1.4-fold excess of AgNO$_3$

III.83. Thermal melting curves of 55 µM C16C19-GGY in the presence of two fold excess of Ag(I) or Cu(I) and five fold excess of TCEP

III.84. The overlay of UV-vis titrations of C16C19-GGY by AgNO$_3$ performed in 0.2 M sodium acetated buffer pH 5.5

III.85. HPSEC results obtained by using series of peptide standards on Superdex 75 HPSEC column under non-denaturating conditions (0.1 M KCl / 0.05 M KH$_2$PO$_4$, pH 7), 0.4 ml/min flow rate. The distribution coefficients ($K_d$) were calculated according to the following expression: $K_d = \frac{V_e - V_o}{V_e - V_o}$, where $V_e$ is the elution volume of the peptide, $V_o$ is the void volume and $V_t$ is the column bed volume.

III.86. HPSEC of the Pt(II)-metalloprotein and Cu(I)/C16C19-GGY used as a standard

III.87. HPSEC of the Au(I)-metalloprotein and Cu(I)/C16C19-GGY used as a standard

III.88. HPSEC results obtained by using series of peptide standards on Superdex 75 HPSEC column under non-denaturating conditions (0.1 M KCl / 0.05 M KH$_2$PO$_4$, pH 7), 0.4
ml/min flow rate. The distribution coefficients (Kd) were calculated according to the following expression: 

$$K_d = \frac{V_d - V_v}{V_e - V_v}$$

where Ve is the elution volume of the peptide, Vo is the void volume and Vt is the column bed volume.

III.89. Circular Dichroism titration of 102μM C16C19-GGY by Au(I) in 0.2 M sodium acetate buffer pH 5.4-5.5. Plot of [θ]_{222} as a function of added Au(I).
<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>III.1. Helical Properties of Metallated Peptide Complexes</td>
<td>52</td>
</tr>
<tr>
<td>III.2. Induction of helicity upon addition of metal ion</td>
<td>60</td>
</tr>
<tr>
<td>III.3. Luminescence properties of Cu(I)-metalloproteins: Luminescence quantum yield of Cu(I)-metalloproteins (298 K)</td>
<td>67</td>
</tr>
<tr>
<td>III.4. Luminescent properties of Cu(I)-metalloproteins: Luminescence lifetimes</td>
<td>89</td>
</tr>
<tr>
<td>III.5. Comparison of metal-binding sites for luminescent copper metalloprotein</td>
<td>95</td>
</tr>
<tr>
<td>III.6. Metal : peptide stochiometries under different conditions</td>
<td>146</td>
</tr>
</tbody>
</table>
CHAPTER I. INTRODUCTION AND BACKGROUND

Metalloproteins play very important roles in many biological systems. They are involved in such fundamental biological processes as metabolism, electron transfer, respiration, homeostasis, oxygen transport, catalysis, photosynthesis, detoxication of radicals, ATP synthesis, and transport and the storage of metal ions.\textsuperscript{1-11} In particular, copper metalloproteins are essential for all living systems which are involved in these biological processes. However, the mechanisms of intracellular copper uptake must be strictly regulated in order to prevent oxidative damage and metabolic disorder.\textsuperscript{1,12} A variety of proteins, called copper transporters or copper chaperones, are involved in the mediation of copper trafficking. They guide copper through the cytoplasm, deliver and insert it into specific physiological partners. Disruption of the mechanisms of copper homeostasis and trafficking pathways in cells leads to significant physiological problems including Menkes syndrome and Wilson disease.\textsuperscript{8-11,13} We are now only beginning to understand these mechanisms of metal ion-trafficking and homeostasis which are well-regulated in biological milieu. An important challenge yet to be overcome is understanding the factors which define metal ion recognition and binding to specific proteins.\textsuperscript{14}

The metal-binding process in vivo may be controlled by either kinetic or thermodynamic factors.\textsuperscript{15} Specific incorporation of a metal ion can be dependent on the concentration of the metal ion present in the biological media \textit{in vivo} and the affinity of a metal-binding site for a specific metal ion.
The thermodynamics of metal-ion selectivity may in turn be influenced by the following factors:

a) the hard-soft/acid-base properties of the metal ion and its binding site

b) the coordination geometry of the metal ion and the geometric arrangement of the ligands involved in the formation of the metal binding

c) the compatibility between relative sizes of the metal ion and its binding site.

To date, only a few studies\textsuperscript{14,16} were performed to address the question of how these factors affect the metal-binding and selectivity in proteins. Caguiat and others\textsuperscript{17} have addressed the question of metal-ion specificity of MerR protein which specifically binds to a Hg(II). The MerR family of transcriptional activators have a trigonal mercury-binding site which responds to the presence of mercury ion at the concentration of $10^{-9}$ M. \textit{In vivo} this protein binds Hg(II) even in the presence of 1000-fold excess of Cd(II) and does not respond to Ag(I).\textsuperscript{17} It was proposed that such unusual specificity is not only governed by the hard-soft/acid-base properties of metal-binding site and ionic radii of metal ions but the correct orientation of the coordinating ligands.\textsuperscript{17}

McCall and co-workers\textsuperscript{14} have described the metal ion affinity and selectivity of a series of variants of human carbonic anhydrase II (CAII).\textsuperscript{14} Histidines at positions 94 and 119 positions were substituted with Asp, Glu, Asn, and Cys residues capable of metal ion binding. It was shown that the affinity of these variants follow the Irving-Williams trend ($\text{Mn}^{2+} < \text{Fe}^{2+} < \text{Co}^{2+} < \text{Ni}^{2+} < \text{Cu}^{2+} \geq \text{Zn}^{2+}$) originally suggested for small molecule chelators. These results indicate that the metal-ion selectivity of the described protein is defined by a combination of hard/soft acid-base properties and crystal field stabilization effects.\textsuperscript{14} In contrast, Pasternak and co-workers have shown that the affinity for divalent transition metal ions of a designed protein called DF2, a dimeric four-helix bundle capable of binding two metal ions, deviates from the
Irving-Williams series. It has been suggested that this occurs due to the limited flexibility of the metal-binding site in DF2 metalloprotein. Therefore, the existence of preorganized metal-binding site of DF2 has a significant effect on geometric preferences of a coordinating metal ion, greatly affecting its metal-ion affinity.\textsuperscript{16}

Similar results were demonstrated by Benson et al. who introduced the minimalist, mononuclear iron binding site, consisting of three histidines, into a thioredoxin scaffold which is a protein naturally devoid of this metal center. The metal binding of first-row transition metal ions were also shown the deviate from the Irving-Williams trend, and this was explained by the rigid first coordination sphere of the protein matrix.\textsuperscript{18} Therefore, these studies emphasized that the geometric arrangement of the ligands involved in the formation of the metal binding site can greatly affect the selectivity of metalloproteins.

Despite the above observations, much remains to be learned about the balance of forces which drive the formation of unique metalloprotein structures as well as the major determinants of metal selectivity in proteins.\textsuperscript{19} It is very important to elucidate how the protein environment or the coordination preferences of metal ions influence the metal-binding, metalloprotein structure, and specificity.\textsuperscript{20} Therefore, significant attention has been focused on investigating the binding properties of metalloproteins in order to understand their biological functions.

There are two extreme cases of metal-protein interactions.\textsuperscript{21} The first is defined as metal-induced protein folding in which metal ion directs and organizes the protein ligands with respect to its preferred coordination geometry.\textsuperscript{22} In such cases, the nature of the specific metal ion plays a dominant role in the metal-binding process. Generally, such metal-binding sites adopt common coordination geometries.\textsuperscript{16,21,23}
In contrast, in the second extreme protein backbone dictate the coordination environment and structure has a dominate role in the metal-protein interactions. It provides a rigid coordination framework which incorporates a metal ion.\textsuperscript{22}

\textbf{1.1. Metal-Induced Folding of Metalloproteins: Zinc Finger Peptides}

Zinc finger domains are perhaps the best-studied example of this type of metal-induced protein-folding.\textsuperscript{23,24} Zinc finger peptides represent the extreme case when the protein folding is coupled to metal-binding, which corresponds to a transition from an unfolded to completely folded protein structure.\textsuperscript{24} Very often such systems provide a unique opportunity to investigate the fundamental issues relating to the protein folding, the mechanisms of metal ion incorporation, and metal-ion specificity.\textsuperscript{22}

Zinc fingers are small metal-binding protein domains originally described in transcription factor IIIA(TFIIIA).\textsuperscript{23,24} These domains are found in many gene regulatory proteins which are involved in the nucleic acid binding and gene regulation.\textsuperscript{24}

It has been found that in the absence of a bound metal ion, zinc-finger proteins are unstructured in the absence of a metal ion and fold in an anti-parallel $\beta$-sheet and $\alpha$-helix upon metal binding.\textsuperscript{25} Such conformational change of metalloproteins can initiate the functional activities.\textsuperscript{19}

A classical metal-binding site of a zinc finger domain consists of 2 histidine and 2 cysteine ligands. Metal binding studies using Co(II) as a spectroscopic probe have revealed that in the folded state the zinc finger proteins have a tetrahedral metal center. It was also shown that zinc finger proteins display tighter binding with Zn(II) over Co(II) ($K_{d}(\text{Co(II)})$/ $K_{d}(\text{Zn(II)})=1.4\times10^3$) which was explained by the strong geometric preferences of Zn(II) for a
tetrahedral binding site. In this case, zinc finger domains can exhibit specific zinc binding and functionality.

Because of its relative simplicity and small molecular weight, the zinc-finger motif has been utilized as a model system to address the fundamental questions of protein structure and function, metal-binding, and protein folding. Several research groups have addressed the following fundamental questions in studying zinc-finger peptides:

- Which role does the protein ligand environment (first coordination shell) have in metal ion recognition and binding, and how does it affect the metal ion specificity?

- What is the role of a second-shell protein environment in metal-directed protein folding; and what is its effect on the metal ion specificity?

It has been shown that in addition to Zn(II), the binding of Cd(II) and Co(II) to zinc-finger peptides can also induce protein folding. Berg et al. has been working on resolving the role of peptide ligands set in (first coordination shell) metal-ion specificity of zinc finger peptides. Studies were performed on a series of peptides containing the following metal-binding ligands: CCHH (natural occurring zinc finger motif), CCHC, and CCCC. Interestingly, these systems have been shown to be very specific to the metal ion. Competition experiments demonstrated that a natural zinc finger motif with a CCHH protein ligand set is selective not only for Zn(II) over Co(II), Ni(II), Mn(II), Fe(II) and also over Cd(II). The comparison of dissociation constants estimated from the competition experiments indicated that the affinity for Cd(II) over Zn(II) increased dramatically as the number of thiolates ligands was increased. This
result indicates that the affinity for Cd(II) over Zn(II) which follows the order CCCC > CCHC > CCHH in a manner consistent with the hard/soft acid/base concept.\textsuperscript{26} This work indicated the particular importance of the His\textsubscript{2}Cys\textsubscript{2} ligand set in the selective Zn(II)-binding of the native zinc-finger domain. Metal binding studies have revealed that the metal-binding specificity is governed by the combination of hard/soft acid/base effects and ligand field stabilization energy electronic effects which are crucial in the determination of specificity of metal-binding sites for zinc over other divalent first-row transition metal ions.\textsuperscript{24,26}

The other fundamental question listed above is the role of second-shell protein environment in metal-induced protein folding and binding specificity.\textsuperscript{23} Scott, Berg, Weiss and others\textsuperscript{23,24,28-32} have studied the contribution of hydrophobic interactions to proper protein folding and the formation of a well-defined three-dimensional protein structure. NMR techniques were used to investigate how the positions of hydrophobic amino acid residues affect the tertiary structure of the zinc finger domains.\textsuperscript{32} The mutations of the conserved hydrophobic amino acids lead to the destabilization of the protein structure. In particular, the Phe residue was found particularly important in proper protein folding as its replacement lead to a significant destabilization of the overall protein structure.\textsuperscript{31,32}

The importance of second shell interactions was also been studied using a minimalist Cys\textsubscript{2}His\textsubscript{2} zinc finger peptide (MZF) having the conserved metal-binding and hydrophobic residues typical for the TFIII-A-like protein domain.\textsuperscript{33} Four different mutants were prepared to probe the importance of hydrophobic residues in metal-binding and metal-ion specificity. Metal-binding studies using cobalt (II) as an optical probe along with the NMR experiments showed that the minimalistic MZF zinc finger peptide adopts a three-dimensional structure very similar to the natural zinc finger protein domains. Metal-binding studies and NOE experiments revealed
that the positions of seven conserved hydrophobic amino acid residues were especially important for correct protein folding. Based on this study, it was suggested that the hydrophobic packing (second-shell effects) along with the metal binding (first-shell effects) play an important role in the protein folding and stabilization of a well-defined protein structure.\(^{33}\)

Marino and Regan\(^ {28}\) considered both first- and the second-shell effects to construct a novel metalloprotein by the incorporation of a tetrahedral-zinc metal-binding site into the B1 domain of the IgG-binding protein. The affinity of the designed protein for Zn(II)-binding was enhanced by introducing hydrogen-bonding interactions to stabilize the metal-coordinating histidine ligand in second shell effects.\(^ {28}\) Therefore, an important step was made towards the design of more complex proteins with predictable structures.

The studies described above demonstrated that zinc-finger proteins are one extreme of metal-protein interactions which is described as a metal-induced peptide folding. The metal-binding and protein-folding process is fully controlled by the zinc ion which directs and organizes the protein ligands with its coordination geometry playing the dominant structural role in the process of protein folding.\(^ {22}\) Structural studies of zinc-finger proteins have also revealed that the first and second shell coordination environments have a particular importance in the formation of a well-defined protein structure and metal-specific binding.

In contrast, in the other extreme case of metal-binding the peptide plays in determining the structure of metalloproteins.\(^ {22,34,35}\) In such case, metal-coordination environment is dictated by the preformed protein scaffold which creates a preorganized rigid coordination sphere.\(^ {22,34,35}\) Usually, such metal binding sites remain unperturbed upon removal of the metal ion. Such performed metal-binding sites often have unusual geometries which to enable a variety of useful
functions, including electron transfer and catalysis. The example of such metal-binding sites is blue copper proteins which modulate their redox properties by changing the geometry of distorted tetrahedral copper site.\(^{36,37}\)

In the most cases the metal protein interactions of native metalloproteins lie in between of these two extremes described above. The synergy of these forces defines the final metalloprotein structure.

Metalloprotein design is a successful approach to study metal-protein interactions and to construct metalloproteins capable of folding into well-defined three-dimensional structures.

I.2. Metalloprotein Design

The ultimate goals of protein design are the engineering of synthetic metalloproteins with specifics chemical functions, well-defined structures, and/or interesting new physical/chemical properties. To achieve these goals designed proteins must fold into a unique, functionally-active three-dimensional structure.\(^{38}\) At this stage, we have a limited ability to predict how a metalloprotein will fold into a particular functionally-active structure and which factors define metalloprotein reactivity. Therefore, such goals present a considerable challenge and the design of new functional proteins “from scratch” is not yet possible.\(^{10}\)

However, the approach of de novo metalloprotein design which combines the fundamental knowledge of protein design and bioinorganic chemistry and can be defined as a design from the first principles, has been used to create new model metalloproteins.\(^{19,22,34,38-40}\) De novo metalloprotein design studies help to elucidate the relationship between the primary protein sequence, the forces which stabilize the final folded three-dimensional structure, and the functions of the metalloproteins.\(^{11,19,21,22,34,38,41}\) This approach utilizes model systems which are
simpler than natural systems but can provide a sufficient structural or functional complexity and are amenable to systematic studies. The approach of the de novo metalloprotein design has utilized two major strategies to construct metalloproteins. The first is to construct minimalistic metalloprotein models, usually based on the α-helical coiled-coil structure. The second involves redesign of naturally occurring proteins to incorporate metal-binding sites into existing protein scaffolds that do not normally bind metal ions.

I.2.1. Structural Design

Studying minimalistic protein models provides insight into the factors which are responsible for the stabilization of the three-dimensional structure of native metalloproteins: the balance of hydrophobic, hydrogen bonding, hydrophilic, van der Waals, and electrostatic interactions, necessary for existing stable tertiary protein structures capable of performing proper functions.

The approach of de novo protein design is now used to design minimalistic model proteins based on the α-helical coiled-coil structure. This motif is a common protein domain found in many native protein systems which can be described as two, three, or four amphipathic α-helices that are wound into a non-covalent left-handed supercoil. The coiled coil structure is driven and stabilized by hydrophobic interactions in a “knob-into-holes” manner in which the hydrophobic side-chains of bulky amino acids (isoleucine, leucine, or valine) are directed into the core of the domain arranged to optimize van der Waals packing.

The coiled coil motif was chosen as a model system for protein design studies because of its structural simplicity. Synthetic coiled-coil sequences generally consist of several seven amino acid residue heptad repeats, denoted by the letters (abcdefg)n. Positions a and d of the
heptad are occupied by hydrophobic amino acids, positions $b$, $c$, and $f$ of the heptad repeat are occupied by hydrophilic residues, exposed to the solvent, and positions $e$ and $g$ are occupied by oppositely-charged residues to form salt bridges between complimentary helices.\textsuperscript{19} A helical wheel diagram (Figure 1) illustrates a parallel two-stranded $\alpha$-helical coiled coil.

Even though the hydrophobic effect is the driving force for the formation of a coiled-coil, the specific conformational properties of coiled-coils depend upon the particular amino acids used to form the heptad repeat\textsuperscript{19,46-48} For example, the sequence studied by Harbury et al.\textsuperscript{48} having the isoleucine and leucine residues placed at the $a$ and $d$ positions, respectively, was shown to form only dimers. It was also shown that the formation of the coiled-coil trimers can be achieved by placing $\beta$-branched amino acid residues, for instance isoleucine, at both the “a” and “d” positions of the heptad. The formation of tetramers is observed when leucine and isoleucine are situated at the heptad “a” and “d” positions, respectively.

![Figure I.1](image)

**Figure I.1.** Schematic views of synthetic coiled-coils. **Top:** primary amino acid sequence of the two helices of the coiled-coil. **Left:** Helices in parallel orientation. **Right:** helical wheel diagram of the coiled coil; view down helical axes of the coiled-coil.
Several research groups have utilized the coiled-coil motif to create metal-binding sites in either solvent-exposed positions or buried in the hydrophobic core of coiled-coils.\textsuperscript{19,34,41,43,44,49-52} One example of creating a solvent-exposed metal-binding site was shown by Hodges and co-workers\textsuperscript{41} who engineered a “hard” lanthanide-metal-binding site on the surface of coiled-coil. A two-stranded disulfide-bridged 35-amino-acid-residue coiled coil with $Q_g V_a G_b A_c L_d Q_e K_f Q_g$ heptad repeat was used for this design. Here, the hydrophobic positions “a” and “d” were occupied by leucine and valine amino acid residues and glutamine amino acid residues occupied “e” and “g” positions. The metal-binding site was designed by substitution of $\gamma$-carboxyglutamic acid for glutamine at positions 15 and 20 of the sequence which correspond to the “e” and “g” positions of a middle heptad repeat. These substitutions resulted in ionic repulsions existing between the negatively charged $\gamma$-carboxyglutamic acids of each peptide strand which
destabilized of the coiled-coil structure. Thus, the apopeptide existed as a random coil in the absence of a metal ion in aqueous solutions. However, in the presence of lanthanide ions, the peptide underwent a transition from a random coil to an \( \alpha \)-helical structure. The folded lanthanide-bound peptide was stabilized by metal binding because the metal ion was chelated by the \( \gamma \)-carboxyglutamic acid-containing side chains of the peptide. The metal ion binding was monitored by CD and nuclear magnetic resonance spectroscopy, which demonstrated the tight binding of hard metal ions, such as LaCl\(_3\) and YbCl\(_3\) to the peptide with 1 : 2 peptide-to-metal ion stoichiometry. The metal dissociation constants were measured to be for La(III) 0.6 ± 0.3 \( \mu \)M, for Yb(III) 0.4 ± 0.2 \( \mu \)M, for Zn(II ) 1.7 ± 0.3 \( \mu \)M, and for Ca(II) 18 ± 2 mM, indicating the higher affinity of the designed peptide for lanthanide ions and zinc ions over calcium.\(^{41}\) This successful design demonstrates an how metal ligation property and secondary structural elements of peptide can induce and stabilize a \emph{de novo} designed metalloprotein.

