Inorg. Chem. **2002**, *41*, 1584–1590

Thermodynamic Studies on the Recognition of Flexible Peptides by Transition-Metal Complexes

Shuguang Sun, Md. Abul Fazal, Bidhan C. Roy, Binita Chandra, and Sanku Mallik*

Department of Chemistry, North Dakota State University, Fargo, North Dakota 58105

Received August 14, 2001

Strong and selective binding to a trihistidine peptide has been achieved employing Cu^{2+}–histidine interactions in aqueous medium (25 mM HEPES buffer, pH 7.0). When the pattern of cupric ions on a complex matched with the pattern of histidines on the peptide, a strong and selective binding was observed. UV–vis spectroscopic studies show that the cupric ions coordinate to the histidines of the peptides. Thermodynamic studies reveal that the binding process is enthalpy driven over the entire range of working temperature (25–40 °C). An enthalpy–entropy compensation effect was also observed.

Peptide recognition is one of the fields of growing interest and active research due to its key role in numerous biological processes, e.g., hormone action, immune response, etc.[1] Strong and selective binding of a peptide to a biological target can lead to a successful drug. Detection of disease marker peptides has the potential of early diagnosis of the disease. In addition, recognition of oligopeptides has been used as an intermediate step toward the recognition of the protein surface.[2] The principles of molecular recognition learned from these model systems are laying the foundation for protein surface recognition.[2b]

Recognition of peptides has attracted considerable attention in recent years, and several groups have reported moderately strong and selective binding to peptides[3] by designed molecules in the aqueous phase. Receptors have been reported for both sequence-selective recognition[4] and recognition of α-helices and β-sheets of peptides.[5] To our knowledge, these synthetic receptors bind to the target peptides through one or more noncovalent interactions, e.g., hydrogen bonding, electrostatics, van der Waals forces, hydrophobic interactions, etc. Usually, the binding constants are comparatively low ($<50{,}000\ M^{-1}$).

Immobilized metal affinity chromatography (IMAC) has been used extensively to separate and purify proteins and peptides.[6] IMAC often uses polymer-bound iminodiacetic acid (IDA) and several other tridentate ligand systems to chelate the metal ions. When borderline metal ions (e.g., Cu^{2+}, Ni^{2+}) are used in IMAC sorbents (in the pH range 4.5−7.5), the absorbents exhibit a preference for nitrogen-containing functional groups (e.g., the imidazole of histidine). IMAC distinguishes proteins and peptides on the basis of the content of histidine residues.[7]

Recognition based on metal−ligand interactions offers several advantages over traditional approaches. Hydrogen-bonding and ion-pair interactions are either very weak or completely absent in a competitive solvent, e.g., water, making them unsuitable for recognition study in the aqueous phase.[8] On the contrary, metal−ligand interactions are quite strong in an aqueous solution. In addition, the strength and kinetics of metal−ligand interactions can be fine-tuned by the choice of the metal ion, the ligand positioning on the metal ions, and the pH of the medium.[9] The metal ions have

* To whom correspondence should be addressed. Phone: (701) 231-8829. Fax: (701) 231-8831. E-mail: Sanku.Mallik@ndsu.nodak.edu.

(1) *Bio-organic Chemistry: Peptides and Proteins*; Hecht, S. M., Ed.; Oxford University Press: New York, 1998.
(2) (a) Peczuh, M. W.; Hamilton, A. D. *Chem. Rev.* **2000**, *100*, 2479−2494. (b) Fazal, A. Md.; Roy, B. C.; Sun, S.; Mallik, S.; Rodgers, K. R. *J. Am. Chem. Soc.* **2001**, *123*, 6283−6290.
(3) (a) Hossain, A. Md.; Schneider, H. J. *J. Am. Chem. Soc.* **1998**, *120*, 11208−11209. (b) Bresloco, R.; Yang, Z.; Ching, R.; Trojandt, G.; Obodel, F. *J. Am. Chem. Soc*. **1998**, *120*, 3536−3537. (c) Hoiki, H.; Still, W. C. *J. Org. Chem.* **1998**, *63*, 904−905. (d) Nagola, S. M.; Kearney, P. C.; Mecozzi, S.; Russell, K.; Dougherty, D. A. *J. Am. Chem. Soc.* **1999**, *121*, 1192−1201.
(4) Maletic, M.; Wennemers, H.; McDonald, D. Q.; Breslow, R.; Still, W. C. *Angew. Chem., Int. Ed. Engl*. **1996**, *35*, 1490−1492.
(5) Schneider, J. P.; Kelly, J. W. *Chem. Rev.* **1995**, *95*, 2169−2187.
(6) Recent examples: (a) Sharma, S.; Agarwal, G. P. *J. Colloid Interface Sci*. **2001**, *243*, 61−72. (b) Ward, M. S.; Shepherd, R. E. *Inorg. Chim. Acta* **2000**, *311*, 57−68. (c) Fitton, V.; Santarelli, X. *J. Chromatogr., B* **2001**, *754*, 135−140. (d) Michael, P.; Torkkeli, T.; Karp, M.; Oker-Blom, C. *J. Biotechnol*. **2001**, *85*, 49−56.
(7) (a) Jiang, W.; Graham, B.; Spiccia, L.; Hearn, M. T. W. *Anal. Biochem.* **1998**, *255*, 47−58. (b) Zachariou, M.; Hearn, M. T. W. *Biochemistry* **1996**, *35*, 201−211. (c) Zachariou, M.; Traverso, R.; Spiccia, L.; Hearn, M. T. W. *Anal. Chem*. **1997**, *69*, 813−822.
(8) Frey, P. A.; Cleland, W. W. *Bioorg. Chem*. **1998**, *2*, 62−66.

10.1021/ic0108742 CCC: $22.00
Published on Web 02/22/2002

well-defined spectroscopic properties and can be used to obtain structural information about the recognition process.[10]

Our goal is to design and to synthesize metal complexes capable of binding strongly to histidine patterns of flexible peptides in water.[11] Herein, we report that a very strong and selective peptide−ligand interaction can be achieved by matching the *pattern* of histidine moieties of a peptide with a *complementary pattern* of cupric ions on a designed receptor.