Several research groups have successfully demonstrated the creation of metalloproteins by the incorporation of metal binding sites into the hydrophobic core of coiled coils.\(^ {19,41,43,49-53}\) Pecoraro and co-workers have been investigating the interplay between the coordination preferences of metal ions versus the conformational properties of the peptide framework in metal-binding studies.\(^ {19,44,49,53-55}\) The metal binding properties of the de novo designed three-stranded \( \alpha \)-helical coiled-coil peptides based on the parent peptide called “TRI” have been described.\(^ {44,49,53,55,56}\) The parent 30-residue peptide TRI has the sequence Ac-G(LKALEEK)\(_4\)G-NH\(_2\) having hydrophobic positions “a” and “d” occupied by leucine residues.\(^ {56}\) The designed peptide was shown to exist as a three-stranded coiled coil at neutral or higher pH and a metal-binding site was engineered into the hydrophobic core by placing a cysteine residue at either position 9 (TRI L9C), 12 (TRI L12C) or 16 (TRI L16C) of the sequence.\(^ {53}\) It was demonstrated
that this three-stranded coiled coil peptide which has a well-defined three dimensional structure in the absence of a metal ion can provide a trigonally symmetric framework which forces such metal ions as, Hg(II), Cd(II), and Pb(II), to adopt an unusual trigonal coordination geometry.\textsuperscript{21,44,53-55} In contrast, Hg(II) usually adopts linear coordination geometry. The only example of trigonal mercury coordination geometry in native systems is metalloregulatory MerR metalloprotein. However, Pecoraro et al. were able to create a synthetic Hg(II)-metalloprotein with a trigonal Hg(II) site. The presence of such metal-binding site was proved by EXAFS studies. This result demonstrates that the coordination preferences of Hg(II) can be controlled by the preorganized trimetric of protein scaffold.\textsuperscript{44,53-55}

To challenge the notion that a highly stable, preorganized peptide scaffold is necessary to enforce uncommon geometries of a metal ion, Pecoraro and co-workers performed binding studies on unstructured, weakly associated peptides. Here, the 23-residue Baby L9C, which is one heptad shorter than the TRI peptide, showed less than 20\% helicity indicating a largely disordered coiled coil in the absence of a metal ion. However, the Baby L9C peptide was able to stabilize a trigonal coordination of Hg(II) within its hydrophobic environment.\textsuperscript{21,53,54} They have shown that a peptide scaffold can bind such metal ions as, Hg(II) or Cd(II) to adopt a trigonal geometry even in the case of using less-structured peptides which lack a rigid peptide backbone. The latter case is also accompanied by the metal-induced folding resulting in a metallopeptide assembly with a well-defined three-stranded $\alpha$-helical bundle.\textsuperscript{49}
The peptide sequences of the TRI family: (Adapted from reference 1)

**BabyTRI** Ac-G LKALEEK LKALEEK LKALEEK G-NH₂

**BabyL9C** Ac-G LKALEEK CKALEEK LKALEEK G-NH₂

**BabyL12C** Ac-G LKALEEK LKALEEK LKALEEK G-NH₂

**TRIL9C** Ac-G LKALEEK CKALEEK LKALEEK LKALEEK G-NH₂

**TRL12C** Ac-G LKALEEK LKALEEK LKALEEK LKALEEK G-NH₂

**GrandL9C** Ac-G LKALEEK CKALEEK LKALEEK LKALEEK LKALEEK G-NH₂

This work shows how the interplay between the conformational preferences of the peptide and the coordination geometry of a metal ion can influence the conformational properties of metalloproteins. From this work the authors demonstrate that the formation of the unusual coordination geometries of the designed metallopeptides can be achieved within both preorganized and relatively unstructured peptides scaffolds.

In related work, Tanaka et al. have described the metal-induced peptide folding of a parallel three-stranded coiled-coil peptide. The authors designed a three-stranded coiled coil peptide having the amino acid sequence YGG(IEKKIEA)₄. A metal-binding site was introduced into the hydrophobic core of the designed peptide by replacing the hydrophobic amino acid residues of the third heptad repeat with one or two histidine residues. These substitutions cause the apopeptide to exist as a random coil structure. However, in the presence of transition metal ions, such as Ni(II), Co(II), Zn(II), and Cu(II), the peptide undergoes a metal-induced self-assembly, to form a triple-stranded-α-helical bundle having a octahedral metal-binding site in the
hydrophobic core comprise from histidine residues. The metal-binding was monitored by circular dichroism spectroscopy and dissociation constants were found to be $K_d = 35 \pm 1 \text{ µM}$ for Co(II), $23 \pm 2 \text{ µM}$ for Zn(II), $17 \pm 1 \text{ µM}$ for Cu(II), and $5 \pm 0.3 \text{ µM}$ for Ni(II).

In order to investigate the metal ion selectivity of this processes, the authors designed the peptide (IZ-AC) having a “soft” metal-binding site in its hydrophobic core. Thus, Ile residues at the $a$ and $d$ positions of the third heptad repeat were substituted with Cys and Ala residues which resulted in the destabilization of the coiled-coil structure. Both amino acid residues were directed toward the hydrophobic interior of the putative coiled-coil. In these experiments, the apopeptide was shown to exist as a random coil in the absence of a metal ion. However, in the presence of such “soft” metal ions as Cd(II), Cu(I), and Hg(II), the unstructured peptide was induced to fold into a three-stranded $\alpha$-helical bundle with a trigonal metal-binding site, as monitored by circular dichroism spectroscopy. In contrast, “harder” metal ions, such as Ni(II), Co(II), and Zn(II) did not show the inducing of peptide self-assembly, which is indicative of metal ion selectivity. Interestingly, these unstructured peptides not only fold into metal-induced three-stranded $\alpha$-helical bundles but force Cd(II) and Hg(II) to adopt a trigonal coordination geometry.
In contrast to work described by Pecoraro,\textsuperscript{21} Hodges,\textsuperscript{40} and Tanaka\textsuperscript{43,51} the work described in this dissertation illustrates how the structures of metalloproteins may be controlled by the coordination chemistry of their inorganic cofactors.

Figure I.3. Helical wheel diagram of the coiled coil; view down helical axes of the coiled-coil. Adapted from reference Li.\textsuperscript{51}
The work described in this dissertation utilizes the principles of *de novo* protein design to create novel synthetic metalloproteins. Similar to the work of Pecoraro \(^{21}\) and Tanaka \(^{43,51}\) the synthetic metalloproteins described in this dissertation were based on the coiled-coil structure. However, the Cys-X-X-Cys metal-binding motif, found in a variety of metalloproteins including rubredoxin, was incorporated into both positions “a” and “d” of the third heptad repeat to create of a rubredoxin-like (Cys)\(_4\) “soft” tetrahedral metal-binding site within the hydrophobic peptide-peptide interface. Similar to work described by Tanaka et al.\(^{43}\) this cysteine-containing random coil apopeptide is capable of binding different transition metal ions, which results in the formation of a metal-bridged self-organized \(\alpha\)-helical bundle.

The system described here differs from those described by Pecoraro, \(^{19,21,44,54,55}\) Hodges, \(^{40}\) and Tanaka\(^{43,51}\) in several principal ways. Firstly, the synthetic metalloproteins presented here utilize the parent apopeptide sequences which have been shown to exist as two-stranded \(\alpha\)-helical coiled-coils, not three-stranded coiled-coils. This cysteine-containing random coil apopeptide is capable of binding different a variety of soft metal ions such as, Cu(I), \(^{52}\) Cd(II), \(^{41}\) Ag(I), Hg(II), Au(I), and Pt(II) which results in the formation of a metal-bridged self-organized \(\alpha\)-helical bundles. The Cd(II) and Hg(II) synthetic metalloproteins exist as peptide dimers, however, Cu(I), Ag(I), and Pt(II)-metalloprotein were shown to exist as 4-helix bundles, and Au(I)-metalloprotein exists as a hexamer. \(^{52}\) Such oligomerization states differ from those predicted by the original design. This is an important result showing that inorganic cofactors can tune the oligomerization state of the peptides. To our knowledge it is a first example of synthetic metalloproteins which displays metal-specific oligomerization states.
Additionally, it is shown that this system displays metal-dependent binding stoichiometry: a 1:1 metal:peptide stoichiometry is observed for the Cu(I) and Ag(I) adducts but the Cd(II) and Hg(II) complexes show a metal:peptide stoichiometry of 1:2.

Significantly, the conformational change from a disordered random coil to a \( \alpha \)-helical coiled-coil occurs upon binding to a variety of soft metal ions including Cd(II), Hg(II), Ag(I), Cu(I), Pt(II), and Au(I) to the C16C19-GGY but not “harder” transition metal ions such as, Fe(II), Co(II), Ni(II) Zn(II), or Pb(II). These results demonstrate that this system displays metal-binding specificity.

The studies described in this dissertation and the work described by Pecoraro\(^{19,21,44,54,55} \) provide insight into the details of metal-protein interactions. Pecoraro et al. were able to create a synthetic Hg(II)-metalloprotein having a trigonal Hg(II) binding site and showed that the coordination preferences of Hg(II) can be controlled by the preorganized protein scaffold.\(^{44,53-55} \) The work presented in this dissertation describes how the structural properties of synthetic metalloproteins can be controlled by inorganic cofactors. These two examples demonstrate the importance of the balance between the binding preferences of inorganic cofactors and conformational preferences of protein matrix.

Such miniature synthetic native-like metalloproteins display interesting rich metal-binding properties and can be regarded as sophisticated models to study metal-binding metal-folding processes and to probe metal-protein interactions and metal ion specificity.
Knowledge gained from studies described above has allowed addressing the next goal of incorporating chemical functionality into the proteins in order to create new functionally active synthetic systems.

1.2.2 Functional Design

Metal-binding sites are found in approximately one-third of all proteins and serve either structural or functional roles. As it was discussed above, in structural sites metal ions can assist, regulate, or even direct protein folding. They also add new functionality to proteins, for example, dioxygen binding, electron transfer, catalysis, metal ion transport and storage. In particular, electron transfer reactions have a particular importance because they play key roles in many biological systems. The functional properties of electron transfer proteins can be fine-tuned by different metal ions or their redox states and the unique geometric arrangement of protein ligands or by changing conformational properties of the protein backbone. For instance, cytochromes $b$ or $c$ which have the same inorganic cofactor (haem iron) are capable of tuning the redox potential ranging from -400 mV to 400 mV. It is therefore, important to investigate the properties of these systems in order to understand the nature of biological reactions. This knowledge will allow develop and engineer novel synthetic systems capable of performing processes currently unknown in nature.

The functional design of metalloproteins targets the creation of new metalloproteins with novel structures, controlled functions, and predictable properties. Such functional design and preparation of synthetic metalloproteins provides the opportunity to recruit new functions into protein structures and will facilitate the creation of novel systems which can find a successful
application in pharmaceuticals, medical diagnostics, catalysis, affinity chromatography, and protein engineering.\textsuperscript{10,60}

Notable examples of incorporating different chemical functionalities are reported to date which demonstrate an important first step towards the creation of functionally active metalloproteins and enzymes.\textsuperscript{19}

One successful example of metalloprotein active site design inserted into preformed protein framework is described by Lombardi, DeGrado and co-workers.\textsuperscript{61} Using a “retrostructural approach” of native carboxylate-bridged bimetallic proteins, the authors designed the C\textsubscript{2} symmetric “Due Ferro” (DF1) protein having a Glu-X-X-His metal-binding motif. DF1 is a homodimer comprised of two non-covalently self-associated helix-loop-helix motifs which forms the well-defined geometry for the dimetal-binding four helix bundle. The design takes into consideration the interactions of the second coordination shell providing hydrogen bonds to the ligating residues resulting in formation of the dinuclear metal-binding site which consists of the two five-coordinate metal ions.\textsuperscript{61} Moreover, this elegant design provided the well-defined and well-characterized metallopeptide architecture which has also displayed catalytic activity capable of oxidizing Fe(II) to Fe(III) under single turnover conditions.\textsuperscript{42} Therefore, this system is a successful step towards engineering metalloenzymes capable of selective catalysis.

Holm and Laplaza have described the successful design of a Ni-Fe\textsubscript{4}S\textsubscript{4} protein based on a helix-loop-helix motif. The created bridged assembly is related to the A-cluster of carbon monoxide dehydrogenase.\textsuperscript{62} The designed peptide scaffold having a helix-loop-helix motif was shown to stabilize the bridged assemblies having two metal cofactors in close proximity to each
These studies opened up the opportunity to design and redesign de novo catalytic sites and provide important knowledge of the roles and functions of metal-binding sites in proteins.

Several research groups have described the synthesis of heme-protein models which usually consist of four-helix bundles that can accommodate a histidine-ligated heme in the hydrophobic environments. The Dutton and Gibney groups\textsuperscript{45,60} have described the design of heme protein maquettes based on four-helical bundle oriented in up-down-up-down directions. One of the particularly important goals of this work is to elucidate how the protein matrix is capable of tuning the redox potential of hemes which can enable a variety of functions such as, electron transfer, oxygen binding, and catalysis.\textsuperscript{35,37}

In other work, McLendon et al.\textsuperscript{19,63} have successful designed peptide-based model systems based on a parallel three-helix bundle which have a redox-active N-terminal ruthenium(II)tris-bipyridine moiety. A electron-acceptor, (Ru(NH$_3$)$_5$)$_3^{3+}$ was attached to a histidine amino acid residue placed at different locations along the $\alpha$-helical chain. It was shown that the drop in electron transfer rate depends on distance between the donor and acceptor exponentially, as it was theoretically predicted.\textsuperscript{64} Therefore, this work demonstrated how electron transfer rates depend on protein structure and that it is possible to design functional protein models with predictable structures and functions.\textsuperscript{64}

The Ogawa group\textsuperscript{65,66} described the design, synthesis, and characterization of new synthetic electron transfer metalloproteins to study electron-transfer process across non-covalent helix-helix interfaces of coiled-coils. A 30-mer peptide sequence was derivatized for electron-transfer studies are based on the IEALEGK heptad repeat. Histidine amino acid residues located at the solvent-exposed positions were used in order to attach electron donor and acceptor, (Ru(trpy)(bpy))$^{2+}$ and (Ru(NH$_3$)$_5$)$_3^{3+}$, respectively, with the electron donor/acceptor separation of
24 Å. The observed electron-transfer rate constant determined by pulse radiolysis was found to be $380 \pm 80 \text{ s}^{-1}$ which is consistent with rates seeing for electron transfer reactions occurring in native systems over similar distances.\textsuperscript{65,66}

In related studies\textsuperscript{67,68} an artificial electron-transfer metalloprotein has been synthesized and characterized in order to study directionality of electron transfer along a helix dipole. The apopeptides were functionalized with $[\text{Ru(bpy)}_2(\text{phen-CIAc})]^{2+}$ and ruthenium pentammine was attached to a histidine residue. The electron transfer rates were identical in both directions, and therefore, no evidence for the electron-transfer directionality was observed.\textsuperscript{67,68}

Zinc-finger domain was also utilized in \textit{de novo} protein design and redesign studies to produce chemical or catalytic functionality into these systems.\textsuperscript{24} Several groups have addressed the question of how the structural role of zinc-binding sites can be transformed into catalytical ones. Berg and co-workers\textsuperscript{30} attempted to engineer a catalytical metal-binding site into a truncated zinc-finger by analogy to a catalytical site of carbonic anhydrase. Carbonic anhydrase is an enzyme having a zinc-binding site in which one coordination position of zinc remains open to engage a fourth-, catalytically active exogenous ligand (water or hydroxide ion).\textsuperscript{30} Even though the designed Zn(II)-metalloprotein showed no catalytic activity, the authors proved that water molecule occupied the fourth coordination position.\textsuperscript{30} Thus, zinc finger peptides are a good model system not only for elucidating different aspects of metal-induced protein folding\textsuperscript{23,25,69} but for designing existing and \textit{de novo} catalytical sites. It provides necessary insights into understanding the roles and functions of metal-binding sites in proteins, and also facilitates the development of improved \textit{de novo} protein design strategies.\textsuperscript{12-15}
The work described in this dissertation has also targeted the important goal of incorporating chemical functionality into de novo designed metalloproteins. The described system not only undergoes the metal-induced peptide folding and exhibits specific metal-binding, but the synthetic Cu(I)-metalloprotein described here displays intense room temperature long-lived (microsecond) luminescence at 600 nm. Such incorporated chemical functionality allows using this *synthetic* metalloprotein as a photoinduced electron-transfer agent in protein-like environment in future studies.

The knowledge gained from these studies can also illuminate the new direction towards creating novel functionally-active systems.
REFERENCES TO CHAPTER I


(2) Barker, P. D. *Current Opinion in Structural Biology* 2003, 13, 490-499.


        5422-5423.
(61) Lombardi, A., Summa, C. M., Geremia, S., Randaccio, L., Pavone, V., DeGrado, W. PNAS
        2000, 97, 6298-6305.
(65) Kornilova, A. Y., Wishart, J. F., Xiao, W. Z., Lasey, R. C., Fedorova, A. V., Shinm, Y.-K.,
CHAPTER II. EXPERIMENTAL

Materials

The F-moc-protected L-amino acid derivatives, 2-(1H-benzotiazol-1-yl)-1,1,2,2,-
tetramethyluronium hexafluorophosphate (HBTU), pipyridine, diisopropylcarbodiimide, and
anhydrous hydroxymethylbenzotiazole (HOBt) were purchased from Peptide International,
tetrakis(acetonitrile)copper(I)hexafluorophosphate and sodium aurothiomalate hydrate were
purchased from Aldrich. 5,5’-dithio-bis(2-nitrobenzoic acid was purchased from Sigma.

General Methods

Reverse-phase HPLC analyses were performed on Vydac semipreperative reversed-phase
Vydac C18-column (10 µM particle size, 10×250 mm) or preparative Vydac C-18 column (10
µM particle size, 22×250 mm) with linear gradient acetonitrile/water mixture as a mobile phase
containing 0.1 (v/v) % trifluoroacetic acid over a course of 90 min using the flow rate of 2
(semipreperative) or 6 (preparative) ml/min. A two pump system (Waters Model 515) equipped
with a Waters Model 994 diode array detector/spectrophotometer having 1 cm cell was used. For
preparative separations, the monitoring wavelengths were set to 210-230 nm and purity of the
collected peptide was verified by analytical HPLC runs. The UV-vis spectra of the purified
peptide were recorded on Hewlett-Packard model 8452A diode array spectrophotometer having a
1cm cell pathlength. The molar extinction coefficient, $\varepsilon(275 \text{ nm}) = 1450 \text{ M}^{-1}\text{cm}^{-1}$, was used to
determine the apopeptide concentration. Static luminescence spectra were obtained with a single
photon counting spectrofluorimeter from Edinburgh Analytical Instruments (FL/FS 900) as
described previously.¹
Synthesis of the C16C19-GGY Peptide

The 32-residue peptide, C16C19-GGY, prepared having the sequence Ac-K(IEALEGK)2(CEACEGK)(IEALEGK)-GGY-amide, was synthesized using the solid-phase methods, on an Applied Biosystems Model 433 A peptide Synthesizer by standard Fmoc chemistry; the 0.25 mmol scale protocol with followed N-terminal capping protection strategy by acetic anhydride. Activation was achieved by diisopropylcarbodiimide (HBTU) and 1-hydroxybenzotriazole (HOBT) in DMF.

Deprotection of the amino acid side chains and cleavage from the resin was performed by a reaction with a mixture of trifluoroacetic acid (88% v/v), phenol (5% v/v), triisopropylsilane (1% v/v), 2-mercaptoethanol (1% v/v) and water (5% v/v) for 2.5 hours at room temperature. The crude peptide was then precipitated in cold anhydrous diethyl ether, collected by vacuum filtration and dried under vacuum. Final purification was achieved by preparative reversed-phase C18 HPLC using as described in the General Methods section. The collected peptide was then lyophilized with followed analysis by MALDI mass-spectroscopy, \( m/z \) (ion), found: 3406.7, calculated: 3407.9.

Circular dichroism spectroscopy.

Circular Dichroism spectra were obtained using an Aviv and Associates model 62 DS circular dichroism spectrometer (Lakewood, NJ) equipped with a thermoelectric temperature controller. A rectangular 1 mm or 5-mm cells were used. Mean residue molar ellipticities were calculated according to the equation:

\[ [\theta] = \frac{[\theta]_{\text{obs}}}{(10^6 l c n)} \]

where \([\theta]_{\text{obs}}\) is the observed ellipticity measured in degrees, \( l \) is the path length of the cell in centimeters, \( c \) is the molar peptide concentration, and \( n \) is the number of amino acid residues in
the peptide. The peptide concentration was determined using the molar extinction coefficient, 
\( \varepsilon(275 \text{ nm}) = 1450 \text{ M}^{-1} \text{cm}^{-1} \). The spectra were obtained as an average of 3-5 scans using 1nm 
wavelength step in the absence and presence of 1-10 fold excess of the following metal ions:
CdCl\(_2\), HgCl\(_2\), CuCl\(_2\), AgNO\(_3\), Pb(NO\(_3\))\(_2\), CoCl\(_2\), Pt(en)(NO\(_3\))\(_2\), sodium aurothiomalate, ZnCl\(_2\) tetrakis(acetonitrile)copper(I)hexafluorophosphate. Metal ion titration was carried out 
anaerobically by monitoring \([\theta]_{222}\) as a function of the metal ion concentration. The peptide 
concentration was 80-240 \(\mu\)M in water or buffer solutions in the presence or absence of TCEP. After the addition of a 1\(\mu\)L aliquot of the stock solution of a metal ion, the solutions were 
allowed to equilibrate for 5 minutes. The titration data were obtained using 0.5 cm path length 
cell as an average of 5 scans and corrected by a blank.