Results and Discussion

Design and Syntheses of the Peptides. To demonstrate the pattern-matching concept for peptide recognition, we have designed and synthesized water-soluble peptides with three histidine moieties at particular distances apart (12−16 Å). This distance corresponds to the inter-histidine distances (His 1, His 7, and His 12 or 14) on the surface of the protein carbonic anhydrase (bovine erythrocyte, Protein Data Bank file name 1G6V.pdb). The peptides were modeled using the software Spartan (version 5.1, Wavefunction Inc., Irvine, CA). The Merck molecular mechanics force field was employed for energy minimization, and then systematic conformational searches were performed to identify the lowest energy conformation(s) of the molecules. The structures of the peptides are shown in Figure 1. The syntheses of the peptides are depicted in Scheme 1 (for detailed synthetic procedures, see the Supporting Information).

From molecular modeling, the distance among the imidazole groups of the histidines was estimated to increase by approximately 4 Å when the peptide length increased by a glycine unit. The inter-histidine distances changed from ∼12 Å for **t_H** to ∼16−18 Å for **t_GH**. The peptide **t_GGH** was designed to probe the role of flexibility in the recognition process (the inter-histidine distance for **t_GGH** was estimated to vary from ∼18 to ∼23 Å). Introduction of another glycine unit resulted in decreased solubility (in 25 mM HEPES buffer at pH 7.0), and the peptide **t_GGGH** was unsuitable for our studies. The peptide **t_AH** has the hydrophobic methyl group while **t_QH** has the hydrophilic amide moiety in the side chain. These two peptides were used to study the effect of hydrophobicity on the recognition process. Replacement of alanine of **t_AH** with a phenylalanine (**t_FH**) caused a further increase in hydrophobicity and made the peptide insoluble in aqueous buffer (pH 7.0). The peptide **t_aH** (estimated inter-histidine distance ∼12−14 Å) was synthesized to study the effect of an additional functional group (COOH) of the peptide on the recognition process. The monohistidine peptides **m_H** and **m_aH** served as the controls for recognition studies.

Design and Syntheses of Metal Complexes. The metal ion was selected on the basis of two criteria: (a) formation of a M·His complex is favored over that of a M·OH at pH 7.0; (b) the kinetics of binding is fast. A comparative study on various transition-metal ions (Co^{2+}, Cu^{2+}, Cd^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}) indicated that Cu^{2+} was the optimum. IDA was chosen as the ligand to chelate the cupric ions. IDA has

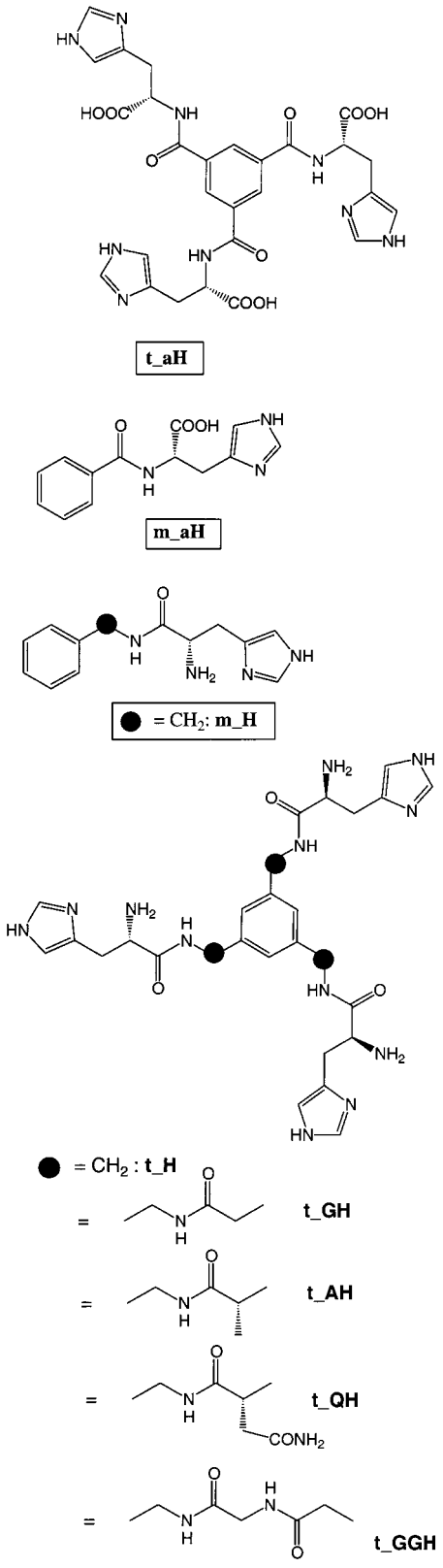
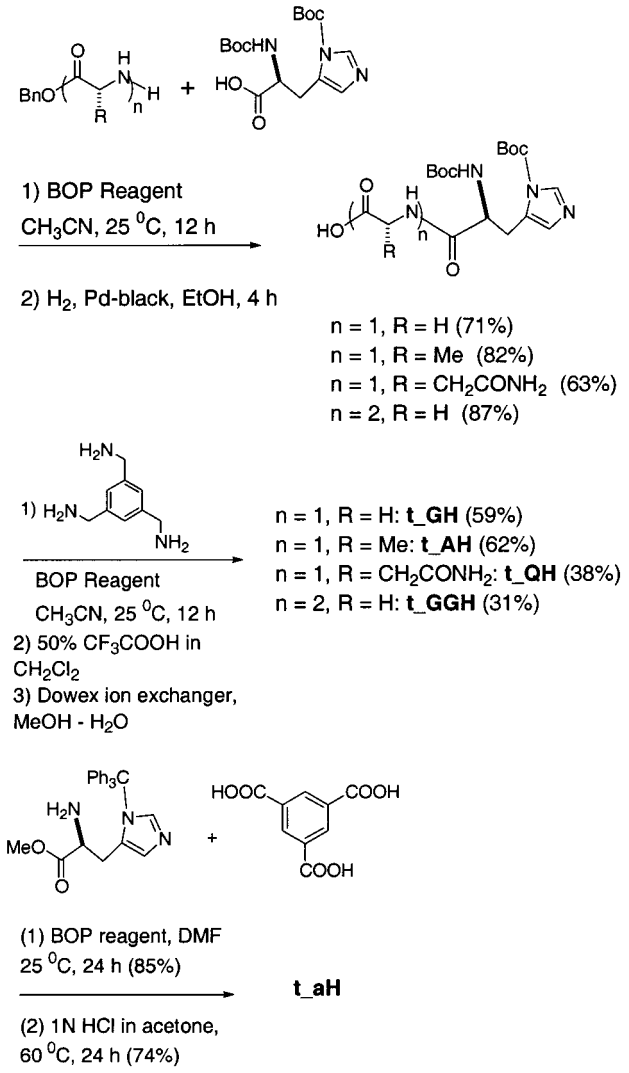