**Steady-State Luminescence Measurements.**

Steady-State luminescence spectra were obtained with a single photon counting 
spectrofluorimeter from Edinburgh Analytical Instruments (FL/FS 900). The excitation was 
accomplished with a 450 Xe lamp optically coupled to a monochromator, and the emission was 
gathered at 90\(^\circ\) and passed through a second monochromator. The luminescence measurements 
were carried out at room temperature using excitation wavelength of 300 nm. The measurements 
were recorded over 400-750 nm range with 340 nm long-pass filter. The samples were argon 
saturated for 10-15 min prior to measurement. The 70-120 \(\mu\)M solutions of C16C19-GGY (or 
C16C19) in water or 0.2 M acetate buffer, pH 5.4-5.5 were used for the emission titrations of 
peptide by Cu (I) in the absence or presence of TCEP.

For determination of metal ion/peptide stoichiometry 70-250 \(\mu\)M C16C19-GGY peptide 
in argon saturated 0.2 M acetate buffer, pH 5.4-5.5 was titrated anaerobically by addition 1 \(\mu\)L
aliquots of 0.01 M solution of a corresponding metal ion. After each aliquot, the samples were
allowed to equilibrate for 5 minutes before the measurement.

Quantum yield of C16C19-GGY-Cu(I) was estimated by comparison with
Ru(bpy)$_3$[(PF$_6$)$_2$ in water for which $\Phi_r = 0.044$.\textsuperscript{2}

**SDS-PAGE gel electrophoresis**

For the SDS-PAGE experiments, a discontinuous gel system was with a 16.5% resolving
gel (containing 40% acrylamide (37.5:1), 3 M Tris-Cl 0.3% SDS pH = 8.45, glycerol, ddH$_2$O,
APS, TEMED) with the combination of a 4% stacking gel (containing acrylamide (37.5:1), 0.5
M Tris pH = 6.8, ddH$_2$O, APS, and TEMED) was used. The plates were prerun for 20 minutes at
20 mA (for 2 plates) in a combination bottom buffer containing 0.1 M Tris base, 0.1 M tricine,
and 0.1% SDS and top buffer containing 0.2 M Tris-Cl pH = 8.9 in the mini-vertical gel system.
After destining in Coomassie blue stain containing 0.25% Coomassie R-50, 50% methanol, and
7.5% glacial acetic acid for 30 minutes the gel was placed in a destaining buffer contained 10%
methanol and 10% glacial acetic acid for 2-4 hours.

**High Performance Size Exclusion Chromatography (HPSEC).**

HPSEC experiments were performed a Superdex 75 Amersham Pharmacia Biotech
column connected to a Waters Model 515 High Performance Liquid Chromatography system
equipped with Waters Model 996 diode array detector. The peptide samples were eluted using
0.1 M KCl / 0.05 M KH$_2$PO$_4$, with 0.2-0.4 ml/min flaw rate and monitored at a wavelength of
230 nm.

**UV-vis titration.**

A Hewlett-Packard model 8452 A diode array spectrophotometer filled with a 1 cm
quartz cell. The spectra were collected over a 190 to 900 nm range. The UV-vis titration of 50-
200 µM C16C19-GGY peptide prepared in aqueous solutions or 0.2 M acetate buffer pH 5.4-5.5 was carried out anaerobically using in the presence or absence of the reducing agents: immobilized Cleland’s REDUCTACRYLTM reagent (Calbiochem) or TCEP. After the addition of 1 µL aliquots of deoxygenated freshly prepared stock solutions of metal ions, the sample was purged with argon and allowed to equilibrate for 5 minutes before each measurement. The peptide concentration was determined using the molar extinction coefficient, $\varepsilon$(275 nm) = 1450 M$^{-1}$cm$^{-1}$.

A Job plot was constructed by measuring the absorbance at 238 nm as a function of the mole fraction of Cd(II) present in solution.

**Emission Lifetime Measurements**

The emission lifetime measurements were carried out using a nitrogen broadband dye laser (2-3 nm fwhm) using fundamental nitrogen excitation (337 nm) or 357 nm (BPBD dye). The emission of copper/peptide complexes was monitored at 600 nm. Lifetime measurements were performed on argon-saturated solutions.

**Ellman’s test**

The spectrophotometric thiol assay (Ellman’s test) was performed to determine the content of free sulfhydryl groups in the apopeptide and the metallated peptide complexes. Calibration for thiol determination was achieved be the spectrophotometric titration of the apopeptide by Ellman’s reagent (5,5’-dithio-bis(2-nitrobenzoic acid)). The samples were prepared in 0.1 M phosphate buffer, pH 7-8.3 and adjusted to a final volume of 1 ml. After the addition of 50 µM 11 mM of Ellman’s reagent prepared in the same buffer the samples were carefully mixed and incubated for 10-15 min at the room temperature. $A_{412}$ was measured for
the solution of the peptide and the copper complex. The amount of free sulfhydryl groups was calculated using the standard value \( \varepsilon = 14,150 \text{ M}^{-1}\text{cm}^{-1} \).

**Analytical ultracentrifugation**

Sedimentation equilibria were performed in collaboration with Dr. Borris Demeler, for Center for Analytical Ultracentrifugation of Macromolecular Assemblies, University of Texas Health Science Center, San Antonio, Texas 78229. The samples were prepared in 40 or 200 mM sodium acetate buffer, pH 5.4 - 5.5 with 2-fold excess of metal ion, in the absence or presence of 5-fold excess of TCEP. In the experiment, 280 nm or 230 nm (for the Cd(II)-metalloprotein) scans were taken at equilibrium from 3 concentrations (200, 320 and 450 µM) and 4 different speeds (50.0, 53.3, 56.7 and 60.0 krpm). The obtained data were globally analyzed with UltraScan (Demeler, B. (2005) UltraScan version 7.0) by fitting to two different models: self-associating and noninteracting models. The fit resulted in a species with a molecular weight of 2.9 kD, and a species with a molecular weight of 12.1 kD and a variance of 2.36 \( \times 10^{-5} \). The “monomer-tetramer” model resulted in a monomer molecular weight of 3.06 kD and a variance of 5.01 \( \times 10^{-5} \). The partial specific volume of C16C19GGY peptide was determined by the method of Cohn and Edsall\(^4\) from the protein sequence of C16C19GGY peptide and found to be 0.7425 ccm/g.

**Redox potentiometry**

Redox potentiometry was performed in combination with UV-vis absorption and steady-state emission spectra at Columbia University, New York, NY. The electrochemical redox potentials of the Cu(I)-metalloprotein were determined by monitoring the decrease of emission intensity at 600 nm upon the excitation at 275 nm, and by monitoring the decrease absorbance
intensity at 310 nm. The titrations were performed in specially designed anaerobic quartz or glass cells with a syringe, equipped with a platinum working and a calomel reference electrode calibrated against freshly prepared quinhydrone in standardized phosphate buffer solution pH 7 (+47 vs SCE). Cu(I)/C16C19-GGY solutions were prepared in 0.2 M sodium acetate buffer, pH 5.5 under nitrogen atmosphere in a glove box and transferred from a glove box to a nitrogen-degassed 1 cm cell in a septum-sealed container. All potentials were measured under N₂ flow at room temperature, vs SCE and converted to NHE by the addition of 244 mV. The following redox mediators were used to facilitate electrode-solution mediation: 1,2-naphtoquinone-4-sulphonate, phenazine methosulfate, 1,2-naphtoquinone, 5-hydroxy-1,4-naphtoquinone, phenazine ethosulfate, methyl-1,4-benzoquinone. The initial potential and spectra were recorded after the addition of mediators to the Cu(I)/C16C19-GGY solution in the redox cell equipped with a small stir bar. The oxidation of Cu(I)-metalloprotein was achieved by addition of small aliquots of the fresh prepared solution of K₂IrCl₆. After the addition of the oxidant the solution was allowed to equilibrate and stir for 1-2 minutes before recording the measurements. After the completion of the titrations, the emission (or absorbance) intensity was plotted against the measured potential, and the data were fit to the single Nernst equation (n=1):

\[
Y = I_{\text{reduced}} + \Delta I \left( \frac{1}{10^{\frac{nF}{RT} (E_h - E_m) / RT}} + 1 \right)
\]

(1)

where \(I_{\text{reduced}}\) is the absorbance intensity at 310 nm or emission intensity at 600 nm corresponding to the reduced Cu(I)-metalloprotein, \(\Delta I\) is the change in absorbance or emission from reduced to oxidized forms, \(E_h\) is the measured potential of the solution, and \(E_m\) is the
midpoint potential, F/RT=60 mV. The number of electrons (n) was set to 1 but other parameters were allowed to vary in the nonlinear fit.

**X-ray absorption spectroscopy**

X-ray absorption spectroscopy was performed in collaboration with Professor Michael J. Maroney and David C. Kennedy, Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003. Copper K-edge XAS data for Cu-C16C19-GGY were collected on beam line X9B at the National Synchrotron Light Source at Brookhaven National Laboratory. The sample for X-ray absorption spectroscopy was prepared with an equimolar Cu(I)/peptide ratio and in the presence of 5-fold excess of TCEP in 0.2 M sodium acetate buffer, pH 5.4 containing 18 % (v/v) of glycerol. The final concentration of Cu(I) in the sample is 1.03 mM. The sample was placed in a polycarbonate sample holder and frozen in liquid nitrogen. The plastic sample holder was then inserted into an aluminum frame that was held near 50K by a He displex cryostat. Data were collected with the ring containing 120-300 mA at 2.8 GeV and using a sagitally focusing Si(111) double-crystal monochromator. Harmonic rejection was accomplished with a focusing mirror left flat. The X-ray energy scale was internally calibrated with reference to the lowest energy inflection point of a Cu foil spectrum (8980.3 eV). X-ray fluorescence data were collected using a solid state array of 13-element germanium detectors (Canberra). X-ray absorption near-edge structure (XANES) data were collected from ca. 8780-9180 eV and X-ray absorption fine structure (EXAFS) were collected from 8780-10000 eV. The integrity of the samples was monitored by comparing XANES data from sequential scans. No significant changes were noted. Upon removal from the sample holder, visual inspection revealed that where the beam had been incident on the sample, the color had changed from colorless to yellow. The data obtained form XANES were analyzed by fitting a baseline to the
pre-edge and edge regions of the spectrum (ca. 8780 – 8960) using a cubic function for the pre-edge and 75% Gaussian and 25% Lorenzian function to fit the rise in fluorescence occurring at the edge. Gaussian functions were added for transitions occurring at lower energy and the areas of the Gaussian fits were taken as peak areas. The XANES analysis was obtained by averaging of ca. 3 scans (39 spectra with edge energy variation ≤ 0.2 eV). EXAFS data were analyzed using the program EXAFS123.\textsuperscript{5} An EXAFS spectrum was generated by averaging of eight scans with edge energy variation ≤ 0.4 eV. The data was summed, background corrected and normalized using a five section cubic spline to fit the background in the pre-edge and post-edge regions. The data were converted to $k$ space using the relationship \[2m_e(E-E_0)\hbar^2]^{1/2}\), using a threshold energy of the absorption edge 8990 eV, and where $m_e$ is the electron mass, $\hbar$ is Plank’s constant divided by $2\pi$ and $E_0$ is the threshold energy of the absorption edge. Least-squares fits of the EXAFS data over a $k$ range of 2-12.5 Å\(^{-1}\) were performed on Fourier-filtered data with a backtransform window = 1 – 4 Å. Best fits were obtained by minimizing the goodness of fit criterion \(GOF = [n\{idp\}/(n\{idp\}-n\{p\})]^{1/2} R\), where $R = \text{avg} [\text{data simulation}/\text{esd(data)}]$, $n(p)$ is the number of varied parameters, and $n(idp)$ is the number of data points for unfiltered refinements or $2(r_{max} – r_{min})(k_{max}-k_{min})\pi$ for filtered refinements). The amplitudes and phase functions were calculated using FEFF 8. The following crystallographically characterized compounds: benzilbisthiosemicarbazonatocopper(II)\textsuperscript{6}, bis(α\textsubscript{2}-thiourea)-tetrakis(thiourea)-di-copper(I)bis(tetrafluoro-borate)\textsuperscript{7}, and tetrakis(N-methylimidazole)copper(I) perchlorate\textsuperscript{8}. In order to determine the first coordination sphere around the copper, additional scatterers were added to fit the FT between 2 and 4 Å. For the initial fit the number of copper atoms was allowed to refine, giving a value of 2.8 Cu atoms. Then to perform the subsequent fits this value was locked to either two or three Cu atoms. The individuals fit were performed allowing the following three free-running parameters
for each shell to vary: the distance ($r$), the disorder parameter ($\sigma^2$) and $E_0$. With the exception of the Cu scattering atoms, the number of scattering atoms in a given fit were set to integer values and not allowed to refine.

REFERENCES FOR CHAPTER II


(4) Cohn, E. J., Edsall, J. T. 1943.


CHAPTER III: RESULTS AND DISCUSSION

III.1. Peptide Design.

The work described in this dissertation illustrates how the structures of metalloproteins may be controlled by the coordination chemistry of their inorganic cofactors. The presented miniature synthetic native-like metalloproteins displays interesting rich metal-binding properties and can be regarded as sophisticated models to study metal-binding metal-folding processes and to probe metal-protein interactions and metal ion specificity.

To date, only a few examples have been reported describing the design of the metal binding sites in the hydrophobic solvent-shielded environment of the peptide within the interior of the coiled coil.\textsuperscript{1-12} It is described in this dissertation this cysteine-containing random coil apopeptide is capable of binding of different metal ions in its hydrophobic interior and that metal-binding is accompanied by folding of the peptide which results in the formation of metal-bridged self-organized $\alpha$-helical bundles. Such metal-binding produces synthetic metalloproteins which display metal-specific oligomerization states and metal-dependant binding stochiometries. the oligomerization states of Cu(I), Ag(I), Au(I), and Pt(II)-metalloproteins were different from ones predicted by original design. This is an important result showing that inorganic cofactors can change the oligomerization state of the peptides. To our knowledge it is a first example of synthetic metalloproteins which displays metal-specific oligomerization states.

The described synthetic metalloproteins are based on coiled-coil motif. The coiled-coil is a very common protein domain in nature the structural properties of which are well-established.\textsuperscript{13-17} This domain consists of supercoiling two, three or four parallel or antiparallel
amphiphatic α-helices. Its sequence consists of several seven-residue heptad repeats, 
\((abcdefg)_n\).\(^{2-4,18,19}\) The parent peptide sequence used in this work was based on the IEALGEK heptad repeat. The \(a\) and \(d\) positions were occupied by hydrophobic amino acid residues isoleucine and leucine; positions \(e\) and \(g\) were filled with opposite charged amino acid residues glutamic acids and lysine, and \(b\), \(c\), and \(f\) positions were occupied by hydrophilic amino acids. This peptide was acetylated on the N-terminus and amidated from the C-terminus for an additional stabilization of the coiled-coil structure.

The circular dichroism spectra of peptides prepared previously by our group\(^{20-23}\) for the electron transfer studies based on the IEALEGK heptad repeat were shown to consist of two negative maxima at 208 and 222 nm and a positive signal located below 200 nm which demonstrates the formation of the α-helical structure. An ellipticity ratio of \(\frac{[\theta]_{222}}{[\theta]_{208}}=1.06\) is indicative of the existence of the α-helical structure and is considered characteristic for a coiled-coil structure. Thermal denaturation studies showed that these self-assembled peptides are very stable in the aqueous solutions producing a melting curve with \(T_m = 65^0\) C. The oligomerization state of peptides was determined by a combination of analytical ultracentrifugation, EPR-spin labeling experiments, SDS-PAGE, and size-exclusion chromatography. The analytical ultracentrifugation results could be fit to a dimer model with the molecular weight of 7.5 kDa. Therefore, the parent peptides were shown to exist as stable two-stranded α-helical coiled coils.\(^{24-27}\)

The work presented in this dissertation is based on studying the metal-binding properties of the peptide C16C19-GGY. The peptide design of C16C19-GGY was based on the peptide sequences described above.\(^7,10\)

In order to engineer the metal-binding site into the peptide, two cysteine residues were introduced into the hydrophobic core. In particular, Ile and Leu residues were substituted with
cysteine residues at $a$ and $d$ positions of the third heptad. These substitutions may result in the possible creation of soft tetrathiolate metal binding site located at the hydrophobic peptide-peptide interface when a two-stranded coiled-coil is formed.

Figure III.1. Wheel representation showing the sequence of C16C19-GGY peptide.
III.2. Synthesis and Characterization of C16C19-GGY Apopeptides

A 32-residue peptide, C16C19-GGY, was prepared using solid-phase peptide synthesis having the following sequence (Figure III.1.).

\[
\text{Ac-K(IEALEGK)(IEALEGK) (CEACEGK)(IEALEGK)-GGY-amide.}
\]

Additionally, the spectroscopic tag GGY was introduced to the peptide sequence for the precise determination of the peptide concentration in aqueous solutions based on the extinction coefficient of tyrosine (\(\varepsilon(275 \text{ nm})=1450 \text{ M}^{-1} \text{ cm}^{-1}\)).\(^{10}\)

The apopeptide C16C19-GGY was purified by preparative and reverse-phase C18 HPLC. The chromatogram of the purified peptide is shown in Figure III.2. The C16C19-GGY peptide was identified by MALDI mass-spectroscopy (\(m/z\) (ion), found: 3408, calculated: 3408.) (Figure III.4). ESI-MS spectrum of C16C19-GGY presented in Figure III.3. corresponds to \((M+3H^+)_{3+}\) (1137 m/z) and \((M+4H^+)_{4+}\) (853 m/z) charge state which is consistent with the calculated molecular mass of the peptide (3408).

![Figure III.2. Analytical chromatogram of the purified C16C19-GGY peptide.](image-url)
Figure III.3. ESI-MS of the purified C16C19-GGY (m/z observed 1137, 853, calculated 1137 (M+3H\(^+\))\(^3+\), 853 (M+4H\(^+\))\(^4+\)).
Figure III.4. MALDI-TOF MS spectrum of the C16C19-GGY (32-mer) (m/z observed: 3408, calculated 3408).
Circular dichroism spectroscopy was utilized to determine the conformation of the apopeptide. Figure III.5. shows that the CD spectrum of the reduced apopeptide in 20 mM sodium acetate buffer (pH 5.4-5.5, 298 K) consists of a large, negative maximum centered at 205 nm with $[\theta]_{205\text{ nm}} = -10,000 \text{ deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$ and positive signal at $\lambda < 200$ nm. These results indicate that the apopeptide exists as a disordered random coil in aqueous solutions which is consistent with previous observations of the related C19 peptide which has only one cysteine amino acid residue at the hydrophobic position “d” of the third heptad repeat and also exists as a disordered random coil in the aqueous solution. In related studies carried out by Pecoraro and co-workers utilizing three-stranded coiled-coils based on TRI family, it was shown that introducing a cysteine in the “d” position usually has destabilizing effect on coiled-coil formation.$^{11,28}$

Fig. III.5. Circular dichroism spectra of aqueous solutions of reduced C16C19-GGY (46 µM) (●) and oxidized apopeptide (▲) (pH ~5-6, 298 K)
Figure. III.6. Circular dichroism spectra of the reduced C16C19-GGY (157 µM) taken in 1 : 1 ethanol/methanol mixture (298 K).

The presence of the random coil structure of C16C19-GGY indicates that the substitution of the hydrophobic amino acid residues Ile and Leu at the “a” and “d” positions of the third heptad repeat for cysteine leads to the destabilization of a two-stranded α-helical coiled-coil which is unable to accommodate the presence of four cysteinyl side-chains within its hydrophobic core.

When dissolved in ethanol or 1 : 1 ethanol/methanol, the conformational properties of the peptide changes dramatically. The circular dichroism spectrum of C16C19-GGY now consists of negative maxima at 208 and 222 nm and a positive signal located below 200 nm, which indicates the presence of an α-helical structure. The ellipticity ratio of $[\theta]_{222}/[\theta]_{208}$ of the CD spectrum is 0.82, which is characteristic of monomeric α-helices$^{19}$ (Figure III.6.). It is known that alcohol solvents stabilize α-helical structure but disturb hydrophobic interactions.
High performance size exclusion chromatography (HPSEC) and SDS-PAGE experiments were performed for determining the oligomerization state of the peptide. Figures III.7. demonstrates the SDS-PAGE experiment which shows the comparison of the molecular weight of C16C19-GGY vs. Pal14C19-GGY oxidized which was used as a reference having a dimeric molecular weight. This 32-residue peptide, having the IEALEGK heptad repeat has a cysteine amino acid residue placed the “d” position of third heptad repeat replacing the hydrophobic leucine amino acid residue. This peptide contains cysteine at the “d” position of the third heptad repeat and was shown to form intramolecular disulfide bond between two α-helices of the coiled-coil upon the oxidation. The oxidation of this peptide produces the cross-linked Pal14C19 oxidized dimer which was used as a standard. Figure III.7. shows that the C16C19-GGY produces the band which corresponds to the monomer species (MW≈3.5 kDa).