Figure 1. Structures of the histidine-containing peptides used in the binding studies.

(9) *Principles of Bioorganic Chemistry*; Lippard, S. J., Berg, J. M., Eds.; University Science Books: Mill Valley, CA, 1994.
(10) *Metal in Biological Systems*; Kendrick, M. J., May, M. T., Plishka, M. J., Robinson K. D., Eds.; Ellis Horwood: Chichester, England, 1992; pp 17−48.
(11) Sun, S.; Fazal, A. Md.; Roy, B. C.; Mallik, S. *Org. Lett.* **2000**, *2*, 911−914.

Scheme 1. Synthesis of the Histidine-Containing Peptides



strong affinity for Cu^{2+} ($K \approx 10^{12}$ M^{-1}),[12] and the resultant complex has been widely used in protein purification by IMAC. Literature reports indicate that the complex will not be demetalated even at high peptide concentrations.[13]

Five Cu^{2+} complexes (**R1**, **R2**, **R3**, **R4**, and **R5**, Figure 2) were designed and synthesized for our studies. The inter-Cu^{2+} distances were estimated by molecular modeling employing the software Spartan. The complexes were energy minimized in the gas phase (with a Merck molecular mechanic force field) followed by systematic conformational searches to locate the lowest energy conformation(s). The distances among the Cu^{2+} ions were estimated to be ~12 Å for **R2**, ~16 Å for **R3**, and ~14−18 Å for **R4** and **R5**. The complex **R1** with one Cu^{2+} ion was used as a control.

The syntheses of these metal complexes have been reported previously.[2b] Elemental analysis indicated that the complexes had the correct number of cupric ions. This was confirmed by determination of Cu^{2+} by UV−vis spectroscopy employing EDTA (for the detailed procedure, see the Supporting Information). The complexes were found to be stable in aqueous solution (25 mM HEPES buffer, pH 7.0, 25 °C) in air for more than a month.

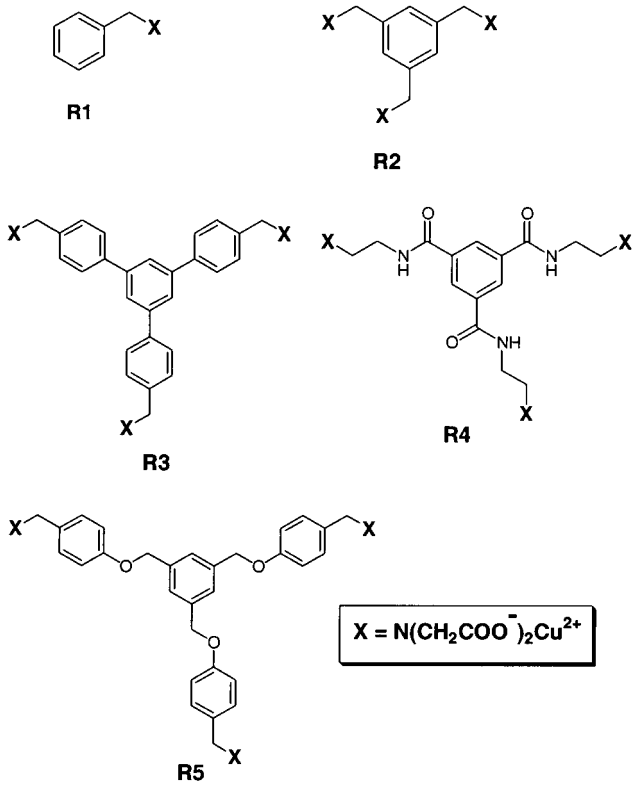


Figure 2. Structures of the metal complexes used in the recognition studies.

Binding Studies by Isothermal Titration Microcalorimetry (ITC). When substances bind, heat is either generated or absorbed. Measurement of this heat by ITC allows the accurate determination of the binding constant (K_{st}), reaction stoichiometry (n), and enthalpy change (ΔH). The method has been used to study small-molecule interactions (including metal−ligand interactions),[14] protein−ligand interactions,[15] protein−protein interactions, and other interactions.[16]

In a typical ITC experiment, a solution of metal complex (0.1−0.5 mM) in the ITC cell was titrated with a peptide (0.80−5.0 mM) solution. The heat of dilution of the peptide was then separately determined by injection of a solution of the appropriate peptide in buffer. The raw titration data were corrected for the heat of dilution of the peptide and processed (by nonlinear regression) using the software provided by the manufacturer (Bindworks 3.0) to get the binding parameters. The "independent set of multiple binding sites" model was