Figure III.7. SDS-PAGE of the C16C19-GGY and Pal14C9 oxidized samples.
HPSEC experiments were performed to confirm the monomeric nature of C16C19-GGY. Figure III.8 shows the HPSEC chromatogram of the C16C19-GGY peptide along with the H21 dimer investigated previously,\textsuperscript{20,21} used as a standard. The C16C19-GGY peptide eluted 4 minutes later comparing to the H21-mer dimer used as a standard. These results suggests that the C16C19-GGY exists as a monomer in aqueous solutions.

![Figure III.8. High performance size exclusion chromatography (HPSEC) chromatogram of the H21-mer and C16C19-GGY monitored at 230 nm. The sample was eluted with 0.2 M sodium acetate buffer pH 5.4, 0.3 ml/min.](image)

Circular dichroism spectroscopy was used to identify the structure of the oxidized form of the C16C19-GGY peptide. Since the peptide contains two cysteine amino acid residues, the oxidized form of the peptide can produce either intra or intermolecular disulfide bonds. Figure III.5. shows the CD spectrum of the oxidized apopeptide, similarly to the CD spectrum of the
reduced form; it consists of a large, negative maximum centered at 205 nm with \( [\theta_{(205\,nm)}] = -7,500\, \text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1} \) and a positive signal at \( \lambda < 200\, \text{nm} \). This spectrum is typical for a disordered random coil peptide structure. SDS-PAGE and HPSEC experiments were performed to determine the oligomerization state of the oxidized form of the C16C19-GGY. It was shown that both the reduced and C16C19-GGY oxidized peptides exist as peptide monomers. Therefore, it can be concluded that the disulfide bond formation of the C16C19-GGY peptide occurs intramolecularly resulting in intra-chain peptide crosslinking between the C16 and C19 residues, and not dimers created by the formation of inter-peptide disulfide bonds.

Reverse-phase HPLC experiments also showed that the oxidized and reduced forms of the peptide are significantly different in retention times. Figure III.9. shows that the oxidized peptide eluted 8 minutes earlier than the reduced peptide. This observation allowed a time course for the oxidation process of the peptide. The oxidation of a 100 µM solution of the C16C19-GGY upon exposure to ambient air was monitored by HPLC and CD experiments. The circular dichroism spectra were measured concurrently to confirm the existence of the random structure of the peptide (Figure III.10.). Aliquots of the solution of the peptide exposed to ambient air were analyzed by reverse-phase HPLC. The absorbance intensity of the peaks which corresponds to the reduced and oxidized forms of the peptide was plotted vs. time, shown in Figure III.11. It can be seen that the absorbance intensity which corresponds to the reduced form of the peptide diminishes with time. In contrast, the absorbance intensity which corresponds to the oxidized form of the peptide increases.

It was shown that after 3 hours of oxidation at ambient conditions about 20% of the peptide was oxidized (Figure III.12.). Therefore, all experiments were performed under anaerobic conditions (see Materials and Methods) to prevent oxidation of an apopeptide.
Figure III.9. Analytical chromatogram of the oxidized C16C19-GGY peptide.

Figure III.10. The circular dichroism spectra of a 100 µM solution of C16C19-GGY peptide after 0, 19, 25, 96, 120, and 196 hours of oxidation by exposure to ambient air.
Figure III.11. The oxidation course of C16C19-GGY monitored by HPLC. 100 µM solution of C16C19-GGY oxidized under aerobic ambient conditions by exposure to air with the following injection to HPLC after 0 – 7 hours.

III.3. Metal-Binding Properties of C16C19-GGY

To analyze the effect of metal binding on the structural properties of C16C19-GGY the following transition metal ions were added to samples of C16C19-GGY: Cd(II), Hg(II), Cu(I), Ag(I), Au(I), Pt(II), Zn(II), Fe(II), Pb(II), Ni(II), or Co(II).

As shown in Figures III. 12 - 17, the circular dichroism spectrum of the reduced C16C19-GGY peptide changes dramatically upon the addition of Cd(II), Hg(II), Cu(I), Ag(I), Au(I), or Pt(II) to the solution of the peptide. The CD spectra of all metal/peptide complexes consist of negative maxima at 208 and 222 nm and a positive signal located below 200 nm, which indicates the presence of α-helices. The CD spectra of the metallated peptide complexes further showed an ellipticity ratio of $\frac{\theta_{222}}{\theta_{208}}$ ranging from 0.97 to 1.22 (Table 1) which falls within the
range generally regarded to indicate the presence of a coiled-coil structure. In particular, the ellipticity ratios of $[\theta_{222}] / [\theta_{208}]$ of the CD spectra obtained in the presence of Cd(II), Ag(I), Au(I), or Cu(I) have similar value of 1.0-1.1 which indicates the formation of the interacting $\alpha$-helical coiled-coils. In contrast, the Hg(II)/peptide complex displayed the ellipticity ratio of $[\theta_{222}] / [\theta_{208}]$ of 1.22 which is somewhat higher than ones observed for the other metallated peptide complexes. Neither Zn(II), Fe(II), Pb(II), Ni(II), nor Co(II) were able to induce peptide folding.

The ellipticity value at 222 nm was used to estimate the helical content of the metal/peptide complexes by comparison to the maximum molar ellipticity ($X_{H\infty}$) calculated for a 32-residue polypeptide:

$$(X_{H\infty}) = (-37,400 \text{ deg cm}^2 \text{ dmol}^{-1})(1-k/n) = -34,500 \text{ deg cm}^2 \text{ dmol}^{-1}$$

where $k$ is a wavelength-dependent constant equal to 2.5 at 222 nm, and $n$ is the number of residues per helix.
Table III.1. Helical Properties of Metallated Peptide Complexes

<table>
<thead>
<tr>
<th></th>
<th>Cd(II)/C16C19GGY</th>
<th>Hg(II)/C16C19GGY</th>
<th>Cu(I)/C16C19GGY</th>
<th>Pt(II)/C16C19GGY</th>
<th>Ag(I)/C16C19GGY</th>
<th>Au(I)/C16C19GGY</th>
</tr>
</thead>
<tbody>
<tr>
<td>[θ&lt;sub&gt;222&lt;/sub&gt;]/[θ&lt;sub&gt;208&lt;/sub&gt;]</td>
<td>0.99</td>
<td>1.15</td>
<td>0.97</td>
<td>1.07</td>
<td>1.13</td>
<td>1.04</td>
</tr>
<tr>
<td>deg cm&lt;sup&gt;2&lt;/sup&gt; dmol&lt;sup&gt;-1&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% helicity</td>
<td>38</td>
<td>46</td>
<td>59</td>
<td>48</td>
<td>70</td>
<td>33</td>
</tr>
</tbody>
</table>

The comparison of the helical content of the metallated peptide complexes is presented in Table 1. The addition of the soft metal ions listed in the table induces considerable helicity in peptide. The Ag(I)/C16C19-GGY complex displayed the highest helical content (70 %) compared to other metallated complexes. The Cu(I)/C16C19-GGY complex displayed 59% of helicity. The Hg(II) and Pt(II)/C16C19-GGY complexes have a similar helicity of ~50%. The Cd(II) and Au(I)/C16C19-GGY complex showed the lowest induction of helicity (36 and 33 %, respectively) as compared to other metal ions.
Figure III.12. Circular dichroism spectrum of C16C19-GGY (107 µM) taken in the absence (■) and presence (●) of a fivefold excess of cadmium chloride (5 mM sodium acetate buffer, pH 5.5-5.6, 298 K).

Figure III.13. Circular dichroism spectrum of C16C19-GGY (107 µM) taken in the absence (■) and presence (●) of a fivefold excess of tetrakis(acetonitrile) copper(I)hexafluorophosphate, (5 mM sodium acetate buffer, pH 5.5-5.6, 298 K).
Figure III.14. Circular dichroism spectrum of C16C19-GGY (107 µM) taken in the absence (▲) and presence (■) of a fivefold excess of silver nitrate (5 mM sodium acetate buffer, pH 5.5-5.6, 298K).

Figure III.15. Circular dichroism spectrum of C16C19-GGY (107 µM) taken in the absence (●) and presence (■) of a fivefold excess of sodium aurothiomalate hydrate, (5 mM sodium acetate buffer, pH 5.5-5.6, 298K).
Figure III.16. Circular dichroism spectrum of C16C19-GGY (138 µM) taken in the absence (●) and presence (■) of a fivefold excess of Pt(en)(NO$_3$)$_2$, (5 mM sodium acetate buffer, pH 5.5-5.6, 298K).

Figure III.17. Circular dichroism spectrum of C16C19-GGY (107 µM) taken in the absence (●) and (■) presence of a fivefold excess of mercury chloride (5 mM sodium acetate buffer, pH 5.5-5.6, 298K).
Incorporation of the metal ions into the peptide was further verified by electronic absorption studies. The UV-vis spectra of the metalloproteins are presented below as difference absorption spectra to eliminate the contribution of the polypeptide chain. Figure III.18. shows the difference spectrum of the Cd(II)-metalloprotein. The formation of a new absorption band centered at 238 nm is observed upon addition of CdCl_2 to the solution of C16C19-GGY. This 238 nm band is attributed to the CysS-Cd(II) ligand-to-metal-charge-transfer transition by analogy with studies of Cd-substituted metallothionein, growth-inhibitory factor, and Cd-rubredoxin.

The Cu(I)-metalloprotein exhibits the broad absorption envelope with a new absorption band centered at 236 nm with a noticeable shoulder at 296 nm and a broad tail that extends to > 375 nm (Figure III.19.). Similar results were reported when Cu(I) was added to metallothionein, growth inhibitory factor, ACE1 transcription factor, which produced a broad spectral envelope in the UV region of the spectrum. By analogy with Cu(I)-metallothioneins and neuronal growth inhibitory factors, the binding of Cu(I) to cysteine residues is known to produce both Cys-thiolate to Cu(I) ligand-to-metal-charge-transfer (LMCT), and metal-centered (MC) transitions. These high-energy bands (230-260 nm) of the UV-vis spectrum of the Cu(I)/C16C19-GGY originate from CysS-Cu ligand-to-metal-charge-transfer transitions and the low-energy features (260-375 nm) in the electronic absorption spectrum of the Cu(I)-metalloprotein can be assigned to metal-localized (MC) transitions.

It is was shown that absorption spectra of small inorganic mononuclear d^{10} Cu(I) halide complexes (for instance, Cu(I)Cl_3^{2-}) revealed only strong electric-dipole allowed charge transfer bands with the lowest energy band at 274 nm. Therefore, the fact that Cu(I)-metalloprotein has
a broad spectral envelope in the UV region up to 375 nm suggests the presence of polynuclear Cu(I)-cluster within the Cu(I)-metalloprotein.

The electronic absorption spectrum of Hg(II)-metalloprotein shows both high-and low-energy transition bands at 236 and 266 nm, respectively. The presence of the spectroscopic features above 230 nm in the UV region of the spectrum of the Hg(II)-metalloprotein are indicative of the presence of mercury thiolates with trigonal or tetrahedral geometry (Figure III.20.).\(^{51,52}\)

The absorption spectrum of Ag(I)-metalloprotein exhibits similar absorption features to Cu(I)-metalloprotein. As shown in Figure III.21., the UV-vis spectrum of the Ag(I)-adduct is characterized by a broad absorption envelope with a new absorption band with a maximum at 230 nm which extends up to 320 nm. The high-energy band (230 nm) is characteristic of CysS–Ag(I) LMCT and low-energy transitions originate from metal-centered (MC) transitions. (Figure III.21.).\(^{53}\)

Figure III.18. UV-vis spectrum of C16C19-GGY/Cd(II) ([C16C19-GGY]=140 µM) taken in the presence of a twofold excess of Cd(II) in 0.2 M sodium acetate buffer pH 5.4-5.5.
Figure III.19. UV-vis spectrum of C16C19-GGY/Cu(I) taken in the presence of a twofold excess of Cu(I), [C16C19-GGY]=84 µM, 5-fold excess of TCEP in 0.2 M sodium acetate buffer pH 5.4-5.5 (298 K).

Figure III.20. UV-vis spectrum of C16C19-GGY/Hg(II) taken in the presence of a twofold excess of Hg(II), [C16C19-GGY]=111µM in 0.2 M sodium acetate buffer pH 5.4-5.5, (298 K).
Figure III.21. UV-vis spectrum of the aqueous solution of C16C19-GGY/Ag(I) taken in the presence of a 1.5-fold excess of Ag(I), [C16C19-GGY]=111µM (298 K).

In summary, the results show that the C16C19-GGY peptide undergoes a metal-induced folding process from a monomeric random coil to an organized, metal-bridged \( \alpha \)-helical coiled-coil upon the addition of Cd(II), Hg(II), Cu(I), Ag(I), Au(I), or Pt(II). The formation of the metal/peptide complexes can be monitored by the appearance of new absorption bands in the UV region which can be assigned to the ligand-to-metal charge-transfer (LMCT) transition of the newly formed M-S bond. \(^{54}\)

Circular dichroism spectra were collected for samples containing C16C19-GGY peptide and the following metal ions: Fe(II), Zn(II), Co(II), and Ni(II). Interestingly, the addition these metal ions to the solution of the peptide did not induce the peptide self-assembly and was not accompanied by the appearance of LMCT bands in UV region. The results presented in Table 2
demonstrate that the metal-induced peptide folding occurs with only soft metal ions, such as Cd(II), Hg(II), Cu(I), Ag(I), Au(I), and Pt(II).

Table III.2. Induction of helicity upon addition of metal ion.

<table>
<thead>
<tr>
<th></th>
<th>Fe(II)</th>
<th>Zn(II)</th>
<th>Co(II)</th>
<th>Ni(II)</th>
<th>Au(I)</th>
<th>Pt(II)</th>
<th>Cu(I)</th>
<th>Ag(I)</th>
<th>Cd(II)</th>
<th>Hg(II)</th>
<th>Pb(II)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Interestingly, the addition of Pb(II) to the apopeptide did not induce the formation of a coiled-coil. However, the addition of Pb(II) to the C16C19-GGY was accompanied by the appearance of an intense charge-transfer band at 225-320 nm (Figure III.22.) indicating of the formation of S-Pb bond. Therefore, these results indicate that Pb(II) can bind to the peptide but was not capable of inducing the conformational change of the peptide into a metal-bridged α-helical bundle as was observed for other soft metal ions. This effect might be explained by the unusual coordination chemistry of lead ion which is strongly influenced by the presence of lone-pair electrons.
In summary, the C16C19-GGY peptide not only exhibits interesting and rich metal-binding properties but also displays selective metal-binding as observed for many naturally occurring metalloproteins and enzymes. This system is can thus be useful in understanding mechanisms of metal induced peptide folding.
III. 4.1. Investigation of the Metal-Binding Properties of the Synthetic Cu(I)/C16C19-GGY Metalloprotein

Copper-containing metalloproteins are involved in a variety of fundamental metabolic processes including transport, storage, and detoxification of essential metal ions; electron transfer, respiration, homeostasis, catalysis, and photosynthesis.\textsuperscript{11,57} Among the various copper metalloproteins there are the metallothioneins\textsuperscript{58-60}, copper chaperones\textsuperscript{61}, and the P-type ATPases.\textsuperscript{62,63} It is known that metallothionein and well as to those of the copper responsive transcription factors, \textit{ACE1}, \textit{AMT}, and \textit{CopY}, and copper chaperones, all contain embedded thiolato metal-binding domains.

The copper-metalloprotein described in the previous section also contains a C-X-X-X-X metal-binding motif in its hydrophobic environment. Therefore, it can be used as a good model system which can help to elucidate the metal-binding properties and specificity of copper-containing metalloproteins. Moreover, the Cu(I)-metalloprotein was shown to exhibit room-temperature luminescence, and therefore, can be a potential photoinduced electron-transfer donor to study electron-transfer process in protein-like environment.

**Determination of the oligomerization state of the Cu(I)-metalloprotein**

Sedimentation equilibrium analysis was used in collaboration with Dr. Borries Demeler at UTHSC to determine the oligomerization state of the Cu-C16C19-GGY adduct. Figure III.23 shows the results obtained within the concentration range 200-450 µM and 4 different speeds (50.0, 53.3, 56.7 and 60.0 krpm). The data were fit to both a two-component ideal noninteracting model and a monomer-tetramer model. The fit resulted in a species with a molecular weight of 2.9 kDa, and a species with a molecular weight of 12.1 kDa. The “monomer-tetramer” model
resulted in a monomer molecular weight of 3.06 kDa and a variance of 5.01 x 10^{-5}. Both analyses indicated the presence of two species that correspond to a peptide monomer and tetramer, respectively.

These results were not anticipated from the sequence of the apopeptide, which was based on a IEALEGK heptad repeat known to form two-stranded coiled-coils. Therefore, this result is important because it shows that Cu(I) not only induces peptide self-assembly, but dictates and changes the nature of the oligomerization state of the peptide: Cu(I) forces the formation of the 4-helix bindle instead of dimeric coiled-coil. In contrast, the systems described before by Pecoraro, Tanaka, and Hodges capable of metal-induced peptide self-assembly were shown to fold into the structure expected by original designs.

Such metal-dependant oligomerization state of the metalloprotein points out how the structures of metalloproteins can be controlled by the directional bonding properties of their inorganic cofactors.
Figure III.23. Determination of molecular weight of the Cu(I)/C16C19-GGY by sedimentation equilibrium ultracentrifugation, (0.2 M sodium acetate buffer, pH 5.4-5.5, 5-fold excess of TCEP).

The oligomerization state of the Cu(I)-metalloprotein was further confirmed by SDS-PAGE electrophoresis. Figure III.24. presents the SDS-PAGE of the Cu(I)/C16C19-GGY complex with comparison to the SDS-page standards has known molecular weight. The Cu(I)/C16C19-GGY complex reveals three bands which correspond to tetramer, dimer, and monomer. The appearance of lower-molecular bands can be explained by partial dissociation of Cu(I)-metalloproteins occurring due to denaturating conditions of the experiment.
Standards     Cu(I)/C16C19-GGY   C16C19-GGY

Figure III.24. SDS-PAGE of the Cu(I)/C16C19-GGY complex, apopeptide monomer, SDS-PAGE polypeptide standards.

**Luminescence properties of Cu(I)-metalloprotein**

Figure III.25. shows that the addition of Cu(I) to a solution of C16C19-GGY upon the excitation at 300 nm results in the appearance of an intense room-temperature luminescence centered at 600 nm which is stable upon standing overnight under ambient conditions.\(^{68}\)

This behavior is similar to that observed for several Cu(I)-containing metalloproteins, including metallothionein and inhibitory growth factor, both low molecular weight, cysteine-rich
metalloproteins. Similar luminescence in ~ 600 nm region have also been reported for the ACE1 transcription factor which exhibited room-temperature luminescence at 619 nm$^{53}$ and for the copper responsive repressor CopY which exhibits room-luminescence centered at 600 nm if metal binding sites are adequately shielded from the bulk solvent by the peptide backbone.$^{69-73}$ The Cox17 copper chaperone from different organisms was also shown to luminesce at ~600 nm.$^{74}$

As a rule, luminescent properties of Cu(I)-metalloproteins have been associated with polynuclear or, in rare cases, binuclear copper(I) clusters within metalloproteins in the environment shielded form solvent.$^{75}$ Cu(I) does not luminesce when it is not bound to cysteines. It was also reported that mononuclear copper(I) containing metalloproteins or Cu(I)-proteins with the exposed metal-binding site, for instance, a variety of copper chaperones (e.g. CopZ), do not display luminescent properties.$^{76-78}$

Figure III.26. presents the excitation spectrum of the luminescence of Cu(I)-adduct which has a maximum at 275 nm. It is known that Cu(I)-metallothioneins exhibit the excitation spectrum with a maximum located in ca. 275 nm. Yeast Cu(I)-metallothionein displays similar excitation spectrum with a maximum centered at 277 nm.$^{79}$

The luminescence quantum yield was determined by comparison with $\text{Ru(bpy)}_3^+\text{[(PF}_6\text{)]}_2$ used as a standard. The luminescence quantum yield of aqueous tris(bipyridyl)ruthenium(II) of 0.044 ± 0.003 was shown to be independent of excitation wavelength from 280 to 560 nm.$^{80}$ The determined value of the luminescence quantum yield was found to be 0.053 which that was about 2.5-5 times higher compared to Cu(I)-metallothioneins from various species and other luminescent Cu(I)-metalloproteins:
**Table III.3: Luminescence properties of Cu(I)-metalloproteins:**

Luminescence quantum yield of Cu(I)-metalloproteins (298 K)

<table>
<thead>
<tr>
<th></th>
<th>Yeast Cu(I)-metallothionein (wild type)</th>
<th>Yeast ACE1 Transcription Factor</th>
<th>Neuronal growth-inhibitory factor (Cu_{4.5}, Zn_{2.5})</th>
<th>Cu_{8}-metallothionein</th>
<th>Cu_{12}-metallothionein</th>
</tr>
</thead>
<tbody>
<tr>
<td>The luminescence quantum yield</td>
<td>0.0006 \textsuperscript{79} (\lambda_{exc} =300 \text{ nm}) (\lambda_{mon} =609 \text{ nm})</td>
<td>0.013 \textsuperscript{81} (\lambda_{exc} =305 \text{ nm}) (\lambda_{mon} =619 \text{ nm})</td>
<td>0.02 \textsuperscript{46} (\lambda_{exc} =300 \text{ nm}) (\lambda_{mon} =570 \text{ nm})</td>
<td>0.009 \textsuperscript{47}</td>
<td>0.011 \textsuperscript{47}</td>
</tr>
</tbody>
</table>

As shown in Table 3 various copper-metalloproteins display similar luminescence properties to Cu(I)-C16C19-GGY. By analogy to Cu-metallothionein, neuronal-growth inhibitory factor, and some copper(I) inorganic compounds, the large energy difference between the excitation and the position of the emission band, referred as a Stokes shift, and long-lived luminescence indicate the presence of spin-forbidden triplet excited state manifold. This low-energy emission band centered at 600 nm was assigned to a triplet cluster-centered, a combination of sulfur-to-copper ligand-to-metal charge transfer bands and mixed d-s transitions.\textsuperscript{79,82} This low energy band was also observed in a variety of copper-metalloproteins which was assigned d\textsuperscript{9,1}\textsuperscript{1} orbital parentage modified by Cu-Cu interactions which take place within Cu(I)-clusters.\textsuperscript{31,83,84} As noted by Ford and co-workers\textsuperscript{82} that the lowest molecular orbital (LUMO) is more likely to be 4s orbitals of copper, however, delocalized over the copper-cluster core. The highest molecular orbital (HOMO) is most likely to be S-Cu LMCT in origin. Large Stokes shifts can occur due to the distortions between the electron density between ground and excited states which take place due to Cu-Cu interactions.\textsuperscript{82}
Figure III.25. Emission spectrum obtained upon addition of Cu(I) the to the peptide solution. Conditions: 120 µM C16C19-GGY in 0.2 M acetate buffer (pH 5.4) containing 730 µM tris-(2-carboxyethyl)phosphine (TCEP) as a reducing agent.