(12) Martell, A. E.; Smith, P. M. *Critical Stability Constants*; Plenum Press: New York, 1975; Vol. 2.
(13) Kronina, V. V.; Wirth, H. J.; Hearn, M. T. W. *J. Chromatogr., A* **1999**, *852*, 261−272.
(14) (a) Berger, M.; Schmidtcher, F. P. *Angew. Chem., Int. Ed.* **1998**, *37*, 2694−2696. (b) Wu, C.; Chen, W.-Y.; Lee, J.-F. *J. Colloid Interface Sci*. **1996**, *183*, 236−242.
(15) (a) Taquet, A.; Labarbe, R.; Houssier, C. *Biochemistry* **1998**, *37*, 9119−9126. (b) Heileman, R. E.; Jennings, R. N.; Linhardt, R. J. *Biochemistry* **1998**, *37*, 15231−15237.
(16) (a) *Applications of Calorimetry in the Biological Sciences*; Ladbury, J. E., Chowdhury, B. Z., Eds.; John-Wiley & Sons: New York, 1998. (b) Jelesarov, I.; Bosshard, H. R. *J. Mol. Recognit*. **1999**, *12*, 3−18. (c) Haq, I.; Trent, J. O.; Chowdhury, B. Z.; Jeskins, T. C. *J. Am. Chem. Soc*. **1999**, *121*, 1768−1779.

used for data analysis. The other binding models did not fit the binding data well and led to large errors.

Optimization of Binding Interactions. Experimental conditions were optimized to achieve the strongest Cu^{2+}-histidine interactions. The monohistidine peptide **m_H** and copper complex **R1** were used to study the effect of variation in pH, buffer concentration, and buffer type on binding affinity.

(1) Effect of pH. The peptides have two potential sites for metal coordination: the α-amino group ($pK_a = 9.2$) and the imidazole moiety ($pK_a = 6.8$) of the histidine residue. The difference in pK_a values of these two binding sites was used to achieve selective Cu^{2+}-imidazole interactions. Calculations showed that, at pH 7.0, the α-amino group remained protonated (> 99%) while the imidazole nitrogen atom was mostly (62%) unprotonated. The pH of the medium controls the donating power of the lone pair of electrons by the imidazole group to Cu^{2+} and directly affects binding capability.[17] To study the pH effect, binding experiments were carried out in the pH range 6.0−8.0 in 25 mM HEPES buffer (Supporting Information). The binding constants were also determined by UV−vis spectrometry (Supporting Information). The binding constants determined by the two methods were closely matched.

At pH 6.0 the binding affinity ($K_{st} = 2{,}900 \pm 600\ M^{-1}$) was a minimum because of protonation (86%) of the imidazole ϵ-nitrogen atom (pH < pK_a).[17] When pH was increased to 7.0, the extent of unprotonated imidazole increased and the binding affinity also increased ($K_{st} = 4{,}500 \pm 800\ M^{-1}$). At higher pH (8.0) the affinity decreased ($K_{st} = 3{,}400 \pm 1200\ M^{-1}$). This decrease in binding constant with increasing pH (beyond 7.0) can be attributed to the change in the overall charge of the complex as a result of coordination of hydroxide ions. At pH 8.0, the net charge of the complex $[Cu(IDA)(OH)^-]$ is −1,[7b] which reduces the affinity of the complex to accept the lone pair from the imidazole group. Similar binding behavior has been reported for the Sepharose-immobilized IDA−Cu^{2+} complex.[17a] Literature reports indicate that multinuclear species containing bridging oxygen atoms are not formed under these conditions.[7b,18]

(2) Effect of Buffer Type. Three buffers (HEPES, MOPS, CHES, 25 mM each) were used to study the effect of buffer type on the binding affinity. The changes in binding parameters for different buffers were not significant (Supporting Information). The HEPES buffer was chosen for our study not only for its good buffering capacity over the required pH range but also for its poor metal ion complexing ability.[19]

(3) Effect of Buffer Concentration. ITC experiments were carried out with HEPES buffer at different buffer concentrations (25−100 mM). The binding affinity was not significantly affected by the buffer concentration (Supporting Information). For the studies reported here, 25 mM HEPES buffer was used.

ITC Results. The control complex **R1** bound weakly to the monohistidine peptides **m_H** and **m_aH** and to all of the trihistidine peptides (except **t_QH**). The stoichiometry indicated that each histidine moiety was binding to one cupric ion. The binding parameters are shown in Table 1 (for studies with complex **R1**, the overall affinities are reported for the trishistidine peptides). The hydrophilic peptide **t_QH** showed moderate affinity for the control complex **R1**. The entropy loss for this interaction was found to be low. We do not have any explanations yet for this observation.

The two control monohistidine peptides **m_H** and **m_aH** had low binding affinity for all metal complexes tested. For some systems (e.g., **m_H·R3** and **m_H·R5**), the binding constants were so low that they could not be determined reliably by ITC. For these systems, only estimates of the binding constants (<1000) have been reported. With the monohistidine containing a free carboxylic acid (**m_aH**), only complexes **R1** and **R2** showed the expected stoichiometry of binding. The other Cu^{2+} complexes possibly form oligomeric species by coordinating to both imidazole and carboxylate groups of **m_aH**. No precipitates were observed after the ITC titrations for these systems. The possibility of the formation of oligomeric species was not investigated further as this does not change the conclusions reached from these studies.

Strong and selective binding was achieved by employing the metal complex **R4**. With the distance-matched peptide **t_GH**, complex **R4** demonstrated a strong binding affinity ($K_{st} = 1.19 \times 10^6\ M^{-1}$). This appears to be due to relatively low loss of entropy upon binding. A shorter peptide (**t_H**, $K_{st} = 104{,}400 \pm 22{,}800\ M^{-1}$) or a longer peptide (**t_GGH**, $K_{st} = 243{,}600 \pm 24{,}500\ M^{-1}$**)** was found to decrease the binding constant. Higher losses of entropy offset the enthalpy gains for these interactions. A hydrophobic side chain on peptide **t_AH** had a devastating effect on the affinity for the metal complexes, again due to unfavorable entropy changes. Introduction of a hydrophilic side chain on peptide **t_QH** also led to large entropy losses and consequently decreased the affinity for the metal complexes. For all of these systems tested, if the metal-ion-free ligands were used, no measurable bindings were detected by ITC under the same experimental conditions.