Figure III.26. Excitation spectrum obtained upon addition of Cu(I) the to the peptide. Conditions: 200 µM C16C19-GGY in the presence of twofold excess of Cu(I).
a) Luminescence quenching by urea

The studies reported for the ACE1 transcription factor which demonstrated almost complete quenching of emission due to denaturation with the 6 M guanidinium chloride. It has been shown that the copper-thiolate cluster within the metallothionein and other luminescent Cu(I)-metalloproteins has to be properly solvent-shielded in order to retain its luminescence properties. Therefore, the hydrophobic, solvent-shielded environment protects the emissive excited states from deactivation by the solvent. For that reason, Cu(I)-metalloproteins with more open, solvent-exposed metal-binding sites usually are weakly or not emissive.

Figure III.27. illustrates the effect of the denaturation on its luminescent properties. Urea is a well known and widely used chemical denaturant which causes the unfolding of metalloproteins. Therefore, it was anticipated that the unfolding of the Cu(I)-metalloprotein will disturb the ability of the peptide backbone to shield the Cu(I)-binding site from the solvent. Figure III.27. demonstrates the decrease of the emission intensity of the Cu-adduct before and after the addition of the denaturant. This higher accessibility of the Cu(I)-luminophore to solvent molecules results in the efficient deactivation of the emissive excited states through a radiationless pathway to the ground state.
Figure III.27. Quenching of emission observed upon addition of urea to the solution of the Cu(I)/C16C19-GGY complex.

**b) Luminescence quenching by molecular oxygen**

Molecular oxygen is a well-known quencher of triplet excited states. Studies performed on the mammalian metallothionein and ACE1 transcription factor indicated that molecular oxygen acts only as a quencher of triplet excited states which leads to partial quenching of luminescence.\(^{36,85-87}\)

Its effect was studied by monitoring the emission spectra of the Cu(I)/C16C19-GGY in the presence and absence of air. Figure III. 28. shows that the emission intensity of the Cu(I)-metalloprotein decreases after saturation the solution of the Cu(I)-metalloprotein with compressed air for ca. 20 minutes. However, when the solution of the Cu(I)/C16C19-GGY was resaturated with argon, the emission intensity was restored. Therefore, molecular oxygen acts as a quencher of the triplet excited states.
Figure III.28. Quenching of emission observed upon the exposure of the solution of the Cu(I)/C16C19-GGY adduct to air. The solution of the argon-saturated solution of Cu(I)/C16C19-GGY was bubbled with compressed air for 17-20 min and de-aerated with argon for 15-20 min with the following recording of the emission spectra.

**Stability of the Cu(I)-metalloprotein to air oxidation**

Whereas the quenching experiments by molecular oxygen have revealed that the exposure to air for a short time interval does not affect the three-dimensional structure of the Cu(I)-metalloprotein, it was important to elucidate long-term stability of the Cu(I)-adduct to air. Figure III.29. demonstrates the change of the molar ellipticity at 222 nm as a function of time upon exposure of a solution of Cu(I)/C16C19-GGY to air at room temperature for 7 hours.

It is noticed that within first 20 minutes a 10 % increase of the molar ellipticity intensity occurs. After this, a steep decrease of the negative emission intensity was observed. By analogy
with metallothionein studies, it is possible that such increase of the negative molar ellipticity after ~ 20 minutes of the Cu(I)-complex formation might be due to some secondary structure effects associated with the rearrangement of Cu(I)-cluster.

The ellipticity value at 222 nm was used to estimate the helical content of the Cu(I)/peptide complex recorded before and after the time course Figure III. 30. as presently described. The values of % helicity for the Cu(I)-metalloprotein before and after 7-hour air exposure were found to be 44 and 27 %, respectively. Therefore, ca. 40 % decrease in the helical content of the Cu(I)-adduct was detected after 7 hours of the aerobic exposure of the Cu(I)-metalloprotein.

Figure III.30. demonstrates the circular dichroism spectra of the Cu(I)/C16C19-GGY complex recorded before and after the time course. The ellipticity ratio of $[\Theta]_{222}/[\Theta]_{208}$ for the Cu(I)-adduct before the time course was found to be 1.02 which is in the range generally regarded to the presence of a coiled-coil structure. However, after exposure to air for 7 hours, this decreased to 0.87 which is more indicative of monomeric $\alpha$-helix.

It is possible that the exposure of the Cu(I)-C16C19-GGY complex to air leads to the oxidation of either cysteines or Cu(I) of the metalloprotein resulting in the disulfide crosslinking. The noticeable decrease in molar ellipticity intensity at 222 nm upon the exposure to air might be associated with the disulfide crosslinking of the peptide and producing the increased content of the random coil oxidized peptide which can be forming throughout the time course that would lead to decrease in the overall helicity. This result is similar to the behavior observed for Cu(I)-metallothionein: it is well documented that metallothioneins are also quite susceptible to air oxidation in case of prolonged aerobic exposure. ACE1 transcription factor was affected by air oxidation as well.
Figure III.29. Stability time course at aerobic conditions: the change in molar ellipticity at 222 nm as a function of time measured for the C16C19-GGY/Cu(I) at room temperature in 33 mM sodium acetate buffer, pH 5.4-5.4. (concentrations: 53 μM C16C19-GGY, 265 Cu(I) μM).

Figure III.30. Circular dichroism spectra recorded before and after exposure to air for 7 hours of the Cu(I)-C16C19-GGY. (53 μM peptide, 265 μM Cu(I), 33 mM sodium acetate buffer, pH = 5.4-5.5).
In the light of the results obtained throughout the time course, it is evident that some precautions should be taken towards the air sensitivity of the Cu(I)-adduct. In order to ensure that the Cu(I)-metalloprotein remains reduced throughout time intervals required to perform the necessary experiments, the reducing agent, TCEP, was introduced to the system. In order to test this CD spectra were obtained in the presence of TCEP. Figure III.31. demonstrates the change of the molar ellipticity at 222 nm as a function of time upon exposure of Cu(I)/C16C19-GGY/TCEP solution to air at room temperature for 7 hours. As observed previously, the first ~25 minutes of air exposure produced a slight increased of the negative ellipticity.

However, the 7 hour time course resulted in only a modest in molar ellipticity. The initial value of $\Theta = -20058$ deg cm$^2$ dmol$^{-1}$ decreased only by 13 %. The ellipticity ratio remained almost constant throughout this period of time changing from $[\Theta_{222}] / [\Theta_{208}] = 1.02$ to 1.13 (Figure III.32.).

Based on these results, it can be concluded that Cu(I)-metalloprotein is stable to air oxidation in the presence of the reducing agent, TCEP.
Figure III.31. Stability time course at aerobic conditions: the change of molar ellipticity at 222 nm as a function of time measured from the Cu(I)/C16C19-GGY/TCEP at room temperature in 30 mM sodium acetate buffer, pH 5.4-5.4. (concentrations: 87 μM C16C19-GGY, 174 Cu(I) μM, 435 μM TCEP).
Figure III.32. Circular dichroism spectra recorded before and after oxidation course of the Cu(I)-C16C19-GGY. (87 μM peptide, 174 μM Cu(I), 435 μM TCEP, 30 mM sodium acetate buffer, pH = 5.4-5.5).

**Thermal stability of Cu(I)-metalloprotein**

Thermal melting analysis was performed on the Cu(I)/C16C190GGY complex (in the presence of TCEP) by monitoring the CD signal at 222 nm as a function of temperature in order to assess the stability of the Cu(I) metalloprotein (Figure III. 33). The melting curve of the Cu(I)/peptide complex exhibited a reversible unfolding behavior with the melting temperature of about 65 °C. The melting temperature of Cu(I)-metalloprotein was shown to be almost identical to the melting temperature of the previously investigated H21-mer peptide whose thermal denaturation studies revealed a melting curve with a melting temperature at 65° C, indicating that the Cu(I)-peptide is very stable in the aqueous solutions.24-27
Figure III.33. Thermal melting curve of 55 \( \mu \)M C16C19-GGY in the presence of two fold excess of Cu(I) and five fold excess of TCEP.

**Effect of potassium ferricyanide**

Oxidative quenching by ferricyanide has been previously observed for mammalian metallothionein.\(^8^5\) Here, a decrease of the emission intensity occurred upon the addition of potassium ferricyanide to the solution of the metallothionein, circular dichroism spectroscopy has also shown that the oxidation process was accompanied by a collapse of the metalloprotein.\(^8^5\)

Figure III.34. illustrates the effect of potassium ferricyanide on the emission properties of the Cu(I)/C16C19-GGY. The emission intensity decreases monotonically upon the addition of potassium ferricyanide. When 1.4 equivalents of ferricyanide per Cu(I) have been added to the solution of the Cu(I)-metalloprotein, the emission intensity decreases by approximately 50%. This effect can be partially reversed in the presence of the reducing agent (TCEP): Figure III.34.
shows that about 20% of the emission intensity can be restored after the addition of the high excess of TCEP.

To investigate the effect of potassium ferricyanide on spectral and structural properties of Cu(I)-metalloprotein, the circular dichroism spectra of the Cu(I)/C16C19-GGY upon the addition of the aliquots of the potassium ferricyanide solution were recorded. Figure III.35 illustrates that ferricyanide has an effect on the structural properties of the Cu(I)-metalloprotein: the addition of ca. 3 mol equivalents/per Cu(I) of ferricyanide to the solution of the copper/peptide complex was accompanied by ca. 50% decrease of the intensity of the CD signal. Moreover, the $\left[\Theta_{222}/\Theta_{208}\right]$ ellipticity ratio increased from 1 to ca. 1.45 which is indicative of some structural changes occurring with the metalloprotein which could not be reversed by the reducing agent.

![Emission spectra](image)

Figure III.34. Emission spectra recorded upon the addition of ferricyanide to a solution of the Cu(I)/C16C19-GGY complex. Cu(I)/C16C19-GGY complex was formed by the equimolar addition of the tetrakis(acetonitrile)Cu(I)hexafluorophosphate without reducing agent in 0.2 M sodium acetate buffer, pH=5.4, $[C16C19-GGY]=197 \mu$M, $\lambda_{\text{exc}}= 300$ nm.
Based on these results, it is concluded that the decreased emission intensity upon the addition of potassium ferricyanide indicates that the oxidation process is accompanied by irreversible changes in the metalloprotein structure. Even though some structural changes occur upon the oxidation of the Cu(I)-metalloprotein, these changes do not result in a complete loss of helical structure.

Figure III.35. Emission spectra recorded upon the addition of ferricyanide and the sequential addition of TCEP to a solution of the Cu(I)/C16C19-GGY complex. Cu(I)/C16C19-GGY complex was formed by the equimolar addition of the tetrakis(acetonitrile)Cu(I)hexafluorophosphate in 0.2 M sodium acetate buffer, pH=5.4, [C16C19-GGY]=58 μM, λ_{exc}= 300 nm.
Figure III.36. Circular dichroism spectra recorded upon the addition of ferricyanide and TCEP to a solution of the Cu(I)/C16C19-GGY complex. Cu(I)/C16C19-GGY complex was formed by the equimolar addition of the tetrakis(acetonitrile)Cu(I)hexafluorophosphate, \([C16C19-GGY]=10 \mu M\), 1 mm cell.

Near UV Circular Dichroism Spectroscopy

Circular dichroism spectroscopy is a sensitive and reliable technique to determine the structural properties of metalloproteins.\(^{47}\) As previously described, it was observed that the addition of Cu(I) (or Cu(II)) to C16C19-GGY produces two negative maxima at 208 and 222 nm in far UV CD spectrum (Figure III.13.) which indicates that Cu(I) induces peptide self-assembly and the C16C19-GGY peptide undergoes a metal-induced conformational change from a random coil to a coiled-coil conformation. The appearance of these bands in far UV CD spectrum was accompanied by the appearance of a new set of signals in near UV CD spectrum. A strong well-resolved negative CD signal at 295 nm (-) with weaker positive signals at 270 nm (+) and 328 nm (+) and isodichroic points at ca. 282 and 310 nm (Figures III.13, 37). The appearance of
such new signals upon metal-binding further verify that Cu(I) was successfully introduced into the metalloprotein. These features are similar to those reported for the CD spectra of Cu(I)-metallothionein derivatives. As it was shown for the neuronal growth-inhibitory factor and metallothioneins containing Cu(I) clusters, the CD bands in the high-energy region (below 280 nm) originate from CysS-Cu(I) ligand-to-metal-charge transfer transition, and the CD bands in the low-energy CD region originate from spin-forbidden 3d-4s metal-centered transitions arising from Cu(I)-Cu(I) interactions taking place within polynuclear Cu(I)-cluster. Therefore, by analogy to these studies since the CD spectrum of Cu(I)-adduct exhibits similar low-energy spectral feature, this indicates that a polynuclear Cu(I)-cluster is present in the metalloprotein.

![Figure III.37](image-url)

Figure III.37. Near UV Circular dichroism spectrum of C16C19-GGY (109 µM) taken in the presence of a twofold excess of tetrakis(acetonitrile) copper(I)hexafluorophosphate and fivefold excess of TCEP in 0.2 M sodium acetate buffer pH 5.4.
Determination of peptide/Cu(I) stoichiometry

The metal stoichiometry of the Cu-peptide was determined by examining the various spectral changes that occur upon the successive addition of Cu(I) (or Cu(II)) to the C16C19-GGY sample. Several anaerobic titrations of C16C19-GGY by Cu(I) were performed by monitoring the change of the emission, absorbance, and CD signal intensity. The Figure III.38. shows that the emission intensity of Cu(I)/C16C19-GGY increases proportionally with the sequential addition of Cu(I) into the peptide solution until a maximum is reached after ca. 0.9 equiv of metal ion was added. The 1:1 peptide : metal stoichiometry observed for the tetrameric Cu(I)-metalloprotein indicates that four Cu(I) centers have been incorporated into the peptide tetramer (Figure III.39). This conclusion is consistent with near UV CD results and earlier observations that luminescent Cu(I) compounds contain polynuclear metal clusters in which metal-metal interactions play an important role in stabilizing the emissive photoexcited state.82

Figure III.38. Emission titration of C16C19-GGY by [Cu(CH3CN)4]PF₆. Emission spectra obtained upon addition of Cu(I) to the 120 µM peptide solution 0.2 M acetate buffer (pH 5.4) containing 730µM tris-(2-carboxyethyl)phosphine (TCEP) as a reducing agent.
Figure III.39. Emission titration of C16C19-GGY by [Cu(CH3CN)4]PF₆. Titration plot of emission intensity (600 nm) vs. equivalents of Cu(I) added.

**UV-visible spectroscopy**

Figure III.40. shows that the addition of tetrakis(acetonitrile)copper(I) to C16C19-GGY produces a new absorption band centered at 236 nm with a noticeable shoulder at 296 nm which and a broad tail that extends to > 375 nm. As described previously, this band was assigned to Cys-S-Cu(I) charge-transfer transition with significant covalent character.⁵³,⁸¹ These data are in a good agreement with absorption spectrum reported for ACE1 transcription factor,⁸⁷ metallothionein,³⁸,³⁹,⁵³ neuronal-growth inhibitory factor,⁴² and several copper chaperones.⁷⁴,⁸⁹,⁹⁰

The absorption intensity at 296 nm increases linearly with increasing amounts of Cu(I) added to the solution of the peptide and saturates after one equivalent of copper ion has been
added. This result agrees the 1 : 1 peptide to metal stochiometry obtained from the emission titration. This result shows that the Cu(I)-metalloprotein exists as a 4-helix bundle which contains four copper centers (Figure III.41).

The absorbance intensity at the 262 nm shoulder is often used to estimate the number of CysS-Cu(I) bonds in the copper(I)-metalloproteins for which \( \varepsilon = \text{ca. } 5500 \text{ M}^{-1}\text{cm}^{-1} \) per cysteine thiolate.\(^{42,44,46,47}\) The intensity of 262 nm LMCT transition (Figure III.40.) gives the estimated value of \( \varepsilon \approx 22,500 \text{ M}^{-1}\text{cm}^{-1} \) which indicates that only four thiolates are involved in the formation of the Cu(I)–cluster. Interestingly, this result implies that four other thiolates remain uncoordinated within the Cu(I)-metalloprotein.

![Absorbance vs Wavelength](image)

Figure III.40. UV titration of 100 \( \mu \text{M} \text{C16C19-GGY} \) by Cu(I) in 0.2 M acetate buffer pH 5.4; 500 \( \mu \text{M} \text{TCEP} \). Difference spectra of the Cu(I)/C16C19-GGY solutions.
Figure III.41. UV titration of 100 µM C16C19-GGY by Cu(I) in 0.2 M acetate buffer pH 5.4. The titration plot of $A_{296}$ vs. equivalents of Cu(I): growth of new absorption band upon Cu(I) addition.

CD titrations further confirm 1 : 1 metal/peptide stoichiometry. As shown in Figure III.42. the intensity of the signal at 222 nm was seen to increase upon successive additions of Cu(I) and reaches a plateau after 1.0 equivalents of metal have been added. However continued additions of Cu(I) after the saturation point (1 equivalent of Cu (I)) begins to reduce the signal intensity at 222 nm to indicate that the subtle conformational changes may be occurring at high loadings of copper. Such behavior indicates that the some conformational changes may be occurring at high concentrations of copper. Similar behavior has been seen in the copper-mediated dimerization of the putative copper chaperone CopZ. 91
Figure III.42. Far UV Circular Dichroism titration Far UV of C16C19-GGY (236 µM) in 0.2 M sodium acetate buffer, pH 5.4 in the presence of 1 mM TCEP. Inset: Plot of $[\theta]_{222\text{ nm}}$ as a function of added Cu(I).

**Luminescence properties: emission lifetime**

Emission lifetime measurements performed at ambient temperatures in argon-saturated solutions show that the luminescence of the Cu-protein follows bi-exponential decay kinetics in which $A_S$, $k_S$ and $A_L$, $k_L$ correspond to the amplitudes and the rate constants for the longer and shorter components:

$$I(t) = A_S \exp(-k_S t) + A_L \exp(-k_L t)$$

A nonlinear least-squares fit of the obtained data yields values of $A_S = 0.0035$, $\tau_S = 1/k_S = 1.18$ µS and $A_L = 0.0049$, $\tau_L = 1/k_L = 8.63$ µS. (Figure III.43.)
Figure III.43. Emission decay of the of Cu(I)-metalloprotein excited at 357 nm monitored at 600 nm. The red line represents the fit of the data to Equation as described in the text. (The Cu(I)/C16C19-GGY complex was formed in 1.5-fold excess of tetrakis(acetonitrile)copper(I) hexafluorophosphate in argon-saturated 0.2 M sodium acetate buffer, pH 5.4, the peptide concentration is 132 μM).

The double exponential decay kinetics indicates that two components with approximately equal amplitudes are responsible for the emitting species. Neither the luminescence lifetimes nor their amplitudes changed significantly upon titrating Cu(I) into a solution of C16C19-GGY within a 0.3-1.5 Cu(I)/peptide ratio. The fact that the luminescence lifetime behavior displays no concentration dependence suggests that these excited state manifolds are not associated with different Cu(I)-binding sites within structurally different adducts.
As it can be seen from Table 4, either single or multiexponential decay kinetics was observed for various Cu(I)-metalloproteins. The luminescence decay of Cu(I)-metallothionein from yeast *Saccharomyces cerevisiae*\(^92\) and rabbit liver\(^93\) follows three-exponential decay kinetics with the relatively long-lived main components. Such multiexponential decay kinetics might indicate the existence more than one excited-state manifolds or the presence of different Cu(I)-binding sites which produce different excited states.