The stoichiometry was found to be less than unity when the distances were not matched. This possibly indicates the formation of oligomeric species among the peptide and the metal complexes. Some peptides (**t_H** and/or **t_GH**) formed precipitates with some complexes (e.g., complex **R3** and/or **R5**). This is probably because of formation of insoluble oligomeric species. No further investigations were made on these systems. The binding constants are summarized in Table 1 and are graphically illustrated in Figure 3.

(17) (a) Chen, W. Y.; Wu, C. F.; Liu, C. C. *J. Colloid Interface Sci.* **1996**, *180*, 135−143. (b) Lin, F. Y.; Chen, W. Y.; Sang, L. C. *J. Colloid Interface Sci*. **1999**, *214*, 373−379.

(18) (a) Roman-Alpisto, M. J.; Martin-Ramos, J. D.; Castineiras-Campos, A.; Bugella-Altamirano, E.; Sicilia-Zafra, A. G.; Gonzalez-Perez, J. M.; Nicols-Guiterrez, J. *Polyhedron* **1999**, *18*, 3341−3351. (b) Castineiras- Campos, A.; Sicilia-Zafra, A. G.; Gonzalez-Perez, J. M.; Nicols-Guiterrez, J.; Chinea, E.; Mederos, A. *Inorg. Chim. Acta* **1996**, *241*, 39−45.

(19) Good, N. E.; Winget, G. D.; Winter, W.; Connally, T. N.; Singh, R. N. N. *Biochemistry* **1966**, *5*, 467−474.

Table 1. Binding Constants, Enthalpy, Entropy, and Stoichiometry of Binding for the Metal Complexes with the Histidine Peptides (25 mM HEPES Buffer, pH 7.0, 25 °C)[a]

		R1	R2	R3	R4	R5
m_H	*n*	1.12 ± 0.11	3.89 ± 0.06		2.63 ± 0.21	
	K_{st}	(4.5 ± 0.82)	(9.5 ± 1.1)		(1.7 ± 0.17)	
	$-\Delta H$	11.6 ± 0.80	6.9 ± 0.21	<1	18.4 ± 2.5	<1
	$-\Delta G$	5.0	5.4		4.4	
	$-T\Delta S$	6.6	1.5		14.0	
t_H	*n*	0.31 ± 0.01	0.92 ± 0.01		0.80 ± 0.02	
	K_{st}	(56.0 ± 11.8)	(104.6 ± 32.9)		(104.4 ± 22.8)	
	$-\Delta H$	22.1 ± 1.5	25.8 ± 0.92	PPT	30.0 ± 1.5	PPT
	$-\Delta G$	6.5	6.8		6.8	
	$-T\Delta S$	15.7	18.9		23.2	
t_GH	*n*	0.44 ± 0.06	0.92 ± 0.01		1.02 ± 0.006	0.50 ± 0.06
	K_{st}	(23.5 ± 2.0)	(32.6 ± 3.7)		(1190.0 ± 130.0)	(346.00 ± 53.0)
	$-\Delta H$	23.8 ± 0.52	30.2 ± 0.7	PPT	20.82 ± 0.21	28.32 ± 0.62
	$-\Delta G$	6.0	6.1		8.3	7.55
	$-T\Delta S$	17.8	24.1		12.6	20.77
t_GGH	*n*	0.36 ± 0.03	1.25 ± 0.03	0.53 ± 0.01	0.68 ± 0.003	
	K_{st}	(17.0 ± 3.0)	(50.3 ± 7.6)	(200.0 ± 45.0)	(243.6 ± 24.5)	
	$-\Delta H$	32.61 ± 3.0	21.9 ± 0.91	25.80 ± 1.0	28.0 ± 0.4	PPT
	$-\Delta G$	5.77	6.4	7.22	7.3	
	$-T\Delta S$	26.84	15.4	18.58	20.72	
t_AH	*n*	0.31 ± 0.01	1.06 ± 0.01	0.52 ± 0.01	0.61 ± 0.04	0.46 ± 0.04
	K_{st}	(3.52 ± 0.90)	(15.4 ± 1.3)	(82.9 ± 10.5)	(5.1 ± 1.0)	(14.10 ± 2.80)
	$-\Delta H$	26.75 ± 3.73	29.6 ± 0.82	23.21 ± 0.93	51.5 ± 6.8	33.67 ± 4.53
	$-\Delta G$	4.83	5.7	6.70	5.0	5.65
	$-T\Delta S$	21.92	23.8	16.51	46.3	28.02
t_QH	*n*	0.37 ± 0.005	0.61 ± 0.02	0.42 ± 0.01	0.47 ± 0.03	
	K_{st}	(72.3 ± 11.3)	(77.0 ± 15.7)	(42.60 ± 5.40)	(41.12 ± 7.5)	
	$-\Delta H$	15.05 ± 0.62	32.71 ± 2.13	27.22 ± 1.25	71.0 ± 6.7	PPT
	$-\Delta G$	6.62	6.7	6.31	6.3	
	$-T\Delta S$	8.43	26.1	20.91	64.7	
m_aH	*n*	0.97 ± 0.04	3.05 ± 0.22	0.42 ± 0.03	0.88 ± 0.01	0.35 ± 0.01
	K_{st}	(1.70 ± 0.15)	(1.38 ± 0.12)	(7.20 ± 1.80)	(4.50 ± 0.90)	(5.10 ± 0.90)
	$-\Delta H$	6.42 ± 0.46	6.81 ± 0.64	3.69 ± 0.62	12.42 ± 0.90	14.09 ± 3.52
	$-\Delta G$	4.40	4.28	5.25	4.98	5.05
	$-T\Delta S$	2.02	2.53		7.44	9.04
t_aH	*n*	0.39 ± 0.02	0.83 ± 0.009	0.50 ± 0.01		0.42 ± 0.005
	K_{st}	(29.8 ± 8.60)	(62.6 ± 9.4)	(15.81 ± 2.42)		(204.80 ± 25.80)
	$-\Delta H$	9.98 ± 0.70	17.22 ± 0.30	20.66 ± 0.71	nf	24.36 ± 0.32
	$-\Delta G$	6.10	6.54	5.72		7.24
	$-T\Delta S$	3.88	10.68	14.94		17.12

[a] Binding constants are reported in thousands ($\times 10^3$). ΔH, ΔG, and $T\Delta S$ are in kcal mol^{-1}. nf = raw data did not fit to the binding models because of an irregular pattern. PPT = precipitation occurred.