The studies on luminescence properties of Cu(I)/C16C19-GGY described previously indicate that the emitting species is associated with the reduced Cu(I) state which has significant triplet character, and is quenched upon exposure to bulk solvent. The incorporation of four Cu(I) into a solvent-shielded environment of a metalloprotein may indicate that the metalloprotein contains a cluster-like cofactor analogous to those found in copper(I)-metallothionein.\(^93,94\) Similar properties have been reported for Cu(I) derivatives of the metal-binding protein, metallothionein\(^93,94\) as well as to those of the copper responsive transcription factors, ACE1, AMT, and CopY, which all contain embedded thiolato metal-binding domains.\(^95\)
Table III.4: Luminescent properties of Cu(I)-metalloproteins: Luminescence lifetimes

<table>
<thead>
<tr>
<th></th>
<th>Cu(I) metallothionein from yeast <em>Saccharomyces cerevisiae</em></th>
<th>Yeast Cu(I)-metallothionein (wild type)</th>
<th>Neuronal growth-inhibitory factor (Cu_{4,5}, Zn_{2,5})</th>
<th>Cu_{11}-MT (rabbit liver)</th>
<th>ACE1 transcription factor of <em>Saccharomyces cerevisiae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>(low molecular weight component)</td>
<td>(high molecular weight component)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Excited state Lifetime (μsec)</strong></td>
<td><strong>τ_1 = 0.44</strong></td>
<td><strong>τ_1 = 1.39</strong></td>
<td><strong>τ_1 = 0.02</strong></td>
<td><strong>τ_1 = 150 {^46}_{46}</strong></td>
<td><strong>τ_1 = ~3.5</strong></td>
</tr>
<tr>
<td></td>
<td><strong>τ_2 = 3.85</strong></td>
<td><strong>τ_2 = 7.69</strong></td>
<td><strong>τ_2 = 0.70</strong></td>
<td><strong>τ_2 = ~3.5</strong></td>
<td><strong>τ_2 = ~3.5</strong></td>
</tr>
<tr>
<td></td>
<td><strong>τ_3 = 11.8 {^92}_{92}</strong></td>
<td><strong>τ_3 = 13.3 {^92}_{92}</strong></td>
<td><strong>τ_3 = 3.5 {^93}_{93}</strong></td>
<td><strong>τ_3 = 13.3 {^93}_{93}</strong></td>
<td><strong>τ_3 = 13.3 {^93}_{93}</strong></td>
</tr>
<tr>
<td></td>
<td>(λ_{exc} = 305 nm) (λ_{em} = 615 nm)</td>
<td>(λ_{exc} = 305 nm) (λ_{em} = 590 nm))</td>
<td>(λ_{exc} = 337 nm) (λ_{em} = 609 nm)</td>
<td>(λ_{exc} = 310 nm) (λ_{em} &gt; 560 nm)</td>
<td>(λ_{exc} = 310 nm) (λ_{em} &gt; 560 nm)</td>
</tr>
<tr>
<td>The properties of the Cu(I)-metalloprotein obtained by Cu(II) addition</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In a further set of experiments, it was noted that the addition of cupric ion (CuCl_2) to solutions of C16C19-GGY produces a metallopeptide with identical luminescence properties to those observed when Cu(I) is added to the peptide solution. In particular, Figure III.44 represents the steady-state emission spectrum obtained upon the addition of Cu(II) into the solution of the peptide. Furthermore, as it is shown in Figure III.45, that such luminescence follows biexponential decay kinetics with τ_1 = 9.07 μsec, and τ_2 = 1.06 μsec with approximately equal amplitudes. This biexponential decay kinetics is nearly identical to one observed for the adduct formed by the addition of Cu(I). These results indicate that the addition of Cu(II)
produces the copper-metalloprotein with almost identical luminescent properties as the previously described Cu(I)-adduct.

As shown in Figure III.46, the addition of Cu(II) to the solution of peptide also produces a dramatic change in CD spectrum of the C16C19-GGY as observed when Cu(I) was added to the peptide. The CD spectrum of both copper/peptide complexes consists of negative maxima at 208 and 222 nm and a positive signal located below 200 nm, which indicates the presence of α-helical coiled-coil.

These results suggest that the added cupric ion can be efficiently reduced to cuprous for which a probable mechanism as suggested for various natural systems, such as CopZ and others, which involves the oxidation of proximal cysteine residues of the apopeptide to the corresponding disulfide cystine product $^{37,96,97}$.

$$2 \text{RSH} + \text{Cu(II)} \rightarrow \text{RS-SR} + \text{Cu(I)}$$

The proposed mechanism is consistent with HPLC studies which show that the reaction of CuCl$_2$ with C16C19-GGY produces not only the luminescent copper protein but also a peptide whose retention time is identical to that of the oxidized C16C19-GGY monomer. (Figure III.47.) These results were confirmed by size exclusion chromatography using which also show that the addition of CuCl$_2$ to the apopeptide produces two species. One peak, with the shorter retention time, eluted at the same position as the metallated peptide formed by addition of Cu(I) and the second peak has identical retention time to the monomeric C16C19-GGY. Therefore, it can be concluded that the addition of Cu(II) to the solution of the peptide results in formation of oxidized monomeric C16C19-GGY with intramolecular disulfide cross-linking.
Figure III.44. Emission spectrum obtained upon addition of Cu(II) to the peptide solution. Conditions: 50 µM C16C19-GGY, 30 µM CuCl₂, (λₑₓc=300 nm, monitoring of emission at 600 nm).

Figure III.45. Emission decay of the copper/C16C19-GGY complex formed by the addition of 2.5-fold excess of CuCl₂ to 41 µM of C16C19-GGY excited at 357 nm and monitored at 600 nm.
Figure III.46. Circular dichroism spectrum of C16C19-GGY (50 µM) taken in the presence (■) of a tenfold excess of CuCl₂.

Figure III.47. Analytical chromatogram of the C16C19-GGY/copper complex formed by the addition of CuCl₂.
X-ray absorption spectroscopy

X-ray absorption spectroscopy (XAS) techniques are useful and powerful tools in the determination of the metalloprotein structures, as they provide information about the coordination environment and geometry of metal-binding sites. XAS has been shown to be especially useful in solving the structures of Cu(I) centers. It is known that Cu(I) centers exhibit an absorption edge at 8983 eV which decreases with increasing Cu(I) coordination number. XAS techniques can also help to detect non-bonded Cu-Cu scattering in multicopper clusters due to Cu-Cu interactions. Moreover, the copper coordination geometry can be elucidated from the determination of Cu(I)-thiolate bond length.\textsuperscript{98}

This technique has been successfully applied to solve the structures of Cu(I) centers including yeast Cu(I) metallothionein (MT)\textsuperscript{99}, a low molecular weight cysteine-rich protein which bind a variety of d\textsuperscript{10} metal ions, including Cu(I) and Cd(II).\textsuperscript{100-104} It has been shown that the Cu(I) center in the yeast metallothionein (Cu\textsuperscript{7(8)}-MT) mostly adopts a trigonal coordination geometry with a mean coordination number of 2.6.\textsuperscript{105} Based on the data obtained from XAS and high-resolution NMR studies five metal ions within the cluster are trigonally coordinated to three sulfurs with the estimated Cu-S distances of 2.242 Å and two coppers may exist in linear coordination.\textsuperscript{57,59,100,101,104}

Sulfur K-edge EXAFS studies of copper-rabbit liver metallothionein studies have revealed that Cu(I) adopts a trigonal geometry in Cu\textsubscript{12}-MT with the Cu-S distance of 2.25 Å.\textsuperscript{101,106,107}

Cu K-edge XAS was applied to solve the structure of Cu(I) clusters in native Cu, Zn neuronal-growth-inhibitory factor.\textsuperscript{35} The estimated value of the Cu-Cu separation was
approximately 2.67 Å and 4 Å and Cu-S distance was 2.26 Å, which was consistent with preliminary trigonal Cu(I) coordination geometry.\textsuperscript{35,108}

Similar results have been obtained for the solved structure of the transcription activator from \textit{Saccharomyces cerevisiae} (ACE1).\textsuperscript{87} Cu K-edge XAS analysis has revealed Cu-Cu backscattering at 2.68 Å within a Cu(I)-cluster which consists of six or seven copper(I) ions. The EXAFS curve-fitting analysis of Cu\textsubscript{6,7}-ACE1 have shown that the Cu-S bond length of 2.26 Å is indicative of trigonal coordination geometry.\textsuperscript{87,108}

The CopY repressor which also exhibited room-temperature luminescence was shown to contain only two copper atoms having trigonal coordination geometry with the Cu-S distance of 2.26 Å in a close proximity.\textsuperscript{109} Therefore, it was proved that the binuclear copper (I)-cofactors also display luminescent properties. Similar results have been obtained for the copper chaperone Cox17 from \textit{Saccharomyces cerevisiae}.

AMT1 transcription factor from \textit{Cnadida glabrata} which was shown to contain a tetranuclear copper-binding cluster which is held together by bridging thiolates with a sort Cu-Cu distance about 2.75 Å in a trigonal coordination geometry and one isolated Zn(II) metal-binding site.\textsuperscript{108}

Similar analyses have been performed to elucidate the coordination cluster environments in a variety of other Cu(I)-thiolate proteins which are presented in Table 6. The data show that the average Cu-S distance in these systems are in the range of 2.24 – 2.26 Å which corresponds to predominately trigonal cooper-thiolate coordination.\textsuperscript{98,110}

All metalloproteins listed in below in Table 5 were shown to contain polynuclear Cu(I)-binding sites I which Cu(I) adopts a trigonal coordination geometry. It has been proposed that in some cases copper(I) favors only trigonal coordination geometry exclusively which implies the
tendency of Cu(I) to bind third exogenous ligand which brings Cu(I) to the preferable trigonal coordination. Therefore, very often the exogenous ligands are necessary to complete Cu(I)-coordination sphere of various proteins.  

Table III.5: Comparison of metal-binding sites for luminescent copper metalloprotein

<table>
<thead>
<tr>
<th>Cu-metalloprotein</th>
<th>Cu-S distance, Å</th>
<th>Cu-Cu distance, Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE1 transcription factor form <em>Saccharomyces cerevisiae</em></td>
<td>2.260</td>
<td>2.68</td>
</tr>
<tr>
<td>Cu-metallothionein from <em>Saccharomyces cerevisiae</em></td>
<td>2.242</td>
<td>2.60</td>
</tr>
<tr>
<td>Native Cu, Zn-neuronal-growth inhibitory factor</td>
<td>2.260</td>
<td>2.67</td>
</tr>
<tr>
<td>Cu-rabbit liver metallothionein</td>
<td>2.25</td>
<td>N/A</td>
</tr>
<tr>
<td>CopY repressor of <em>Enterococcus hirae</em></td>
<td>2.26</td>
<td>2.69</td>
</tr>
<tr>
<td>Cox17 copper chaperone from <em>Saccharomyces cerevisiae</em></td>
<td>2.26</td>
<td>2.70</td>
</tr>
<tr>
<td>AMT1 transcription factor from <em>Cnadida glabrata</em></td>
<td>2.26</td>
<td>2.75</td>
</tr>
</tbody>
</table>

X-ray absorption spectroscopy (XAS) was performed in collaboration with Prof. Michael Maroney, University of Massachusetts, Amherst.
The Cu K-edge X-ray absorption near-edge (XANES) spectrum of Cu-C16C19-GGY (Figure 47) shows a Cu edge with an energy appropriate for a Cu(I) center \((E= 8987.0 \text{ eV})\). The Cu(I)/C16C19-GGY spectrum shows no the 1s→3d transition near 8980 eV typical of Cu(II) sites and exhibits a pre-edge peak at 8983.5 eV consistent with a 1s→4p transition observed for Cu(I) centers in a variety of Cu(I)-metalloproteins.\(^{35,100,101,115}\) The shape of the peak observed for Cu-C16C19-GGY indicates a three-coordinate site, as the intensity is too weak for a linear two-coordinate geometry and the energy is too low for a four-coordinate site.\(^{115}\) EXAFS analysis (Figure III.49.) is consistent with a Cu(I) site having a N(O)S\(_2\) ligand donor set. The best fit for the data over the range of 1-4 Å (uncorrected for phase shifts) consists of one N- and two S-donors at distances of 1.89(1) and 2.22(2) Å, respectively. The data also reveal the presence of additional scatterers in the second and third coordination spheres of the Cu centers, indicating the presence of a Cu cluster. These data can be fit with two Cu atoms at a distance of 2.88(1) Å, and with Cu and S atoms at 3.91(1) and 3.54(3) Å, respectively. The Cu-S distance of 2.22(2) Å is somewhat shorter then the distances described for Cu(I)-metalloproteins, however, it is still considered symptomatic of trigonal coordination geometry for Cu(I).\(^{100}\)

The results are consistent with the formation of a Cu(I) cluster containing a Cu\(_4\)S\(_4\) ring, where each Cu is bridged by the side chains of two cysteine residues and has terminal N/O ligation. However, such a model indicates that only half of the available cysteine residues are ligated to metal atoms. Such mixed N(O)-Cu-S coordination is quite common in metalloproteins. It is also well documented that N/O-donor ligation usually arises from solvent or lysine residues.\(^{116}\)

The Ellman’s test was performed to test further this hypothesis. DTNB titrations repeated in triplicate revealed that the apoprotein contains 2 free cysteines per peptide monomer. In
contrast to this result, DTNB titration of the Cu(I)/peptide complex indicated that only one thiol group per monomer was accessible for the reaction with DTNB. Therefore, the titration of free thiol groups with DTNB confirms the presence of one free thiol per peptide chain. These results are in agreement with the estimated number of thiolates involved in the formation of the Cu(I)-metalloprotein obtained from the magnitude of the 262-nm LMCT transition.

The proposed Cu(I) cofactor strongly resembles the structure of a recently reported synthetic Cu₄S₄ cluster containing Cu(I) in a trigonal NS₂-donor environment (Figure 48). The Cu-S distances (2.22(2) Å) are similar to the average Cu-S distances in the synthetic cluster (2.21(2) Å) and are significantly longer than those for linear two-coordinate CuS₂ complexes found in transcription factors (~2.15 Å).

The Cu-N(O) scatterer observed in Cu-C16C19-GGY may be derived from glutamate residues found in the peptide or from coordination of solvent. The Cu-N(O) distance is shorter than that in the synthetic cluster (2.09(1) Å) but similar to that found in the trigonal model compound, [Cu(1,2-Me₂Im)₃]PF₆ (Figure III.49.) (1.89 Å). The Cu-Cu distances (2.88(1) and 3.91(1) Å) found for Cu-C16C19-GGY are also similar to those observed in the synthetic cluster (avg. 2.70(4) and 3.81(3) Å). The two Cu-Cu distances observed are appropriate for a dihedral angle formed by intersecting Cu₃ planes that is ~134°, which describes a Cu₄ configuration that is distorted 18% from square planar (180°) toward tetrahedral (70°) geometry.

The results show that the addition of Cu(I) to the random coil C16C19-GGY peptide produces a self-organized 4-helix bundle (Figure III.50.) which is likely driven by the formation of the luminescent Cu₄S₄ cluster.
Figure III.48. Copper K-edge XAS data of Cu(I)-C16C19-GGY. Top: The XANES spectrum; bottom: Fourier-transformed; and insert: Fourier-filtered EXAFS spectrum data (shown as circles and fit as a solid line).\textsuperscript{68}

Figure III.49. Synthetic Cu$_4$S$_4$ cluster containing Cu(I) in a trigonal NS$_2$-donor environment.\textsuperscript{119}
The Cu(I)/Cu(II) redox cycle is a fundamental one-electron transfer process which occurs many in biological systems such as, superoxide dismutase, cytochrome c oxidase, multicopper oxidase and others. As, it was demonstrated above, the synthetic Cu(I)-metalloprotein is a useful model system with an incorporated native-like transition cofactor buried within its hydrophobic core which can enable the studies of the electron-transfer process in protein-like environment.

The reduction potentials of the of the Cu(I)/C16C19-GGY complex were determined by performing redox titrations in combination with UV-vis absorption and/or steady-state emission spectra. Figure III.51. shows UV-visible spectra of the Cu(I)-metalloprotein as it was titrated.
with small aliquots of the oxidant \( \text{K}_2\text{IrCl}_6 \). After the addition of each aliquot the solution was vigorously stirred and allowed to equilibrate for several minutes before each measurement. The disappearance of the absorption band at 310 nm was monitored with the simultaneous recording the cell potential with platinum and calomel reference electrodes. The decrease of the absorption band at 310 nm upon addition of the oxidant occurs concomitantly with the increase of absorbance at 370-500 nm in the UV-visible spectrum. The occurrence of isosbestic point around 350 nm indicates the presence of two species in solution. The absorbance intensity was plotted versus the measured potential resulting in the Nernst curve shown in Figure III.52. which was fit to a single \( n=1 \) Nernst equation with a midpoint of \( 99 \text{ mV vs SCE} \) (343 mV vs NHE).

\[
Y = I_{\text{reduced}} + \Delta I \left( \frac{1}{10^{\frac{nF(E_h-E_m)}{RT}} + 1} \right)
\]

where \( I_{\text{reduced}} \) is the absorbance intensity at 310 nm or emission intensity at 600 nm corresponding to the reduced Cu(I)-metalloprotein, \( \Delta I \) is the change in absorbance or emission from reduced to oxidized forms, \( E_h \) is the measured potential of the solution, and \( E_m \) is the midpoint potential, \( F/RT=60 \text{ mV} \).

The difference spectrum corresponding to Cu(II)-adduct is presented in Figure III.53.
Figure III.51. UV-visible spectra of 186 μM Cu(I)-metalloprotein in 0.2 M sodium acetate buffer pH 5.5 in anaerobic cell upon the oxidation by titration with K$_2$IrCl$_6$.

Figure III.52. Redox titration curve of Cu(I)-metalloprotein monitored by optical spectroscopy. Absorbance at 310 nm is plotted against the measured redox potential vs. SCE and the obtained data were fit to the single Nernst equation curve (n=1).
The electrochemical reduction potential of the Cu(I)-metalloprotein was verified by performing the redox titrations by monitoring the decrease of the emission intensity of the Cu(I)-metalloprotein at 600 nm upon the excitation at 275 nm with the simultaneous recording the cell potential with platinum and calomel standard electrodes (Figures III.54. and 56.). The titrations were performed in duplicate. Figure III. 55. and 57. demonstrates the redox titration of Cu(I) metalloprotein obtained by plotting the emission intensity at 600 nm as a function of redox potential. The data were fit to a single n=1 Nernst curve with a midpoint of 95 mV vs SCE (339 mV vs NHE) and 110 mV vs SCE (354 mV vs NHE).

It should be noted that the redox titration obtained by monitoring of the decrease in emission intensity of the Cu(I)/C16C19-GGY complex was not reversible. Even though the values of the measured potentials were changing towards the reduced state of Cu(I) upon the addition of the reducing agent (TCEP) to the solution of the Cu(I)-metalloprotein, the emission
intensity was not restored. Therefore, the midpoint redox potential of the Cu(I)-metalloprotein obtained by three independent experiments by monitoring either the changes in absorbance or emission was found to be 345±8 mV vs NHE.

Figure III.54. Emission spectra of 112 μM Cu(I)-metalloprotein in 0.2 M sodium acetate buffer pH 5.5 in anaerobic cell upon the oxidation by titration with K₂IrCl₆.

Figure III.55. Redox titration curve of Cu(I)-metalloprotein. Plot of the emission intensity at 600 nm is plotted against the measured redox potential vs. SCE and the obtained data were fit to the single Nernst equation curve (n=1).
Figure III.56. Emission spectra of 120 μM Cu(I)-metalloprotein in 0.2 M sodium acetate buffer pH 5.5 in anaerobic cell upon the oxidation by titration with K₂IrCl₆.

Figure III.57. Redox titration curve of Cu(I)-metalloprotein. Plot of the emission intensity at 600 nm is plotted against the measured redox potential vs. SCE and the obtained data were fit to the single Nernst equation curve (n=1).
**Effect of TCEP on Cu(I)-binding**

TCEP is widely used as a reducing agent in a variety of biological studies. In some cases the utilization of reducing agents is necessary, especially if a protein contains cysteine residues in a close proximity which makes it readily susceptible to oxidation.

As demonstrated above, the Cu(I)-metalloprotein is sensitive to air oxidation. Thus, precautions should be taken when handling the Cu(I)-adduct. This effect can be minimized in the presence of TCEP. However, several studies of metal-binding stoichiometry of the copper chaperone CopZ and related systems have revealed that the addition of reducing agents, other thiol-containing or TCEP in particular, in metal-binding studies can greatly affect the metal-binding stoichiometry. It has been proposed that TCEP acts as a coordinating molecule for Cu(I). X-ray absorption and NMR studies performed on a CopZ copper chaperone having the C-X-X-C metal-motif revealed that different stoichiometries can be observed for metal/chaperone complexes depending upon the experimental conditions used. In particular, it was demonstrated that the presence of an exogenous molecules (TCEP or DTT) which can act as a third ligand and can greatly affect the stoichiometry of metal/protein complex: 1 : 2 metal – CopZ stoichiometry is be disrupted and form a 1 : 1 monomeric complex instead of a dimer. It was proposed that coordinating molecules (DTT, TCEP, or glutathione) can occupy the copper(I) coordination site which leads to change of the Cu/protein stoichiometry. Three-coordinate Cu(I)-CopZ metal-binding site was also confirmed by XAS studies.