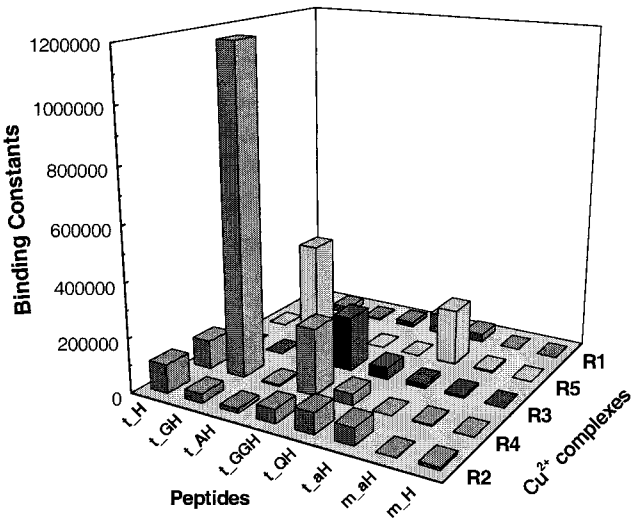


Figure 3. Binding constants of the model peptides with the metal complexes (25 mM HEPES buffer, pH 7.0, 25 °C).

Binding Studies with UV−Vis Spectrometry. UV−vis spectrophotometry has been extensively used to study peptide−receptor[24] and porphyrin−nucleotide interactions.[20] Peptide−metal chelation has also been studied by UV−vis spectrometry.[21] For our studies, the system with highest affinity (**t_GH.R4**) was studied in detail by UV−vis spectrometry.

The electronic spectrum of **R4** contains a broad band in the visible region with a maximum of 728 nm. Upon addition of **t_GH** to the solution, this maximum is blue-shifted (Supporting Information). This is a consequence of ligand field transition of the Cu^{2+} ions upon coordination with the nitrogen atom of the imidazole.[22] In addition, a visual observation of a darker blue solution was indicative of a greater number of nitrogen donors contributing to the ligand field after addition of **t_GH**. The changes in the absorption maximum were plotted as a function of the molar concentration ratio of the peptide to Cu^{2+} complex (i.e., **t_GH:R4**, Supporting Information) to generate the binding isotherm. The shift in the absorption maximum increased with increasing molar ratio of **t_GH** to complex **R4**, and an approximate plateau was observed at a 1:1 molar ratio with an absorption maximum of 673 nm.

(20) (a) Sirish, M.; Scheider, H. J. *J. Chem. Soc., Chem. Commun*. **1999**, 907−908. (b) Sirish, M.; Scheider, H. J. *J. Chem. Soc., Chem. Commun*. **2000**, 23−24.
(21) Chen, Y.; Pasquinelli, R.; Ataai, M.; Koepsel, R. R.; Kortes, R. A.; Shepherd, R. E. *Inorg. Chem*. **2000**, *39*, 1180−1186.
(22) (a) Sigel, H.; Martin, R. B. *Chem. Rev*. **1982**, *82*, 385−426. (b) Bernardncci, E.; Schwindinger, W. F.; Hughey, J. L.; Jespersen, K. K.; Schugar, H. J. *J. Am. Chem. Soc*. **1981**, *103*, 1686−1691.

Table 2. Binding Constants, Stoichiometry, Enthalpy, Entropy, and Free Energy Change of Peptide **t_H** and Cu^{2+} Complex **R2** at Different Temperatures (25 mM HEPES buffer, pH 7.0)[a]

temp, K	n	K_{st}, $\times 10^3$ M^{-1}	ΔH, kcal mol^{-1}	ΔG, kcal mol^{-1}	$T\Delta S$, kcal mol^{-1}
298	0.92 ± 0.01	104.6 ± 32.2	-25.92 ± 0.52	-6.84	-18.08
303	0.92 ± 0.01	80.3 ± 18.9	-27.33 ± 0.54	-6.79	-20.54
308	1.03 ± 0.02	62.7 ± 6.8	-28.94 ± 0.17	-6.76	-22.18
313	0.95 ± 0.03	49.5 ± 6.8	-30.15 ± 1.12	-6.72	-23.43

[a] Binding constants are reported in thousand ($\times 10^3$).

It has been reported that the coordination of a second imidazole to the Cu^{2+} center is characterized by a further larger blue shift (absorption maximum 628 nm).[23] Further addition of **t_GH** (up to 1.7 equiv of complex **R4**) did not produce any appreciable change. These UV−vis titration studies suggested that the peptide **t_GH** and complex **R4** bind in a 1:1 fashion. The presence of an isobestic point (510 nm) also indicated the formation of a 1:1 complex between **t_GH** and **R4**. Analysis of the titration curve[24] corroborated the binding constant measured by ITC. Solution-phase EPR studies (9.44 GHz, 25 °C) on the complex **t_GH.R4** ($g = 2.26$, data not shown) showed that the imino nitrogen atom of IDA was bound to the cupric ions and only one histidine was binding to each cupric ion.[2b]

Thermodynamic Studies. Although complex **R4** showed the highest affinity for the distance-matched peptide **t_GH** at 25 °C, this system was found to be unsuited for thermodynamic studies by ITC. At lower temperatures ($T < 20$ °C), the solubility of **R4** was very low in HEPES buffer (pH 7.0). When the temperature was increased to 30 °C or higher, ITC studies indicated that the stoichiometry of binding of **R4** with **t_GH** was substantially different from 1 (0.58 at 30 °C). Both **R4** and **t_GH** are fairly flexible; when the temperature is increased, they probably form oligomeric species. Because of these problems, thermodynamic studies are described in detail here for the binding of the complex **R2** with its distance-matched trihistidine peptide **t_GH**.

The thermal stability of the **t_H**, **R2**, and **t_H·R2** complexes were first studied by temperature-controlled UV−vis spectrophotometry (the absorbance at 330 nm was followed). Neither of the species under investigation showed any appreciable change in absorbance up to 60 °C. A temperature range of 25−40 °C was selected for thermodynamic studies with ITC. The results are shown in Table 2.

The association constant decreased progressively with increasing temperature from 25 to 40 °C. This decrease may be due to greater entropic losses at higher temperatures. The stoichiometry (n) was found to remain constant for the entire experimental temperature range. A relatively constant stoichiometry suggested that the interaction mode between peptide **t_H** and complex **R2** did not change with temperature.

The thermodynamic parameters for these interactions are summarized in Table 2 and plotted against temperature in the Supporting Information. The binding is driven strongly by the enthalpy component; i.e., the binding enthalpy is negative and contributes favorably to the binding energy. For the entire range of working temperature, the binding entropy is negative and contributes unfavorably. The negative ΔG values remained almost independent of temperature.

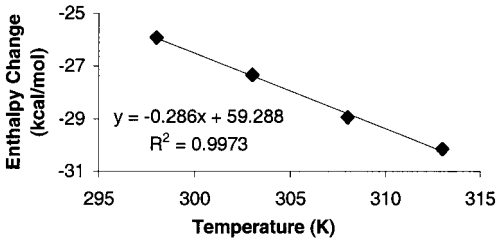


Figure 4. Effect of temperature on the enthalpy change for the peptide **t_H** and Cu^{2+} complex **R2** (25 mM HEPES buffer, pH 7.0).

The change in heat capacity was determined by plotting the enthalpy change (ΔH) at different temperatures and fitting a straight line through these data points (Figure 4). The heat capacity change (ΔC_p) determined from the slope of this straight line was -0.29 kcal mol^{-1}.

The change in heat capacity (ΔC_p) is one of the most valuable thermodynamic parameters for detecting structural changes of interacting molecules, especially for peptides and proteins. Several authors[25] have reported that the exposure of nonpolar surface areas to water results in a positive increase in the ΔC_p value whereas the opposite (i.e., the burial of nonpolar surface areas from water) results in a negative value of ΔC_p. ΔC_p values in the range of -0.1 to -1.0 kcal mol^{-1} K^{-1} have been reported for many protein−ligand,[26] antigen−antibody,[27] peptide−protein,[28] and peptide−antibody[29] interactions.

Enthalpy−entropy compensation is associated with solvent reorganization accompanying receptor−ligand interactions.[30] A plot of ΔH vs $T\Delta S$ is shown in Figure 5. The straight line fitted through these points has a slope of 0.8. Although the data points are slightly scattered, they however demonstrate the enthalpy−entropy compensation effect. The complete compensation is indicated by a slope of unity. In our study the slope is less than unity, which suggests that the free energy of binding is more sensitive to changes in entropy.[30]

(23) Dhal, P.; Arnold, F. H. *Macromolecules* **1992**, *25*, 7051−7059.
(24) *Binding Constants*; Connors, K. A., Ed.; Wiley-Interscience: New York, 1987.
(25) (a) Spolar, R. S.; Livingstone, J. R.; Record, M. T., Jr. *Biochemistry* **1992**, *31*, 3947−3955. (b) Livingstone, J. R.; Spolar, R. S.; Record, M. T., Jr. *Biochemistry* **1991**, *30*, 4237−4244. (c) Murphy, K. P.; Gill, S. J. *J. Mol. Biol.* **1991**, *222*, 699−709.
(26) (a) Lin, Z.; Schwarz, F. P.; Eisenstein, E. *J. Biol. Chem.* **1995**, *270*, 1011−1014. (b) Kozlov, A. G.; Lohman, T. M. *Biochemistry* **1999**, *38*, 7388−7397.
(27) Torigoe, H.; Nakayama, T.; Imazato, M.; Shimada, I.; Arata, Y.; Sarai, A. *J. Biol. Chem.* **1995**, *270*, 22218−22222.
(28) McNemar, C.; Snow, M. E.; Windsor, W. T.; Prongay, A.; Mui, P.; Zhang, R.; Durkin, J.; Le, H. V.; Weber, P. C. *Biochemistry* **1997**, *36*, 10006−10014.
(29) Swaminathan, C. P.; Nandi, A.; Visweswariah, S. S.; Sourila, A. *J. Biol. Chem.* **1999**, *274*, 31272−31278.
(30) (a) Swaminathan, C. P.; Sourila, N.; Sourila, A. *J. Am. Chem. Soc.* **1998**, *120*, 5153−5159. (b) Lumry, R.; Rajender, S. *Biopolymers* **1970**, *9*, 1125−1127.
(31) (a) Keown, M. B.; Henry, A. J.; Ghirlando, R.; Sitton, B. J.; Gould, H. J. *Biochemistry* **1998**, *37*, 8863−8869. (b) Faiman, G. A.; Horouitz, A. *J. Biol. Chem.* **1997**, *272*, 31407−31411.