Moreover, the HAH1 copper chaperone several was also shown to adopt a linear Cu(I) coordination geometry in the absence of a potentially coordinating molecules. However, XAS studies were used to show that a trigonal coordination geometry in the presence of a weak ligand
present in solution (TCEP). Therefore, it was suggested that very often copper(I) favors trigonal coordination geometry which implies the tendency of Cu(I) to bind third exogenous ligand. Therefore, it was suggested that very often copper(I) favors trigonal coordination geometry which implies the tendency of Cu(I) to bind third exogenous ligand. 

A series of experiments was performed to determine if the metal-binding or structural properties of the Cu(I)-C16C19-GGY are affected by the presence of TCEP. It was important to determine if TCEP has any effect on the Cu(I)/peptide stoichiometry of the Cu(I)/C16C19-GGY complex. As several studies have shown that the experimental conditions can greatly affect the metal/protein stoichiometry of EhCopZ and HAH1 and related systems, i.e. small coordinating molecule present in the solution can greatly affect the form of the complex. Therefore, two UV-vis titration were performed in the absence and presence of TCEP. As can be seen from Figure III.58, the UV titration curves display similar behavior with the saturation occurring at ca. 1 equivalent of Cu(I) per peptide. Thus, Cu(I)/C16C19-GGY complexes in the presence and in absence of TCEP displays identical metal : peptide stoichiometry. Importantly, HPSEC experiments showed that the oligomerization state is not affected by the presence of TCEP. Both Cu(I) complexes (formed in the presence and absence of TCEP) exist as peptide tetramers which incorporate four copper ions.
Figure III.58. The UV-vis titration of C16C19-GGY by tetrakis(acetonitrile)Cu(I)hexafluorophosphate in the presence and absence of TCEP.

Even though it has been confirmed that the presence of the potentially coordinating molecule, TCEP, does not affect the metal : peptide stoichiometry of the Cu(I)-metalloprotein, it was important to determine if TCEP has an effect on the Cu(I)/peptide luminescence properties. Figure III.59. presents steady-state emission spectra recorded before and after the addition of the reducing agent. The intensity of the emission recorded after the addition of the excess of TCEP decreases by ca. 10% compared to the initial emission intensity. This relatively small decrease in emission intensity suggests that there is no significant effect of TCEP on the luminescent properties of the Cu-metalloprotein, and therefore, it can be implied that the overall structure of the luminescent tetranuclear Cu(I)-cluster was not disturbed by the presence of the reducing agent.
Emission lifetime measurements of the Cu-metalloprotein in the presence of 5-fold excess of TCEP were performed at ambient temperatures in argon-saturated solutions to further verify that the presence of the reducing agent does not disturb the three-dimensional structure of the Cu(I)-cofactor or its luminescent properties. Figure III.60. demonstrates that the luminescence of the Cu-protein also follows bi-exponential decay kinetics in which \( A_S, k_S \) and \( A_L, k_L \) correspond to the amplitudes and the rate constants for the longer and shorter components.

A nonlinear least-squares fit of the obtained data yields values of \( A_S = 0.031, \tau_S = 1/k_S = 1.01 \mu S \) and \( A_L = 0.029, \tau_L = 1/k_L = 8.70 \mu S \): \( I(t) = A_S \exp(-k_S t) + A_L \exp(-k_L t) \). The fit of the data obtained for the Cu(I)-adduct prepared in the absence of TCEP is listed for comparison (\( A_S = 0.00035, \tau_S = 1/k_S = 1.18 \mu S \) and \( A_L = 0.00049, \tau_L = 1/k_L = 8.63 \mu S \). Therefore, TCEP does not affect the emission lifetimes of the Cu(I)-C16C19-GGY complex.

![Graph](image)

Figure III.59. The comparison of the emission intensity of the Cu(I)-C16C19-GGY in the absence or presence of TCEP, \( \lambda_{exc} = 600 \text{ nm} \).
Figure III.60. Emission decay of the Cu(I)-metalloprotein excited at 357 nm monitored at 600 nm. The red line represents the fit of the data to Equation as described in the text. (The Cu(I)/C16C19-GGY complex was formed in argon-saturated aqueous solution in 5-fold excess of tetrakis(acetonitrile)copper(I) hexafluorophosphate in the presence of TCEP, the peptide concentration is 114 μM).

From the set of experiments described above, it can be concluded that TCEP affects neither the luminescent properties of Cu(I)-metalloprotein nor the stochiometry of Cu(I)/C16C19-GGY complex. Therefore, TCEP does not compete for copper ions or at most acts as a very weak ligand that does not affect structural and optical properties of the Cu(I)-metalloprotein.
Summary

In summary, the Cys-X-X-Cys metal-binding motif, found in a variety of metalloproteins has been incorporated into both positions “a” and “d” positions of the third heptad repeat of a coiled-coil peptide sequence. This results in formation of a “soft” metal-binding site within the hydrophobic peptide-peptide interface of the coiled-coil. This cysteine-containing random coil apopeptide is capable of binding copper(I) which results in the formation of a metal-bridged self-organized α-helical bundle. It was shown that the synthetic Cu(I)-metalloprotein exists as a 4-helix bundle which contains a cyclic Cu4S4 cofactor in which each Cu(I) atom is bridged by two cysteine residues and has a terminal N/O ligand. Therefore, the copper(I) cofactor not only induces peptide self-assembly, but also directs and changes the oligomerization state of the peptide. That illustrates how the structures of metalloproteins may be controlled by the coordination chemistry of their inorganic cofactors.

The work described here has also targeted another important goal of de novo metalloprotein design – incorporating chemical functionality into de novo designed metalloproteins to construct new synthetic metalloproteins with useful functions. It was shown that the Cu(I)-metalloprotein displays intense room temperature long-lived (microsecond) luminescence at 600 nm. Such incorporated chemical functionality allows using this synthetic metalloprotein as a photoinduced electron-transfer agent in future studies.

Significantly, the designed Cu(I)-metalloprotein to our knowledge is the first example of the creation of a synthetic Cu(I)-metalloprotein with introduced chemical functionality into native-like protein scaffold obtained by incorporating the copper(I) cofactor into its hydrophobic interior.
III. 4.2. Investigation of the Metal-Binding Properties of the Synthetic Cd(II)/C16C19-GGY Metalloprotein

HPSEC and SDS-PAGE electrophoresis were used to study the oligomerization state of the Cd(II)/C16C19-GGY complex. Figure III.61. presents denaturating SDS-PAGE electrophoresis results which show the presence of both peptide monomers and dimers when excess Cd$^{2+}$ is added to a solution of C16C19-GGY. Figure III.62. also demonstrates the calibration performed by using different peptide standards characterized previously. As it was determined from calibration, the calculated molecular weight of the Cd(II)-metalloprotein is ~6.9 kDa. This value is nearly identical to the calculated molecular weight of the Cd(II)-adduct: 6.9 kDa. Thus, high performance size-exclusion chromatography (HPSEC) and SDS-PAGE results indicate that the Cd(II)-metalloprotein likely exists as a metal-bridged, two-stranded $\alpha$-helical coiled-coil.
Figure III.61. SDS-PAGE electrophoresis shows that the Cd(II)/C16C19-GGY complex exists as a monomer and a dimer peptide, respectively.
Figure III.62. HPSEC results obtained by using series of peptide standards on Superdex 75 HPSEC column under non-denaturating conditions (0.1 M KCl / 0.05 M K$_2$HPO$_4$, pH 7), 0.4 ml/min flow rate. The distribution coefficients (Kd) were calculated according to the following expression: Kd = Ve - Vo / Vt - Ve, where Ve is the elution volume of the peptide, Vo is the void volume and Vt is the column bed volume.

Figure III.63. presents the absorbance difference UV spectra of Cd(II) metalloprotein which shows the growth of a new absorption band centered at 238 nm upon the addition of Cd(II). Vasak et al. have assigned the absorption band in the 230-250 nm region of Cd(II)-substituted metallothionein or Cd(II)-substituted rubredoxin to a RS$^-$ → Cd$^{2+}$ ligand-to-metal charge transfer band using the concept of semiempirical optical electronegativity of Jørgensen for charge-transfer excitations. Based on this work, the new band in Cd(II)/C16C19-GGY centered at 238 nm can be assigned to the ligand-to-metal charge-transfer (LMCT) transition of the newly formed Cd-S bond.
The metal-peptide stoichiometry of the Cd(II)/peptide complex was studied by UV-vis and circular dichroism spectroscopy. Figure III.63. demonstrates the sequential increase of the absorption intensity at 238 nm upon successive additions of CdCl₂. As seen, the plot of A₂₃₈ vs. equivalents of Cd(II) represents the subsequent increase of the absorbance intensity upon addition of Cd(II) and the saturation after 0.5 equivalents of Cd(II) added per peptide, which corresponds to a 2 : 1 C₁₆C₁₉-GGY/Cd(II) ratio (Figure III.64.). This result was supported by a Job plot which was constructed by measuring the absorbance at 238 nm as a function of the mole fraction of Cd(II) present in solution. (Figure III.65.) These results indicate the formation of a 1 : 2 metal : peptide complex.

Figure III.63. UV–Vis titration in which successive additions of CdCl₂ were made to a 100 µM solution of C₁₆C₁₉ immobilized Cleland’s REDUCTACRYLTM reagent (Calbiochem).
Figure III.64. The plot of $A_{238}$ vs. equivalents of Cd(II): growth of new absorption band upon Cd(II) addition.

Figure III.64. The Job plot obtained by monitoring the absorbance intensity at 238 nm as a function of the mole fraction of Cd(II).
The metal binding stoichiometry was further studied by circular dichroism titrations which were shown to produce somewhat similar results. The intensity of molar ellipticity of the at 222 nm increases with increasing amounts of Cd(II) up to 0.5 equivalents of Cd(II) added to solution of the peptide with the following saturation after 0.5 equivalents have been added. (Figure III.66.)

Together the HPSEC, SDS-PAGE results indicate that in contrast to the behavior of the Cu (I)-metalloprotein which exists as a 4-helix bundle, the Cd(II)-metalloprotein exists a dimer. UV and CD results show one Cd ion incorporated into the peptide dimer, compared to Cu-metalloprotein which has four copper centers per peptide tetramer.

![Figure III.66. Plot of normalized ellipticity at 222 nm as a function of Cd(II) addition.](image)

The comparative metal-binding analysis performed for metallated derivatives of metallothionein showed that these proteins bind Cu(I) and Ag(I) ions with higher stochiometries than such divalent transition metal ions as, Cd(II) and Hg(II). Furthermore, metallothioneins
were found to bind 7 Cd(II) ions and 12 Cu(I) ions.\textsuperscript{75} Cd(II)-substituted metallothionein (Cd(II)-MT) studies have revealed that Cd(II) preferably adopts tetrahedral coordination geometry with thiolate ligands in contrast to the Cu(I)-metallothionein which has a trigonal metal-binding site.\textsuperscript{124,125} It is suggested that the Cd(II) coordination preferences are responsible for a such difference in a stochiometry compared to Cu(I)-metalloprotein. It was observed that the near UV circular dichroism spectrum of the Cd(II)/peptide complex has a noticeable difference compare to the apopeptide which does not have any significant signals in the 240-300 nm region, i.e. the chirality of the apopeptide framework do not contribute to the near UV CD region.\textsuperscript{106} Figure III.67. shows that the CD spectrum of the metallated peptide complex consists of a positive maximum at 242 nm (~ 900 deg cm\(^2\) dmol\(^{-1}\)), a zero crossing at 250 nm, and a less-pronounced negative maximum at 257 nm with the molar ellipticity value of -300 deg cm\(^2\) dmol\(^{-1}\). Therefore, the appearance of the CD bands upon addition of the metal ion indicates the successful incorporation of Cd(II) into the metal-binding site. Near UV CD spectra have been observed for the Cd(II)-substituted metallothionein and Cd(II)-derivative of rubredoxin.\textsuperscript{123,126} The near UV CD spectra of these metalloproteins were characterized by the appearance of the positive band at ca. 240-250 nm. These excitations have been assigned to optically active CysS-Cd(II) ligand-to-metal-charge-transfer-transitions in a chiral protein environment.\textsuperscript{9,31,33,40,45}
Therefore, taken together CD and UV-vis titrations, HPSEC, and SDS-PAGE results along with the Job plot show that the cadmium ion induces peptide self-assembly resulting in the formation of a well-organized metal-bridged $\alpha$-helical dimeric metalloprotein with the of a 2:1 peptide : metal stoichiometry.

**Effect of TCEP on Cd(II)-binding**

Even though the experiments described above were performed under anaerobic conditions and precautions were taken to prevent the oxidation of the peptide, another set of the experiments were performed in the presence of the reducing agent, TCEP in order to eliminate any possible consequences of peptide oxidation. As it was demonstrated above from the Job plot,
CD, and UV-vis spectroscopy studies, the metal-binding occurs with 0.5 : 1 Cd(II)/C16C19-GGY stochiometry. Figure III.68. presents the overlay of two UV-vis titrations performed in the presence or absence reducing agent, TCEP. Interestingly, the titration plot obtained from the titration of C16C19-GGY by Cd(II) in the presence of TCEP, displays a 1 : 1 peptide to Cd(II) stochiometry, in contrast to the 1 : 2 metal to peptide stochiometry when the reducing agent was not added. Therefore, the presence of TCEP in the solution does indeed have a significant effect on the formation of the Cd(II)/C16C19-GGY complex. These results are in parallel with the studies performed on the metallochaperone family which have demonstrated that the metal-binding stochiometry can be greatly affected by the presence of a coordinating molecule. Cd(II)-substituted EhCopZ and related systems have revealed that the addition of reducing agents, TCEP in particular, in metal-binding studies can greatly affect the metal-binding stochiometry.

These results show that in contrast to the Cu(I)/C16C19-GGY complex, the Cd(II)-metalloprotein is sensitive to the presence of the reducing agent which can occupy the cadmium coordination position that changes the stochiometry of Cd(II)/C16C19-GGY complex.
Figure III.68. The UV-vis titration of C16C19-GGY by CdCl₂ in the presence and absence of TCEP.

**III. 4.3. Investigation of the Metal-Binding Properties of the Synthetic Hg(II)/C16C19-GGY Metalloprotein**

High performance size exclusion chromatography technique was used to determine the oligomerization state of the Hg(II)/C16C19-GGY complex. Figure III.69. demonstrates the calibration performed by using varies standards characterized previously. As it was determined from calibration, the calculated molecular weight of the Hg(II)-metalloprotein is ~6.7 kDa. This value is nearly close to the calculated molecular weight of the Hg(II)-dimer: 6.9 kDa. Thus, high performance size-exclusion chromatography (HPSEC) results indicate that the Hg(II)-metalloprotein likely exists as a dimeric metal-bridged, two-stranded α-helical coiled-coil. Hg(II)/peptide stochiometry was further studied by the examining new UV-vis and CD absorption features.
Figure III. 69. HPSEC results obtained by using series of peptide standards on Superdex 75 HPSEC column under non-denaturating conditions (0.1 M KCl / 0.05 M KH$_2$PO$_4$, pH 7), 0.4 ml/min flow rate. The distribution coefficients (Kd) were calculated according to the following expression: Kd =Ve-Vo/Vt-Vo, where Ve is the elution volume of the peptide, Vo is the void volume and Vt is the column bed volume.

Hg(II) can adopt a variety of coordination geometries with thiolate ligands, the most common geometries being linear digonal, trigonal and tetrahedral.$^{12}$ The preferable coordination geometry of Hg(II) with small organic compounds is generally linear in aqueous solutions.$^{1,51,127}$ Several studies of Hg(II) protein/peptide complexes revealed that the Hg(II) complexes with different coordination environments have noticeable differences in the number and position of the S-Hg LMCT bands. Mercury thiolates with linear coordination geometry display only one well-resolved LMCT band centered at 230 nm with an extinction coefficient of $4 \times 10^3$ M$^{-1}$ cm$^{-1}$. As it was shown for Mer R metalloregulatory metalloprotein and trigonal Hg(SR)$_3$ complexes based on TRI family, the difference spectra of mercuric thiolates reveal a characteristic low energy transitions at $\sim$ 265 ($\Delta\varepsilon=12 \times 10^3$ M$^{-1}$ cm$^{-1}$) and $\sim$ 290
nm ($\Delta \varepsilon = 5.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) distinguishable from the higher energy transition centered at ~230 nm with molar extinction coefficient of $21 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. The trigonal coordination geometry of Hg(S-Cys)$_3$ in Mer R metalloregulatory protein and TRI peptides with Hg(S-Cys)$_3$ metal-binding site confirmed by EXAFS studies. The difference absorption spectra of tetrahedral Hg(II) thiolates usually have two well-resolved bands at about 230 and 257 nm with molar extinction coefficients of $22 \times 10^3$ and $12 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, respectively, and a less-resolved band at 284 nm with extinction coefficient of $20 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. Therefore, based on these spectroscopic signatures, it is possible to identify different geometries of mercury thiolates.

As described before, circular dichroism and UV-vis spectroscopic titrations were carried out to evaluate the structural changes accompanying the formation of a newly formed Hg/C16C19-GGY complex. Figure III.70. presents the difference absorption spectra obtained upon the sequential addition of small aliquots of Hg(II) into the solution of the peptide. The difference spectra show that at low Hg(II)/peptide ratios (0.09-0.35 equivalents of HgCl$_2$) a new well resolved absorption band can be seen centered at 236 nm with a broad tail that extends to > 260 nm. The wavelength and extinction coefficient for this transition are similar to those observed for Hg(II) thiolate with linear coordination geometry. Therefore, it is possible that at low Hg(II)/peptide loadings mercury adopts the most preferable linear geometry. However, when the concentration of Hg(II) is increased beyond the 0.35 equivalents of HgCl$_2$, a new species grows having new transitions at 236, 266 nm and 290 nm. Figure III.71. shows that the higher energy absorption band at 236 nm increases linearly with increasing amounts of Hg(II) added to the solution of the peptide and reaches a plateau after 0.5 equivalents of metal ions have been added.
Extinction coefficients for the LMCT transitions from Hg(II)/C16C19-GGY difference spectra were calculated assuming the molecular weight of the metalloprotein corresponding to the dimer: $\varepsilon_{(230\ nm)} = 21,545$, $\varepsilon_{(270\ nm)} = 10,958$, and $\varepsilon_{(290\ nm)} = 4,142\ \text{M}^{-1}\ \text{cm}^{-1}$ which are in the good agreement with extinction coefficients previously reported for the MerR protein and the TRI family.\textsuperscript{3,127,128}

It has been demonstrated that the Hg(II)/C16C19-GGY complex exist as a peptide dimer with the metal/peptide stoichiometry of 1 : 2 which suggests the presence of one metal center incorporated into the metalloprotein. However, the Hg(II)-coordination geometry changes depending upon increasing of the mercury : peptide ratio: at lower Hg(II)/peptide ratio Hg(II) adopts a linear geometry, however, at high Hg(II)/ratio adopts a trigonal coordination geometry.

Similar change of the Hg(II) coordination geometry has been reported before by Stillman and co-workers who has described the Hg(II)-binding to rabbit liver metallothionein.\textsuperscript{52} The formation of Hg(II)-adducts was monitored by circular dichroism, magnetic circular dichroism, and optical spectroscopy, and the formation of two different species in which Hg(II) adopts different (trigonal and tetrahedral) coordination geometry has been postulated. It has been shown that Hg(II) coordination geometry changes thorough the Hg(II)-titration: Hg\textsubscript{7}-MT species were shown to contain mercury ions with tetrahedral coordination geometry, however, further addition of mercury causes the formation of Hg\textsubscript{11}-species having trigonal Hg(II) metal-binding sites.\textsuperscript{52}

Circular dichroism experiments indicate that the formation of the Hg(II)-C16C19-GGY metalloprotein is accompanied by a conformational change of C16C19GGY from a random coil to a coiled-coil. Therefore, the stoichiometry of the Hg(II) metalloprotein was further studied by circular dichroism spectroscopy. Metal-binding experiments were performed by the sequential
addition of the aliquots of concentrated HgCl$_2$ solution to the solution of C16C19-GGY. The induction of secondary structure upon addition of Hg(II) was measured by monitoring molar ellipticity at 222 nm. Figure III.72. shows the induction of helicity as a function of HgCl$_2$ concentration which demonstrates the linear increase in $[\theta]_{222}$ with increased equivalents of Hg(II) added to the solution that reaches a plateau after 0.5 equivalents of metal have been added. The data are similar to what was observed for cadmium metalloprotein but significantly different from Cu(I)/C16C19-GGY complex which revealed 1 : 1 stochiometry.