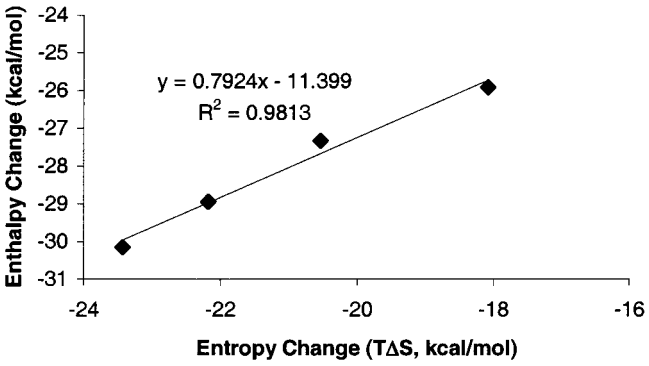


Figure 5. Enthalpy−entropy compensation plot for the interaction of peptide **t_H** and Cu^{2+} complex **R2** (25 mM HEPES buffer, pH 7.0, temperature 25−40 °C).

Conclusions

We have demonstrated that a histidine-containing peptide (**t_GH**) can be bound strongly and selectively by using complementary polyvalent metal−ligand interactions (complex **R4**). Comparison of binding constants reported in Table 1 indicates that complex **R4** favors **t_GH** compared to other peptides differing by only one amino acid unit (selectivity 230:1 with **t_AH**, 30:1 with **t_QH**), a shorter peptide (selectivity 10:1 with **t_H**), or a longer peptide (selectivity 5:1 for **t_GGH**). These findings can be applied for designing transition-metal complexes for recognition of more complex systems (e.g., proteins).

Experimental Section

Equipment. Calorimetric studies were performed with an isothermal titration calorimeter (ITC-4200, Calorimetry Sciences Corp., Provo, UT) linked to a PC for data acquisition and analysis. In addition to the internal temperature controller an external water bath was used to maintain the desired temperature (±0.01 °C). A continuous stream of dry nitrogen was used to prevent moisture condensation in the unit. Absorption spectra were recorded using an Ultrascope 4000 (Pharmacia Biotech) UV−vis spectrophotometer. Quartz cuvettes of 10 mm path length were used for all routine studies. The temperature effect on UV−vis spectra was studied using a Beckmann-60 unit fitted with a constant-temperature accessory. Quartz cuvettes with a path length of 5 mm were employed for this study. An analytical balance (Analytical plus, Ohaus AP 250D) was used for weighing the samples (accuracy 0.1 mg).

Isothermal Titration Calorimetry. Calorimetric measurements were conducted in aqueous solution (25 mM HEPES buffer, pH 7.0). Peptide samples (0.80−5.0 mM) were routinely centrifuged prior to the titration and were examined for precipitates, if any, after the titration. All solutions were thoroughly degassed by stirring (15 min) under reduced pressure before use. The ITC cell was filled with the Cu^{2+} complex solution, and the peptide solution was loaded into the syringe. For all experiments, freshly prepared peptide and Cu^{2+} complex solutions were used. An equilibration period of 30−45 min was used before the experiment was started.

A typical titration consisted of injecting 5 μL of ligand solution (42 aliquots) into the cell with an interval of 5.0 min to ensure that the titration peak returned to the baseline prior to the next injection. To achieve a homogeneous mixing in the cell, the stirrer speed was kept constant at 300 rpm. The heats of dilution were determined under identical conditions by injecting the appropriate peptide solution into the ITC cell containing only the sample buffer. For every experiment, the heat of dilution of the peptide was determined and subtracted from the sample titration data before processing. The temperature-dependent binding studies were conducted after the instrument had been equilibrated overnight at the required temperature.

The titration data were analyzed using the software provided by the manufacturer (Bind Works 3.0). The corrected binding isotherms were fitted using least-squares regression to obtain the association constant (K_{st}), the number of peptide molecules bound to per Cu^{2+} complex (stoichiometry, n), and the enthalpy change associated with the interaction (ΔH). The independent binding model (a single set of identical sites) was used. Attempted fits using either nonequivalent or cooperative binding models resulted in poor curve fitting. All parameter uncertainties were evaluated at the 95% confidence level and reported as standard deviations calculated as half the difference between derived upper and lower limits.

Once K_{st} was obtained directly from the curve fitting, the free energy change for binding (ΔG) was calculated from the following relationship:

$$\Delta G = -RT \ln K_{st}$$

where R = universal gas constant (1.987 cal mol^{-1} K^{-1}) and T = absolute temperature. The entropy change (ΔS) was calculated from the equation

$$\Delta G = \Delta H - T\Delta S$$

The change in heat capacity at constant pressure (ΔC_p) was calculated from

$$\Delta C_p = (\Delta H_{T_2} - \Delta H_{T_1})/(T_2 - T_1)$$

UV−Vis Spectrometry. Solutions of peptide (0.2 mM) and Cu^{2+} complexes (0.5 mM) were prepared in 25 mM HEPES buffer (pH 7.0). For routine analysis at room temperature, the absorption spectra were recorded in the 200−800 nm range against the blank (HEPES buffer). UV absorption titration profiles were recorded by adding aliquots of peptide to the cell containing a known initial concentration of Cu^{2+} complex. To adjust working solutions to a fixed mole ratio (peptide/Cu^{2+} complex), appropriate volumes of prepared solutions were mixed. All measurements were made after sample equilibration for at least 15 min.

Acknowledgment. We thank Prof. M. Lakshman (City College of CUNY, New York) for helping with EPR and temperature-controlled UV−vis experiments. This work was supported by an NSF-CAREER award (Grant CHE-9896083), an NGMS-AREA grant (NIH, 1R15 59594-01), and a COBRE award from NCRR (NIH, Grant 1P20 RR15566-01). The microcalorimeter was purchased through an NSF EPSCoR award to North Dakota.

Supporting Information Available: Experimental details for the synthesis of the peptides, UV−vis spectral data for optimization of pH for the binding constant determinations, procedure for Cu^{2+} estimation with EDTA titration, ITC data for optimization of binding interactions (effect of pH, buffer type, and buffer concentration), UV−vis titration of peptide **t_GH** and Cu^{2+} complex **R4**, and plots of the data reported in Table 2. This material is available free of charge via the Internet at http://pubs.acs.org.

IC0108742