As was observed for the Cd(II)/peptide complex the near UV circular dichroism spectrum changes dramatically upon the addition of Hg(II) to the apopeptide. The CD spectrum of the apopeptide shows only features below 240 nm associated with the secondary structure of the peptide and no signals in the 240-300 nm region UV CD region. Figure III.73. shows that the CD spectrum of the metallated peptide complex consists of a strong positive maximum at 247 nm (~1955 deg cm$^2$ dmol$^{-1}$) and a second well-resolved LMCT excitation at 268 nm with the molar ellipticity value of 1424 deg cm$^2$ dmol$^{-1}$. These transitions originated from the S-Hg LMCT excitations in chiral protein environment were also observed for other Hg(II) systems with the trigonal Hg(II)-geometry.$^{11,12,52,126}$ It is well documented that the mercury thiolates with linear coordination geometry do not have significant LMCT transitions in far UV region above 240 nm.$^{52}$
Figure III.70. UV–Vis titration in which successive additions of HgCl$_2$ were made to a 111 µM solution of C16C19-GGY, pH 5.4-5.5.

Figure III.71. UV-vis Titration of 111 µM C16C19-GGY by HgCl$_2$ at pH 5.4-5.5
Figure III.72. Circular Dichroism titration of C16C19-GGY (103 µM) by HgCl₂.

Figure III.73. Near UV Circular dichroism spectrum of C16C19-GGY (175 µM) taken in the presence of a twofold excess of HgCl₂ in 20 mM sodium acetate buffer pH 5.4.
This result further suggests that at higher Hg(II)/peptide ratios the metalloprotein accommodates trigonal metal binding site. Significantly, there are only few examples of such unusual tridentate mercury/thiolate coordination geometry and to our knowledge the Hg(II)/C16C19-GGY is only a third example demonstrating the three coordinate mercury binding site. Until now only MerR metalloregulatory protein\textsuperscript{51} and the designed Hg(II) peptide complexes described by Pecoraro and co-workers\textsuperscript{1,7,12} were shown to adopt Hg(II) in trigonal coordination geometry. This system can be a good model system to study the interplay between the geometric preferences of metal ion vs. the protein conformation.

Similarly to the Cd(II)-adduct, the Hg(II)-metalloprotein displays a dimeric oligomerization state and 1 : 2 metal/peptide stoichiometry. These structural properties were very different from the Cu(I)-metalloprotein. As shown above, from UV and CD titrations, such divalent transition metal ions as, Cd(II) and Hg(II) bind to the peptide with 1 : 2 metal peptide stoichiometry compared to the 1 : 1 copper : peptide stoichiometry. Moreover, Cd(II) and Hg(II)-binding leads to the formation of the peptide dimer, in contrast to the 4-helix bundle induced by Cu(I). Such difference in the metal : peptide stoichiometry and oligomerization states may be explained by the strong tendency of Cu(I)-cofactor to form cluster-like structures which drives the formation of the tetramer which can adopt 4 copper ions.
Effect of TCEP

As it was demonstrated for Cd(II) and Cu(I) complexes, the presence of reducing reagent can have a great effect on the metal/peptide stochiometry. It was shown that the stochiometry of the Cd(II)-complex changes dramatically when TCEP is present in solution. In contrast, the Cu(I)-adduct does not display any change in peptide/metal stochiometry in the presence or absence of TCEP. In order to elucidate the effect of reducing agent (TCEP) on the coordination Hg(II) metal-binding properties, two different titration sets were performed in the presence and absence of TCEP, respectively. Figure III.74. demonstrates the overlay of CD titrations of C16C19-GGY performed in the absence of presence of the reducing agent. A 1 : 2 metal/peptide stochiometry has been observed for the CD titration when TCEP was not present in solution. In contrast, a 1.5 : 1 metal : peptide ratio was detected in the presence of excess TCEP. Therefore, similar, to the result observed for the Cd(II)-system, TCEP affected the coordination properties of mercury to result in a change of the metal/peptide stochiometry. A similar result was observed for the UV-vis titration of the apopeptide by Hg(II). Figure III.75. presents the overlay of two titrations performed in the presence and absence of TCEP. This overlay demonstrates that the metal : peptide stochiometry has changed from 1 : 2 to 3 : 2 for the titrations performed in the absence or presence of TCEP, respectively. This result in a good agreement with the CD titration described above. Figure III.76. shows that the result of the titration plot obtained in water or sodium acetate buffer are nearly identical. Therefore, this change of the stochiometry occurs due to the presence of TCEP but not buffer. Based on these results, it is suggested that similar to Cd(II)/C16C19-GGY complex, TCEP acts as a coordinating molecule in case of Hg(II)-binding that greatly affect the stochiometry of the metallated peptide complex. In both cases, the metal :
peptide stochiometry has increased if the metal-peptide complex was formed in the presence of TCEP.

Figure III.74. CD of C16C19-GGY by HgCl$_2$ in the presence or absence of TCEP. Plot of normalized ellipticity at 222 nm as a function of Hg(II) addition.

Figure III.75. The overlay of UV-vis titrations of C16C19-GGY by HgCl$_2$ in the presence or absence of TCEP.
Figure III.76. The overlay of UV-vis titrations of C16C19-GGY by HgCl₂ performed in water or 0.2 M sodium acetated buffer pH 5.5.
III. 4.4. Investigation of the Metal-Binding Properties of the Ag(I), Pt(II), and Au (I)/C16C19-GGY Synthetic Metalloproteins

High performance size exclusion chromatography was used to study the oligomerization state of the Ag(I)/C16C19-GGY complex. Figure III.77. demonstrates the calibration performed by using varies standards characterized previously. It was determined from calibration that the calculated molecular weight of the Ag(I)-metalloprotein is ~11.6 kDa. This result indicates that the Ag(I)-metalloprotein exists as a tetramer. Figure III.78. demonstrates the overlay of the HPSEC chromatograms for the Cu(I)/C16C19-GGY complex, the molecular weight of which corresponds to tetramer, as determined by AUC, and the Ag(I)/C16C19-GGY complex. As it can be seen, the Ag(I)/C16C19-GGY complex co-elutes with the tetrameric standard. Thus, similarly to the Cu(I)-adduct, Ag(I)-metalloprotein exists a peptide a tetrameric metal-bridged, four-stranded α-helical coiled-coil.
Figure III.77. HPSEC results obtained by using series of peptide standards on Superdex 75 HPSEC column under non-denaturating conditions (0.1 M KCl / 0.05 M KH₂PO₄, pH 7), 0.4 ml/min flow rate. The distribution coefficients (Kd) were calculated according to the following expression: Kd = \( \frac{V_e - V_o}{V_e - V_t} \), where \( V_e \) is the elution volume of the peptide, \( V_o \) is the void volume and \( V_t \) is the column bed volume.
Figure III.78. HPSEC of the Ag(I)-metalloprotein and Cu(I)/C16C19-GGY used as a standard.

The metal-peptide stoichiometry of the silver/peptide complex was examined by UV-vis spectroscopy. Metal binding was monitored by measuring the increase in absorbance of a new UV absorption band. As shown in Figure III.79, the addition of Ag(I) to C16C19-GGY produces a new absorption band at 230 nm with a broad tail that extends up to 320 nm. Such a spectral envelope was observed for silver-derivatives of metallothionein: the UV-vis spectrum of which also revealed a broad absorption band with a broad tail extended up to 380 nm centered at ca. 230-240 nm which was assigned to the S-Ag(I) ligand-to-metal-charge-transfer transitions.

Figure III.80. shows that the absorption intensity at 230 nm increases linearly with increasing amounts of Ag(I) added to the solution of the peptide and saturates after one equivalent of copper ion has been added.
The binding of Ag(I) was also analyzed by circular dichroism spectroscopy. Similar to the metal-binding results obtained for the Cu(I)/peptide complex, the $\alpha$-helicity was increased upon the addition of AgNO$_3$ into the solution of peptide up to 1 equivalent of Ag(I) added and reaches a plateau after 1.0 equivalents of metal have been added (Figure III.81.). This result confirms the 1 : 1 peptide to metal stochiometry obtained from the UV-vis titration. This observation is consistent with the metal-binding studies performed on mammalian metallothionein which have shown that metallothionein binds Ag(I) ions with the same stochiometry as Cu(I).

The near and far UV circular dichroism spectra change dramatically upon the addition of Ag(I) to the apopeptide. Similarly to the Cu(I)/C16C19GGY complex, the appearance of the bands at far UV CD spectrum was accompanied by the appearance of a new set of signals in near UV CD spectrum. Figure III.82. shows that the CD spectrum of the metallated peptide complex has of a strong well-resolved positive CD signal at 286 nm with the molar ellipticity value of $+322 \text{ deg cm}^2 \text{ dmol}^{-1}$, a negative maximum at 258 nm ($\sim +330 \text{ deg cm}^2 \text{ dmol}^{-1}$), and a zero crossing at 267 nm. This result further verifies that Ag(I) was successfully introduced into the metalloprotein. These features are similar to those reported for the CD spectra of Ag(I)-metallothionein derivatives, which have characteristic signals: (+) 240 nm, (-) 260 nm, and (+) 280 nm.$^{129-131}$ By analogy to Ag(I)-substituted mammalian metallothionein, the CD bands in the low-energy CD region can be assigned to metal cluster-centered transitions arising from Ag(I)-Ag(I) interactions taking place within polynuclear Ag(I)-cluster.$^{42,47,84,130,131}$ Therefore, these spectrophotometric features of the Ag(I) metalloprotein obtained upon addition of Ag(I) to the peptide provide a valuable information about the fingerprint of the specific near UV CD bands.$^{106}$
Figure III.79. UV titration of 117 µM C16C19-GGY by Ag (I).

Figure III.80. UV titration of 117 µM C16C19-GGY by Ag (I). The titration plot of $A_{230}$ vs. equivalents of Ag(I): growth of new absorption band upon Ag(I) addition.
Figure III.81. Circular Dichroism titration of 165 µM C16C19-GGY by AgNO₃. Plot of $[\theta]_{222}$ as a function of added Ag(I).

Figure III.82. Near UV Circular dichroism spectrum of C16C19-GGY (165 µM) taken in the presence of a 1.4-fold excess of AgNO₃.
In contrast to the behavior previously seen for the Cd\textsuperscript{132} and Hg proteins, but similarly to the Cu(I)/C16C19-GGY complex, a 1:1 peptide : metal stochiometry was observed for Ag(I) metalloprotein. Since the size exclusion chromatography experiments showed that the Ag(I) metalloprotein eluted with the same retention time as Cu(I) metalloprotein, four Ag(I) centers have been incorporated into the peptide tetramer.

Traditionally, it has been proposed that Ag(I) serves as an isostructural and isoelectronic probe for Cu(I) in metallothioneins systems. Moreover, it has been postulated that Ag(I) displays similar coordination geometry as Cu(I) and therefore, Ag(I)-substituted metallothioneins has been used as a structural analog of Cu(I)-MT in NMR and other structural studies.\textsuperscript{34,103} The recent finding described by Gonzalez-Duarte and co-workers have provided new insight into the structural aspects of Ag(I)-binding to metallothionein and the suitability of Ag(I) as a structural probe for Cu(I). Mass-spectral, CD, and UV-vis analysis of Ag(I) and Cu(I) binding thorough Zn(II) replacement from recombinant mouse metallothionein1 (Zn-MT) have revealed that Ag(I) and Cu(I) bind similarly to Zn(II)-MT only at the first stages of the Zn(II)/M(I)-replacement which result in the formation of M(I)\textsubscript{3}Zn\textsubscript{2}-\alpha-MT species. It has been shown that the newly formed Ag(I) and Cu(I)-metallothioneins displayed similar CD fingerprint in near UV CD region, and therefore, it was concluded that Ag(I), Cu(I)-MT have comparable three-dimensional structures.\textsuperscript{131} However, further additions of M(I) to Zn(II)-metallothioneins resulted in the formation of different species (M(I)\textsubscript{9} -\alphaMT) with drastic differences in their CD spectra. Thus, this result indicates that Cu(I) and Ag(I) bind differently at high M(I) loading, and Ag(I) can not be considered as an adequate probe for Cu(I).\textsuperscript{102,103,130,131} Structural dissimilarities of Cu(I) and Ag(I)-metallopeptides can arise due to different coordination preferences and ionic radii of the metal ions.\textsuperscript{102} The preferred coordination geometry for Ag(I)-thiolates is linear with a preferred
coordination number of two,¹³³ in contrast to Cu(I) which usually tends to favor trigonal coordination geometry in proteins.¹⁰²

As mentioned above, the CD bands in the low-energy CD region suggest the existence of polynuclear Ag(I) cluster⁴²,⁴⁷,⁸⁴,¹³⁰,¹³¹ within the four-helix Ag(I)-metalloprotein. However, in the light of the results described above, a comparison of the CD spectra of Cu(I) and Ag(I)/C₁₆C₁₉-GGY show that the CD spectra display no similarities, and therefore, it is suggested that the structures of these metallated M(I) clusters are very different.

Taking into account the fact the preferred coordination geometry for Ag(I)-thiolates is linear with a preferred coordination number of two,¹³³ and that the structure of Ag(I)-adduct must be different from Cu(I) cofactor which was shown with trigonal coordination geometry, it is suggested that Ag(I) adopts linear geometry.
Figure III.83. Thermal melting curves of 55 μM C16C19-GGY in the presence of two fold excess of Ag(I) or Cu(I) and five fold excess of TCEP.

**Thermal stability**

The Ag(I)/C16C19-GGY complex was analyzed by thermal denaturation performed by monitoring the CD signal at 222 nm to assess the stability of the Ag(I) metalloprotein. The melting curve of the Ag(I)/peptide complex exhibited reversible behavior with the melting temperature of about 65 °C (Figure III.83.). The value of the melting temperature of Ag(I)-metalloprotein (70 °C) was higher that the melting temperature of the previously investigated Cu(I)-metalloprotein and self-assembled H21-mer apopeptide.24-27
**Effect of TCEP**

As it was observed for the Cu(I)-metallopeptide, TCEP did not have a significant effect in the metal binding properties. Figure III.84. indicates ca. a 1 : 1 stoichiometry in the presence and absence of TCEP.

Figure III.84. The overlay of UV-vis titrations of C16C19-GGY by AgNO₃ performed in 0.2 M sodium acetated buffer pH 5.5.
Pt(II) and Au(I)-Metalloproteins

Figure III.85. demonstrates the calibration performed by using varies peptide standards characterized previously. As it was determined from calibration, the observed molecular weight of the Pt(II)-metalloprotein is ~11 kDa. Figure III.86. presents the HPSEC chromatogram of the Pt(II)-metalloprotein. As it has been shown for Ag(I)-metalloprotein, the Pt(II)-C16C19-GGY complex co-eluted with the tetrameric Cu(I)-adduct which indicates that the Pt(II)-metalloprotein also exists as a peptide tetramer.

Figure III.85. HPSEC results obtained by using series of peptide standards on Superdex 75 HPSEC column under non-denaturating conditions (0.1 M KCl / 0.05 M KH$_2$PO$_4$, pH 7), 0.4 ml/min flow rate. The distribution coefficients (Kd) were calculated according to the following expression: Kd = Ve-Vo/Ve-Vo, where Ve is the elution volume of the peptide, Vo is the void volume and Vt is the column bed volume.
Figure III.86. HPSEC of the Pt(II)-metalloprotein and Cu(I)/C16C19-GGY used as a standard.

In contrast to results observed for the Cu(I), Ag(I), and Pt(II)-peptide complexes, the Au(I)-metalloprotein displays higher molecular weight. Figure III.87. represents that the HPSEC chromatogram of Au(I)-metalloprotein in comparison to the tetrameric Cu(I)-metalloprotein. The molecular weight of Au(I)/C16C19-GGY determined from HPSEC calibration is 21.4 kDa (Figure III.88.). These results indicate that Au(I)-C16C19-GGY predominantly exists as hexamer. Such oligomerization state differs from one predicted by original design.

This is an important result showing that different inorganic cofactors can change the oligomerization state of the peptide. The synthetic metalloproteins described here display metal-specific oligomerization states and can exist as dimers, tetramers, or even hexamers. This can be
explained by the tendency of such metal ions as, Cu(I), Pt(II), Ag(I), and Au(I) form cluster-like structures which can drive the formation of higher-molecular weight species.

Figure III.87. HPSEC of the Au(I)-metalloprotein and Cu(I)/C16C19-GGY used as a standard.
Figure III.88. HPSEC results obtained by using series of peptide standards on Superdex 75 HPSEC column under non-denaturating conditions (0.1 M KCl / 0.05 M KH₂PO₄, pH 7), 0.4 ml/min flow rate. The distribution coefficients (Kd) were calculated according to the following expression: \( K_d = \frac{V_e - V_o}{V_t - V_e} \), where \( V_e \) is the elution volume of the peptide, \( V_o \) is the void volume and \( V_t \) is the column bed volume.

Circular dichroism titrations were carried out to evaluate the structural changes accompanying the formation of a newly formed Au/C16C19-GGY complex. The behavior was slightly different to what obtained for Cu(I) and Ag(I) metalloproteins. As shown in Figure III.89, at low loading of Au(I) (0.1-0.5 equivalents of Ag(I)) the ellipticity signals at 222 nm did not change significantly. However, as the concentration of Au(I) was increased beyond 0.5 equivalents, the dramatic change was observed in the ellipticity signals at 222 nm which flattened out after 1 equivalent of Au(I) added to the solution of the peptide as it was similarly observed for Cu(I) and Ag(I) metalloproteins.
Table 6 summarizes the effect of the reducing agent on the metal-binding properties. It is demonstrated that TCEP has a dramatic effect on the formation of the Cd(II) and Hg(II)-complexes, and has no effect on the formation of the Cu(I), Ag(I), and Au(I)/C16C19-GGY.
Table III.6. Metal : peptide stochiometries under different conditions

<table>
<thead>
<tr>
<th>Metal Ions</th>
<th>0.2 M sodium acetate buffer pH 5.5 no TCEP</th>
<th>0.2 M sodium acetate buffer pH 5.5 5-fold excess of TCEP</th>
<th>Water No TCEP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UV-vis</td>
<td>CD</td>
<td>UV-vis</td>
</tr>
<tr>
<td>Cd(II)</td>
<td>1 : 2</td>
<td>-</td>
<td>1 : 1</td>
</tr>
<tr>
<td>Cu(I)</td>
<td>1 : 1</td>
<td>-</td>
<td>1 : 1</td>
</tr>
<tr>
<td>Hg(II)</td>
<td>1 : 2</td>
<td>-</td>
<td>1.5 : 1 (Hg(II) : peptide)</td>
</tr>
<tr>
<td>Ag(I)</td>
<td>-</td>
<td>-</td>
<td>1 : 1</td>
</tr>
<tr>
<td>Au(I)</td>
<td>-</td>
<td>~1 : 1</td>
<td>-</td>
</tr>
</tbody>
</table>
CONCLUSIONS

The work presented in this dissertation describes metal-binding properties of synthetic self-assembled metalloproteins which were based on the de novo designed peptide C16C19-GGY. This peptide was designed such that a Cys-X-X-Cys metal-binding domain was placed at positions 16-19 of its sequence and it was anticipated that metal binding might result in the formation of a two-stranded coiled-coil.

This cysteine-containing random coil apopeptide is capable of binding a variety of soft metal ions which results in the formation of a metal-bridged self-organized α-helical bundles. It is noted that the binding is observed for the binding of Cd(II), Hg(II), Ag(I), Au(I), Pt(II), and Cu(I), but not Fe(II), Co(II), Ni(II) Zn(II), or Pb(II). A 1:1 metal:peptide stoichiometry is observed for the Cu(I) and Ag(I) adducts but the Cd(II) and Hg(II) complexes show a metal:peptide stoichiometry of 1:2. Therefore, this system displays metal-dependant binding stoichiometry.

It was shown that such binding produces a metal-specific oligomerization state of metalloproteins: synthetic Cu(I), Ag(I), and Pt(II)-metalloproteins exist as 4-helix bundles, in contrast to Cd(II) and Hg(II) metalloproteins which exist as two-stranded coiled-coils. This illustrates how the structures of metalloproteins may be controlled by the coordination chemistry of their inorganic cofactors.

It was also shown that the Cu(I)-metalloprotein described here displays intense room temperature long-lived (microsecond) luminescence at 600 nm. Such incorporated chemical functionality allows using this synthetic metalloprotein as a photoinduced electron-transfer agent in future studies.
REFERENCES FOR CHAPTER III


6196.


14696-14705.


(30) Henehan, C. J., Pountney, D. L., Zerbe, Vasak, M. Protein Sci. 2; 1756–1764.

Protein Sci. 1993, 2, 1756-1764.


*113*, 9354-9358.


(105) Pickering, I. J.; George, G. N.; Dameron, C. T.; Kurz, B.; Winge, D. R.; Dance, I. G. 


(110) Brown, K. R.; Keller, G. L.; Pickering, I. J.; Harris, H. H.; George, G. N.; Winge, D. 


432-433.


(130) Bofill, R., Palacios, O., Capdevila, M., Cols, N., Gonzalez-Duarte, R., Atrian, S., Gonzalez-Duarte, P. J. Inorg. Biochem. 1999, 73, 57-64.

